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Original Article

Cytomegalovirus PCR Analysis of Serum and Ocular Swab Samples in Patients with Inflammatory Eye Disease

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Abstract

Background and Aims: Cytomegalovirus (CMV) infection is very common in the population. Virus belongs to the family Herpesviridae, whose representatives are characterized by the ability to cause the human body's latent persistent infections. The goal of the study is to assess the CMVV infection frequency and PCR method in the choice of CMV in inflammatory eye disease, Comparing with CMV presence in Eye Infection and the control group.

Materials and Methods: Primers were designed for conserved regions of the CMV genome. We have used PCR to rapid, accurate detection of EBV DNA in blood and from eye swabs as well as pp65 antigenemia. We have chosen to study patients with eye inflammation or infection symptom.

Results: CMV DNA was detectable in three 21 out of 130 control samples of serum (16.5%). CMV infection was seen in 9 out of 20 (45%) patients' serum samples. Compared with the controls, the presence of EBV DNA was only significantly increased in samples of the patient group. 13 out of 20 (65%) patients in patients and 19 out of 130 (14.61%) of the control group had detectable CMV DNA in their ocular swab.

Conclusion: We have presented evidence for the presence of CMV sequences in normal and eye inflammation samples with PCR. CMV serology was available for a large number of individuals. The prevalence of CMV in ocular disease samples varied dramatically that this wide range may be due to variations and inconsistency in the techniques used to detect the virus and its Components, as well as geography and genetic susceptibility.

Keywords: cytomegalovirus, CMV, PCR analysis, serum and ocular swab, inflammatory eye disease

Introduction

Cytomegalovirus (CMV) virus infection is very common in the community (1-5). These viruses belong to the family Herpesviridae, whose representatives are characterized by the ability to cause the latent persistent infections (4-7). The source of infection is an infected human (6-10). CMV is lymphotropic agent and Infections can be transmitted through air-mist, contact, sexual contact, and blood transfusions (7, 10-13). CMV infects people of all ages can be congenital or acquired. Congenital CMV infection can cause fetal or infant problem - usually violates the lungs, kidneys, liver and other organs or other systems (1-3, 5, 7, 10).

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Human cytomegalovirus (CMV) can cause a minority case of infectious mononucleosis (14, 15). This virus can detected in body fluids including tears, saliva, blood, and urine, a person can becomes infected with CMV by direct contact with infected body fluids (3, 16-18). Cytomegalovirus is most commonly transmitted through kissing and sexual intercourse. It can also be transferred from an infected mother to her unborn child (fetus, embryo) (1, 2, 7, 10, 12). This virus is often "silent" because the signs and symptoms cannot be felt by the person infected (19-21). However, it can cause life-threatening illness in infants, HIV patients, transplant recipients, and those with weak immune systems (3, 5, 6, 7, 17). For those with weak immune systems, cytomegalovirus can cause more serious illnesses such as inflammations of the retina and pneumonia, esophagus, liver, large intestine, and brain disorder (3, 5, 17). Approximately 90% of the human population has been infected with cytomegalovirus by the time they reach adulthood, but most are unaware of the infection (6, 9, 22, 23). Once a person becomes infected with cytomegalovirus, the virus stays in body fluids throughout host lifetime (19-23). In addition, these infections affect some blood diseases. Normally, people with healthy immune may have CMV and EBV infections without complications (6, 9, 22, 23). One of the most pressing problems is the CMV infections are it effects in transplant recipients (24-26). Infections have the ability to go into a latent state. Cytomegalovirus belongs to the Herpesviridae family. Herpesvirus is one of the widespread human viral infectious agents that cause latent infections with recurrent viral activation. Their replication takes place in the cell nucleus. The viruses leave the cell nucleus and into the cytoplasm through nuclear membrane (4-7). Cytomegalovirus infection is widespread throughout the world. In developed countries, 50% -70% of the general population has antibodies against CMV (6, 9, 22, 23). The only source of infection is human. In women illness of a primary infection during pregnancy, the risk of transmission to the fetus is about 50%. Postnatal CMV infection can occur due to viruses in cervical secretions, but most often the source of infection is the mother's milk. CMV cans Infects B lymphocytes as well as epithelial cells (1, 2, 4, 6). CMV causes infectious mononucleosis, pneumonia, hepatitis, hematological changes. Often CMV infection may be asymptomatic or subclinical and people with the infection can distribute it. Of the 10% -20% of healthy individuals' CMV can be distinguished in nasopharyngeal mucus. Individual groups and people living in asocial conditions, where there are close contacts may be endemic foci of infection (6, 9, 14, 1522, 23). The goal of study is to assess CMV infection frequency and PCR method in the choice of patient who have Eye Infection and Comparing CMV infection frequency in Eye Infection and control group.

Methods

CMV infection diagnosis is based on clinical symptoms, which must be confirmed by laboratory tests. CMV pp65 protein is determined in the patient infected neutrophils by IF method. CMV pp65 antigen can be assessed qualitatively or quantitatively. Antigenemia test has equivalent diagnostic sensitivity and specificity to the PCR method. Compared with cell culture, molecular methods are more sensitive and highly specific. CMV DNA determined by polymerase chain reaction method in the blood, bone marrow, bronchial secretions and other body fluids and secretions. CMV is possible to identify by DNA and hybridization [17, 29].

CMV pp65 Antigenemia and CMV PCR methods comparison. We conducted studies whose objective was to assess the qualitative CMV pp65 Antigenemia test diagnostic possibilities for early detecting clinically manifested CMV disease in eye infection. It was the observation and investigation of 20 patients with eye discharge. A positive Antigenemia test for 7 patients showed. Qualitative CMV pp65 Antigenemia is not sufficiently useful for the diagnosis of CMV eye disease patients as part of a positive Antigenemia test. Therefore, the study data, more accurate quantitative antigenemia test,

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which is significantly better correlated with clinical CMV disease and appropriate for disease control after detection (antiviral treatment application of the active virus replication phase, before the advent of disease symptoms). It has also been carried out the PCR method for prognostic and diagnostic capability assessment and early detection of CMV disease in patients with eye infection. However CMV by PCR had a sensitivity of 92.3% and specificity - 54.5%, or slightly smaller than the qualitative CMV pp65 method, the corresponding index is equal to 100% and 82.6% for PCR and pp65 positive predictive methods are comparable (respectively - 70.6% and 69.2%), and PCR negative results. Research has shown that quantitative PCR method allows the determination of CMV DNA on average two weeks earlier from CMV pp65 method. It is important to identify CMV infection as early as possible. On the other hand, studies have revealed that the PCR method is too sensitive to allow reliably distinguish patients with the risk of CMV disease. The research result also concluded that the quantitative PCR specificity and positive predictive value is significantly smaller than pp65 method, when the virus is found in a small amount of DNA. When virus found in a large enough quantity, the PCR specificity and positive predictive value is closer to CMV pp65 method specificity and positive predictive value. It is also important to note that the CMV pp65 antigenemia testing requires a large amount of neutrophil cells, so the sensitivity decreases in neutropenic status patients. While virologic test is accurate, but is not suitable because it takes a long time to wait for the result, it is difficult and costly investigation procedure, and in addition, it is sometimes difficult or impossible to grown the pathogen. There is a rapid test for CMV, but the results may not be obtained as early as after 24-48 hours after infection. Serological tests are accurate, but antibodies can be detected as early as one week after the start of infection. In the analysis of serological testing methods, should be noted that the complement fixation test for these reactions are of low sensitivity, the result may be a false negative, in addition, the test takes a lot of time and work. Immunoassay tests are accurate, but the IgM test is not reliable enough to acute or recent infection diagnostics.

The patients studied groups and the number of inquiries. All 150 samples were divided into two groups. The first group includes individuals who have experienced eye inflammation, and the second group - individuals, inexperienced the infection. Group I consisted of 20 eye infection experienced in individuals. Group II for individuals of CMV DNA tests were carried out according to clinical symptoms, targeted, usually - once on suspicion of any infection. CMV DNA test is carried out by PCR method using Research thermal cycler. Isolation of nucleic acids in the laboratory is done manually, but in case of a large number of samples, extraction is carried out with the Qiagen kit. Venous blood is taken into the test tube with EDTA. The study sampled 5-10 ml of blood. Immediately after sample collection tubes are mixed. Blood samples delivered to the laboratory on the same day after collection (if not possible, then the sample must be stored at -20 C). Laboratory checks whether the sample was taken correctly or it intact during transportation to the laboratory. Blood samples recorded and given a unique identification number, which is then further research appears in the register or the PCR test. The study confirms adherence to the Declaration of Helsinki. Consent to remaining in the research, obtained as soon as possible from the subject or a legally authorized representative. All swab samples were collected within maximum six hours to arrive at our laboratory and manipulated within maximum 1 hour at room temperature or Swabs were kept at 4°C for 1-3 hours until processed or aliquoted and stock at -70°C until later examination. Swabs were immersed in distilled water as eluent and resulted suspension was used for total DNA extraction. DNA was isolated using the QIAamp Tissue Kit (Qiagen Company). Extracted DNA samples were dissolved in 200 µl of sterile TRIS-EDTA, pH 8 and visualized by electrophoresis on 0.7% agarose gel.

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DNA purification. Isolation of nucleic acids in the laboratory is done manually, but in case of a large number of samples, extraction is carried out with the Qiagen kit. Venous blood is taken into the test tube with EDTA. The study sampled 5-10 ml of blood. Immediately after sample collection tubes are mixed. Blood samples delivered to the laboratory on the same day after collection (if not possible, then the sample must be stored at -20 C). Laboratory checks whether the sample was taken correctly or it intact during transportation to the laboratory. Blood samples recorded and given a unique identification number, which is then further research appears in the register or the PCR test. Viral nucleic acid extraction procedure and PCR performed in a separate room to prevent contamination of clean area of PCR reaction products. Nucleic acid purification and PCR reagents assembly phases are carried out "clean". We Used QIAamp DNA Blood Mini Kit. The kit contains: lyophilized proteinase (QIAGEN Protease); Lizzie ready solution (Buffer AL); washing buffer (Buffer AW1); washing buffer (Buffer AW2); DNA elution buffer (Buffer AE); internal control (IC). All reagents may be stored at room temperature, diluted proteinase other than that at + 2-8 ° C, and the internal control that is stored - 20 ° C. Additional Reagents is ethanol (96% to 100%); distilled water. DNA extraction is performed at room temperature (+ 20-24C).

PCR reagents. PCR takes place by the following reaction components: isolated DNA, primers (oligonucleotide), Taq DNA polymerase, available nucleotides (dNTPs), and reaction buffer. All the reaction regents are then added to a PCR tube for holding the PCR reaction. Then the tube is placed in a special thermal cycler, which ensures the necessary reaction conditions. All components (except for isolated DNA) are prepared and submitted by commercial kits.

Polymerase Chain Reaction. PCR for the detection of CMV was performed by using oligonucleotide primers from the HindIII-X fragment region (400bp) (TABLE-1). These primers checked for not to amplify other herpesviruses or cellular DNA. A no-target control reaction tube received 50 ml of reaction mixture only. The tubes were overlaid with 2 drops of mineral oil and were subjected to 35 cycles of amplification (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) by using a DNA thermal cycler. After the cycling was completed, the amplified PCR products were electrophoresed on an agarose gel (1.5%) and were visualized with UV light as a single band by staining with ethidium bromide (10 mg/ml). No other bands aside from the product were visualized. The PCR amplification of sample DNA was performed for 35 cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min) on a DNA thermal cycler with Taq polymerase. Primer sequences were derived from the specific region of the CMV genome. The immortalized human foreskin epithelial cell line RHEK-l and sterile deionized water served as negative controls; the approved EBV-positive, served as a positive control. The PCR products electrophoresed in 1.8% agarose gels. The 50 µl PCR mixture contained: 50 mM KCl, 10 mM TRIS (pH 8.3), 10 µg of bovine serum albumin, 1.75 mM MgCl2, 0.75 pmol of each primer, 0.2 pmol of dUTP (Fermentas), 0.2 pmol each of dGTP, dATP, dCTP (Fermentas), 1.5 unit of Taq DNA Polymerase (Fermentas), 5 µl of isolated DNA solution, and distilled H2O was added to a total volume of 50 µl.

Sensitivity of PCR. The sensitivity of the PCR was tested by serial tenfold dilutions of the extracted positive control DNA of both CMV in sterile Milli Q water. PCR was performed on the diluted samples and the sensitivity was determined. The sensitivity of two nucleic acid extraction protocols was measured by determining PCRD50 as the highest dilutions of the sample that gave a 50% detection rate by PCR. To further evaluate the sensitivity of PCR on clinical specimens, we performed PCR analysis on dilutions of previously quantified stocks of patient-derived CMV particles. CMV stocks were subject to 2-fold serial dilution using distilled water as a diluent. To determine the maximum dilution of whole, virus particles detectable by the assay, the diluted samples were subjected to PCR. Purified CMV target DNA standard (provided by the kit) were

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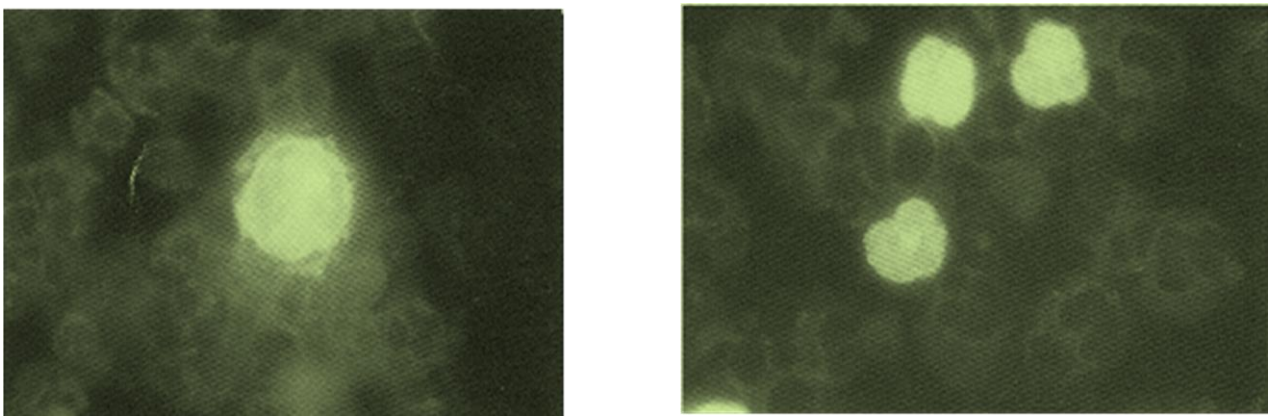
found to be detectable at a minimum level of 20 copies in 4 out of 4 reactions, with the highest detected crossing point values being 27.92.

Specificity of PCR. Since high rates of false-positive negatively affect the interpretation of the PCR observed sensitivity, must first present the specificity results in the study. The specificity of the developed PCR test verifies by testing several DNA samples extracted from different sources including bacteria, Fungal over the intended PCR. The cross reactivity between the each of the primer sets, herpes group of viruses were also determined by testing the primers against varicella zoster virus DNA (Oka vaccine strain), and standard or laboratory isolates of EBV DNA, HHV-6.

Statistical analysis. Mann–Whitney U-test was used for the statistical analysis to assess the association between antigenemia and PCR results. The results were expressed as medians and P- values <0.05 were considered significant. Pearson correlation and linear regression were used to compare EBV PCR results. Data were analyzed by mean values and standard deviations for frequency and percentages for qualitative categorical variables. SPSS version 13.0 was used to analyze the data. A P value of _ .05 and 95% confidence intervals was accepted as statistically significant.

Result

The table shows that the Group I of individuals with CMV DNA found, a rate of 45.0% of the group studied individuals. Group II of CMV DNA was detected in only 16.15% of individuals. Thus, the CMV infection group I individuals were found in a relatively 3.0 times the number of individuals (45.0% / 16.15%). In assessing both indicators can be concluded that CMV DNA infection is more frequent in group I than Group II. CMV DNA was detectable in 21 out of 130 control samples of serum (16.15%). No DNA of other infectious agents such as Toxoplasma gondii, HSV-1, and VZV could be detected except EBV (5 out of 130) in these controls. None of the controls were known to use any immunosuppressive therapy. CMV infection was seen in 9 out of 20 (45%) patients' serum samples. Compared with the controls, the presence of CMV DNA was significantly increased in samples of the patient group. 7 out of 20 (35%) in patients and 4 out of 130 (3.07%) of the control group had detectable CMV DNA in their ocular swab. In non out of these patients another infectious agent could be detected either by measurement of local antibody production or by PCR. The details of patients demography were as follows. CMV DNA was detectable in three 21 out of 130 control samples of serum (16.5%). CMV infection was seen in 9 out of 20 (45%)



Positive Control slide

Positive Test slide

Fig. 1. Detection of CMV pp65 in human peripheral blood cells

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Table1: Summarized test Results.

Test	Result	Number	Percent
PCR	+	174	38.4
Pp٦٥ antigen	+	60	13. 2 5
Only PCR	+	118	2 6
Only pp٦٥	+	4	0.88
Both	+	56	1 2 .37

Table 2: Demographics data of sample patients

Gender		
Male	50	
Female	50	
Race		
Iranian	100	
Age (mean)		
Male	42 (18–58)	
Female	37 (19–65)	
History of approved disease (n = 20)		
Ocular	20	
Cutaneous(cold sores)	5	
Genital	0	
Education	ND[1]	
EBV antibody	Positive	120(80)
	Negative	30(20)

ND: not determined

patients' serum samples. Compared with the controls, the presence of EBV DNA was only significantly increased in samples of the patient group. 13 out of 20 (65%) patients in patients and 19 out of 130 (14.61%) of the control group had detectable CMV DNA in their ocular swab.

The mean age of the patients was 42 (18–58) for men and 37 (19–65) years in the female. The male to female ratio was 1/1. 7 patients had used the antiviral agent within 12 months of the tear sampling. Next, we analyzed the total positive rate of tear PCR assay and the conditional positive rates, according to the methods used to collect swab and the methods used to detect CMV. The total incidence rate of positive PCR was 35% (7 out of 20) in the

Table 3: used EBV primer and PCR methods

Primer pair	$[Mg^{2+}]$ (M)	Annealing temperature (°C)	MELT TEMP	GC CONTENT	Product size (base pairs)
5-GGATCCGCATGGCATTCACGTATGT-3	0.002	55°C	61.4 °C	52 %	400
5-GAATTCAGTGGATAACCTGCGGCGA-3	0.003	55°C	61.1 °C	50 %	240

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swab. Finally, we investigated whether clinical factors affect the positive rates of swab PCR. There was no significant statistical difference in the positive PCR rates between the patients by sex, but higher ages showed higher virus detection. Finally, the positive PCR rates were no greater in the patients without previous administration of anti-herpesidal medication than in the patients with previous administration of the antiviral agent within 12 months before the sampling ($P< 0.001$). From these data, it can be said that EBV infection is more frequent in Group I to II, and the difference between the two groups is as pronounced as in the case of CMV infection at previous study. According to the literature, the incidence of CMV infection is high, especially among young people. Transplant recipients can of contracting CMV infection from the donor. Because of administration and undergoing immunosuppressant treatment in Post-transplant period, the recipient is in consequence of manifestation of various infections, including CMV, EBV and other. Some of the drugs used after transplant, can cause latent CMV infection regeneration. Thus, CMV infection is widespread in society, caused by various diseases, but more of these infections occur in recipients' post-transplant period due to poor and inadequate immunity. The results of this study confirm the literature analysis. The study noted that the EBV infection was more frequent (as compared to CMV) in both groups, but especially - in Group II. A very significant difference between the groups was observed by comparing the data of CMV infection (individuals, who was found to CMV infection, the percentage of Group I and II differ triple). Comparing the data of EBV infection, the difference is also, but not as bright as CMV infection.

Discussion

This study evaluates the results of the analysis of CMV pp65 antigen detection against PCR assays, detecting DNA of cytomegalovirus in ocular fluid, in relation to the clinical diagnosis of CMV in serum and ocular samples in patients with eye inflammation. According to the literature, antibodies against CMV infection have a 50% -70% of people, and the EBV - 95% of young people less than 25 years of age and 50% of individuals fewer than 5 years of age(6, 9, 22, 23). Thus, CMV infections of people in the population are less frequent than those of EBV infection, especially among young people(27-30). The analysis of these data as a percentage showed that 45.0% AND 35%of the first group of individuals at one time it was established CMV DNA and / or CMV pp65 (at least one TEST). Meanwhile, in Group II of CMV DNA and /or CMV pp65 (at least one of test) at least once, it was detected in only 16.5 % and 11.53% of individuals. Thus, in Group I of infection was found in relatively 3 or more times larger number of individuals than in Group II. In total 150 individuals were tested for CMV infection agent. The Group I has been studied in 20 individuals, while in the second group 130 individuals. Due to weak immunity violated recipients may have CMV and EBV infections, because the body's cells are susceptible to CMV and EBV infections, as well as these viruses have a specific protection mechanism against host immunity, so this infection is very common among people (14, 15, 17,21, 23-27). Also, these infections in patients can received after blood transfusion or from a donor. The results of this study confirm the literature data (3, 12, 14, 17, 25). In McCann et al. study, PCR analysis used to examine patients with CMV retinitis and reported that the sensitivity of PCR 48% and 95% in treated and non-treated patients respectively(31, 32). The findings of the PCR assay require a rational and level-headed interpretation, especially in analyzing Herpesviridae family (CMV, HSV-1, VZV, and EBV) (32-34). When PCR result was positive it confirmed the clinical diagnosis (3, 16, 17). Whether the presence of concomitant CMV influences the course of intraocular inflammation due to other causes such as Toxoplasma, VZV, HSV or EBV remains speculative (35).

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