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COMPARISON OF SEROLOGICAL AND MOLECULAR METHODS IN THE DIAGNOSIS OF CYTOMEGALOVIRUS INFECTIONS IN DIALYSIS PATIENTS

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Summary

Introduction. Cytomegalovirus is the most common cause of infections in the post-transplantation period. A reliable and timely laboratory diagnosis of cytomegalovirus infection in patients on dialysis and in the post-transplantation period is significant because of the possibility of preventing or mitigating the effects of cytomegalovirus disease. The main objective of this study was to compare serological and molecular polymerase chain reaction methods to determine the presence of cytomegalovirus in the blood of dialysis patients. Material and Methods. The study included 28 dialysis patients, potential renal transplant recipients. All patients were evaluated for the presence of cytomegalovirus in the blood by a quantitative polymerase chain reaction method as well as in the serum for the presence of anti-cytomegalovirus Immunoglobulin G and Immunoglobulin M antibodies. Results. According to the comparative enzyme-linked immunosorbent assay for detecting antibodies in dialysis patients, 96.4% were once exposed to the virus, while 7.1% showed current infection not confirmed by polymerase chain reaction test. No statistically significant association was found between the positive finding of anti-cytomegalovirus Immunoglobulin M antibodies and the findings of the polymerase chain reaction cytomegalovirus method when Chi-square (χ²) and Fisher's correlation tests were conducted (p > 0.05). Conclusion. Due to 7.1% false positives results for the presence of anti-cytomegalovirus Immunoglobulin M antibodies in the serum of immunocompromised dialysis patients, not confirmed by polymerase chain reaction test, serological techniques are not reliable in detecting active cytomegalovirus infection causing positive finding of anti-cytomegalovirus Immunoglobulin M, so confirmation of cytomegalovirus deoxyribonucleic acid by polymerase chain reaction method is required. Key words: Cytomegalovirus; Cytomegalovirus Infections; Polymerase Chain Reaction; Diagnosis; Kidney Transplantation; Renal Dialysis; Enzyme-Linked Immunosorbent Assay; Serotyping

Sažetak

Uvod. Citomegalovirus je najčešći uzrok infekcija u posttransplantacionom periodu. Pouzdana i pravovremena laboratorijska dijagnoza infekcije citomegalovirusom kod pacijenata na dijalizi i u postransplantacionom periodu je značajna zbog mogućnosti prevencije ili ublažavanja posledica citomegalovirusne bolesti. Glavni cilj ovog istraživanja je poredjenje seroloških sa molekularnim metodom lančane reakcije polimeraze za određivanje prisustva citomegalovirusa u krvi pacijenata na dijalizi. Materijal i metode. Ispitivanjem je bilo obuhvaćeno 28 pacijenata na dijalizi, potencijalnih recipijenata za transplantaciju bubrega. Svim pacijentima je određivano prisustvo citomegalovirusa u krvi kvantitativnom metodom lančane reakcije polimeraze, a paralelno u serumu i prisustvo antitela na citomegalovirus, imunoglobulina G i imunoglobulina M. Rezultati. Uporednom enzim-imunoesejometrom detekcije antitela kod pacijenata na dijalizi 96,4% je nekada bilo izloženo virusu, dok je 7,1% pokazalo trenutnu infekciju koja nije potvrđena testom lančane reakcije polimeraze. Upotrebom χ² i Fisherov testa nije dokazana statistički značajna povezanost između pozitivnog nalaza antitela imunoglobulina M na citomegalovirus i nalaza citomegalovirusa metodom lančane reakcije polimeraze (p > 0,05). Zaključak. Zbog 7,1% lažno pozitivnih nalaza na prisustvo antitela imunoglobulina M na citomegalovirusu u serumu i lančane reakcije polimeraze (p > 0,05) je potrebna korak navedeniji pristup u cilju potvrde odgovarajuće dijagnoze i sanacije bolesti.

Ključne reči: citomegalovirus; citomegalovirusne infekcije; PCR; dijagnoza; transplantacija bubrega; bubrežna dijaliza; ELISA; serotipizacija

Introduction

Cytomegalovirus (CMV) belongs to the herpes family and it is also known as human herpes virus 5 (HHV-5). The CMV can be transmitted in utero, perinatally, horizontally (direct or indirect transmission) and also by tissue or organ transplantation [1]. Peripheral blood mononuclear cells and endothelial cells are the main site of infection. The infection happens in immunocompetent as well as in immuno-
nocompromised persons and the level of viremia is not the only crucial factor for the onset of symptoms [2]. The most important interaction for the onset of symptoms is between the virus itself and the immune system. In immunocompetent people, CMV causes asymptomatic infections or infections with a mild clinical presentation, but it persists in the organism of a person who has once been infected, often for a lifetime. The newborns, immunocompromised persons, patients with transplanted organs, and persons affected by acquired immunodeficiency syndrome (AIDS) represent high risk groups for the development of serious CMV infections, leading to an increased rate of morbidity and mortality. In developed countries, 75% of adult population is CMV immunoglobulin G (IgG) positive, and in underdeveloped areas, the number rises up to 100% [3]. Severe clinical manifestations of CMV infection include retinitis, gastroenteritis, hepatitis, encephalitis, esophagitis, enterocolitis, pancreatitis and pneumonia [2].

Essentially, CMV infection development depends on two basic factors: immunosuppression intensity and transplanted organ type [4]. The CMV is the most common cause of infections in the post-transplant period [5]. In solid organs and bone marrow recipients, CMV infection can occur in several ways: as a primary infection, as a secondary infection (reactivation of latent virus) or as a superinfection (reinfection with another viral strain). Infections most commonly occur after the transplantation of heart and lungs (39%), liver and pancreas (29%), heart (25%) and kidneys (8%). The greatest risk for CMV infection is the combination of seronegative recipients and seropositive donors [6]. When donors are seropositive and recipients seronegative, the risk is enormous and it goes up to 90%.

Viral (CMV) infection does not imply its clinical presentation. In solid organ recipients, active CMV infection (virus replication) happens in 60–100% of patients, and without preventive antiviral treatment, clinical manifestations of CMV disease are present in 20–60% of patients with active infection. Recipients of solid organ transplants are the most vulnerable during two to four months after the transplantation [7]. Without preventive antiviral treatment, about half of bone marrow recipients develop active CMV infection and 20–25% of these patients develop CMV infection with clinical manifestations [8].

Detection and quantification of CMV in blood is significant for patient monitoring and making the decision when to initiate antiviral therapy [9]. Today, there are many commercial immunoenzymatic tests which detect immunoglobulin M (IgM) and IgG antibodies to CMV [10]. Serological tests are frequently used to determine patient’s immune status prior to tissue and organ transplantation; however, it is insufficient and inconceivable to diagnose CMV infection with clinical presentation in immunocompromised persons without confirmatory molecular tests [11, 12]. In the case of solid organ transplant (especially renal transplants) or bone marrow transplant recipients, detection of CMV in urine may contribute to identify high-risk patients, although this is not a reliable prognostic marker for CMV disease occurrence [13]. The main aim of this research paper was to compare serological methods with molecular polymerase chain reaction (PCR) method in the context of CMV presence determination in the blood of dialysis patients.

Material and Methods

This research was conducted during 2016, at the Laboratory for viral and PCR testing of the Laboratory Medicine Center, Clinical Center of Vojvodina in Novi Sad. The research protocol was approved by the Ethics Committee of the Clinical Center of Vojvodina in Novi Sad.

The research included 28 dialysis patients from the Dialysis Department of the Clinic of Nephrology and Clinical Immunology of the Clinical Center of Vojvodina in Novi Sad. All patients needed dialysis due to chronic kidney disease and were potential organ recipients.

Blood samples were taken from every patient and were stored in test tubes with ethylenediaminetetraacetic acid (EDTA) which served as an anticoagulant. Immediately after blood collection, the test tubes were centrifuged at 3000 – 4000 rpm for 10 minutes; blood plasma was separated and used for PCR analysis.

The CMV was determined in every patient by using quantitative PCR method, as well as by blood serum anti-CMV IgG and IgM antibodies (quantitative). Serological status of patients (anti-CMV IgG and IgM antibodies) was determined by enzyme-linked immunosorbent assay (ELISA) which was carried out using EUROIMMUN analyzer I 2-P (EUROIMMUN AG, Luebeck, Germany) according to manufacturer’s protocol. The cups for anti-CMV IgG and IgM antibodies were coated by the inactivated cell lysate of Human Fetal Lung Fibroblasts Cells - Medical Research Council cell strain 5 (MRC-5) that were infected by “AD169” strain of human cytomegalovirus.

COBAS AMPLICOR analyzer (Roche Diagnostics, USA) in combination with manual and automated isolation of CMV deoxyribonucleic acid (DNA) was used for CMV viremia detection by PCR method. Total Nucleic Acid Isolation (TNAI) commercial kit (Roche Diagnostics, USA) was used for automatic nuclear CMV DNA isolation from individual patient’s blood samples, which was carried out on COBAS AmpliPrep analyzer (Roche Diagnostics, USA). COBAS AmpliPrep analyzer uses magnetic particles and it consists of four basic steps: cell lysis, nucleic acid...
stabilization and deproteinization, nucleic acid binding to magnetic particle surface, rinsing of unbound material, and purified nucleic acid elution.

Manual isolation of CMV DNA was done using COBAS AMPLICOR CMV MONITOR test protocol (Roche Diagnostics, USA) according to manufacturer’s instructions [14]. COBAS AMPLICOR CMV MONITOR TEST is based on four basic processes: sample preparation, PCR amplification of target DNA by using specific primers complementary to CMV DNA, hybridization of amplified products and oligonucleotide probes which are specific for target sequence, and colorimetric detection of probes which are attached to the amplified product.

Isolation of CMV DNA is done directly from blood plasma by viral particles lysis, which includes chorthropic agent and then the DNA is precipitated by ethanol. COBAS AMPLICOR CMV CMV test uses biotinylated specific primers LC342C and LC383, in order to define a sequence consisting of 362 nucleotides which is located within the polymerase gene at amino terminus of CMV DNA. Target DNA fragment, located within the human CMV polymerase gene, is CMV-specific and it is not homologous to ones found in other herpes family members.

Correlation between measured parameters was determined by $\chi^2$ and Fisher’s test (Statistica software, version 8.0). Statistical significance was set at $p \leq 0.05$. The results were presented in tables and graphs.

**Results**

The ELISA method of antibody detection in potential organ recipients through renal transplantation showed that there were 27 (96.4%) positive patients and 1 (3.6%) patient who was negative for anti-CMV IgG antibodies in the serum. Only 2 (7.1%) patients were positive for anti-CMV IgM antibodies, and 26 (92.9%) had negative results (Graph 1). Comparative analysis of results showed that the majority of patients (27) were sometimes exposed to the virus, while only a small number (2) of patients had an ongoing infection confirmed by the PCR test (Table 1). The statistical processing of results by $\chi^2$ and Fisher’s correlation test showed that there was no statistically significant correlation between the findings of positive anti-CMV IgM antibodies and the PCR CMV DNA test result (Chi-square, $p = 0.1498$; Fisher exact $p$, one-tailed, $p = 0.2455$).

The percentage of PCR success with manual DNA isolation was 92.9%. Among 28 tested patients, 2 samples were incorrect, while the percentage of PCR success with automatic DNA isolation was 100%.

The PCR method yielded no positive results with either manual or automatic isolation of CMV DNA. According to the molecular (PCR) method with manual DNA isolation, of 28 patients 26 (92.9%) were negative for viral DNA, while the same method with automatic isolation showed that all 28 (100%) patients were negative for viral DNA.

**Graph 1.** Serological status (anti-CMV IgG and IgM antibodies) of dialysis patients in %

| Antibody Status | Percentage |
|-----------------|------------|
| IgM Ab/IgM At   | 7.10       |
| IgG Ab/IgG At   | 96.40      |

Legend: IgM Ab - immunoglobulin M antibodies; IgG Ab - immunoglobulin G antibodies

Statistical processing of results ($\chi^2$ and Fisher’s correlation test) showed that there was no statistically significant correlation between CMV detection by serological (ELISA) method and molecular (PCR) method ($p > 0.05$) (Table 1).

**Discussion**

Laboratory methods for the diagnosis of disseminated infections and active tissue/organ damage, caused by human CMV, include cultivation of peripheral blood leukocytes for virus isolation, pathohistological analysis of biopsy samples, and blood analysis based on pp65 antigen measuring and CMV detection by PCR [15]. Analysis of corresponding cell culture is not convenient for prognostic assessment; it takes 48 hours to 3 weeks to cultivate cells and it shows limited application especially in people with a compromised immune response. Analysis of pp65 antigen is very demanding and blood samples need to be introduced in this procedure not later than 6 hours since blood collection because the titer of this antigen decreases when blood is stored for a longer period of time.

Today, there are many commercial enzyme-linked immunosorbent assays which determine anti-CMV IgM and IgG antibodies. Serological diagnostics is used for immune status determination of dialysis patients who are potential organ recipients, while the diagnosis of CMV infection with clinical manifestations in immunocompromised persons is very insufficient and implausible without confirmatory molecular tests [16].

In our research, ELISA method for antibody detection in potential renal transplant recipients showed that there were 27 (96.4%) positive patients and 1 (3.6%) negative patient for anti-CMV IgG antibodies in the serum. Only 2 (7.1%) patients were positive for anti-CMV IgM antibodies and 26 (92.9%) were negative. Vilibic et al. [5] obtained
similar results – among 255 tested hemodialysis patients, 254 (99.6%) were positive for anti-CMV IgG antibodies and only 1 (0.4%) was positive for anti-CMV IgM antibodies in the serum according to ELISA method. Results of our research are in accordance with literature data, although samples were different. If the patient was anti-CMV IgM antibodies positive, that implied primary CMV infection, but these antibodies appeared rarely in dialysis patients (2/28), which can also be seen in results. A positive anti-CMV IgM antibodies test may arise suspicion and indicate that there is an active infection, although it is not a proof; it may also indicate antibody lag after recent infection considering that antibodies persist in blood serum up to 6 months after active infection and also the test can be false positive, because PCR method showed that these patients were negative for CMV DNA. A positive finding of anti-CMV IgM antibodies and negative PCR of CMV DNA in the same patient can be explained by the lagging of antibodies in the blood after the primary infection. Positive IgG antibody titer indicated that 27 out of 28 patients were exposed to CMV infection at a certain point of life. In CMV IgG seropositive recipients, there is a danger of virus reactivation (latent infection) [7]. The presence of IgG antibodies is therefore a marker of potential infection, and although a seropositive recipient is an immune person in immunological context, this term cannot be used to implicate protection from endogenous or exogenous infection. In CMV infection, IgM antibody synthesis is rare, but a rise in IgG antibodies titer is present [2]. Antibodies of IgM class are produced in primary infection, but not in recurrent infection in immunocompetent persons and they stay in the organism for 3 – 4 months. However, some immunocompetent persons may have detectable IgM antibodies during the recurrent infection. Immunocompromised persons sometimes do not produce IgM antibodies during the primary infection, and one third of them have detectable IgM antibodies in the recurrent infection [8].

When it comes to monitoring of immunocompromised potential recipients, serological techniques do not represent a method of choice because of altered and incomplete humoral immune response, late appearance of antibodies during infection, as well as the fact that their presence does not confirm an active infection, which additionally decreases the reliability of serological diagnostic results. Numerous studies showed an association between CMV viral load and the presence of CMV disease. The CMV disease is clearly defined by a high viral load, which implicates a significant role of virus in disease pathogenesis. Studies of immunocompromised patients showed that PCR detection of CMV DNA is used for reliable prognosis of further disease course and its clinical outcome and it also enables monitoring of antiviral therapy efficacy and indirect assessment of virus resistance to given therapy. Quantitative CMV DNA level determination showed that high viral load as well as its rise in time implies bad prognosis. PCR, as a reference method, is a specific, sensitive, fast and reliable method for viremia determination and possible disease occurrence or development in immunocompromised patients. Clinical significance of PCR method lies in early detection of CMV in blood. Positive PCR test for CMV in blood has a high positive predictive value for symptomatic infection appearance, while a negative PCR test has a high negative predictive value for CMV disease development, because it excludes virus replication in blood. Gärtner et al. evaluated TNAI method of CMV DNA isolation on COBAS AmpliPrep analyzer in contrast to COBAS AMPLICOR CMV MONITOR test [14]. Amplification was done on COBAS AMPLICOR analyzer. Specificity of these methods was assessed based on 100 healthy individuals’ tests. All examinees were negative for CMV DNA presence (specificity of 100%). The sensitivity test was done by testing EDTA blood plasma of CMV positive transplant recipients. Out of a total of 30 samples, 4 were
negative with either manual or automatic DNA isolation, probably due to a known fact that CMV detection in blood plasma during CMV replication is limited to a shorter period of time compared to CMV antigen or DNA detection in leukocytes. Three samples were positive in multiple repetitions of TNAI isolation method and negative in multiple repetitions of manual isolation. The TNAI method confirmed higher level of sensitivity in clinical samples. However, there is a strong correlation in viral titers in 23 positive samples between TNAI method and manual DNA isolation method (r² = 0.98). In our study, there is also an agreement between these methods, 92.86% and 100% success, with automatic TNAI isolation being more successful. Our results correlate with both isolation methods.

Adani et al. [17] used ELISA method of antibody detection in blood plasma samples of renal transplant recipients, and confirmed anti-CMV IgG antibodies in 100% of examinees, and only 6% of anti-CMV IgM positive examinees. Serological diagnostics is not the real indicator of CMV infection, because of frequent virus reactivation in immunocompromised people. Immunocompromised persons sometimes do not produce IgM antibodies during the primary infection, and one third has detectable IgM antibodies in the recurring infection. For CMV DNA detection, Adani et al. [17] used a real-time PCR method. Out of 98 examinees, 32 (32.7%) had a positive CMV DNA finding. A negative anti-CMV IgM antibody test and a positive PCR test in the same patient can be explained by a later production of antibodies because of immunosuppression. In 4 patients, no CMV DNA was detected, and they tested positive for IgM antibodies, which can be explained by antibodies lag after primary infection as noticed in some healthy individuals. Out of 6 patients who had high CMV DNA concentration, which was in correlation with their clinical status, two died in a year. Virus concentration in the initial phase of an active infection, as well as the rate of its increase, correlates with CMV disease in transplant recipients. Patients with a lower DNA concentration did not have disease symptoms.

If a potential recipient is PCR CMV DNA positive, the therapy should immediately be initiated and he/she cannot receive an organ until tests are negative [18]. If patients are PCR DNA negative and IgM antibodies negative, but IgG antibodies positive, as in the majority of obtained results, they are good candidates for organ transplantation. After the transplantation, or just before it, immunosuppressive therapy is given in order to avoid organ rejection, and reactivation of CMV infection is expected due to a reduced body defense mechanism.

Conclusion

Due to 7.1% of false positives for anti-cytomegalovirus immunoglobulin M antibodies in the serum of immunocompromised dialysis patients, not confirmed by a polymerase chain reaction test, serological techniques are not reliable in detecting active cytomegalovirus infection causing positive findings of anti-cytomegalovirus immunoglobulin M antibodies, so confirmation of cytomegalovirus deoxyribonucleic acid by polymerase chain reaction method is required.

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