

Comparison of ELISA, Antigenemia Assay and Nested PCR Monitoring Techniques for Detection of Cytomegalovirus Infection in Renal Transplantation Patients

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Abstract

Human cytomegalovirus (HCMV) can be transmitted through blood transfusion and organ transplantation and could be cause of some complication in solid-organ transplant recipients. Current study is aimed to compare the sensitivity and Specificity of ELISA, Antigenemia assay and nested PCR methods to detection of Cytomegalovirus infection in renal transplantation patients.

In this study blood samples were collected from 200 renal transplant recipients' patients.

DNA was extracted by commercial kit and Nested PCR was done by 2 pairs of internal and external primers. Anti CMV antibodies (IgM and IgG) were detected by ELISA and CMV-pp65 antigenemia assay (Ag) was used to detect CMV antigens. The sensitivity and Specificity of each test and all the methods together were evaluated, and SPSS software was used to analysis of data.

From 200 patients, 193 (96.5%) were positive for CMV antibodies with the Specificity of 100 and sensitivity of 97.76%. 120 (60%) and 25 (12.5) samples were positive by nested PCR and Ag assay with the Specificity of 94.49 and 78.12 and sensitivity of 94.49 and 78.12, respectively.

In the case of early diagnosis of the disease, nested PCR diagnose the infection 14 years earlier than Ag assay and was consistently positive, whereas false negative results were frequently observed with the pp65 Ag assay. The sensitivity and specificity of the two methods combined detection for CMV infection were 96.76% and 99.89%. ELISA can be used as a screening reliable detection test for CMV infection in recipient especially when PCR is unavailable.

Combination of ELISA and CMV-PCR methods, provide a more effective method to monitor CMV infection.

Keywords: Cytomegalovirus; Infection; Renal transplantation; Antigenemia assay

Introduction

Cytomegalovirus (CMV) is one of the most important causes of complication in solid-organ transplant recipients and this infection has a significant effect on transplant outcome and is common in renal transplant recipients [1,2]. CMV inclusions on renal biopsy are unquestionably direct evidence of tissue invasiveness or CMV nephropathy. There are no specific symptoms of CMV infection after renal transplantation in patients, but this infection causes many

diverse clinical outcomes. Therefore, it is difficult to accurately diagnose CMV infection. It is essential to use different laboratory diagnostic methods to diagnose CMV infection [3,4]. Therefore, the availability of a sensitive test capable of detecting CMV at an early stage of infection is so essential. The antigenemia assay (Ag) is a reference method commonly used for CMV virus quantification in blood specimens. pp65 antigen (pp65 Ag) is measured by the quantitation of positive leukocyte nuclei and has been validated in clinical trials with immunosuppressed patients [5,6].

This test is limited to detection of the virus in leukocytes and the demonstration of positive-staining signals in the nuclei of leukocytes indicates a positive result. The test is also quantitative and closely

correlating to the viremia and the severity of disease in immunosuppressed populations [7, 8]. Anti CMV IgG indicate the incidence of infection in past but IgM is a sign of the current infection [9]. However, studies have shown poor correlation of results obtained with different commercial kits for IgM testing [10]. In addition, assays for IgM antibody lack specificity for primary infection because of false-positive results, as IgM can persist for months after primary infection, and also IgM can be positive in reactivated CMV infections [11,12]. Due to the limitations of the IgM assays, IgG avidity assays are utilized in some populations to help distinguish primary from non-primary infection.

Molecular methods such as nested PCR, can detect the DNA of the CMV in both active and passive phases of the infection with a high sensitivity and specificity [13,14]. Considering to the limitation and deficiency of the different methods above, in this study we decided to compare the efficiency of three common methods for detecting CMV infection in Iranian renal transplant recipient patients in order to find an improved method to make a rapid and efficient diagnostic test.

Material and Methods

Patients and sampling

During 2014-2015, a total of 200 renal transplant recipients including 150 males and 50 females with a mean age of 33 (12-62) years were analyzed in this study were. Ten ml of blood sample were collected from all patients; 5 ml of blood samples were centrifuged at 2500 rpm for 5 min and serum were separated and transferred to a new tube. Blood and serum samples were maintained in -20°C until to use.

ELISA test

The commercial CMV-IgG/IgM ELISA kit (Diagnostic- EIA gen Biochem, Italy) was used for serologic CMV-IgG/IgM detection. Briefly, at first the control samples including the positive and negative control provided by the ELISA kit and patient serum specimens were diluted 1: 50 in sample diluents, and applied to the wells of a 96-well plate coated with anti-IgG/IgM, and then incubated for 30 min at 37°C. Then the samples were incubated with the diluted CMV-IgG/IgM Enzyme Tracer for 15 min at 37°C. Then the 96-well plate was washed with wash buffer for 4 times. Subsequently, 100 mL of the chromogen was added into each well and incubated for 10 min at 37°C. Finally; stop solution was used to stop the reaction. The absorbance of each the plate was read at 600-650 nm.a

Antigenemia assay

The CMV-pp65 antigenemia was detected by indirect immunofluorescence assay according to the following protocol: at first Polymorphonuclear cells were separated from heparinized blood by dextran solution (0.06) then Red blood cells were destructed in the cell suspension and cell count was performed by hemocytometer. Cells were fixed on slide and hybridized with monoclonal antibody against pp65 antigen. Fluorescein isothiocyanate (FITC) was used as the secondary antibody conjugate and the slide was studied by Fluorescence Microscope to report positive signals.

DNA extraction and polymerase chain reaction

DNA was extracted from 2 ml of blood sample of each patients using DNA extraction kit (QIAamp DNA Mini Kit) according to the manufacturer's instructions. PCR reaction mixture containing Master Mix: 10 µl, primer F: 0.5 µl, primer R: 0.5 µl, DNA 5 µl, 1 U Taq DNA polymerase and DNase free water to a final volume. The sequence of primers was as follows: forward primer, F: 5'-TCGCGCCCGAAGAGG -3' And reverse primer, R: 5'-CGGCCGGATTGTGGATT-3' After initial DNA denaturation at 94°C for 3 min, 35 cycles of DNA amplification were performed (93°C for 50 s, 57°C for 40 s, 72°C for 60 s) followed by terminal product extension at 72°C for 5 min. After amplification, PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

Statistical analysis

Statistical analysis was performed using SPSS version- 11 software and the Student t-test was used to compare results between different groups, in all calculating the difference was considered significant $P < 0.05$. Cohen's kappa coefficient is used to measures inter-rater agreement for qualitative tests.

Results

Out of 200 renal transplant recipient patients, 193 patients (96.5%) (145 male and 48 female) were positive at least by one test. The clinical manifestations of CMV infection were appeared 4-6 months after surgery. There was no relation between CMV infection, age and gender of the patients ($P = 0.183$).

Results of different CMV infection monitoring methods

A total of 200 recipients were analysed by nested PCR, Anti-CMV IgG and IgM and antigen detection of CMV-pp65 testing within 6 months post transplantation. The sensitivity and specificity of the three detection methods are demonstrated in Table 1. From all 200 patients, 193 (96.5%), 120 (60%) and 25 (12.5%) samples were positive for nested PCR, serology and antigen detection of pp65 tests, respectively. All 25 pp65 antigenemia-positive samples were also positive by the N-PCR test. However, 95 (79%) of the samples positive by the N-PCR were negative by the pp65 Ag assay (Table 1). Serology test was the most sensitive tests with the sensitivity of the 94.49. One sample that was negative by serological tests was positive by molecular method. Cohen's kappa coefficient is used to measures the agreement between two by two tests. The most agreement is calculated between Serology-PP65 Antigen ($P = 0.405$, Kappa=0.84).

Discussion

Current study in undertaken to assessed antigen and antibody detection and nested PCR tests to detection of MV in patients with renal transplant in Iranian population.

The total infective rate of CMV was 96.5% within 6 months post-operation in all 200 patients renal transplant recipient patients, which this rate of infection was higher than other related researches like Ram-Peddi, on the United States population and Wujun Xue on China population [15,16]. In addition, we found that the ELISA results of relationship between the CMV serology status and CMV infection in our research group was in agreement with other primary reports [17]. From all 200 patients, 193 (96.5%), 120 (60%) and 25 (12.5%) samples

were positive for nested PCR, serology and antigen detection of pp65 tests, respectively.

Result		Male	Female	Sensitivity	Specificity	P-value	Kappa
Nested PCR		65 (32.5%)	55 (27.5%)	91.9	100	0.000	-
Serology	Ig M	74 (37%)	46 (23%)	94.49	90.26	0.000	-
	Ig G	145 (72.5%)	48 (24%)				-
PP65 Antigene		15 (7.5%)	10 (5%)	78.12	96.43	0.000	-
Nested PCR-Serology		146 (73%)	48 (24%)	96.08	100	0.041	0.53
Nested PCR-PP65 Antigene		65 (32.5%)	55 (27.5%)	91.9	100	0.217	0.47
Serology-PP65 Antigene		145 (72.5%)	46 (23%)	89.19	93.51	0.405	0.84

Table 1: The results of ELISA, Antigenemia Assay and Nested PCR Monitoring techniques for Detection of Cytomegalovirus Infection in Renal Transplantation Patients.

In this study, 145 patients were positive for anti-CMV antibody and 74 patients were positive for anti-CMV antigens. These results indicated that, 71 patients were positive for only anti-CMV antibody and showed that these patients were infected in the past. Based on many studies, Antibodies against an infection, can prevent the incidences of infection. In other words, the presence of antibodies against CMV should prevent to existence antibody in the blood sample, which, if this happens, indicates an infection with another strains virus.

Among serological methods for detecting CMV-antibody such as anti-complementary immune fluorescence assay indirect immune fluorescence assay and complement combined method test ELISA is able to produce quick results and it can distinguish between IgG and IgM. However, the disadvantage of ELISA is the possibility of false-positive results due to contamination from cellular proteins. On the other hand, a specific CMV-IgM-antibody may not appear in the whole Time of CMV infection, all these problems limit the use of ELISA. In our present study, we observed the higher specificity and sensitivity in CMV detection by the CMV-IgG/IgM antibody after 6 months of pre-operation these findings are in agreement with the findings of Sandra Helena Alves [18].

CMV antigen detection method uses monoclonal antibodies to assay CMV-pp65 protein and other antigen components and then determines the number of cells infected with CMV. At present, human CMV-pp65 antigenemia is considered as an important early diagnostic method with high sensitivity and specificity for active CMV infection detection. This method can also provide information for clinical therapy for patients infected with CMV infections [19,20]. In this study we observed the CMV-pp65 in 6 samples that the CMV-PCR and serology results of them were negative and the sensitivity and specificity of this method was lower than the other two methods. False

positive result of this method is reported such as many previous studies [21]. However, CMV antigen detection still is the primary method to determine CMV infection in most laboratories. These results suggest that the negative result of serology test is not a rejection of the disease.

PCR provides a very effective detection method for CMV-DNA in renal transplant recipients after transplantation. We found that CMV-PCR has a higher specificity than CMV-pp65 antigenemia but lower sensitivity than serology detection. Previous studies showed that PCR correlated with CMV infection and can predict late-onset relapse infection [12,22]. The two detection methods had no significant dependability ($p=0.05$) and the rate was 20.2% which is lower than the concordance reported by Ksouri et al [23]. The combination of CMV-PCR and CMV-pp65 methods can increase sensitivity (91.9%). There are many factors which influence the effectiveness of CMV-pp65 detection, such as specificity of monoclonal antibody pre-treatment of samples, and amount of the antibody expression. PCR and IgM antibody are reported as the most effective diagnostic methods for active CMV infection in transplant recipients [24-26].

Conclusion

In conclusion, our observations showed that the implementation of nested-PCR assay that can reproducibly quantify low levels of CMV DNA in plasma and would improve the monitoring of CMV infection. CMV-pp65 and PCR complement each other to increase the detection rate of CMV diseases, providing a more effective method to monitor CMV infection and guide the clinical therapy of CMV infection. To our knowledge, this is the first validation of a plasma-based PCR assay's showing higher sensitivity over the pp65 assay.

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Conflict of Interest

Authors have no conflict of interest.

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