

Pre-Symptomatic Human Cytomegalovirus Disease Diagnosis in Renal Transplant Recipients by the Virus DNA PCR

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Abstract

Human cytomegalovirus (HCMV, CMV) is a major infectious complication of renal transplantation. The objective of this survey was to optimize and establish a polymerase chain reaction (PCR) technique for rapid and early detection of CMV disease in renal transplant recipients. In a cross sectional study, a total of eighty-one EDTA-blood samples were collected as simple nonrandomized (sequential) weekly from thirty-seven renal transplant recipients during a 1-6 months period after their transplantation in Kidney Transplant Center of Shaheed Labbafinejad Hospital of Tehran. Peripheral blood leukocytes (PBLs) were isolated and DNA was extracted. HCMV DNA in PBLs was detected by PCR using a conserved set of primers. Amplified fragment was confirmed by restriction fragment length polymorphism (RFLP) and sequencing. Correlation between PCR results and patients' data was analyzed. Twelve patients from thirty-seven renal transplant recipients had positive samples containing HCMV DNA in PBLs (32.4%), whereas, five of them showed symptomatic CMV disease (13.5%) and seven of them did not show symptomatic CMV disease, but had some signs of pre-symptomatic CMV disease. Twenty-five patients had negative PCR results, and all of them did not have symptomatic CMV disease. Considering type one error ($\alpha = 0.05$), a nonparametric Fisher's exact test showed a good correlation between two variables of positive PCR results and symptomatic CMV disease in renal transplant recipients ($P=0.002$). In conclusion, establishing methods for early detection of HCMV DNA, even prior to showing symptomatic CMV disease, has been shown to be an effective way for starting antiviral therapy, prior to patients' experience of symptomatic CMV disease.

Keywords: *Human cytomegalovirus (HCMV), Renal transplant recipient, Iran*

Introduction

Human cytomegalovirus (HCMV) is one of the eight herpesviruses that are pathogenic for human. HCMV is a major infectious complication of renal transplantation (1-3) and CMV disease in renal transplant recipients has a significant impact on morbidity and mortality and graft survival (4). Many infected patients develop symptomatic CMV disease, manifested by pneumonia, hepatitis, gastrointestinal ulcers, a non-specific febrile illness associated with leu-

kopenia and thrombocytopenia, or less commonly retinitis. Patients with CMV pneumonia or disseminated infection often die (5-7).

Currently, several routine diagnostic tests are available for direct or indirect detection of HCMV infection. Direct detection of HCMV by cultivation of permissive human fibroblast cells with samples from blood or urine is a sensitive method, but results are obtained rather late. The polymerase chain reaction (PCR) technique has recently been applied to the de-

tection of various viral pathogens. These tests are recognized as rapid, sensitive and reliable tests for detection of viral DNA or RNA (8). PCR has been shown to be an extremely sensitive method for detecting small amounts of cytomegalovirus DNA in peripheral blood leukocytes (PBLs) of renal transplant recipients (9). Therefore, early diagnosis of CMV infection and a correct differentiation between CMV diseases from allograft rejection remains of great importance, especially because the antiviral drug, is now available and new preventive strategies named "preemptive therapy" have been used to decrease the incidence of active CMV disease post-transplantation (10-12).

In the present study, a PCR technique was optimized and established for rapid and early detection of HCMV in renal transplant recipients, in order to detect CMV infection in pre-symptomatic stage of CMV disease.

Materials and Methods

In a cross sectional study, thirty-seven consecutive renal transplant recipients were studied for a period of 1-6 months after their transplantation. The patients consisted of 23 (62%) males and 14 (38%) females with a mean age of 33 y (range 10–56). All of the patients were transplanted in Kidney Transplant Center in Shaheed Labbafinejad Hospital, Tehran, Iran. The presence of CMV IgG and IgM antibodies in the donors and patients were determined by an enzyme-linked immunosorbent assay (ELISA) technique before transplantation.

Eighty-one EDTA anticoagulant whole-blood samples (10 ml) were obtained from patients and processed immediately after being drawn. PBLs isolation and DNA extraction was performed as described elsewhere (13). Briefly, isolated PBLs were boiled in 100 µl of 50 mM NaOH for 20 min, then 20 µl of 1 M Tris-HCl pH 7.6 was added to mixture and subsequently centrifuged at 6000 rpm for 2 min; the supernatant was used for PCR. Primer sequences (forward: 5-ggA TCC gCA Tgg CAT TCA

CgT ATg T-3, reverse: 5-gAA TTC AgT ggA TAA CCT gCg gCg A-3) were selected from a conserved region of the fourth exon of the HCMV immediate early (IE) gene, located in the *Hind III-X* fragment of the AD-169 strain (14,15). Optimized PCR reaction (25 µl) consisted of 1X PCR buffer, 2.5 mM MgCl₂, 1 U Taq DNA polymerase (CinnaGen, Tehran, Iran), 0.2 mM dNTPs (Fermentas, Vilnius, Lithuania), 5% DMSO, 500 µg/mL gelatin, 0.5 µM primer forward and reverse, and 5 µl extracted DNA. PCR program consisted of 95° C 10 min, followed by 35 cycles 95° C 45 s, 58° C 50 s, 72° C 45 s with a final extension at 72° C 5 min.

PCR product was confirmed by restriction fragment length polymorphism (RFLP) method described previously (16). HCMV isolated from infected patients and confirmed by immunofluorescent staining technique (IF) and clone-sequencing method (17) served as positive controls. PBLs DNA extracted from healthy and normal fetuses (embryo-cord bloods), born from CMV IgG and IgM negative mothers, served as negative controls. Negative and positive controls were included in each run.

In this study, the diagnosis of the symptomatic CMV disease was based on previous reported surveys at the International CMV Workshop (7, 10) with a minor modification. Briefly, in this study, symptomatic CMV disease was defined as the occurrence of leukopenia (WBC < 5×10³/µl) with thrombocytopenia (PLT < 150×10³/µl) in combination with detecting of HCMV DNA in patients PBLs using PCR and one or more of the following clinical or abnormal laboratory signs: fever (body temperature ≥ 38° C) for one day or more, pneumonitis, gastrointestinal ulceration or hemorrhage, petechiae, creatinine rise (level of serum creatinine > 1.5 mg/dl), liver enzymes rise (alanine aminotransferase [ALT] and aspartate aminotransferase [AST] > 45 U/L).

The association between positive results of PCR and existence of symptomatic CMV disease was statistically assessed using non-para-

metric Fisher's exact test (SPSS software, Version 11.0).

Results

A 406 bp fragment was successfully amplified using specific primers, located within a highly conserved region of HCMV *IE* gene (Fig. 1). The specificity of amplified fragments was confirmed by RFLP and sequencing methods (17). In this study, nobody was HCMV IgM positive or had a fourfold rise in HCMV IgG titer. All patients had HCMV IgG in low titer, which indicated old infection by the virus.

Twelve patients (12/37) had positive samples containing HCMV DNA in PBLs and twenty-five patients (25/37) had negative HCMV PCR results. From total of thirty-seven patients, thirty-two (32/37) had not symptomatic CMV disease and only five patients (5/37) showed symptomatic CMV disease, which also had positive HCMV PCR results during 1 to 3 months after their transplantation (Table 1). Four out of five symptomatic CMV disease patients were treated successfully with antiviral agents such as gancyclovir i.v. Table 2 shows the characteristics of five symptomatic CMV disease patients. One patient out of five symptomatic CMV disease patients died due to severe CMV disease despite administration of antiviral drugs. This patient had elevated level of serum creatinine (level of serum creatinine > 5.8 mg/dl), thrombocytopenia (PLT < 10000/μl), leukopenia (WBC < 2000/μl), fever more than one day, severe pneumonitis, gastrointestinal ulceration and hemorrhage and petechiae.

It is noteworthy to mention that seven patients had positive HCMV PCR results, but did not show symptomatic CMV disease. Some of these patients (4/7) had at least one or two of clinical and/or abnormal laboratory sings of symptomatic CMV disease. Therefore, this may demonstrate the ability of early detection (pre-symptomatic detection) of HCMV disease by developed in-house PCR. Additionally, non-parametric statistical Fisher's exact test showed

a significant association ($P=0.002$) between positive HCMV PCR results and symptomatic CMV disease in renal transplant recipients.

M 1 2 3 4 5 6 7 8 9 10

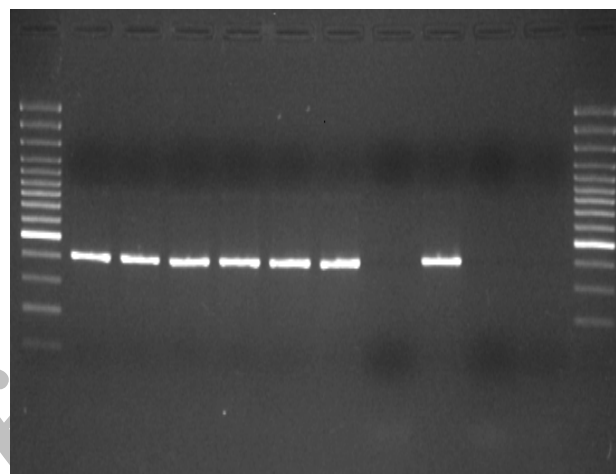


Fig. 1: HCMV PCR results (406 bp) on 1.5% agarose gel. Lanes 1-6 show positive PCR in six patients; Lane 7 shows negative PCR result in a patient with asymptomatic CMV infection; Lane 8: Positive control; Lane 9: Negative control; Lane 10: Blank (no DNA added); M: 100 bp DNA size marker

Table 1: Association between PCR results and symptomatic CMV disease

PCR results	No. of symptomatic CMV disease (%)		
	Positive	Negative	Total
Positive	5 (13.5%)	7 (18.9%)	12 (32.4%)
Negative	0	25 (67.5%)	25 (67.5%)
Total	5 (13.5%)	32 (86.5%)	37 (100%)

Table 2: Characteristics of five renal transplant recipients with symptomatic CMV disease

Sex, Age	Follow-up(s)	Clinical sings	Abnormal laboratory signs
Female,30	4	Fever	Leukopenia, Thrombocytopenia
Female,51	3	Fever, GI, Petechiae, Severe Pneumonia	Leukopenia, Thrombocytopenia, Rise of serum creatinine
Male,42	2	Fever	Leukopenia, Thrombocytopenia, Rise of serum creatinine and AST
Male,44	2	Fever	Leukopenia, Thrombocytopenia, Rise of serum AST
Male,40	1	Fever, Pneumonia	Leukopenia, Thrombocytopenia, Rise of serum creatinine

Abb.: CMV: Cytomegalovirus, AST: Aspartate aminotransferase, GI: Gastrointestinal involvement

Discussion

Depending on pre-transplantation immunity and the degree of post-transplantation immunosuppression, CMV infection in solid organ transplant (SOT) recipients causes a wide range of clinical manifestations, from asymptomatic infection to potentially lethal CMV disease. Currently, CMV infection is diagnosed by virus culture assay or by a rise in anti-CMV antibody titer. SOT patients often have delayed or completely absent antibody responses and CMV cultures can take up to some weeks to yield definitive results; moreover, virus culture techniques may fail due to toxic effects by the specimens (1, 4). However, an early diagnosis allows adequate clinical management, antiviral treatment with immunoglobulins or drugs such as gancyclovir and foscarnet or the modification of immunosuppressive regimens. Thereby, rapid, simple and cost-benefit laboratory tests are essential diagnostic tools.

PCR, which can be used to selectively amplify and detect specific DNA sequences, is known to be a rapid, specific and sensitive method for detection of HCMV DNA in various kinds of specimens and it is increasingly being used in SOT. PCR techniques can detect CMV DNA in PBLs (18-20), as well as showing the earliest positive signal of CMV replication, in whole blood (21) or CMV RNA in leukocytes (22).

CMV DNA can also be detected in cell-free body fluids such as serum (23, 24) and plasma (25, 26), though CMV is a cell-associated virus. Therefore, one of the standard methods for detection of viral nucleic acids is PCR technique; that has been used to diagnose CMV infection pre-symptomatically. With advancements in molecular biotechnology, PCR can be performed quickly and provides quantitative information too. Although, quantitative PCR assays are attractive tests for the diagnosis of established CMV disease, qualitative assays may be sufficient for the early diagnosis of CMV disease (27-29). Analysis and virologic follow-up of antiviral therapy has shown that qualitative monitoring of PBLs for CMV DNA is a sensitive virologic parameter to evaluate treatment efficacy (30). In the present study, seven patients have shown PCR positive results without showing symptomatic CMV disease. However, some of them showed some signs of the CMV disease. These results demonstrate that the qualitative PCR can help in predicting pre-symptomatic CMV disease and distinguish early phase of CMV disease in renal transplant patients. Likewise, in Evans and coworkers study (31) qualitative CMV PCR detected CMV DNA in serum before clinical onset in 70% of liver transplant recipients. Interestingly, in another survey (32), no significant difference

was found between the CMV qualitative and quantitative PCR for the early diagnosis of CMV infection before development of disease. Similarly, in Lao and colleagues study (33), using a qualitative PCR, half of patients showed CMV positive results prior to disease onset and could be treated by preemptive therapy strategy. Moreover, negative qualitative CMV PCR assay can be an accurate negative predictor for CMV disease in kidney transplant recipients (34).

In a study of 148 specimens from liver transplant recipients, primers directed to the *Hind III-X* fragment region of CMV, detected target DNA were the most sensitive compared with primer pairs directed towards *EcoRI* fragment D gene and the immediate-early antigen 1 (*IEA 1*) gene and the major immediate-early (*MIE*) gene, from CMV AD-169 strain, respectively (15). In this study, a set of primer was used, which were directed to *Hind III-X* fragment region of DNA from CMV. This region is specific for CMV and no band was visible when PCR with DNA of other herpesviruses such as herpes simplex virus types 1 and 2, varicella-zoster virus (VZV) as well as hepatitis B virus (HBV) was performed. Therefore, using these primers makes the results of PCR with PBLs more optimal for diagnosis and appropriate future preemptive therapy. Moreover, results of PCR amplicon sequencing also confirmed presence of HCMV DNA (GenBank accession number AY327403).

In our study, all patients and donors had CMV IgG with a low titer, which indicated previous infection by the virus; this may explain CMV disease in five renal transplant recipients and positive PCR results in seven patients with asymptomatic CMV disease. Since CMV positivity can be the result of reactivation of the latent virus due to immunosuppression regimes after transplantation. It may also indicate super-infection by the virus in patients who received infected graft and/or blood products by transfusion and/or by nosocomial infection after transplantation. In fact, the kidney can act as a vehi-

cle for transmission of CMV, and CMV-seronegative recipients receiving a kidney from a CMV-seropositive donor are at the highest risk for development of symptomatic CMV disease (primary infection) after organ transplantation (35,36).

Recently, numerous prevention and treatment strategies have been used to decrease the incidence of active CMV disease post-transplantation. One strategy is to give all patients prophylactic antiviral therapy (universal prophylaxis). Although, this has been shown to prevent CMV disease, it results in unnecessary and costly administration of i.v and/or p.o antiviral therapy to a large group of patients who may have never developed CMV disease. But, the side effects of antiviral drugs prevent their prophylactic application and therefore a reliable and accurate diagnosis such as PCR is required. In this regard, another approach is the prophylactic antiviral agents administration in CMV-seronegative recipients (high risk patients) of renal allograft from CMV-seropositive donor after transplantation that has been beneficial in decreasing the incidence and severity of disease (6, 11, 37). In this study, no CMV-seronegative recipients were transplanted by CMV-seropositive donor's kidney. Another approach, referred to as "Preemptive therapy", involves screening patients routinely for evidence of CMV disease before symptoms developed with initiation of antiviral therapy in those with CMV disease to prevent the development of active CMV disease (38). The major advantage of preemptive therapy is that only patients at high risk of developing active CMV disease receive antiviral medication (39, 40). Therefore, the establishing of rapid and simple methods in clinical laboratories in order to screen renal transplant recipients for the presence of CMV disease is crucial for beginning to preemptive therapy by antiviral agents. In this study, in seven positive PCR patients with asymptomatic CMV disease, detection of HCMV DNA in PBLs has been shown to allow early pre-symptomatic detection of CMV disease. On the other hand, continuous

monitoring of these seven patients that had positive PCR results and asymptomatic CMV disease, which is a prerequisite for rational antiviral therapeutic strategies, seems to be possible with our assay.

In conclusion, this study showed that in renal transplant patients with symptomatic CMV disease, detection of CMV DNA using PBLs obtained from EDTA-blood for PCR was a valid technique. This makes the assay a suitable method for screening CMV disease. Meanwhile, detection of HCMV DNA in PBLs allows rapid and early pre-symptomatic diagnosis of CMV disease in renal transplant recipients.

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