Active CMV and EBV infections in renal transplant recipients with unexplained fever and elevated serum creatinine

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ABSTRACT
Proper identification of active cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infections are helpful for monitoring antiviral treatment in transplant recipients. Qualitative and quantitative CMV, EBV DNA PCR techniques in the context of serological tests are performed for early detection and differentiation of active and latent CMV and EBV infections in renal transplantation. Basically, 129 renal transplanted recipients monitored carefully and hospitalized for unexplained elevated creatinine levels or high fever and 21 of their donors were studied. CMV DNA was detected in 63.5% of the febrile episodes following transplantation and in 46.42% of readmitted patients using qualitative PCR method. In the first group, 15% of the patients and in the second group 42.85% of the patients had copy numbers more than cutoff point (900 copies/mL). Cutoff point had 100% sensitivity and 82.5% specificity for active and symptomatic CMV infection. Only 15.5% of the subjects were positive for EBV infection by qualitative PCR method. Among them 5% had >2000 copies/mL and were symptomatic. One subject with a history of three times hospitalization had higher EBV viral load and developed post-transplant lymphoproliferative disorder. CMV load was significantly correlated with elevated creatinine levels (OR = 3.1, p = 0.006), abnormal heart sounds (OR = 4.7, p = 0.02) and hypertension (OR = 3.6, p = 0.03). Only qRT-PCR could differentiate between latent and active infections and might be clinically useful for monitoring symptomatic CMV and EBV infections and initiation of the antiviral therapy. Elevated creatinine levels, hypertension, and abnormal heart sounds could be considered as main manifestations of HCMV infection in kidney recipients.

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ARTICLE HISTORY
Received 23 June 2015
Accepted 9 July 2016
Published online 29 July 2016

KEYWORDS
Cytomegalovirus; Epstein-Barr virus; Q-PCR; renal transplant patients

Introduction
Viral infections are life threatening in immunocompromised individuals, particularly in pregnant women and renal transplant patients. Among these viral infections, human cytomegalovirus (CMV) is more important for its complications in fetuses and transplants. Furthermore, Epstein-Barr virus (EBV) in renal transplants may cause acute or chronic infection toward malignancy in the long time. Renal transplant patients are more susceptible to CMV and EBV infection due to receiving immunosuppressive drugs. CMV induces more cell-mediated immunosuppression, and causes severe complications such as prolonged fever, leukopenia, hepatitis, colitis, retinitis, allograft injury, and increased susceptibility to opportunistic infections, particularly in the early post-transplant period. Thus, an early reliable diagnosis of CMV infection is very important for an appropriate treatment of transplant patients and reducing the graft complications. Therefore, it is necessary to have a very precise and reliable test for the diagnosis of active CMV and EBV for appropriate decisions.

The prevalence of EBV infection in general population is high and it has been shown that more than 90% of adults in developing countries are seropositive. The post-transplant lymphoproliferative disorder (PTLD) resulted from EBV infection is a major problem in renal transplant patients, particularly in patients who are EBV seronegative at the time of transplantation.

In some reports, it has been demonstrated that the incidence of CMV disease among all kidney recipients is around 63% over the first 100 days following transplantation. The incidence of CMV disease was nearly three-fold higher among seronegative organ transplant recipients compared to seropositive donors.
However, in Khorasan provinces, more than 98.5% of the adult population is seropositive for CMV (this study and Baradaran H, Rezaee S.A.R, Unpublished data).

After transplantation CMV may be activated and replicated, which might cause prolonged hospitalization, increasing the post-transplant costs and the threatening of new kidney and the life of recipient. Therefore, the prevention of CMV disease is a major goal in the management of kidney transplant patients and a reliable and sensitive laboratory techniques for diagnosis of CMV infection before the onset of symptoms will be pivotal. Moreover, such techniques increase our ability to detect CMV infection rapidly and lead to the use of anti-CMV therapy when it is necessary. Detection of CMV infection using molecular techniques such as CMV antigenemia (Ag), qualitative PCR, and quantitative PCR assays introduced new tools for early diagnosis of the infection. Qualitative PCR methods are unable to differentiate between latent or non-productive infections and recurrent or productive infections, and do not correlate with active disease. There has been a good association between the antigenemia and quantitative PCR assays; however, CMV antigenemia test is labor intensive, time consuming, and requires processing of samples within a few hours. Furthermore, researchers have demonstrated that using quantitative PCR assay in the plasma or leukocytes, CMV infection can be detected even earlier. Therefore, quantitative methods may be more clinically useful, as higher CMV DNA load predictably correlates with higher levels of replication in CMV disease.

A prospective study was carried out in the only transplant center in Khorasan provinces with a population of more than 6.2 million (census 2006). The qualitative and quantitative CMV, EBV DNA PCR techniques were evaluated in the context of serological tests for early detection and differentiation of active and latent CMV and EBV infections in renal transplantation.

Materials and methods

Study population

One hundred and twenty-nine kidney transplant patients and 21 of their living donors were enrolled in this study. There were two different groups of renal transplants in the study: Group I consisted of 60 recipients with high fever or elevated creatinine levels without any clear reason during first 20 days of their hospitalization following transplantation and group II consisted 69 recipients who were readmitted to the hospital due to similar signs and symptoms at least one year after transplantation. EBV and CMV IgG antibody titers were assessed using Abcam-IgG Human ELISA kit (Cambridge, MA) according to the manufacturer’s instructions. Moreover, anti-HIV (Dia.pro, Italy), anti-HCV (Dia.pro, Italy), and hepatitis B surface antigen (Radim, Italy) were evaluated by ELISA according to the manufacturers’ instructions. The results of CMV, EBV, HIV, HBV, and HCV serological tests at the time of renal allograft surgery and renal function at the onset of signs and symptoms were recorded for all recipients. Renal transplant recipients received an immunosuppressive regimen consisted of cyclosporine (Neoral®), Mycophenolate mofetile (Cellcept®), and Prednisolone. The study was approved by Ethics Committee of Mashhad University of Medical Science (No: 87489; 93.475020). All experiments were carried out in compliance with the relevant laws and guidelines in accordance with the ethical standards of the Declaration of Helsinki. Recipients’ variables such as age, sex, blood group, routine para-clinical assessment, clinical outcomes, and graft function were evaluated. CMV and EBV viral loads were assessed in the plasma and identification of their impact on clinical outcome in renal transplant recipients were the main objectives of the study.

Data collection

Patients’ demographic, clinical and laboratory data were collected from patients in the transplantation wards. The demographic information such as sex, age, and residence, medical history including, the cause of end-stage renal disease (ESRD), the duration of dialysis, and their para-clinical findings such as CBC differentiation, ABO blood groups as well as other viral infections (HBS, HIV and HTLV-I) and anti CMV and EBV IgG and IgM at the time of admission were collected. Moreover, the clinical and para-clinical records during hospitalization were collected after kidney allograft reception.

DNA extraction

CMV and EBV DNA were isolated from 200µl plasma samples using High Pure Viral Nucleic Acid Kit (Roche Applied Science, Penzberg, Germany). Purification was carried out according to the manufacturer’s instructions and the purified nucleic acid was then eluted in 50µl of low salt elution buffer.

Qualitative PCR

Conventional PCR was performed using a PCR thermal cycler machine (Astec, Tokyo, Japan) with specific primers for CMV; sense: 5'-CGGTGGAGATACTGCTGGTC and Antisense: 5'-CAAGGTGCTCCGTGATGAAC-3, and
for EBV; sense: 5′-CTCTGGTAGTGTGATTTGGACC-3 and Antisense: 5′-GTGAAgTCACAAACAAGCCC-3. Three microliters of the extracted DNA was mixed with 17 µl PCR mix (Genetbio, South Korea) and 35 amplification cycles were carried out in the thermocycler machine.

**CMV and EBV quantitative real-time PCR**

CMV quantification was performed by a Taqman CMV/RG kit (Qiagen, Hilden, Germany) and EBV by an EBV qRT-PCR TaqMan kit (Primer Design, York, UK) according to the manufacturers’ instructions. Internal control supplied with the kit was added to each sample prior to the purification step and was co-purified with the sample DNA. Viral DNA purification efficiency was analyzed by dual-color detection system supplied with the kit. Amplifications were performed in 20-µl volumes including 4 µl DNA, 10 pmol of each primer, 4 pmol of probe and its TaqMan master mix. Q-6000 Rotor-Gene machine (Qiagene, Germany) was used for real-time performance and then the results were analyzed by Rotor Gene 6000 software.

**Statistical analysis**

Mann–Whitney’s U-test was used to compare the mean CMV viral load between groups. Chi-square was used to evaluate correlations between CMV viral load and clinical symptoms. Data were statistically analyzed using the SPSS statistics software package (SPSS, Armonk, NY). The results were considered significant if p values were <0.05.

**Results**

**Demographic data and clinical features**

The total kidney allograft recipients in the study were 129 subjects (55 female, 74 male) with the mean age of 36.99 years (range, 9–66 years old) and 21 subjects of their living donors consisted of 3 female and 18 male. The main etiology of ESRD was hypertension, diabetes, and glomerulonephritis in 21.7%, 19.3%, and 15% of individuals, respectively. The other causes were pyelonephritis, acute glomerulonephritis, polycystic kidney disease, and systemic lupus erythematosus (SLE). Most of the donors and recipients (more than 98.5%) were anti-CMV IgG positive; only two recipients were CMV seronegative. Out of 129 kidney recipients with high fever and elevated creatinine levels, 41% had spontaneous remission after a transient time, while 59% showed one or more clinical manifestations such as high fever (30%), rejection signs (13.3%), rise in blood creatinine levels (31.8%), and dyspnea (11.6%). 12.4% of patients had urinary tract infection (UTI), 3.1% abdominal pain, 14% hypertension, 3.1% fatigue and weakness, 3.1% hyperlipidemia and 5.4% nausea and diarrhea, in which 9.3% under-went graft nephrectomy.

**Qualitative PCR results**

In addition to the CMV and EBV serological tests, qualitative PCR and quantitative real-time PCR were carried out for all recipients and donors. CMV DNA detected in 42.8% of kidney donors by qualitative PCR assay consisted of one female (4.8%) and eight males (38%). Furthermore, EBV DNA was found in one female (4.8%) and three males (14.3%) of kidney donors.

Of 78 kidney recipients in group I (following transplantation), CMV DNA was detected by conventional PCR in 47 of them (63.5%) during onset of symptoms and 12 recipients (15.5%) were positive for EBV infection (five female and seven male). In group II (readmitted patients), 46.42% of subjects were positive for CMV using conventional PCR. Taken together, CMV DNA was detected in 63.5% of group I, and in 46.42% of readmitted patients, group II, using conventional PCR. However, in the first group only 15% of patients and in the second group 42.85% of patients had copy numbers more than cutoff point of this study (900 copies per ml) by qRT-PCR (see further).

**Quantitative real-time PCR results**

Quantitative real-time PCR was performed on CMV qualitative PCR positive samples. An arbitrary cutoff point as 900 copies per ml was selected to determine the positive predictive value and negative predictive value of the qRT-PCR for the patients with sensitivity of 100% and specificity of 87.5%. In addition, CMV viral load was undetectable or lower than 100 copy/mL (according to the sensitivity of the kit) in 7 subjects with weak positive PCR results. Among 21 donors, only one subject had 2450 CMV copies/mL, but this person and his recipient did not have any CMV-related symptoms.

The cutoff point of EBV qRT-PCR assay for symptomatic EBV infection was 2000 copies/mL and only four subjects (5%) had >2000 EBV copies/mL and were symptomatic. However, one subject in group two had high EBV viral load with clinical signs of fever and elevated creatinine, which was not detected earlier by conventional methods. She had been hospitalized three times due to very high temperature and at the last time she ended up with PTLD. In the present study, the EBV viral load for this subject was 5400 copies/mL that was
reported to the transplant wards. Table 1 shows a summary of EBV DNA load in kidney recipients (Table 1).

As shown in Table 2, fifteen percent of the recipients possessed a peak of viral load, which means all of the subjects were positive for CMV infection by qualitative PCR. Although CMV load (median DNA copies/mL of CMV DNA) in one case with related symptoms remained lower than cutoff, our related study in detection of EBV load for the same patients proved that she had got high peak of EBV load, which has cross symptoms with CMV infection. All of these patients have developed related symptoms of CMV infection such as fever, acute rejection, rise in blood creatinine level, and abnormal heart sounds.
Our data showed a significant correlation in CMV load between fever and rise in blood creatinine level after transplantation. On the other hand, the same study of these patients proved that there is a significant correlation between CMV and EBV load in kidney transplant patients.

The mean CMV viral load of symptomatic patients in group I and group II were 432,590 and 3,651,800 copies/mL, respectively. According to univariate analysis, CMV viral load was significantly correlated with increased creatinine levels ($r = 0.264; p = 0.003$), abnormal heart sounds ($r = 0.280; p = 0.001$), and hypertension ($r = 0.248; p = 0.005$). In patients who underwent graft nephrectomy, CMV viral load was significantly associated with elevated creatinine levels ($r = 0.485; p = 0.007$). Moreover, CMV viral load was significantly associated with fever, elevated creatinine levels, abnormal heart sounds, hypertension, in patients who were infected with CMV following transplantation during their hospitalization ($r = 0.202; p = 0.04, r = 0.254; p = 0.01, r = 0.266; p = 0.008, r = 0.237; p = 0.01$, respectively). However, in group II patients, CMV viral load was significantly correlated just with UTI and abnormal heart sounds ($r = 0.271; p = 0.04, r = 0.283; p = 0.03$, respectively). On multinomial regression analysis, higher CMV viral load was significantly associated with increased creatinine levels (OR = 3.1, $p = 0.006$), abnormal heart sounds (OR = 4.7; $p = 0.02$), and hypertension (OR = 3.6; $p = 0.03$). However, there was no difference in the mean CMV viral load between patients with acute rejection, who underwent graft nephrectomy, and who showed partial spontaneous remission.

**Discussion**

The results of this study demonstrated a very high level of CMV (98.5%) and EBV (68.5%) seropositivity in transplant patients. Thus, serological tests in this region maybe in the developing countries are not suitable for diagnosis or monitoring of CMV infections. Molecular techniques such as qualitative PCR could not differentiate between CMV and EBV latency and activity. For instance, there were more than 63.5% CMV positive subjects after 20 days hospitalization following transplantation, while only 15% were symptomatic and had a CMV copy number more than the cutoff point (> 900 copies/mL). More than 15.5% of subjects were EBV positive in qualitative PCR, while only 5% had EBV copy number more than the cutoff points of this study. Quantification of viruses by real-time PCR, for example CMV and EBV in body fluid has been shown to be useful in clinical contexts for monitoring of transplant recipients and antiviral therapy, in which higher viral loads have been found to be associated with increase of disease complication. Due to the high sensitivity of these assays, herpes viruses such as CMV and EBV are also detectable in a substantial number of patients with asymptomatic infection who never progress to disease. Therefore, quantitative PCR-based assays might be too sensitive for clinical purposes. In contrast to antigene- 

mia assays, in qRT-PCR tests the small volume of starting material but not delay between sampling may contribute to loss of sensitivity in low copies of virus. Therefore, should be careful in applying this method for qualitative purposes. Real-time PCR has improved accuracy and significantly decreased the time needed for amplification, detection and quantification. Sample preparation is the only time-consuming and labor-intensive part of this automated assay. Moreover, rapid thermocycling and simultaneous detection characteristics and its specificity of resulted cutoff (≥ 900 copies) are the advantages of real-time PCR TaqMan method. In the present study, cutoff point of 900 copies per ml provides a reliable tool for diagnosis of active CMV infection and can be used as a predictive biomarker for monitoring of transplant patients. The measurement of viral load by qRT-PCR appears to be an important tool in the prediction/diagnosis of active CMV disease in immuno-compromised transplant subjects, for differentiating latent from active infection and for monitoring anti-CMV compared to conventional PCR assay, which cannot accurately identify patients at the risk of developing CMV-related symptoms/disease. The data in present research are in agreement with those of other studies by demonstrating the efficiencies of detecting CMV infections among kidney transplant patients. It has also been proved that generally active CMV infection occurred in 30–70% of transplant recipients with a mortality rate of 5%. Since effective anti-CMV treatment is available and the number of CMV copies well correlated with the severity of clinical symptoms, early detection of CMV DNA in the blood is of great importance in identifying those patients who are at risk of infection and disease progression.

Some studies reported co-infection of CMV/EBV. Nevertheless, these data should be taken cautiously, because other factors, such as underlying diseases, regi- men of immunosuppressive therapy, HLA matching and source of donor kidney may also influence the outcome of CMV infection/reactivation. It was also observed that clinical manifestations of CMV infection were often associated with graft rejection, elevated creatinine levels, and rarely hypertension. However, it has been difficult to define whether the CMV infection is the causative agent for these episodes. The results of this study...
demonstrated that in bivariate analysis the CMV viral load was significantly correlated with increased creatinine levels, abnormal heart sounds and hypertension. Furthermore, the current study showed that although, patients in group I, following transplantation, had fever, increased creatinine levels, abnormal heart sounds and hypertension, in group II, UTI and abnormal heart sounds were more common which were rarely reported previously. More importantly, multinomial regression analysis showed that high CMV viral load (>900 copies/mL in plasma) was significantly associated with rise in creatinine levels, hypertension, and abnormal heart sounds. Taken together, CMV active infection in kidney transplant subjects was associated with fever, elevated creatinine levels, abnormal heart sounds and hypertension. In consistent with the results of current study, Pour-Reza-Gholi also reported that CMV infection in 100 kidney recipients is significantly associated with fever, elevated creatinine and abnormal heart sounds.34

A comprehensive study in the United States National Health and Nutrition Examination Survey (NHANES) has introduced CMV as a possible pathogen for causing hypertension. However, in clinical studies there was no strong evidence that CMV is a significant cause of hypertension. CMV infection may induce oxidative stress and endothelial dysfunction. Endothelial dysfunction and inflammation are two key mechanisms in the development and progression of hypertension, which may happen in CMV infections.35 The results of our study demonstrated statistically significant association between CMV loads in active infection and blood hypertension. In the current study, most of the allograft rejections have occurred in the early post transplantation phase; however, no significant differences were observed between the mean CMV or EBV loads in patients with renal dysfunction or underwent graft rejection and patients who have been relatively treated. In other words, CMV infection did not contribute in the allograft rejection. Clearly, a bidirectional interaction exists between the virus and clinical symptoms, as they can be amplified by the ability of active CMV infection to induce inflammation and endothelial changes. The same inflammatory episodes in active EBV and CMV infections may intensify clinical symptoms and make the situation more complex. Therefore, more studies should be conducted to clarify the exact impacts of active CMV on clinical manifestations in renal allograft transplantation.

In summary, this study demonstrated that serological tests were not suitable for the diagnosis of CMV infections in this region due to high seropositivity, and qualitative PCR assays were unable to differentiate between latency and proliferative phase of infections. In contrast, quantitative real-time PCR was accurate, rapid, and labor-saving for identification and monitoring of active and symptomatic CMV and EBV infection and therapy. A cutoff point of 900 copies per ml was found to be an appropriate for the presence of CMV active infections and 2000 copies per ml for EBV active infection by the qRT-PCR TaqMan method for the initiation of antiviral therapy. The high correlation demonstrated between the qRT-PCR results in CMV and EBV infections and their clinical manifestations introduce the TaqMan method as an accurate laboratory tool for monitoring of, particularly, CMV infections in transplant recipients. Fever, increased creatinine levels, abnormal heart sounds, and hypertension could be considered as clinical outcome of active CMV infection in kidney-transplant patients.

Acknowledgements
We thank Dr. Kiarash Ghazvini for proofreading and editing the article.

Disclosure statement
All the authors have declared no competing interest.

Funding
Mashhad University of Medical Sciences, the Vice Chancellor of Research, granted this study (No: MUMS-87489).

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