Comparing Serology, Antigenemia Assay and Polymerase Chain Reaction for the Diagnosis of Cytomegalovirus Infection in Renal Transplant Patients


Abstract
Background: Cytomegalovirus (CMV) disease is responsible for significant morbidity and mortality following renal transplantation. Currently serology is the only method widely available in our country. Newer methods like early CMV pp65 antigenemia assay and CMV DNA amplification can diagnose CMV disease in its very early period.
Aim: The aim of our study was to compare serologic method with antigenemia assay and CMV DNA amplification to diagnose CMV.
Methods: Seventy-three renal transplant recipients (from 7 centres) with clinical suspicion of CMV disease were studied prospectively. The diagnosis of CMV infection was suspected on the basis of fever and leucopenia.
Result and Discussion: Three tests were done in all 73 patients and in 22 healthy subjects (control group). The sensitivity and specificity of serological test (CMV IgM) was 72.97 and 62.06%; of antigenemia assay was 89.18 and 100% and of PCR was 100 and 72.41%.
Conclusion: Antigenemia assay is a sensitive and specific test for early and rapid diagnosis of CMV infection. Qualitative PCR is a sensitive marker but has low specificity.

INTRODUCTION
Cytomegalovirus (CMV) infection is a major infectious complication of transplant recipients, causing significant morbidity and mortality.1 The effects of CMV infection in transplant recipients may be classified as direct and indirect. Direct effects seen as clinical manifestations of active CMV disease include fever, leukopenia, hepatitis, colitis, and retinitis. Indirect effects are more subtle and lead to allograft injury or loss and increased susceptibility to other infectious diseases, and decreased survival of the patient.2

Serological markers like CMV IgG and IgM antibody assay do not differentiate between latent and active infection due to delayed seroconversion. Moreover IgM titres may not be raised to the detectable level.3 Molecular assays like PCR also do not differentiate between active and latent infection.4 Hence there is a need to develop a more reliable test which can diagnose active disease.

Various studies have reported that detection of the polymorphonuclear leukocytes expressing the CMV tegument pp65 protein by the antigenemia assay, can be used to detect active CMV disease and to monitor antiviral treatment.5 This has prompted many laboratories to use the antigenemia assay as their standard of care for detecting active CMV disease in the transplant recipients.3 The present study was undertaken to compare the three methodologies.

PATIENTS AND METHODS
Subjects
Seventy-three consecutive renal transplant patients with clinical suspicion of active CMV disease seen between January 2000 and March 2002 were included. Triple drug immunosuppression (cyclosporine (CSA), azathioprine, and
prednisolone) was used in most of the patients. In some cases, mycophenolate mofetil (MMF) was used instead of azathioprine.

Twenty-two healthy volunteers formed the control group.

Definition of CMV Disease: Diagnosis of CMV disease was considered in cases of fever and leucopenia where no other obvious cause could be identified and, if there was a response to reduction in immunosuppression and or ganciclovir therapy. The diagnosis was also made when there was a clear cut histologic evidence on biopsy of involved organ (e.g. GI tract).

Samples

5 ml blood was collected in plain tube for serology and PCR and 5ml blood was collected in EDTA for antigenemia assay. All the samples were collected at the same time.

CMV serology: The IMX (Abbott, Abbot Park, I11. USA) microparticle enzyme immunoassays commercial kit was used for estimating IgG and IgM in serum samples. The instructions of the manufacturer were followed for performance and interpretation of the assays. The IgM positive samples in the screening test were confirmed by the rheumatoid factor neutralization format as recommended.

CMV antigenemia assay: The CMV antigenemia assay for lower matrix protein (pp65) was performed using CMV-vue (FITC) immunofluorescence kit (INCSTAR, Stillwater, Minn.). Dextran sedimentation procedure was used to prepare leukocytes. Twenty-five microlitres of cell suspension was spotted onto slides, air dried, and fixed to microscopic slides. The fixed cells were incubated with CMV FITC-vue monoclonal antibodies directed against the CMV lower matrix, early structural protein pp65. This was followed by an immunofluorescence staining with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin G (IgG). Positively stained cells were viewed by light microscopy.

CMV DNA amplification assay:

DNA was extracted from serum samples using Qiamp blood kit (Qiagen).

DNA amplification was carried out using primers obtained from the region as reported by Barber et al.6 Upstream 5’-AGACCTTCATGCAGATCT CC- 3’ (2037-2057) 20mer and Downstream 5’-GTTGCTACGCACATGATC-3’ (2280-2300) 20 mer (Bio Analysis, Italy). Amplification mixtures comprised of 20µl extracted DNA; 10pmol/µl of each oligo upstream and downstream primers; 200µM each of deoxynucleoside triphosphates; 1U of Taq polymerase (Banglore Genei, India), and 1X Taq assay buffers. Sterile distilled water was added to make up the volume to 100µl. The following amplification conditions were carried out in Techne Thermalcycler, initial denaturation at 94 °C/1min, 40 cycles each of denaturation at 94°C/1min primer annealing at 53°C/1min and primer extension at 72°C/1min and final extension at 72°C for 10 min. The amplified products were electrophoresed on an ethidium bromide stained 2% agarose gel to visualize a 263 base pair amplified product (Fig. 1). The amplified products were also confirmed by performing liquid hybridization assay i.e. (DEIA) DNA enzyme immuno assay (Diasorin) as described earlier.7

Statistical analysis:

Data measured on continuous scale (e.g. age) were expressed as mean ± standard deviation (SD) and compared using ANOVA. Categorical data (e.g. sex) were expressed as percentages and compared using chi squared test. All tests were carried out at 5% level of significance.

The performance of the three methods used, was statistically analysed using the following definitions: Sensitivity was calculated as the proportion of the determinations done during disease episodes that tested positive; Specificity was defined as the proportion of the determinations done during disease free episodes that tested negative; Positive predictive value was calculated as the proportion of “test positive” determination that occurred on patients that developed disease; Negative predictive value was defined as the proportion of “test negative” determinations that occurred on patients that did not develop disease.

RESULTS

Of the 73 patients suspected to have CMV disease, 37 were confirmed to have CMV disease as defined in the methods. In 36 cases, the fever and leucopenia could be explained on the basis of other obvious causes (e.g. bacterial/fungal pneumonia, septicemia). Table 1 shows age and sex distribution of 37 patients with CMV disease, 36 patients with other causes of fever and leucopenia and of control subjects. There was no significant difference between the three groups. Out of 37 patients with CMV disease, 6 patients had no specific organ involvement that could be documented. In remaining 31 cases the organs involved were lung, gastrointestinal tract, liver and nasal cavity. In 5 cases this
was confirmed histopathologically [colon (2), esophagus (1), liver (1), nasal cavity (1)]. All of these cases responded to reduction in immunosuppression with or without ganciclovir.

Table 2 shows results of the three methods used in patients with and without CMV disease and in control subjects. In three cases of CMV disease antigenemia assay could have been negative due to delay in processing (samples came from other cities). In one case it may have been negative due to leucopenia. Table 3 shows sensitivity, specificity, negative predictive value and positive predictive value of the three methods used.

**DISCUSSION**

CMV infection is widespread in India with serological evidence of exposure being close to 100% in adults. In our institution we have screened about 320 recipients and an equal number of donors for CMV IgG and found all of them to be positive for CMV IgG (unpublished data). Therefore, in this population, CMV infection among renal transplant recipients is likely to be either a reactivation or superinfection and only rarely primary infection.

Occurrence of CMV disease is associated with increased morbidity and mortality. Thus, CMV assays that are sufficiently sensitive and specific are required for optimal surveillance and management of CMV infection in kidney transplant patient. In this study, we compared three assays and tested their sensitivities, specificities, positive predictive values and negative predictive values in seventy three renal transplant patients.

Serological methods for determining antibody responses to CMV have commonly been used in our country. A key diagnostic issue for CMV infection is distinguishing active disease from latent infection. Before transplantation, serological markers, IgG and IgM against CMV are reasonable as a base line and can be used in order to adjust for the risk of CMV disease. However, after transplantation they have only confirmatory value. In some cases antibodies may not develop due to immunosuppression, or develop after the disease is already cured, or may persist for years. Studies conducted by Flechner et al and Priester et al have reported that serological assays lack the usefulness in diagnosing CMV reactivation in transplant patients. Similar findings have been observed in our study. If only CMV serology had been used in our patients, the incidence of CMV disease would have been (falsely) reduced by 14% because 10 of 37 patients with CMV disease had no serologic response during the study. On the other hand 22 patients without disease had mounted a positive response.

A number of studies have demonstrated the utility of CMV pp65 antigenemia assay for the diagnosis of CMV infection after solid organ transplantation. CMV antigenemia assay is useful for detecting early or active CMV infection with a reported sensitivity of 50-97% and specificity of 71-100%. In the present study sensitivity was observed to be 89.18% with a high specificity of 100%. Of the 37 patients with active disease, 33 samples were picked up by antigen assay.

As few studies have reported that storage of whole blood samples in EDTA at 4°C or 24 hours Room temperature (RT) yielded essentially equivalent results we extended our study to three samples from outside the city limit. However all these three samples were found to be negative, though they all had CMV disease. This false negativity could be attributed to factors like improper storage and delay in transportation. One patient from our centre with active CMV disease was positive by tissue biopsy but was negative by antigenemia assay. This could be due to peripheral leucopenia. Except for these four cases we found a very good correlation between this assay and diagnosis of active CMV disease. Similar results showing better sensitivity of the CMV antigenemia assay have been reported by Gerna et al.

The advantage of antigenemia assay is that it can be completed within 5 hour after sampling and the level is easily assessed by counting the marked cells in a population. The major limitation of this test is the need for rapid processing of the specimens to avoid autolysis of blood leukocytes, which
leads to false-negative results.

There have been many studies evaluating the clinical utility of PCR based assays for the detection of CMV DNA.\textsuperscript{5,6,14} Many of these investigators have shown that although the assays may be sensitive, they often lack clinical specificity as was observed in our study. In the present study PCR had 100% sensitivity with 72.41% specificity. Due to high sensitivity of DNA amplification, CMV is also detectable in a substantial number of patients with asymptomatic infection who never progress to active CMV disease. The finding that qualitative PCR was positive in virtually all situations in which there was no evidence of CMV activation suggests that a negative result could be uniquely useful in ruling out active CMV infection.

**CONCLUSION**

We conclude that serological method is less sensitive due to delayed seroconversion and less specific. Antigenemia assay is a sensitive and specific test currently available for active CMV infection. False negative reports could be avoided by processing the samples within 2 hours of collection. Qualitative DNA amplification is a sensitive marker but has a low specificity. The development and application of sensitive diagnostic assays such as PCR and pp65 antigenemia has helped to increase our understanding of the incidence and course of CMV infection in renal transplant patients. But further study and patient stratification based on the available quantitative diagnostic assay is needed to avoid high rate of overtreatment.

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**REFERENCES**


