

# Determination of human cytomegalovirus pp65 antigenemia among renal transplant patients

S. Moses, J. Malathi, N. R. Singha, R. Bagyalakshmi, H. N. Madhavan

L and T Microbiology Research Center, Kamal Nayan Bajaj Research Centre, Vision Research Foundation, Sankara Nethralaya 41, College Road, Chennai, India

## ABSTRACT

Human cytomegalovirus (HCMV) is an important cause of morbidity and mortality in immunosuppressed transplant recipients. Isolation of HCMV from peripheral blood leukocytes (PBLs) is considered a reliable marker of disseminated HCMV infection. HCMV pp65 antigenemia is widely used for monitoring CMV infection and guiding preemptive therapy. The aim of this study was to compare pp65 antigenemia with culture technique for detection of HCMV in PBLs among kidney transplant patients and also to determine the threshold value of significant pp65 antigenemia. Fifty-one peripheral blood samples from post-renal transplant patients collected during August 2009 to March 2011 were processed for pp65 antigenemia assay. These were also tested for isolation of the virus by inoculation into human corneal fibroblast cells. The results of pp65 antigenemia and culture were compared to determine the clinical significance of pp65 antigenemia. HCMV was isolated in 21 cases. On comparing the pp65 antigenemia results with that of the viral isolation, a mean of 23 cells was determined to yield a positive isolation of HCMV. The values of pp65 antigenemia and isolation results were correlated (paired *t*-test,  $P = 0.0029$ ). A pp65 count of 23 and above was considered significant in our clinical settings since we found that these clinical specimens yield positive culture result.

**Key words:** Corneal fibroblast culture, human cytomegalovirus, threshold pp65 antigenemia count

## Introduction

More than 80% of the adult population worldwide is infected with human cytomegalovirus (HCMV). Primary infections occur early in the childhood and may cause exanthema subitum. HCMV infection is an important cause of morbidity and mortality in immunosuppressed organ transplant recipients.<sup>[1-3]</sup>

As the diagnosis of HCMV infections cannot be made reliably on clinical grounds alone, laboratory confirmation is required. The most specific laboratory method for

diagnosis of HCMV infection is isolation of the virus in culture. Isolation of HCMV from blood leukocytes (CMV viremia) is considered the most reliable marker of disseminated CMV infection, and predicts invasive CMV disease.<sup>[4-8]</sup> HCMV can infect a number of cell types, namely, fibroblasts, epithelial and endothelial cells, and smooth muscle cells.<sup>[9]</sup> In particular, HCMV has been shown to infect and replicate in endothelial cells of the vascular tree. HCMV is also present in peripheral myeloid cells of 50-90% of normal individuals. In disseminated infection, cytomegalic endothelial cells may circulate in the peripheral blood, and virus dissemination is mediated by peripheral blood leukocytes (PBLs) carrying infectious virus acquired from infected endothelium and transmitting the infection to uninfected endothelial cells.<sup>[10-12]</sup> Isolation of HCMV from human PBLs has been documented. Successful isolation was reported for various leukocyte subpopulations such as granulocytes, T lymphocytes, or monocytes.<sup>[13,14]</sup> pp65 antigen of HCMV is recognized by more than 70% of HCMV-specific circulating T lymphocytes (CTLs). As pp65 is processed and presented before endogenous viral replication, pp65-specific CTLs may initiate the HCMV spread.<sup>[15-20]</sup>

The detection of HCMV pp65 antigenemia is widely used

### Address for correspondence:

Dr. Madhavan HN, L and T Microbiology Research Center, Vision Research Foundation, Sankara Nethralaya 18, College Road, Chennai - 600 006, India. E-mail: drhnm@snmail.org

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for monitoring CMV infection and guiding preemptive therapy in patients at risk of developing HCMV disease, as a semiquantitative analysis of HCMV viral replication.<sup>[21,22]</sup> The CMV pp65 antigenemia assay, which quantitates the number of HCMV-infected leukocytes in peripheral blood, has proven efficacy in the detection and monitoring of this virus infection in immunocompromised patients.<sup>[23-26]</sup> This study was undertaken to determine the significant pp65 antigenemia count among renal transplant patients, by evaluating the test against viral isolation.

## Materials and Methods

### Samples

Peripheral blood samples from renal transplant recipients were collected in 2 ml EDTA vacutainer tubes and were transported immediately to the laboratory. All the patients included in the study were symptomatic and referred for laboratory investigation. All specimens were transported in their native form without any transport medium. The blood specimens were processed immediately for pp65. The study was approved by the research and ethics committees of our institute.

### Antigenemia assay

The pp65 antigenemia assay was carried out on smears containing  $2 \times 10^5$  leukocytes prepared from 5 ml of EDTA anticoagulated blood within 6 hours of receipt of the specimen. Smears were fixed in methanol for 10 minutes. Immunofluorescence staining was carried out on the smears using a pp65 staining kit obtained from Argene SA, France. The smears were stained with mouse monoclonal antibody (Argene SA, France) and examined under a fluorescent microscope (Optiphot, Nikon, Japan) with a blue filter.

### Establishment of corneal fibroblast cultures

The stromal layer of the donor was cut into large pieces and placed in tissue culture flasks. The tissues were nourished with Dulbecco's minimum essential medium (DMEM) and F12 medium combination (Gibco, Invitrogen, New York, USA). The medium was supplemented with 15% fetal calf serum (Hi-Media, India), and fibroblast growth factor (Sigma, USA) at  $1 \mu\text{g}/\text{ml}$  concentration. At the end of 24 hours and 48 hours, the outgrowth of fibroblast from the tissue was noted. Upon confluency, the cells were trypsinized onto 12-well tissue culture plates for growth of cells for subsequent inoculation of leukocytes for isolation of HCMV.

### Viral culture

The cultured human corneal fibroblast cell line established from one donor eye and not more than 3-5 passages maintained in DMEM was used for the study. The leukocytes isolated from pp65 antigenemia-positive blood

samples were inoculated onto a monolayer of cells on 12-well tissue culture plates (BD Falcon, USA) for the isolation of HCMV. The plates were rocked gently on a rocking machine for 1 hour at room temperature. The cells were incubated at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator. The cultures were observed for cytopathic effect (CPE). The cultures showing CPE typically of HCMV were harvested using cell scrapers and stored at  $-80^\circ\text{C}$ . Forty-nine randomly selected pp65-positive PBLs were tested for isolation of HCMV. Uninoculated corneal fibroblast cultures were also maintained to rule out nonspecific changes.

### Real-time PCR assay

Real-time PCR (RT-PCR) targeting the morphologically transforming region *mtr II* sequence was applied onto the DNA extracted from the culture isolates in the rotor gene RT-PCR machine (QIAGEN, 5 Plex) using primers and thermal profile described earlier.<sup>[27]</sup> The intra- and inter-assay reproducibility were evaluated using triplicates of plasmid dilutions ( $10^1$ ,  $10^3$ , and  $10^5$ ) corresponding to an input of  $2.5 \times 10^3$ ,  $2.5 \times 10^5$ , and  $2.5 \times 10^7$  copies/ml per reaction in the same and four independent runs, respectively. RT-PCR for the quantification of CMV was performed on the viral isolates that propagated on corneal fibroblast culture. Uninoculated corneal fibroblast culture DNA was used as the culture control to rule out the presence of viral markers in each lot of the cell lines. Reagent controls were also included in each run of the RT-PCR assay.

## Results

### pp65 antigenemia assay

Among the 51 prepared PBLs, 49 specimens were positive for pp65 antigenemia. Based on the results, the specimens were grouped into four (groups A-D). Patients in group A had a cell count ranging from 2 to 10; group B had a count ranging from 11 to 25 cells, group C had a count ranging from 26 to 50 cells and group D had a cell count  $>50$ .

### Comparison of pp65 antigenemia assay and viral isolation

Among the 49 peripheral blood specimens used for inoculation, the cultures showed megaloblastic changes in 21 (42.86%) specimens [Figure 1]. Patients under group A had a cell count ranging from 2 to 10 cells, with a mean 6.3. This group of patients had an isolation rate of 20%. Group B had a cell count ranging from 10 to 25 cells, with a mean of 15.2, and there were 7 (28%) isolates from this group of patients. Group C had a count ranging from 25 to 50 cells with a mean cell count of 39.3, and 4 (66.67%) specimens of the virus were isolated. Group D had a cell count  $>50$  cells with a mean count of 229.11, and cultures were positive in 8 (88.89%) specimens

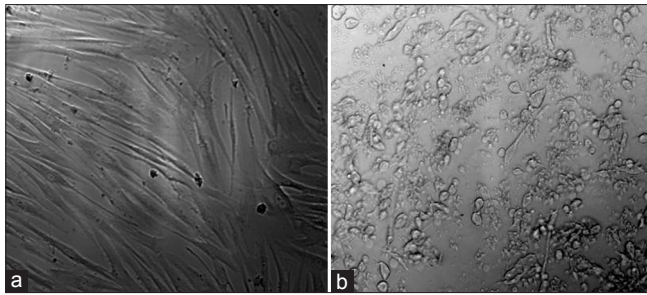


Figure 1: (a) Uninfected cell control. (b) Infected fibroblast cultures

[Table 1]. Isolation of the virus was further confirmed on RT-PCR assay performed on the cell culture isolates.

Inactivation of the virus was ruled out as all the isolates were stored at  $-80^{\circ}\text{C}$  until processed. Each lot of fibroblast cell culture without exposure to leukocytes was harvested and DNA extracted from them was subjected to rule out the presence of endogenous HCMV infection.

#### RT-PCR assay

The RT-PCR assay performed on the DNA extracted from the culture harvests revealed copy numbers ranging from 1 to 7.4 lakh copies/ml of DNA. The range of copy numbers varied for each group (group A 10-431 copies/ml, group B 369-7.1 lakh copies/ml, group C 328-7.04 lakh copies/ml, and group D 1734-3.2 lakh copies/ml).

The mean values of all four groups were correlated with the positivity of HCMV in culture. The results were not statistically significant (paired *t*-test  $P = 0.7$ ). However, when the actual pp65 antigenemia value of the 49 patients was correlated with the corresponding culture results, by the paired *t*-test the results were statistically significant, indicating a strong correlation between the pp65 antigenemia value and the culture result ( $P = 0.0029$ ).

We evaluated the most likely value of pp65 antigenemia that yields culture positive results. It was found that a count of 22.5 and above has more probability of HCMV isolation in our renal transplant patients. A count of 23 cells per 200000 leukocytes is considered as significant

#### Discussion

Gregory *et al.*,<sup>[28]</sup> compared three different assays designed for the rapid and sensitive detection of CMV in blood: quantitative shell vial culture (QSVC), PCR, and the pp65 antigenemia assay. It was found that PCR was the most sensitive of the three, allowing recognition of CMV in blood specimens from a larger number of patients and at an earlier time after transplantation than the other

**Table 1: Results of patient groups based on pp65 antigenemia assay and culture isolation**

Groups	pp65 cell count range (no. of cells/ $2 \times 10^5$ )	No. of patients	No. of culture positives (%)
A	1-10	8	2 (20)
B	11-25	19	7 (28)
C	26-50	5	4 (66.7)
D	>50	9	9 (88.9)

The value of pp65 antigenemia and isolation of HCMV from fibroblast culture had a positive correlation (paired *t*-test  $P=0.0029$ )

assays, and PCR also remained positive for the longest time after transplantation. Quantitative shell vial culture and the antigenemia assay were comparable both in the number of patients in whom CMV was detected in blood and in the time to detection. The advantage of culture was highlighted by the observation that when antiviral therapy was used, QSVC tended to become negative first, while PCR stayed positive the longest. The study finally concluded that the pp65 antigenemia assay and shell vial culture are quantifiable and comparable in sensitivity. Either is recommended for rapid detection of CMV in blood specimens from solid-organ transplant recipients.<sup>[28]</sup>

Reina *et al.*<sup>[29]</sup> concluded that the inoculation of all of the leukocytes extracted from blood samples in the SVC seems to produce a slight increase in the sensitivity of the cell culture and that the SVC becomes positive before the antigenemia for the detection of CMV in peripheral blood, especially in the first blood sample.<sup>[29]</sup>

In another study, assays that analyzed 10,000 leukocytes had moderate analytical sensitivity, thereby exhibiting moderate positive predictive value (PPV) and specificity for the prediction of CMV disease in patients after stem cell transplantation. The sensitivity of RT-PCR depends on the assay conditions. PCR tests with high sensitivity have a low PPV and specificity, and threshold values need to be evaluated under clinically relevant conditions to obtain an appropriate specificity and PPV as close to 100% as possible to avoid unnecessary CMV treatment. In addition to the question of sensitivity, the availability of patient specimens may influence the choice of a test. pp65 antigen detection requires leukocytes from freshly collected blood samples, which cannot be stored or frozen. In contrast, RT-PCR can be performed with small volumes of plasma, which may be stored frozen for long periods without loss of DNA integrity.<sup>[30]</sup>

Ksouri, *et al.* have described that polymorphonuclear leukocytes (PMNLs)-based assays are more efficient in monitoring CMV reactivation, but for high-risk patients with graft-versus-host disease, more sensitive RT-PCR assays must be carried out.<sup>[31]</sup> A standardized RT-PCR assay is more appropriate than the antigen assay for

detecting CMV. It allowed earlier diagnosis of active CMV infection and monitoring of the response to anti-CMV treatment.<sup>[32]</sup> The PCR detected all clinically significant CMV infections (>10 positive cells in pp65 test) and infections which required antiviral treatment. Other authors have described that the quantitative PCR appears to be a suitable alternative to diagnose and monitor CMV infections in transplant patients.<sup>[33]</sup> Therefore, we undertook this study to know the value of pp65 antigenemia using the culture technique.

Human fibroblasts have represented the conventional cell substrate for recovery of HCMV from clinical samples since the beginning of medical virology. Due to the increasing need for recovery of HCMV from the blood of immunocompromised patients, in our study, we have demonstrated the successful isolation of HCMV from human corneal fibroblast cultures. We have also demonstrated the correlation of pp65 antigenemia count of renal transplant patients and isolation of HCMV from the leukocytes of the same group.

The isolation of HCMV is not easy. As the experiment of isolation needs to be carried out with the same batch of fibroblast culture, we performed the viral isolation on 51 consecutive pp65 antigenemia-positive peripheral blood specimens. We would also like to state that the rate of isolation in the manuscript is 41.17% using the human fibroblast cell culture. In a study conducted by Veal *et al.*,<sup>[34]</sup> the HCMV isolation rate was 16.9%. In another study, Mazzulli *et al.* compared the isolation of HCMV and pp65 antigenemia assay and reported an isolation rate of 40.89%. The HCMV isolation rate reported in our study correlated with the results of Mazzulli *et al.*<sup>[35]</sup>

Human corneal fibroblast cultures have proven to be the best substrate for the isolation of the virus in this study. It has been proved that HCMV can be isolated from 42.85% of the pp65 antigenemia-positive individuals, but in our laboratory setup the cutoff value was 23 cells/ $2 \times 10^5$  cells.

From the literature it is evident that the cutoff pp65 value of 10 is considered to be significant in renal transplant patients,<sup>[33,36,37]</sup> except a report made by Gerna *et al.* in which it is suggested that a pp65 antigenemia count of 100 was considered significant.<sup>[38]</sup> In India, the seroprevalence of HCMV is reported to be very high among normal population and renal transplant patients.<sup>[39-41]</sup> In our earlier report, we reported the rate of positivity of HCMV to be very high among the transplant patients.<sup>[27]</sup> Therefore a pp65 antigenemia count of 10 reported based on the

Western literature<sup>[42-46]</sup> may not reflect the true situation of replication of HCMV in PBLs in Indian patients. It should also be borne in mind that pp65 antigenemia assay is subjective as it is prone to the visual errors of individuals reporting on the smears. Therefore, we attempted to estimate the significant pp65 antigenemia count that would have viable HCMV in the peripheral blood.

Despite the fact that pp65 antigenemia is the most valuable tool in the diagnosis of HCMV infections, the viral isolation has also been a useful tool in diagnostics of HCMV disease.

In conclusion, HCMV was successfully cultivated for the first time in India on human corneal fibroblast culture. A pp65 count of 23 was determined to be the value of clinical significance as it gives culture positive results among renal transplant patients.

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The study was approved by the Research and Ethics Subcommittee of our institute (study code no: 34-2003/P).

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