

**CLINICAL UTILITY OF QUANTITATIVE CYTOMEGALOVIRUS VIRAL LOAD
DETERMINATION FOR PREDICTING CYTOMEGALOVIRUS DISEASE IN
LIVER TRANSPLANT RECIPIENTS**

BY

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**A thesis submitted in conformity with the requirements for the degree of
Masters of Science,
Graduate Department of Community Health
University of Toronto**

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Title: Clinical Utility of Quantitative Cytomegalovirus Viral Load Determination for Predicting Cytomegalovirus Disease in Liver Transplant Recipients

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ABSTRACT

The early detection of cytomegalovirus (CMV) reactivation after liver transplantation may form the basis of a pre-emptive strategy for prevention of active CMV disease. We prospectively analyzed the clinical utility of weekly CMV plasma viral load determinations by quantitative PCR and the antigenemia assay in predicting CMV disease in 97 liver transplant recipients. CMV disease occurred in 21/97 (21.7%) patients a mean of 60 days post-transplant. Using a threshold of >400 copies/ml plasma, PCR had a sensitivity of 100%, specificity 47.4%, positive predictive value (PPV) 34.4 % and negative predictive value (NPV) 100% for prediction of CMV disease. Respective values for a positive antigenemia (threshold > 0 positive cells per 150,000 examined) were 95.2%, 55.3%, 37.0% and 97.7 %. Different cut-off points for a positive test were analyzed using receiver-operating characteristic curves. The optimal cut-off for viral load was in the range of 2000-5000 copies/ml (sensitivity 85.7%, specificity 86.8%, PPV 64.3%, NPV 95.7% for > 5000 copies/ml). The optimal cut-off for antigenemia was in the range of 4-6 positive cells/slide. Mean peak viral load in symptomatic patients was 73,715 copies per/ml compared to 3615 copies/ml in patients with asymptomatic CMV reactivation ($p < 0.001$). In a multivariate logistic regression analysis of risk factors for CMV disease (CMV serostatus, acute rejection, and induction immunosuppression), peak viral load and peak antigenemia emerged as the only significant independent predictors of CMV disease (for PCR, OR=1.40 per 1000 copy/ml increase in viral load, $p=0.0001$; for antigenemia OR=1.17 per 1 positive cell/slide). Plasma viral load by quantitative PCR is a useful test for predicting CMV disease, is at least as sensitive and specific as antigenemia, and could be employed as a marker in a pre-emptive strategy.

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1. STATEMENT OF OBJECTIVES

The primary objective of this study was to determine the clinical utility of the quantitative cytomegalovirus (CMV) polymerase chain reaction (PCR) (CMV viral load), and the CMV antigenemia assay in predicting the development of active CMV disease in liver transplant recipients. Specific questions to be answered include:

- 1. Can quantitative CMV PCR and/or the CMV antigenemia assay be used to predict which patients will develop active CMV disease and could therefore be targeted for anti-CMV prophylaxis?**
- 2. What is the utility of quantitative PCR and the CMV antigenemia assay for the diagnosis of active CMV disease and how do they compare to each other?**
- 3. Are these quantitative CMV assays useful for monitoring patients' responses to anti-CMV therapy?**

The answers to these questions may permit the development of a targeted and more cost-effective strategy for predicting which patients are at the highest risk for CMV disease and tailoring patient specific therapy to prevent serious complications from CMV.

2. BACKGROUND

A) CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is a double-stranded DNA virus belonging to the herpesvirus family. Infection with CMV is common in the population, whereas disease is relatively rare in immunocompetent hosts. In these later patients CMV may occasionally cause a mononucleosis syndrome similar to Epstein-Barr virus (5). Cytomegalovirus shares with other herpesviruses the unique capacity to remain latent in tissues after the host recovers from an acute infection, hence, the saying "once infected, always infected" (1,2). The sites of CMV latency are not precisely known, but they include the circulating peripheral mononuclear leukocytes and possibly polymorphonuclear leukocytes (2,3). More recent evidence suggests that latent CMV is widely distributed in different cells and various tissues of normal seropositive individuals (3,4). It is among the various groups of immunosuppressed patients such as recipients of organ transplants, patients with AIDS, immature neonates, that CMV causes its most significant disease syndromes.

Seroprevalence studies show that infection with this virus is widespread. Depending on the socioeconomic condition of the population, the prevalence of antibodies in adults ranges from 40 – 100 % (6). The virus may be transmitted by several routes including transplacental transfer with consequent *in utero* infections, infection at the time of birth by exposure to infected secretions,

person to person spread by infected respiratory secretions in neonates, sexual transmission in adults, or transmission via blood products or transplanted organs (7-11).

B) LIVER TRANSPLANTATION

During the past decade, solid organ transplantation has advanced rapidly to the forefront of therapies available for patients with end-stage organ disease. Advances in immunosuppression, refinement of surgical techniques, new methods of organ procurement and preservation, improved peri-operative patient care, and new agents for prophylaxis and treatment of opportunistic infection have all contributed significantly to successful progress in this field (12). Liver transplantation, in particular, has had a dramatic impact on the treatment of patients with end-stage liver disease. Despite these advances, infection remains the most common life-threatening complication of long-term immunosuppressive therapy. Of particular importance after transplantation is the reactivation and subsequent infection with several viruses, of which cytomegalovirus is the most common.

C) CYTOMEGALOVIRUS INFECTION AND DISEASE

Cytomegalovirus is one of the most important opportunistic infections complicating solid organ and bone marrow transplantation. Active CMV disease

typically occurs with the first 3 months after transplantation and may result in substantial morbidity and mortality in transplant patients (13,14). For example, CMV pneumonitis has been associated with a mortality of 30-50% in bone marrow transplant recipients despite aggressive combination treatment with ganciclovir and immunoglobulin (15,16). Transplant recipients may acquire CMV from the donor organ or blood products, or may develop infection due to reactivation of endogenous latent virus (13,14). CMV infection is defined as the isolation of CMV from body fluids or tissue specimens or can be diagnosed on the basis of positive serology. Patients with CMV infection may go on to develop active CMV disease manifest as symptomatic end-organ involvement. Invasive CMV disease often has a propensity to affect the transplanted organ. Therefore, CMV hepatitis seems to be most severe in liver transplant recipients, CMV pneumonitis occurs most commonly in lung and heart-lung transplant recipients and CMV myocarditis has only been recognized in heart transplant recipients (17). Another form of CMV disease commonly recognized in solid organ transplant recipients is referred to as 'CMV viral syndrome' (13). This syndrome usually begins with fever and symptoms of anorexia and malaise usually accompanied by arthralgias and myalgias. Patients typically develop hematological abnormalities including leukopenia and thrombocytopenia. Another common form of CMV disease occurs with gastrointestinal involvement (18). CMV disease of the gastrointestinal tract may result in a wide spectrum of pathology ranging from diffuse inflammation with functional disturbances to ulceration, hemorrhage and even perforation.

In addition to directly attributable morbidity, CMV may also have an immunomodulatory effect, and active CMV disease has been found to be an independent risk factor for the development of other infectious complications such as bacteremia (19), invasive fungal disease (20) and Epstein-Barr Virus related post-transplant lymphoproliferative disease (21). CMV has also been implicated as a cause of acute and chronic allograft injury (See Figure 1). It is hypothesized, that CMV may play a crucial role in chronic graft vasculopathy resulting in lesions such as the vanishing bile duct syndrome in liver transplants, bronchiolitis obliterans in lung transplants and accelerated coronary artery disease in cardiac transplants (22,23). Given the potential for adverse consequences of CMV disease, and the potential for a poor therapeutic response to established disease, strategies aimed at preventing the development of active disease are preferable.

D) DETERMINANTS OF THE RISK OF CMV DISEASE

The risk of CMV infection is related to pre-transplant donor (D) and recipient (R) CMV serology. D+/R- transplants are at highest risk of CMV infection, with symptomatic CMV disease occurring in up to 80% of liver transplants and 60% of kidney transplants (24-27). This is usually primary symptomatic disease. The next highest risk group is the D+/R+ followed by the D-/R+ patients. CMV disease rates may range from 6-55% in these patients depending on additional risk factors (14,24,25,28). In D-/R- transplants, the risk of active CMV disease generally occurs from receipt of blood products that are CMV positive. Use of CMV seronegative

blood products significantly reduces CMV disease rates in this subgroup (29-31). Other risk factors for CMV include the type of transplant, the degree of immunosuppression, and the occurrence of acute rejection. Of particular importance is the use of antilymphocyte antibody preparations for the treatment of acute rejection, which results in a substantial increase in the incidence of CMV disease (25).

E) CMV PREVENTION

Numerous prophylactic and preventative strategies have been employed to decrease the incidence of active CMV disease post-transplantation. Preventative strategies can generally be divided into one of two categories: i) Universal prophylaxis, and ii) Pre-emptive therapy. These two strategies differ fundamentally in their approach to prevention of CMV disease post-transplantation.

i) Universal prophylaxis

This strategy is to give all patients at risk of CMV prophylactic intravenous or oral anti-viral therapy. This usually involves antiviral therapy for the D+/R-, D+/R+, and D-/R+ subgroups of patients. As noted previously, D-/R- patients are at low risk of CMV disease as long as they receive seronegative blood products (29-31). The antiviral agent is usually administered for a period of three months post-transplant which corresponds to the peak period of risk for the development of CMV disease. In a randomized control trial comparing universal prophylaxis using intravenous ganciclovir versus high-dose oral acyclovir until day 100 post liver transplant, active

CMV disease developed in only 1/124 (0.8%) of patients receiving ganciclovir vs. 12/126 (10%) of patients receiving acyclovir (32). In another trial oral ganciclovir for 98 days post-transplant was compared with placebo in 304 liver transplant recipients (33). The 6-month incidence of CMV disease was 7/150 (4.8%) in the ganciclovir group vs. 29/154 (18.9%) in the placebo group ($p < 0.001$). Therefore, universal prophylaxis, usually with intravenous or oral ganciclovir, has been shown to be quite effective for the prevention of CMV disease in solid organ transplant recipients, (33-34). However, there are several disadvantages to this strategy. These include the unnecessary administration of intravenous or oral antiviral therapy to a large group of patients who may never develop CMV disease. Adverse effects due to ganciclovir (neutropenia), the risks and costs associated with prolonged intravenous administration, and the potential for emergence of antiviral resistance are major disadvantages of this prophylaxis strategy.

ii) Pre-emptive therapy

Another approach to preventing CMV disease is to screen patients routinely for evidence of CMV infection before symptoms develop. Such screening would utilize one or more of a variety of available laboratory methods to detect CMV reactivation in the earliest stages before the patient develops active symptomatic CMV disease. Antiviral therapy would then be initiated only in those with CMV infection in order to prevent the development of active CMV disease. This strategy is commonly referred to as "pre-emptive therapy" (13,14). Ganciclovir is the most logical antiviral agent for employment in a pre-emptive strategy. The major

advantage of pre-emptive therapy is that only patients at high risk of developing active CMV disease receive antiviral medication, thus sparing the majority of patients from potential adverse effects from ganciclovir. Among these, ganciclovir induced neutropenia may lead to an increased incidence of bacterial and fungal infections. Other potential advantages of a pre-emptive strategy include cost-savings due to decreased drug utilization. Such a strategy may also limit the emergence of anti-viral resistance. Verdonck et al. (35) studied the value of collecting serial blood samples for CMV antigenemia (a method of detecting CMV in leukocytes) with a two week course of pre-emptive ganciclovir in patients who tested positive. This study was conducted in a group of 41 allogeneic bone marrow transplant recipients. No case of active CMV disease occurred using this method. Singh et al. (36) stratified liver transplant recipients into "at-risk" groups based on the basis of cultures of the buffy coat and urine every 2 to 4 weeks for 24 weeks post-transplant and demonstrated that administration of pre-emptive ganciclovir to those with asymptomatic viruria or viremia significantly reduced the attack rate of CMV disease.

The employment of pre-emptive therapy has led to the evaluation of numerous diagnostic methods for early detection of CMV and subsequent pre-emptive therapy in those with positive test results in order to prevent the development of active CMV disease. In order for a diagnostic test to be useful for pre-emptive therapy, it must have good positive and negative predictive values for the subsequent development of CMV disease. The ideal test should be relatively

simple, well standardized, not too costly, and have a quick turnaround time. The test should also become positive sufficiently in advance of the development of active disease such that the physician would have time to initiate pre-emptive therapy.

Tests currently available to detect CMV include culture-based methods, serology, polymerase chain reaction (PCR), and the CMV antigenemia assay. Cultures for CMV may be done from urine, throat, blood, or other samples. Although relatively easy to perform, culture methods have generally been disappointing in terms of predicting CMV disease (28,37). In a study analyzing the prognostic significance of untreated viremia in liver transplant recipients, only 32% of patients with organ involvement had preceding viremia (28). Also, positive predictive values for viremia were only 56% in the D+/R- group and even lower in the D+/R+ group and the D-/R+ group (22% and 11% respectively) (28).

Testing for CMV using qualitative rather than quantitative PCR for following patients after transplantation have demonstrated very high sensitivity and negative predictive values (38-40). However, due to the overly sensitive nature of this test, specificity and positive predictive values (PPV) are less than optimal especially in low-risk subgroups (i.e. in D+/R+ and D-/R+ patients). It is clear that a pre-emptive strategy based on monitoring by culture methods or qualitative PCR would be less than ideal. The most useful CMV diagnostic test would therefore be one that would accurately predict the development of CMV disease thereby providing a more precise guide for pre-emptive therapy and spare the majority of patients from

unnecessary anti-CMV therapy. Quantitative testing for CMV may prove more useful than conventional qualitative tests by providing more accurate predictive values and by allowing physicians to follow trends over time. Currently, there are two available methods of CMV quantification: the CMV antigenemia assay and quantitative CMV PCR testing.

The CMV antigenemia assay is a rapid quantitative assay for the direct detection of CMV antigens in peripheral blood polymorphonuclear leukocytes (PMNs) (41,42). The validity of antigenemia testing has been well evaluated in previous studies although performance depends on whether the test is used to diagnose CMV disease or be a measure of CMV reactivation. Performance may also depend on the laboratory, since many of these assays are 'home-grown'. Antigenemia is sensitive for the diagnosis of CMV disease although it lacks specificity (39-42). Antigenemia is both sensitive and specific for demonstrating CMV reactivation. We have demonstrated this for our specific antigenemia assay by evaluating the test in transplant patients not at risk of CMV disease (D-/R-subgroup). Only 1/39 bone marrow transplant recipients had a positive (presumably false positive) antigenemia result only on a single occasion (1 positive test out of 395 tests)(56).

Quantitative PCR employs standard PCR technology but allows viral load determination (viral copies per ml) by analyzing the strength of signal detection. Less information exists on the validity of this particular plasma based quantitative

PCR test since it has just recently become commercially available. Studies evaluating home-grown quantitative PCR have shown a good correlation between viral load and the development of CMV disease (45,46). The test has not been evaluated in a large control group not at risk for CMV disease.

To properly assess the predictive value of a test, several important study conditions must be satisfied: 1) a large enough sample size should be studied with a sufficient number of outcomes (CMV disease); 2) results of testing should be kept blinded and not used in clinical decision making; 3) ideally, patients should not be receiving any form of CMV prophylaxis during the monitoring period and 4) the study population should be relatively homogenous since predictive values may differ depending on the organ transplanted.

As previously stated, an alternative to standard qualitative PCR tests is the use of a quantitative nucleic acid assay that allows the measurement of the number of viral copies/ml of CMV DNA. Precise viral quantification may result in improved predictive values for PCR assays and therefore serve as a more useful guide to pre-emptive therapy than currently used tests. Data in liver transplant recipients have demonstrated that high CMV viral loads as obtained by quantitative PCR are independently associated with a higher risk of CMV disease and that quantification of CMV DNA in blood has the potential to differentiate between asymptomatic CMV infection and symptomatic CMV disease (43-46). For example, in a study by Macartney et al. (46) using a DNA hybrid capture method, 14/15 patients who

developed CMV disease had CMV DNA levels greater than 50pg/ml while in 86 patients who did not develop CMV disease, only 1 had a DNA level above this cut-off. It is clear that viral quantification may prove quite useful in predicting CMV disease in transplant patients. Other potential uses of quantitative PCR for viral load measurements may include following response to therapy in patients with active CMV disease and potentially predicting disease recurrence in patients who have already had one episode of active CMV disease.

Under the current CMV prophylaxis protocol at The Toronto Hospital, only D+/R- liver transplant patients receive prophylactic intravenous ganciclovir until 12 weeks post-transplant. As previously stated, these patients are at highest risk for developing CMV disease (up to 80% may develop active disease) and therefore universal prophylaxis is employed in this subgroup. Patients who are D+/R+, D-/R+ and D-/R- receive no specific anti-CMV prophylaxis and do not undergo routine monitoring for CMV infection. Based on previous surveillance data at our institution, approximately 20-25% of patients still develop active CMV disease.

In summary, quantitative methods for detecting CMV such as the CMV antigenemia assay and the quantitative PCR test may allow for more accurate prediction of CMV disease in liver transplant recipients and therefore could serve as a useful guide to pre-emptive therapy. Subsequent prevention of CMV disease would alleviate significant morbidity and could result in substantial cost saving.

3. METHODS

A) STUDY POPULATION:

Consecutive patients undergoing liver transplantation at The Toronto Hospital, Toronto, Canada, were enrolled. This center is a university affiliated teaching hospital with a well-established multi-organ transplant program. The Toronto Hospital has considerable experience in liver transplantation and a comprehensive program for the management and treatment of these patients.

B) INCLUSION AND EXCLUSION CRITERIA

INCLUSION CRITERIA

Male or female patients who fulfill the following criteria were eligible for inclusion into this study:

- i) Recipients of a liver transplant
- ii) Able to give written informed consent
- iii) Are willing and able to comply with the protocol
- iv) Age > 16 years

EXCLUSION CRITERIA

The following patients were not eligible for inclusion in the study:

- i) Patients unwilling or unable to give informed consent
- ii) Active CMV disease at the time of study enrollment
- iii) Patients who are seronegative for CMV and receive a liver from a seronegative donor.

C) STUDY DESIGN:

This study was a prospective, observational cohort study in liver transplant recipients. Ethics approval was obtained from The Toronto Hospital ethics committee (Appendix IV). Prior to transplantation, all patients and donors were routinely screened for CMV antibodies as per the standard of care (Abbott AxSYM™ enzyme immunoassay, Abbott Laboratories Ltd, Abbott Park, IL). Patients were then assessed by the principal investigator to determine eligibility. Once consent was obtained from patients, the baseline clinical data was collected as outlined in Appendix III. Patients had 10 mls of blood drawn (2 EDTA lavender top tubes) at regular intervals beginning 2 weeks post-transplant until 12 weeks post-transplant according to the study protocol as outlined in appendix I. Blood samples were taken at every clinic visit. In the majority of patients, this entailed weekly blood sampling for the first 6 weeks post-transplant and then every 2 weeks until week 12. Since

patients had routine bloodwork performed at each clinic visit, at no time was blood drawn exclusively for the purposes of the study. This strategy was used to minimize patient discomfort and to make the study as clinically applicable as possible. Patients who have a prolonged initial hospitalization had bloodwork performed weekly at the time of other routine bloodwork. The first 12 weeks post-transplant was chosen as the period for sample collection because it represents the peak "at-risk" period for the development of active CMV disease (13,14). This also represents the period during which transplant recipients have frequent routine follow-up visits and bloodwork as part of the standard of care.

As previously stated, the current CMV prevention strategy at the study center is to administer ganciclovir (5 mg/kg intravenously once daily or 1000 mg by mouth three times per day) to the D+/R- subgroup of patients until 12 weeks post-transplant. CMV disease is unusual in these patients while receiving ganciclovir but does occur after discontinuation of the drug (32-34). Therefore in this subgroup of patients, blood samples were collected at two week intervals for a six week period after the ganciclovir is discontinued (from week 12 to week 18 post-transplant) and no sampling was done while the patient is on anti-CMV prophylaxis. We did not feel it was clinically practical to extend the period of monitoring past 18 weeks since routine clinic visits occur at a much lower frequency in most patients after this point. Since we wished to determine if these tests would be useful in the clinical setting, we chose 18 weeks as the end of CMV laboratory monitoring for this subgroup.

All blood samples had antigenemia testing and CMV viral load testing. Since routine monitoring was not a part of the standard management in this group of

patients, the treating physician was blinded as to the results of testing. Also, all testing was done by technologists blinded to the clinical status of the patient. Since CMV antigenemia is a routinely available clinical test, antigenemia results which were requested by the treating physician for the purpose of diagnosing symptomatic CMV disease were provided to assist in patient management. Since quantitative PCR is still investigational, these results were not made available to the treating physician even if requested. The quantitative PCR testing was performed in batches to save on reagent and labor costs. As previously stated, patients who are D-/R- for CMV have a very low rate of CMV disease provided they are given CMV negative blood products. Therefore this group of patients was not included in the monitoring protocol.

D) OUTCOMES:

Primary outcome:

Patients were followed for the development of active CMV disease within the first 6 months after transplantation (primary outcome). CMV disease was defined according to standard clinical criteria using case definitions outlined below (13,47). CMV infection was defined as the presence of detectable CMV virus by antigenemia, shell vial culture of blood, or a positive PCR test regardless of clinical manifestations.

CASE DEFINITIONS OF ACTIVE CMV DISEASE:

CMV HEPATITIS:

1. Typical clinical picture: fever with elevated transaminases (AST and ALT > 2 X normal) and
2. Biopsy evidence: CMV inclusion bodies seen on liver biopsy histopathology or positive CMV culture of liver tissue.

CMV GASTROINTESTINAL DISEASE:

1. Typical clinical picture: Gastrointestinal symptoms consisting of diarrhea and/or abdominal pain with no other etiology found. Alternatively, gastrointestinal disease may present as ulceration(s) in the GI tract.
2. Biopsy evidence: same as above

CMV PNEUMONITIS:

1. **Typical clinical picture:** fever, shortness of breath, with interstitial pulmonary infiltrates.
2. **Biopsy/culture evidence:** Bronchoscopy specimen culture positive or CMV inclusion bodies seen, in absence of other etiology.

CMV VIRAL SYNDROME:

Definition: fever (temperature > 38°C) with no other etiological explanation plus 3 of the 4 following criteria:

1. **Leukopenia** with WBC < 3000/ μ l or thrombocytopenia with platelets < 100,000/ μ l on two separate measurements.
2. **Arthralgias / myalgias.**
3. **Blood culture positive for CMV.**
4. **Response to ganciclovir therapy within 48-72 hours (defervescence of fever).**

To avoid diagnostic incorporation bias, the antigenemia or PCR results were not included in any of the criteria for the diagnosis of active CMV disease.

E) LABORATORY METHODS

The laboratory studies were performed at the virology laboratory at The Toronto Hospital in Toronto, Canada and at the virology laboratory at the Massachusetts General Hospital, Boston, United States. Ten mls. of EDTA treated blood were collected at regular intervals (2 lavender top tubes) post-transplant as specified above from each patient enrolled in the study. Samples were transported to the lab within 4-6 hours for processing as follows:

CMV Quantitative PCR Assay

All PCR assays were performed using appropriate precautions and in separate areas to avoid contamination. EDTA blood samples were centrifuged and plasma removed for storage at minus 70⁰C until further testing. PCR testing was done in batches in order to save on reagents and for efficiency. Part of the PCR testing was done at the Massachusetts General Hospital (since the PCR machine needed for this test was initially available at this hospital) and part at the Toronto General Hospital (Approximately two-thirds of testing done at former institute). Quantitative CMV PCR was performed according to manufacturers instructions using the Cobas Amplicor CMV Monitor test (Roche Diagnostic Systems, Inc., Branchburg, NJ, USA). Briefly, 100µl of plasma was added to 400µl of Cobas Amplicor lysis buffer and incubated at 60⁰C for 10 minutes. Then 500µl of isopropyl alcohol was added and the specimen centrifuged at 13000 x G for 15 minutes. The supernatant was

removed and 70% ethanol added to the cell pellet. Tubes were then centrifuged again at 13000 x G for 5 minutes. Supernatant were then removed and the DNA pellet resuspended in specimen diluent. Then 50µl of this solution was added to 50µl of PCR master mix. Amplification and detection were all conducted using the Cobas Amplicor system as per manufacturers instructions. Results were recorded as number of viral copies per ml. The lower limit of detection was approximately 400 copies/ml. For the purposes of the primary analysis, this level of viral load was considered a positive test result.

CMV Antigenemia assay:

Specimens for antigenemia testing may degrade quickly, and therefore should be processed within 2 hours of collection. Delays in processing can lead to an erroneously negative result. For this reason a system was set up such that bloodwork collected in the morning clinic was received and processed in the virology lab before 11:00 am. Preparation and staining of polymorphonuclear leukocytes (PMNL) cytopins was carried out according to methods previously described.(41,42) The PMNL fraction of leukocytes was obtained using 5% dextran sedimentation. Contaminating RBCs were lysed using an ammonium chloride solution and the cells were washed twice in PBS. The number of PMNL were counted and cytopsin preparations made using 100 µl of a suspension of 2.0×10^6 cells /ml. The slides were fixed in formaldehyde and stained using monoclonal antibodies directed against the pp65 lower matrix phosphoprotein of CMV. An immunofluorescence technique was used. The number of antigen positive cells

were recorded and expressed as the number of positive cells per 150,000 cells examined (positive cells per slide). For the primary analysis a result of ≥ 1 positive cell per slide was considered a positive test result.

F) SAMPLE SIZE CALCULATIONS

The original study sample size estimates were based on the study being performed at two sites: London, Ontario and Toronto, Ontario. However, problems with blinding and contamination at the London site resulted in that center being dropped from the study. The study period was therefore prolonged by a few months to allow adequate enrollment. The total number of liver transplants performed at the Toronto Hospital is approximately 85 per year. From previous surveillance data at this institution, it was expected approximately 5-10 of these patients would be CMV seronegative and receive an organ from a seronegative donor. Since these patients are at a lower risk of CMV disease, they were not included in the study. Therefore 75-80 patients per year were expected to be available for enrollment. It was expected from previous surveillance data at our institution that the rate of active CMV disease in this group of patients would be approximately 20-25%. It was predicted that in those patients with a positive antigenemia or a positive quantitative PCR result, at least 50% would develop subsequent CMV disease (38-40,48-52). Based on previous studies, patients with negative assays were estimated to have a disease rate of between 0-10%. As a conservative estimate, at least one quarter of patients were expected to have a positive antigenemia or viral load at some point.

Therefore with a 95% confidence level ($\alpha=0.05$) and a power of 80% ($\beta=0.20$), a total sample size of 60 patients was calculated. This sample size would be sufficient to detect a 5-fold relative risk of developing CMV disease in patients who had a positive test. Since the above PPV and NPV are estimates, and the true predictive values were not known, the aim was to enroll 90 patients over a one and a half year period to ensure an adequate number of outcomes and adequate power.

G) ANALYSIS

i) Diagnosis of CMV disease:

To assess levels of viremia in patients with active CMV disease, peak viral load and antigenemia levels were compared in patients who developed symptoms (CMV disease) versus asymptomatic patients with a positive PCR test or a positive antigenemia test respectively. Peak viral load in patients with symptomatic disease were also compared to all asymptomatic patients regardless of whether they had a positive test result or not. All comparisons were done using the Mann-Whitney U test.

ii) Prediction of CMV disease

For prediction, the sensitivity, specificity, and positive and negative predictive values (PPV, NPV) were calculated for the ability of these assays to predict CMV disease using 2 x 2 tables. Since prediction of subsequent CMV

disease was the goal, only test results taken > 3 days prior to the development of active disease were used for this analysis. The > 3 day cut-off was chosen *a priori* because it was felt that if one of these tests were to be employed in a pre-emptive strategy in the clinical setting, this amount of warning time would be sufficient to start pre-emptive anti-viral therapy prior to the development of symptoms. For the initial analysis, a positive PCR test was defined as a viral load above the lower limit of detection for the assay (approximately 400 copies/ml) and a positive antigenemia was defined as ≥ 1 positive cell per slide. Since these assays are quantitative, sensitivity and specificity for different cut-off levels were calculated and used to generate ROC curves (receiver-operating characteristic curves)(53). Receiver operating characteristic (ROC) curves were generated by plotting the sensitivity of the test against 1-specificity using various positive cut-off points. An ideal diagnostic test would have a sensitivity and specificity of 1.0 and therefore would include the maximum area under a ROC curve.

iii) Risk factors for CMV disease

Risk factors for the development of CMV disease were assessed using a corrected χ^2 or Fisher's exact test for categorical variables. Continuous variables were analyzed using a Mann-Whitney U test. Factors analyzed in addition to peak viral load and peak antigenemia included acute rejection, immunosuppressive therapy, antilymphocyte products, and pre-transplant donor/recipient CMV serostatus. For the multivariate analysis, variables that were associated with CMV disease ($p < 0.10$) on univariate analysis were included

and analyzed using a logistic regression model. For the multivariate model, peak antigenemia and peak viral load were analyzed in two separate models since there was a strong correlation between these two variables. No significant interactions were identified between the variables used for the multivariate analysis. All database entries and statistical analysis was performed using SPSS version 7.5.

H) ETHICAL CONSIDERATIONS

There was virtually no risk to the patients as a result of enrollment in this study. Other than the minimal risk of a venipuncture, there were no invasive procedures, interventions or changes in patient management. In addition, at no time was bloodwork taken for the sole purpose of the study. Patients were managed as per the liver transplantation protocols and no changes were made to the current CMV prevention protocol. CMV disease was managed as per the responsible physician using standard therapy. Patient identifying data was available only to study personnel and was kept strictly confidential.

4. RESULTS

A) ENROLLMENT AND BASELINE DATA

A total of 111 transplant patients were evaluated for enrollment. Seven patients were excluded because they did not meet study criteria (Donor and recipient CMV seronegative prior to transplant). Three patients died in the immediate post-transplant period before more than a single sample could be obtained and these patients were excluded from the analysis. Consent could not be obtained or was refused by an additional 4 patients. A total of 97 patients (57 male, 40 female) were enrolled and provided data for analysis. A total of 640 samples were collected (median 6 per patient; range 3-15). All patients were followed until death or 6 months post-transplant. 9/97 (9.3%) patients died within the first 6 months at a mean of 136 days (range 42 – 173 days). No patient died from CMV disease. Underlying disease included hepatitis C (n=32), hepatitis B (n=7), primary sclerosing cholangitis (n=10), primary biliary cirrhosis (n=4), alcoholic liver disease (n=6), cryptogenic cirrhosis (n=16), and others (n=22) (see Table 1). Mean age was 51.2 years (median 51 years ; range 18 – 68 years). Induction immunosuppression consisted of either cyclosporin / prednisone (n=48), tacrolimus / prednisone (n=2), or cyclosporin / prednisone plus either mycophenolate mofetil or immuran (n=38). For the purpose of analysis, the above immunosuppression groups were divided into double and triple induction therapy. Pre-transplant donor (D) and recipient (R) CMV serostatus was as follows: D+/R-: n =12, D+/R+: n=40, and D-/R+: n=45. CMV

infection occurred in 61/97 (62.9%) and was symptomatic (CMV disease) in 21/97 (21.6%) of patients. CMV disease was manifest as CMV hepatitis (n=5), CMV gastrointestinal disease (n=4), and CMV viral syndrome (n=12). CMV disease occurred at a mean of 60.3 days post-transplant (median 46 days; range 22-150 days). Characteristic viral load and antigenemia patterns of 4 symptomatic patients are shown in Figures 2a and 2b.

B) DIAGNOSIS OF ACTIVE DISEASE

Peak plasma viral load and antigenemia levels are shown in Figure 3 and Figure 4 in patients with asymptomatic CMV infection (n=40) and those with symptomatic CMV disease (n=21) (peak level at time of diagnosis). The mean peak viral load was 73,715 copies/ml (median 55,100; range 9230 – 195,000 copies/ml) in patients with CMV disease compared to 3615 copies/ml (median 1820; range 328-15,900 copies/ml) in those with asymptomatic CMV infection and 1903 copies/ml (median 400; range 0-15,900) in all patients without CMV disease (p<0.001). Mean peak antigenemia level was also increased in patients with CMV disease vs. asymptomatic CMV infection (121.8 vs 6.4 positive cells/slide; p<0.001) and compared to all patients without CMV disease ((121.8 vs. 2.9 positive cells/slide; p<0.001).

C) PREDICTION OF CMV DISEASE

The prediction of CMV disease was the primary objective of this study. Test results taken at least > 3 days prior to CMV disease development were analyzed for their ability to predict active CMV disease. When the lower limit of detection for the PCR assay (~ 400 copies/ml) was used as the cut-off value for defining a positive test, the sensitivity was 100%, specificity 47.4%, PPV 34.4 % and NPV 100% for the prediction of CMV disease. PCR was able to predict the development of disease in all 21 patients. Increasing the cut-off value resulted in improved specificity and PPV but decreased sensitivity and NPV as shown in the ROC curve in Figure 5. The optimal cut-off value for predicting CMV disease was in the range of 2000-5000 copies/ml. At > 2000 copies/ml the PPV for PCR was 50% and the NPV was 96.6 %. Nineteen of 21 (90.5%) cases of CMV disease would have been predicted. At > 5000 copies/ml the PPV increased to 64.3% and the NPV decreased to 95.7%. Eighteen of 21 (85.7%) of cases of CMV disease would have been predicted.

The sensitivity, specificity, PPV and NPV for antigenemia (positive ≥ 1 cell/slide) was 95.2, 55.3, 37.0 and 97.7 % respectively. Antigenemia predicted 20/21 cases of CMV disease. Sensitivity and specificity for different levels of antigenemia are shown in the second ROC curve in Figure 6. The optimal cut-off for predicting CMV disease was in the range of 4-6 positive cells/ slide. At > 4 positive cells/slide the PPV for antigenemia was 50.0% and the NPV was 96.6 %. Nineteen of 21 (90.5%) cases of disease would have been predicted. At > 6 positive cells/slide the PPV increased to 60.7% and the NPV decreased to 94.2 % with 17/21 (81%) cases of CMV disease predicted.

Choosing the optimal cut-off point based on the examination of the ROC curve for a particular test requires several important considerations (57). Higher cut-off values will result in improved specificity for the test but sensitivity will suffer, and vice versa. The ideal ROC curve would intersect the top right hand corner at which point specificity and sensitivity would both be 100%. Unfortunately such tests are rare in clinical medicine. When choosing a cut-off point on a ROC curve, one of the most important factors to consider is the purpose of the test. For example, in a screening test, sensitivity is usually quite important and therefore a cut-off point further along the curve would most likely be chosen. One must also take into account the consequences of a positive test – i.e. is there effective treatment that can alter the outcome of the disease and are there consequences to labeling the patient as having a particular disease? One must also take into account the results of a false negative test. For example, if the disease is missed will it have disastrous consequences for the patient?

In this situation, the purpose of the test is to screen patients to detect asymptomatic infection and prevent the development of disease. There exists an intervention (ganciclovir) that can change the natural history of infection (i.e. prevent the development of symptomatic disease) although side effects may be troublesome. The consequences of missing a case of disease are not disastrous as demonstrated in this cohort of patients (all 21 patients responded to ganciclovir therapy with no patient dying from CMV disease). Therefore a test cut-off with fairly high sensitivity and reasonable specificity is appropriate. At a cut-off of 5000 copies/ml or 6 positive cells/slide only 3-4 cases of disease would have been

missed, and most patients would have been spared unnecessary ganciclovir therapy. Using a higher cut-off would have resulted in a clinically unacceptable number of cases being missed (for example 6/21(28.6%) cases missed with a cut-off >7000 copies/ml and 8/21 (38.1%) missed for cut-off>10,000 copies/ml). A lower cut-off point would result in many false-positives leading to unnecessary ganciclovir therapy in a pre-emptive strategy.

The time from the first positive PCR to the development of active disease (lead-time) was 21.5 ± 17.0 days (median 14 days, range 8 – 83 days). Lead-time for the antigenemia assay was slightly lower at 18.4 ± 15.3 days (median 14 days; range 0-71 days) ($p=0.052$ compared to lead time for PCR assay; paired t-test).

D) RISK FACTORS FOR CMV DISEASE / MULTIVARIATE ANALYSIS

Peak viral load levels and peak antigenemia levels were analyzed for their ability to predict CMV disease in a multivariate logistic regression model which included other risk factors for CMV disease. Since the predictive value was being assessed, only levels of viral load and antigenemia prior to the development of CMV disease were used. Variables with a p value of <0.10 on univariate analysis were included in the multivariate logistic regression. Variables that were analyzed in the univariate analysis included induction immunosuppression, pre-transplant serostatus, the use anti-lymphocyte antibody, and acute rejection. These variables were chosen because they have been associated with the development of CMV disease in previous studies (14,15) and because they are in keeping with the proposed

pathogenesis of CMV disease following transplantation (figure 1). In the univariate analysis, peak viral load, peak antigenemia, the development of acute rejection, and the use of three vs. two drugs for induction immunosuppression were significant risk factors for the development of CMV disease.

The multivariate model included CMV serostatus, induction immunosuppression, acute rejection, and the viral load or antigenemia (variable with a $p < 0.10$ on univariate analysis). Two separate multivariate analyses were performed, the first with peak viral load and the second with peak antigenemia. In the first analysis, peak viral load was the only significant predictor of CMV disease ($p = 0.0001$; OR = 1.40 for every 1000 copies/ml increase in viral load; 95% CI 1.11-1.49). In the second analysis, peak antigenemia was the only significant predictor of CMV disease ($p = 0.0007$; OR = 1.17 for every 1 positive cell/slide increase in antigenemia; 95% CI 1.07-1.27).

Since peak viral load and peak antigenemia can by definition only be determined retrospectively, a more operational multivariate analysis would include a prospectively evaluable viral load and antigenemia cut point. Two additional multivariate models were done using the same variables as previous but including the categorical variables viral load > 5000 copies/ml and antigenemia > 6 cells/slide respectively. These were chosen as cut-off points based on the ROC curves for each test. Results of the multivariate analyses are shown in Table 6. Again both viral load and antigenemia were highly significant independent predictors of CMV disease in both analyses with odds ratios of 33.13 (CI 7.32-149.74) and 31.45 (CI 5.73-172.68) respectively ($p \leq 0.0001$).

E) COMPARISON OF PCR AND ANTIGENEMIA

The CMV viral load (by PCR) was highly correlated with the level of CMV antigenemia ($r=0.80$; $p<0.01$) as was the peak viral load and antigenemia in each patient ($r=0.86$; $p<0.01$). Eight patients had a positive viral load with consistently negative antigenemia assay. The viral load was low in these patients (mean 677 copies/ml; median 541 copies/ml; range 328 – 1530). None of these patients developed symptomatic CMV disease. Two patients had a positive antigenemia with consistently negative viral loads. In both patients, the antigenemia was positive only on a single occasion and at a level of 1 positive cell/ slide, and neither patient developed active disease.

F) RESPONSE TO THERAPY

All patients diagnosed with CMV disease received treatment with intravenous ganciclovir for a minimum of two weeks. Patients had follow-up viral loads and CMV antigenemia testing on a regular basis after commencing treatment. Of 21 patients, 18 cleared their CMV as documented by negative PCR and antigenemia. Mean time to first negative viral load was 41.5 days (median 33.5 days; range 9-90 days) and mean time to first negative antigenemia was 23.7 days (median 20; range 9-60 days) ($p = 0.01$ compared to viral load; paired t-test). Two patients who did not clear their antigenemia and PCR, had recurrent CMV disease (CMV viral syndrome) at 39 days and 48 days respectively after the first episode of CMV disease. Both patients responded to

a repeat course of ganciclovir. A third patient died of unrelated causes (recurrent hepatocellular carcinoma) prior to clearing CMV. No autopsy was carried out. One additional patient had recurrent CMV disease (CMV retinitis) 6 months after an episode of CMV colitis. Mean viral load at the onset of CMV disease was 142,200 copies/ml in the patients who recurred vs. 62,300 copies/ml in those who did not ($p= 0.047$). Peak antigenemia was not significantly different in the two groups (148 vs. 117 positive cells/slide; $p=0.53$).

5. DISCUSSION

Options for prevention of CMV disease after organ transplantation include universal prophylaxis or pre-emptive therapy. Although universal intravenous or oral ganciclovir prophylaxis for approximately 12 weeks post-transplant has been shown to be effective for the prevention of CMV disease (32,33), disadvantages to this strategy include over-treatment with potential adverse effects of anti-viral therapy, high cost, and the potential for emergence of ganciclovir resistance. This is especially true in patients who are D-/R+ or D+/R+ who have a significantly lower incidence of CMV disease compared to the D+/R- subgroup of patients. The latter group has a sufficiently high risk of CMV disease (up to 80%) to mandate routine universal prophylaxis (24). CMV disease in lower risk subgroups may occur in only 10-25% of patients and therefore a pre-emptive strategy targeting only those patients likely to develop disease would be more useful than universal prophylaxis.

A) INTERPRETATION OF FINDINGS

For a diagnostic test to be employed in a pre-emptive strategy, it must accurately predict which patients will and won't develop active CMV disease. In this cohort of 97 liver transplant recipients, we examined the utility of plasma viral load measurement by quantitative PCR compared with the antigenemia assay for predicting CMV disease. The plasma viral load was very sensitive for predicting the development of CMV disease. All 21 patients who developed CMV disease had a

plasma viral load exceeding the lower limit of detection (~ 400 copies/ml) which preceded the onset of symptoms by a mean of 21.5 days. Antigenemia was slightly less sensitive but was able to predict the development of disease in 20/21 patients a mean of 18.4 days prior to symptoms. Both assays were not very specific and PPVs were 34.4% and 37.9% respectively. This reflects the fact that many patients will spontaneously clear low-level CMV replication. Increasing the cut-off value for a positive test resulted in improved specificity with decreased sensitivity as shown in the ROC curves in Figures 5 and 6. The optimal cut-off for which to initiate pre-emptive therapy was in the range of 2000-5000 copies/ml for quantitative PCR, and 4-6 positive cells per 150,000 for the antigenemia assay. These higher cut-off values would still allow one to predict most cases of CMV disease, while improving the specificity and PPV for these tests.

In a multivariate logistic regression analysis, the only significant predictors of CMV disease were the peak viral load by quantitative PCR and the peak antigenemia level (analyzed in separate logistic regression models). Peak viral load and antigenemia levels were also significantly higher in patients with symptomatic CMV disease vs. asymptomatic CMV infection as shown in Figures 3 and 4. The increased risk of CMV associated with acute rejection episodes and heavier immunosuppressive regimens, was explainable by an increase in CMV viral load with subsequent development of disease. Donor and recipient CMV serostatus was not a significant risk factor for CMV disease. This was likely because the high risk D+/R- group received 12 weeks of ganciclovir prophylaxis resulting in a disease rate

similar to the low risk D-/R+ group. The use of antilymphocyte product was not associated with CMV disease as has been shown in previous studies evaluating OKT3 for the treatment of acute rejection (25). This may be because antilymphocyte product was used for treatment of acute rejection in only two patients, while the remaining patients received antilymphocyte product (usually rabbit anti-thymocyte serum) for induction immunosuppression.

B) COMPARISON WITH OTHER METHODS

Other methods that have been evaluated for predicting CMV disease include culture based tests, and qualitative PCR. Culture methods have generally been disappointing in predicting CMV disease (28,37). Badley et al. (28) studied the predictive value of routine CMV blood cultures in 126 consecutive liver transplant recipients. Of these, 29 patients (23%) had end-organ CMV disease. However, viremia preceded the onset of disease in only 9/29 (31%) of patients. Untreated viremia was followed by CMV disease in only 32 % of patients (PPV 32%) and the test appeared most useful in the D+/R- subgroup of patients who were not routinely given prophylaxis in this study. PPV values were even lower in the D+/R+ group and the D-/R+ group (22% and 11% respectively). In another study of 156 liver transplant patients, positive and negative predictive values were only 26% and 74% for urine cultures and 32% and 76% for throat cultures (37). Therefore, although evidence exists that a pre-emptive strategy using one or more of these culture based tests could reduce rates of CMV disease (36), a more sensitive and specific

test should be able to reduce rates of CMV disease even further making culture based tests of minimal use in a pre-emptive strategy for CMV prevention.

In studies following patients with sequential qualitative PCR testing, positive predictive values (PPV) are in the range of 45-75% (38-40) when evaluated in high disease prevalence patient groups. The best PPV is in patients with very high rates of CMV disease, i.e. in D+/R- transplants; in all other groups of patients positive predictive values for qualitative PCR are quite poor (in the range of 25-45%) (38,39). Studies evaluating the CMV antigenemia assay have reported positive predictive values in the range of 57-72% and negative predictive values of 95-100% (39,48-52) However, methodological problems with these studies include: a) relatively small number of patients (39,48), b) too few outcomes, especially in lower risk subgroups (48,49), c) administration of some form of anti-CMV prophylaxis during the period of monitoring (39), d) potential lack of blinding, and e) a heterogeneous patient populations (i.e. combining liver, heart, lung, kidney transplants). Our results confirm the high sensitivity and NPV of the antigenemia assay. We observed a lower specificity and PPV than previously reported, possibly due to evaluation of a lower risk group with a decreased prevalence of CMV disease.

Cope et al (45) determined serial viral loads in 47 liver transplant recipients of whom 20 had CMV disease. He determined that peak viral load was a significant independent risk factor for the development of CMV disease illustrating the central role of viral load in the pathogenesis of CMV disease. However, the predictive value was not assessed in that study. Mendez et al (54) examined the utility of quantitative PCR in 43 liver transplant recipients. Again, viral load was found to be significantly

higher in patients with active CMV disease compared with asymptomatic CMV infection. However, the predictive value of PCR in the low risk D+/R+ and D-/R+ groups could not be assessed due to a low number of outcomes of CMV disease. Roberts et al. (43) serially tested 50 renal transplant recipients, 23 of whom developed active CMV disease. They found that by using a threshold of >1000 copies per 100,000 leukocytes, the sensitivity of their assay was 65% and specificity 59% for subsequent prediction of disease. The results of this study suggest that the plasma based PCR assay which we utilized has somewhat better predictive value. For example, at a cut-off of > 5000 copies per/ml the sensitivity and specificity of the test are 85.7% and 86.8% respectively. However, since the two assays use different methodologies they are not directly comparable. The PCR assay we utilized has the advantage of being commercially available and would permit standardized testing across laboratories.

C) STRENGTHS AND LIMITATIONS

Our study had several strengths. First, patients were not given any routine anti-viral prophylaxis except for the high risk D+/R- subgroup. This latter subgroup received oral or intravenous ganciclovir for 12 weeks post-transplant. Therefore, monitoring by PCR and antigenemia was only performed for a period of 6-8 weeks after ganciclovir was discontinued. Second, the results of all antigenemia testing, and PCR testing were not revealed to the treating physician, and therefore, patients did not receive pre-emptive therapy based on these results. This allowed for a true assessment of the predictive value of these tests. Finally, solid organ transplants

other than liver recipients were not included in this study, resulting in a more homogeneous study sample. Limitations of our study include the relatively small number of events (21 cases of CMV disease). This did not permit an analysis of differences in CMV development according to underlying pre-transplant liver disease or on the basis of exact immunosuppressive regimens (the latter was divided into two and three drug group, with antilymphocyte product use analyzed separately).

D) MINIMIZATION OF BIAS

Studies aimed at investigating the efficacy of diagnostic tests have often produced misleading results. Tests that were initially regarded as valuable were later rejected as worthless when used in actual clinical practice. Biases that occur in the study design and implementation are one of the important reasons for this. For any test, when the table of results is created to calculate the statistics, two things must be determined about the patient: 1) the status of the test as positive or negative, and 2) the status of the disease as present or absent. If these two determinations are not made independently, several biased or erroneous statistical associations may give the test a falsely high efficacy (55). The most common biases that affect studies evaluating diagnostic tests include:

i) Diagnostic incorporation bias

This type of bias occurs when the result of the test is actually incorporated into the evidence used to diagnose the disease (55). Since the evidence used for the diagnosis should be independent of the test result, such incorporations will bias the

apparent accuracy of the test (make the test seem more accurate than it really is). This could have been a potential problem in this study since CMV antigenemia is commonly employed as a test to diagnose active CMV disease. In cases of end-organ CMV disease (hepatitis and colitis in this study), the diagnosis is independent of the results of blood tests (diagnosis confirmed by tissue biopsy) and therefore diagnostic incorporation bias was not felt to be a significant problem. However, the diagnosis of CMV viral syndrome is often more subjective and usually based on clinical symptoms and the demonstration of CMV replication (usually within the blood). The test most commonly employed at this institution to aid in this diagnosis is actually the CMV antigenemia assay. To avoid this type of bias, the definition of CMV viral syndrome was instead based on a group of clinical symptoms that are seen with CMV, a clinical response to anti-viral therapy, and on the basis of the CMV blood culture test rather than the antigenemia or PCR test. Neither of these latter tests was incorporated into the diagnostic criteria for the purposes of this study.

ii) Work-up bias

This type of bias occurs when the results of a test affect the subsequent clinical work-up needed to establish the diagnosis of a disease (55). Therefore, a positive test result may make the treating physician look intensely for a disease that would otherwise be undetected, and a negative result may cause the diagnosis to be missed because the additional tests are not ordered. This problem in the differential intensity of the diagnostic work-up can be avoided if the test result is not known

when the work-up for disease is done. This type of bias can lead to underdiagnosis but not to overdiagnosis. The statistical consequence is a high a falsely high sensitivity and negative predictive value for the test under evaluation. In this study, the PCR test results were at no time known to the physician and therefore could not be used for clinical decision making. However, the antigenemia test was available when physicians suspected clinical disease which may have resulted in pursuing tests such as biopsies. However, it is unlikely that any cases of CMV disease were missed because of this type of bias, since the natural history of invasive CMV disease usually results in progressive illness eventually leading to a diagnosis.

iii) Diagnostic-review bias

After the diagnostic work-up has been completed, a second type of bias can occur if the result of the test affects the subjective review of the data that establish the diagnosis. This bias can cause overdiagnosis as well as under diagnosis and can be avoided by blind interpretation of the data used to establish the diagnosis (55).

iv) Test-review bias

The preceding types of bias can occur when the test is done before the diagnosis is established. Test-review bias can arise if the test is done after the diagnosis is established. A test that is interpreted subjectively can be biased by the knowledge of the diagnosis (55). This was not felt to be an issue in this study since all testing was done blinded to the patients' clinical status.

E) RESPONSE TO THERAPY

All 21 patients with active CMV disease were treated with intravenous ganciclovir with good clinical response. Plasma viral load took significantly longer to fall below the detection threshold compared with the time required for the antigenemia assay to become negative (41.5 days vs. 23.7 days; $p = 0.01$). This may reflect increased sensitivity of plasma PCR compared to the antigenemia assay or may be because the former detects plasma DNA, while the CMV antigenemia assay stains for neutrophil associated pp65 CMV matrix protein. Recurrent CMV disease was noted in 2 of 3 patients who failed to clear their virus both by antigenemia and PCR, suggesting that both these tests are useful for monitoring therapeutic response. Also, the viral load at onset of disease was significantly higher in the 3 patients that recurred vs. those who did not (142,200 vs. 62,300 copies/ml; $p=0.047$), while antigenemia level at onset of disease was not significantly different in these two groups. Therefore, quantitative PCR may be useful for identifying a subset of patients with CMV disease and very high viral loads who should receive more prolonged antiviral therapy or undergo closer monitoring for recurrent CMV.

F) CONCLUSIONS

In summary, CMV plasma viral load measurement by a quantitative PCR was useful for predicting the development of CMV disease in a cohort of liver transplant recipients. It appears to be similar to, or slightly more sensitive than the antigenemia assay. Either assay would be useful for using in a pre-emptive strategy using positive cut-off values that optimize sensitivity and specificity for the prediction of CMV disease. Also, in a multivariate analysis, the circulating viral burden as measured by quantitative PCR or CMV antigenemia seems to be the most important predictor of subsequent CMV disease development.

G) FUTURE DIRECTIONS

Further studies should focus on using one of these tests in a pre-emptive strategy to determine the efficacy for prevention of CMV disease. Ideally this should be in the form of randomized controlled trials comparing different pre-emptive strategies or a strategy of pre-emptive therapy vs. universal prophylaxis. The current study is insufficient in itself to recommend the use of a specific pre-emptive strategy for CMV prevention. Instead it lays the groundwork for the development of further clinical trials to evaluate effectiveness of pre-emptive vs. universal strategies. Factors that need to be further evaluated include the choice, duration and route of an anti-CMV drug used in a pre-emptive strategy.

Although the relative efficacy of different strategies will need to be determined, the cost-effectiveness of a specific strategy is also of major importance. The major additional costs involved in a pre-emptive strategy are those of the

monitoring test itself. The cost per antigenemia test (including labor) is in the range of \$20-40 while the PCR assay is closer to \$100 per test. Performing multiple routine tests on every patient could prove very expensive. However, if a pre-emptive strategy resulted in fewer cases of CMV disease (with the subsequent costs of treatment and diagnosis), the pre-emptive strategy could prove significantly less expensive than no preventative strategy. On the other hand, a universal prophylaxis study would likely prevent most cases of disease and not require the additional costs of laboratory monitoring. However, ganciclovir prophylaxis, either given intravenously or orally, is extremely expensive (\$4500 - \$7000 for a 12 week course), and this would have to be taken into account. Clearly many factors come into play in a cost-effectiveness analysis of different strategies for CMV prevention and these need to be analyzed in prospective comparative trials. Only then, will the relative efficacy and costs of different strategies be known and thus allow physicians to recommend general policies for CMV prevention after organ transplantation.

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Table 1: Baseline characteristics of patients.

Variable	Number of patients (%) (n=97)
Age (mean \pm S.D.)	51.2 \pm 10.3
Sex (M/F)	57/40
Underlying liver disease (%)	
Hepatitis C	32 (33.0)
Hepatitis B	7 (7.2)
Primary sclerosing cholangitis	10 (10.3)
Primary biliary cirrhosis	4 (4.1)
Alcoholic cirrhosis	6 (6.2)
Cryptogenic cirrhosis	16 (16.5)
Other	22 (22.7)
Donor and recipient CMV status (%)	
D+/R-	12 (12.4)
D-/R+	45 (46.4)
D+/R+	40 (41.2)
Induction Immunosuppression (%)	
Cyclosporin / prednisone	56 (57.8)
Tacrolimus / prednisone	2 (2.1)
Cyclosporin / prednisone + MMF or immuran (triple therapy)	39 (40.2)

Table2: The occurrence of cytomegalovirus (CMV) disease based on recipient and donor pre-transplant CMV serology.

CMV serostatus*	CMV disease (%)	No CMV disease (%)	Total Patients
D+/R-	2 (16.7)	10 (83.3)	12
D-/R+	6 (13.3)	39 (86.7)	45
D+/R+	13 (32.5)	27 (67.5)	40

D = Donor pre-transplant CMV serology; R = recipient pre-transplant CMV serology.

TABLE 3: Type of CMV disease in study patients.

CMV serostatus*	Type of CMV disease			
	Viral syndrome	CMV hepatitis	CMV colitis	Total
D+/R-	2	0	0	2
D-/R+	3	2	0	5
D+/R+	6	4	4	14

N=21

***D = Donor pre-transplant CMV serology; R = recipient pre-transplant CMV serology.**

TABLE 4: Univariate analysis of risk factors for the development of active cytomegalovirus (CMV) disease.

Factor	CMV disease (n=21)	No disease (n=76)	P-value
CMV serostatus (N %)			
D-/R+	6 (28.6)	39 (51.3)	
D+/R-	2 (9.5)	10 (13.2)	
D+/R+	13 (61.9)	27 (35.5)	0.091
Peak viral load (prior to disease) Mean \pm S.E.	33624 \pm 10126	1902 \pm 389	< 0.001
Peak antigenemia (prior to disease) Mean \pm S.E.	39.5 \pm 22.2	2.9 \pm 0.6	< 0.001
Acute rejection (N%)	10 (47.6)	17 (22.4)	0.044
Induction immunosuppression (N%)			
Double Therapy	7 (33.3)	51 (67.1)	
Triple therapy	14 (66.6)	25 (32.9)	0.011
Antilymphocyte product (N %)	6 (28.6)	14 (18.4)	0.48

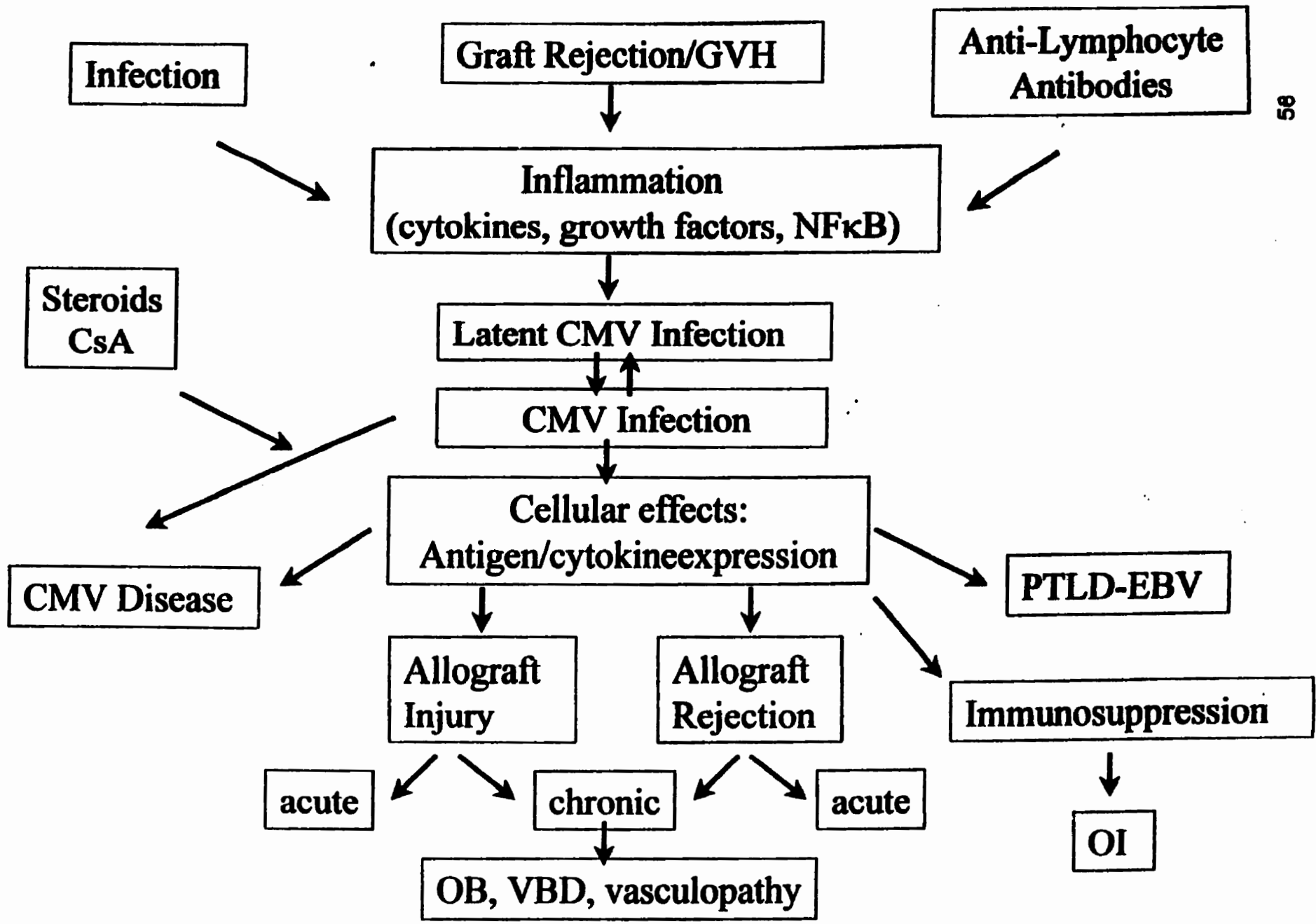
TABLE 5: Multivariable analysis of risk factors for the development of active cytomegalovirus (CMV) disease.

Factor	P value	OR (95% CI)
CMV serostatus		
D-/R+	P = 0.57	
D+/R-		
D+/R+		
Peak viral load		
(prior to disease)	P = 0.0001	OR = 1.40 (1.11-
Mean ± S.E.		1.49) †
Peak antigenemia		
(prior to disease)	P = 0.0007	OR = 1.17 (1.07-
Mean ± S.E.		1.27) ‡
Acute rejection		
	P = 0.35	
Induction immunosuppression		
Double Therapy	P = 0.11	
Triple therapy		

† Odds ratio for every 1000 copies/ml increase in viral load; ‡ Odds ratio for every 1 positive cell/slide increase in antigenemia.

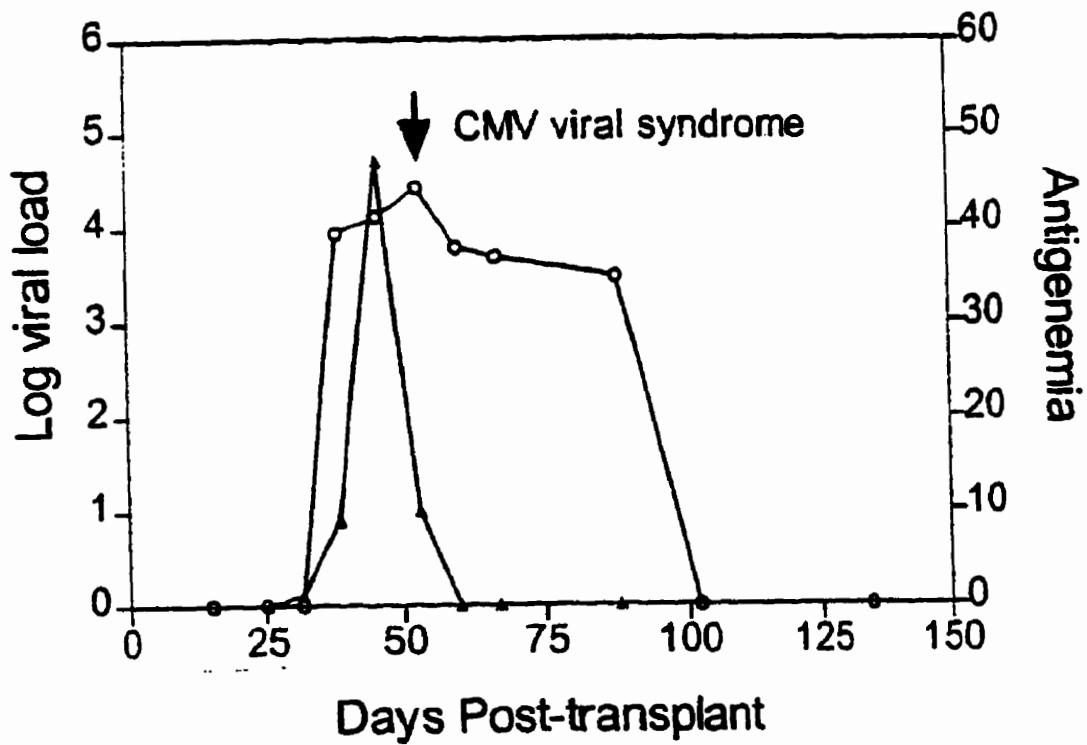
Table 6: Multivariable analysis of risk factors for the development of active cytomegalovirus (CMV) disease using operational definitions of viral load and antigenemia (viral load cut-off >5000 copies/ml or antigenemia > 6 positive cells/slide).

Factor	P value	OR (95% CI)
CMV serostatus		
D-/R+	P = 0.41	
D+/R-		
D+/R+		
Viral load > 5000 (prior to disease)	P < 0.0001	OR = 33.13 (7.32-149.74)
Antigenemia > 6 (prior to disease)	P = 0.0001	OR = 31.45 (5.73-172.68)
Acute rejection	P = 0.48	
Induction immunosuppression		
Double Therapy vs. Triple therapy	P= 0.03	OR = 4.85 (1.16-20.30)

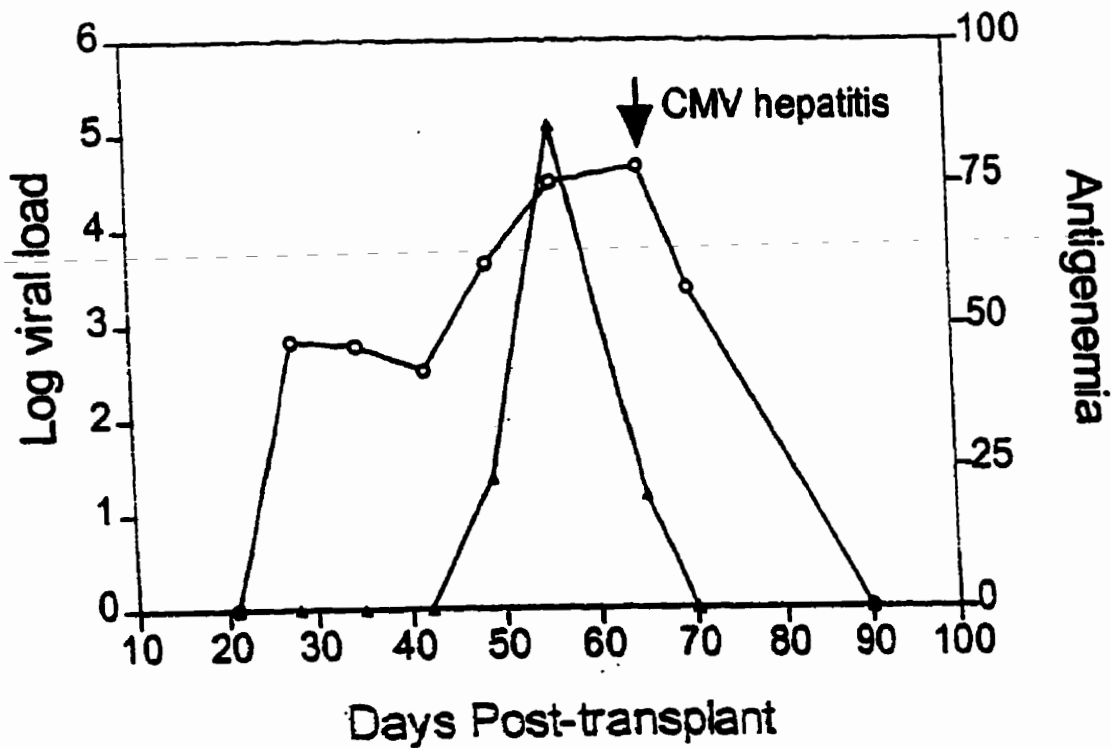


four patients who developed active CMV disease (shown by arrow). CMV viral load shown in open circles (-O-) in log copies/ml. CMV antigenemia shown in closed triangles (-▲-) in number of positive cells/slide.

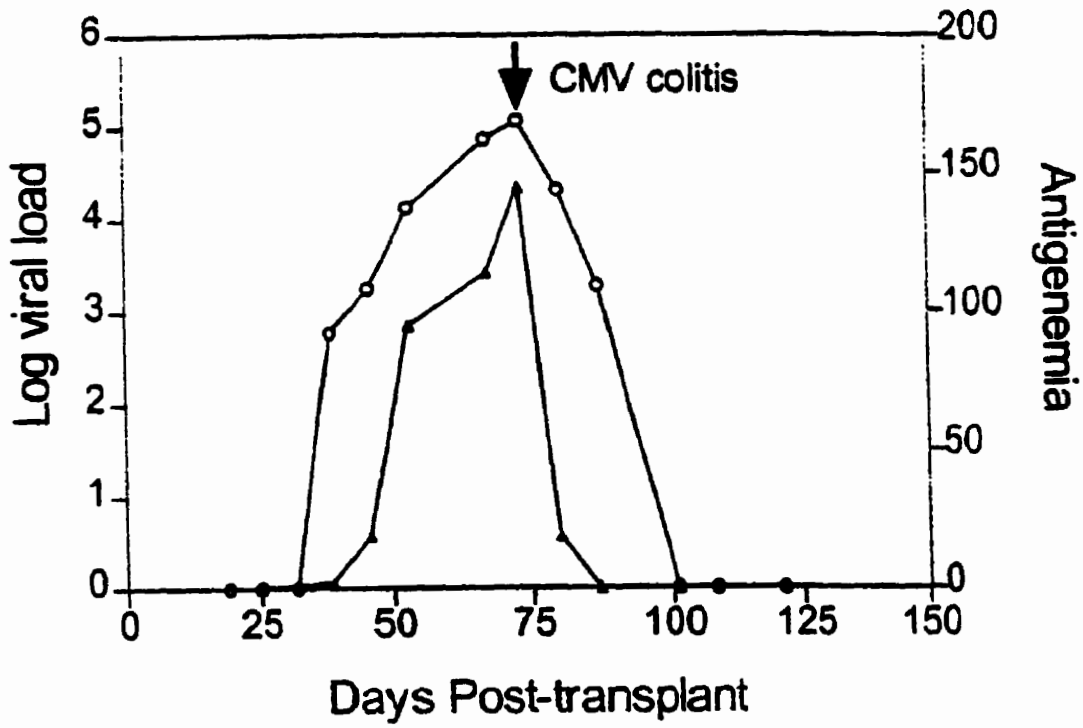
PATIENT 1



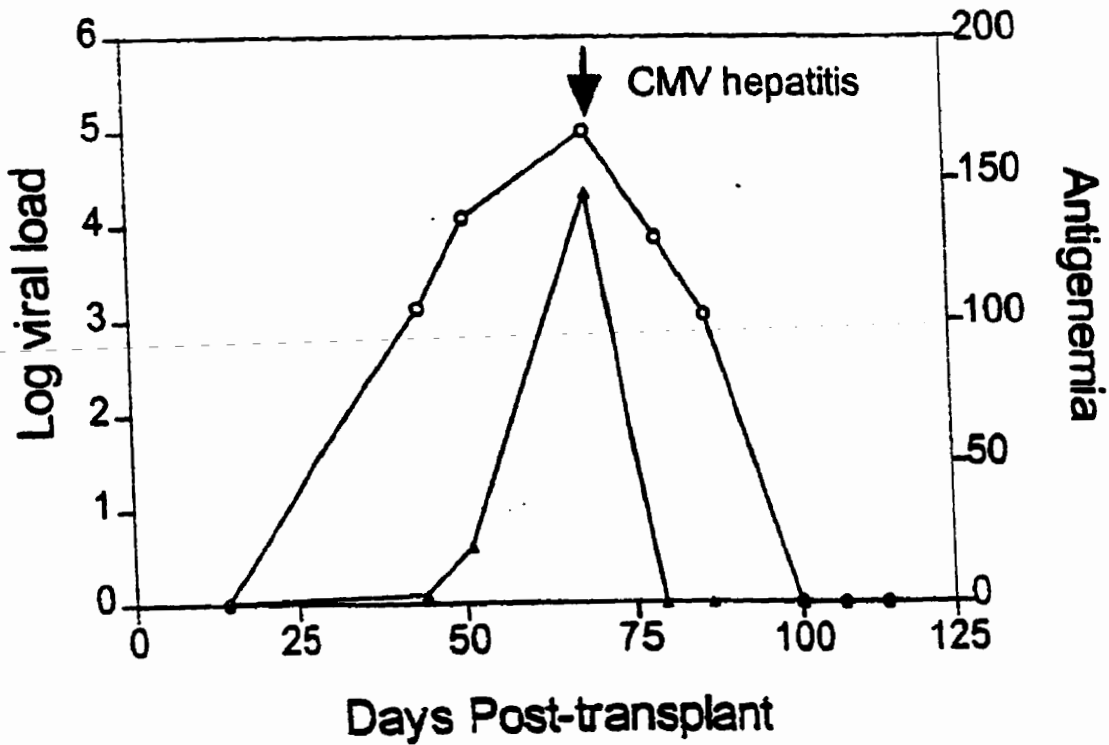
PATIENT 2



PATIENT 3



PATIENT 4



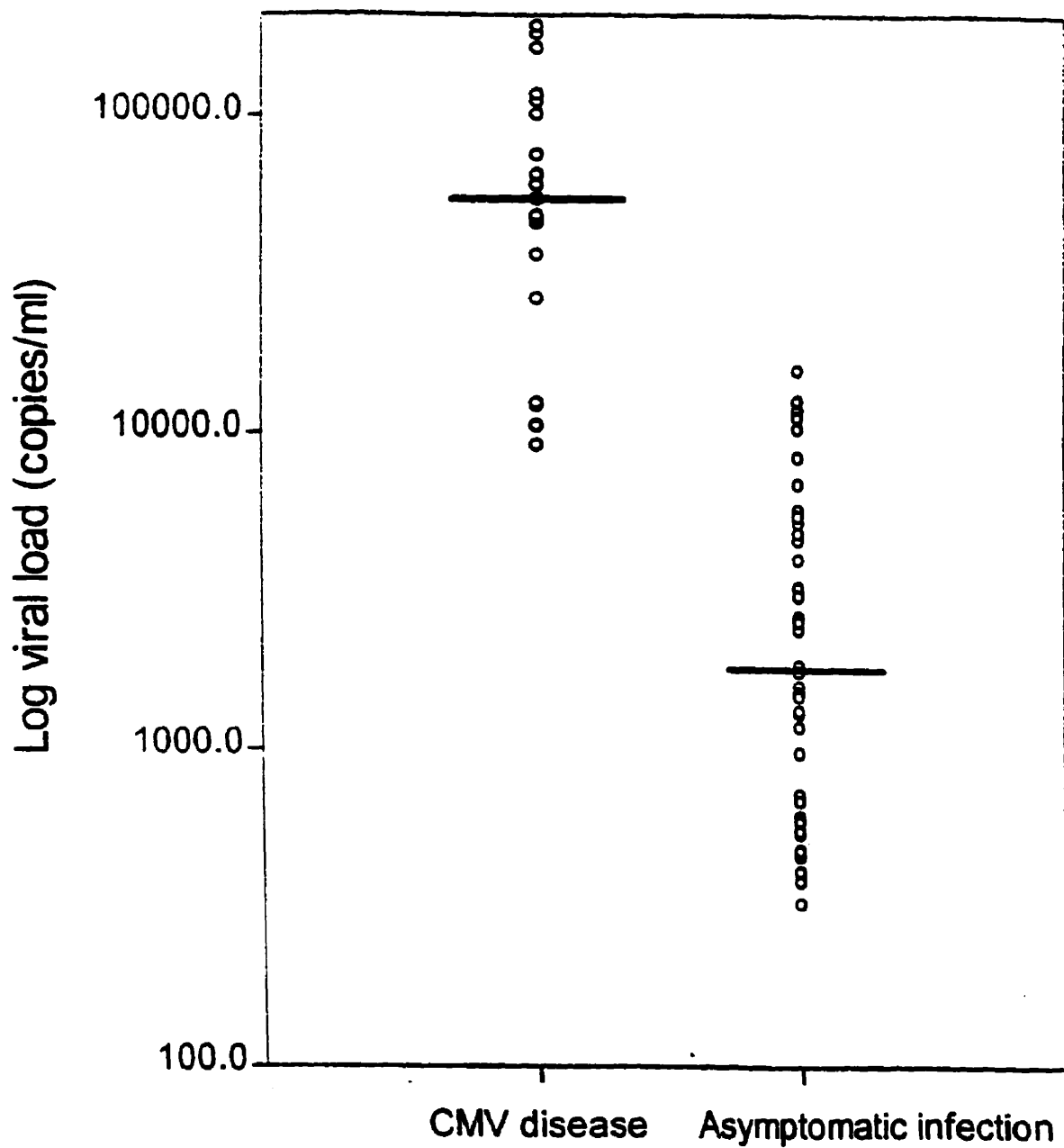


Figure 3: Peak CMV viral load (quantitative PCR) (copies/ml) in patients with active CMV disease and asymptomatic CMV infection. Horizontal bar indicates median viral load.

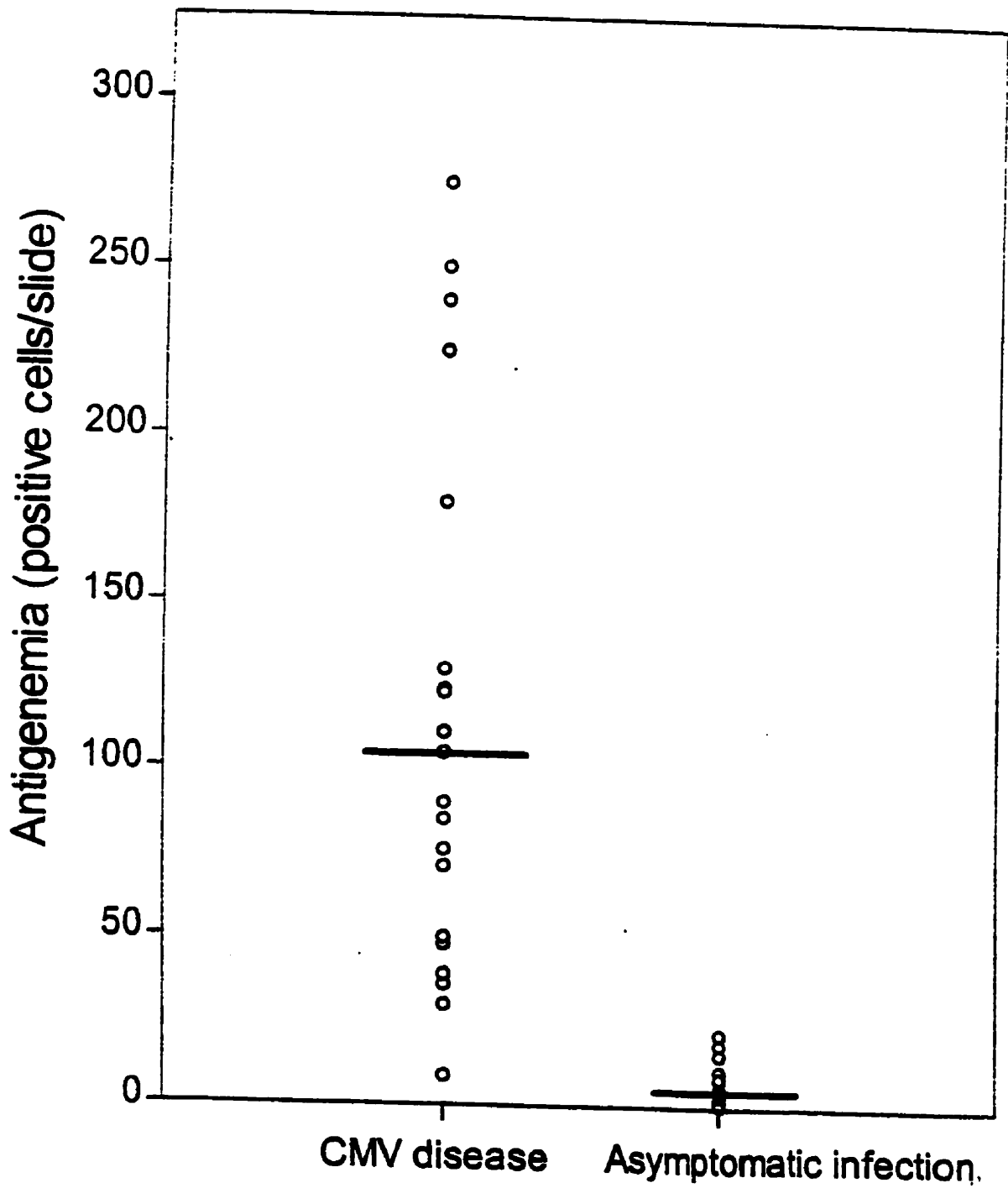


Figure 4: Peak CMV antigenemia levels (positive cells/slide) in patients with active CMV disease and asymptomatic CMV infection. Horizontal bar indicates median antigenemia level.

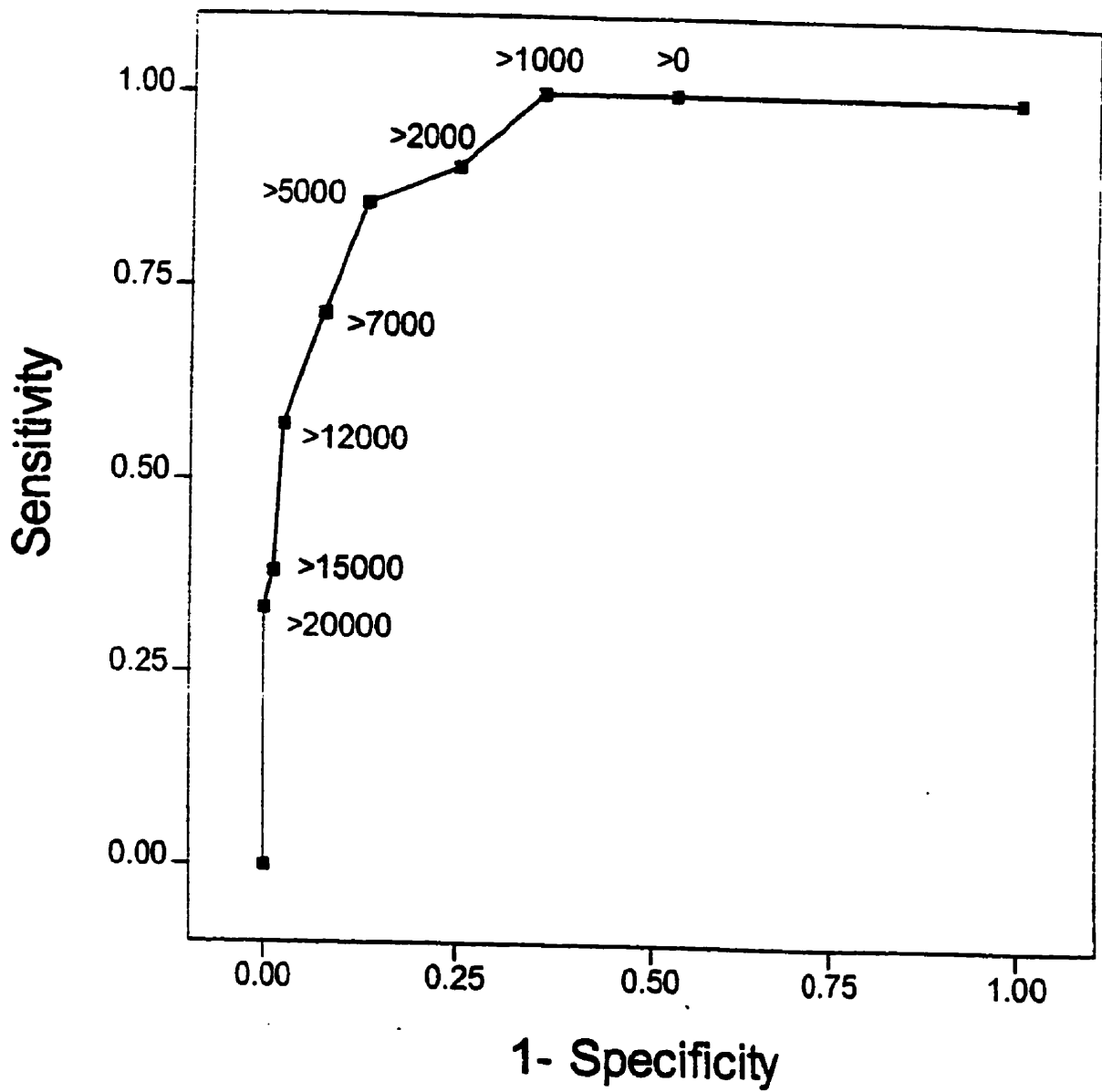


Figure 5: Receiver-operator characteristic (ROC) curves graphing sensitivity vs. 1-specificity for the prediction of CMV disease using different positive cut-off values for quantitative PCR (viral loads shown in copies/ml).

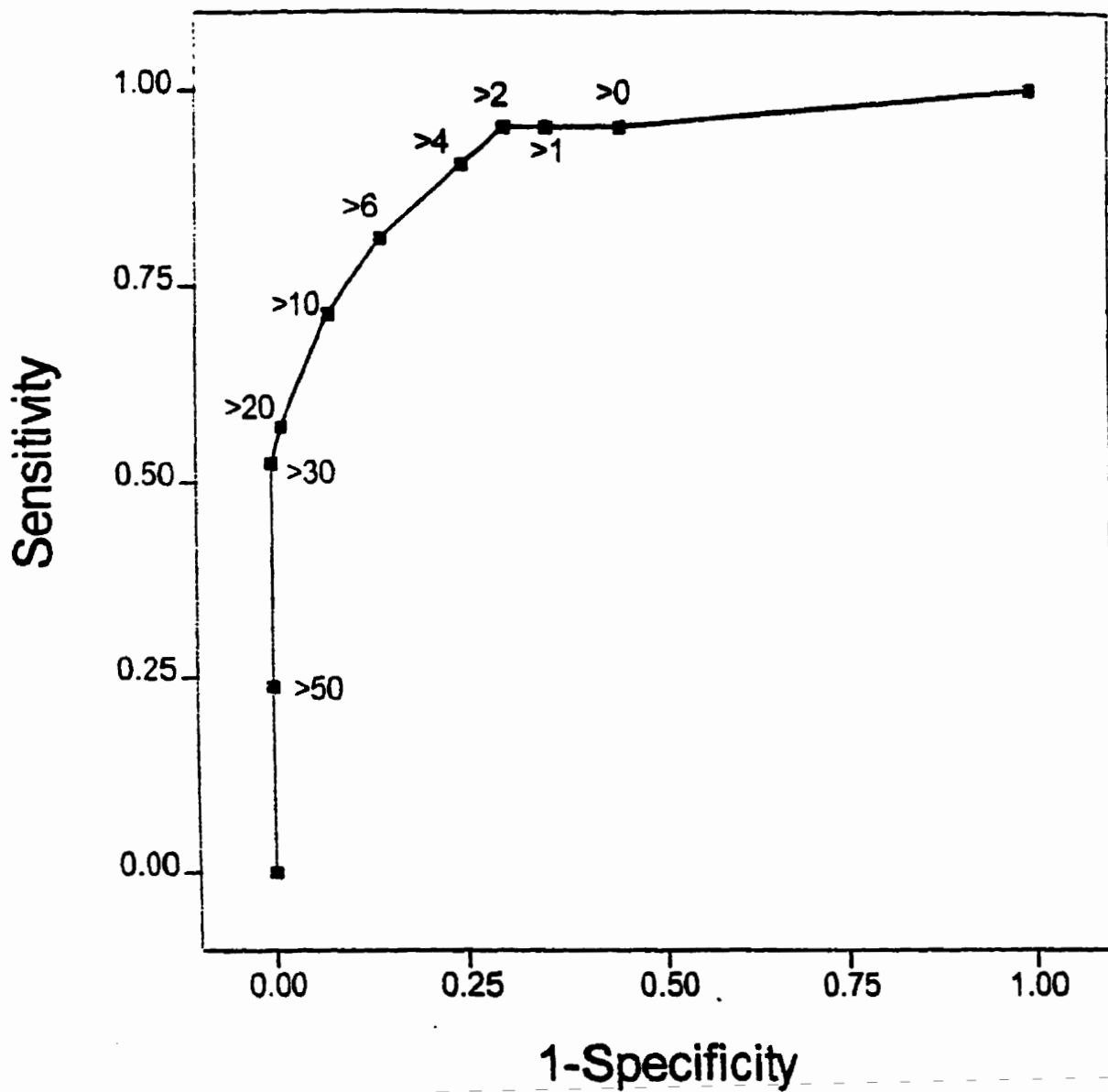


Figure 6: Receiver-operator characteristic (ROC) curves graphing sensitivity vs. 1-specificity for the prediction of CMV disease using different positive cut-off values for CMV antigenemia (number of positive cells/slide).

APPENDIX I : STUDY SCHEDULE

BLOODWORK:

PRE-TRANSPLANT:

- Donor (D) and recipient (R) CMV serology

POST-TRANSPLANT:

D+/R+, D-/R+

Week 2-12: 10 mls of EDTA blood at every clinic visit.

- CMV antigenemia
- CMV quantitative PCR

D+/R-:

Receive IV ganciclovir 5mg/kg once daily or p.o. ganciclovir 1g t.i.d. for 12 weeks post transplant

- Bloodwork at week 12, 14, 16, 18.
- CMV antigenemia and quantitative PCR testing

APPENDIX II : CONSENT FORM

TITLE OF RESEARCH PROJECT: CLINICAL UTILITY OF CYTOMEGALOVIRUS VIRAL LOAD DETERMINATION FOR PREDICTING CYTOMEGALOVIRUS DISEASE IN LIVER TRANSPLANT RECIPIENTS

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Purpose of Research

Cytomegalovirus (CMV) is a common cause of illness in patients who have undergone a liver transplant. Serious infections due to CMV can affect many parts of the body including the lungs, the gut, and the liver. Although there are medications to treat these infections, they may cause potentially serious side effects, and are not always effective in curing the infection.

Some groups of patients at a very high risk of getting CMV infection receive an intravenous antiviral medication (ganciclovir) to prevent the infection before it occurs. Recently, however, new blood tests have been developed which shows promise in diagnosing CMV infection earlier, before patients develop any symptoms. These tests are called the CMV antigenemia assay and the quantitative PCR test. They require approximately 2 teaspoons of blood to perform.

The purpose of this study is to determine if these tests can reliably predict who will develop serious infections due to CMV. This could then serve as a guide for early treatment.

Description of Research

If you consent to participate in this study, beginning 2 weeks after your transplantation, we will collect an additional 10 ml (2 teaspoons) of blood from you. This will be done every time you visit the clinic until 12 weeks after your transplant. You will otherwise continue to receive the usual standard care by your doctor.

Potential Harms

Taking blood is briefly uncomfortable, but not dangerous. When you have blood drawn, you may have some bruising where it is taken. This may take several days to go away. Every effort will be made to collect blood for the study at times when you may be having other routine blood tests.

Potential Benefits

You may not benefit directly from participating in this study. However, the information learned in this study may help other patients with similar conditions in the future.

Confidentiality

Confidentiality will be respected and no information that discloses your identity will be released or published without consent. Access to study records will be limited to the physicians and research staff only.

Participation

Participation in this research is strictly voluntary. If you chose not to participate, you will continue to have access to quality care. You can withdraw from the study at any time and again, you will continue to have access to quality care.

I agree to participate in the above study:

Patient Name

Signature

Date

Witness

Signature

Date

APPENDIX III: SAMPLE DATA COLLECTION FORM

BASELINE DATA

PATIENT NAME _____

STUDY #

HOSPITAL # _____

Date of transplant: (d/m/y) _____

Demographic Data:

Recipient : Age _____ Sex M F Race _____

Donor: Age _____ Sex M F Race _____

Underlying disease _____

Retransplant: Y N

Status: 1 2 3 4

Fulminant: Y N

CMV Serology Pre-transplant: Donor + -

Recipient + -

ABO blood type: Donor _____

Recipient _____

STUDY # _____

WEEKLY FOLLOW-UP DATA

DATE (d/m/y) _____

Fever >38.5 in last week (Y/N):

New symptoms/signs or complications in last week:

MICROBIOLOGY/PATHOLOGY RESULTS IN PREVIOUS WEEK:

Virology:

Bacteriology:

Pathology:

DRUGS:

Antimicrobials:

Immunosuppressives: