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ORIGINAL ARTICLE

A prospective study comparing cytomegalovirus antigenemia, DNAemia and RNAemia tests in guiding pre-emptive therapy in thoracic organ transplant recipients

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Summary

We evaluated the usefulness of DNAemia and mRNAemia tests in guiding the pre-emptive therapy against cytomegalovirus (CMV) infections in thoracic organ transplant recipients using antigenemia test as the reference. Seven lung (LTR) and 14 heart (HTR) transplant recipients were prospectively monitored for CMV by antigenemia, DNAemia (Cobas Amplicor PCR Monitor) and pp67-mRNAemia (NASBA) tests. However, only the antigenemia test guided pre-emptive therapy with cut-off levels of ≥2 and ≥5–10 pp65-positive leukocytes/50 000 leukocytes in the LTRs and HTRs, respectively. CMV DNAemia was detected in 26/28 (93%) and RNAemia in 17/28 (61%) of the CMV antigenemias requiring antiviral therapy ($P = 0.01$). Optimal DNAemia levels (sensitivity/specificity) estimated from receiver-operating characteristic curve to achieve maximal sum of sensitivity and specificity were 400 (75.9/92.7%), 850 (91.3/91.3%) and 1250 (100/91.5%) copies/ml for the antigenemia of 2, 5 and 10 pp65-positive leukocytes, respectively. The sensitivities of nucleic acid sequence-based amplification (NASBA) were 25.9%, 43.5% and 56.3% in detecting the same cut-off levels of antigenemia. In thoracic organ transplant recipients, the Cobas PCR assay is comparable with the antigenemia test in guiding pre-emptive therapy against CMV infections when threshold levels of over 5 pp65-antigen-positive leukocytes are used as the reference. In contrast, the low sensitivity of NASBA limits its usefulness in the guidance of pre-emptive therapy.

Introduction

Cytomegalovirus (CMV) remains a major viral pathogen in heart transplant recipients (HTR) and lung transplant recipients (LTR), despite advances in diagnostic techniques and the development of antiviral agents [1–3]. To prevent and treat CMV infections, antiviral agents may be administered either to all recipients considered to be at high risk for CMV infection (prophylaxis), when a positive laboratory test or a certain quantity of virus in blood is detected (pre-emptive therapy) or to treat symptomatic CMV infection (rescue therapy) [4]. Ganciclovir prophylaxis has been shown to be effective in LTRs and HTRs [2,3,5–10], but delayed CMV infections frequently occur after cessation of

prophylaxis necessitating the surveillance of CMV even when anti-CMV prophylaxis is initially used [8,10–12].

Pre-emptive therapy needs to be guided by a convenient, reliable and timely diagnostic surveillance test that will identify CMV infection quickly enough to prevent CMV disease. The surveillance test should also be useful in monitoring the response to antiviral therapy. Traditionally, CMV pp65 antigenemia test has been used for surveillance of CMV infection and guidance of antiviral therapy in many institutions including our own. A number of previous studies have proved antigenemia test reliable in predicting CMV disease and guiding pre-emptive therapy, but the cut-off level for the initiation of therapy varies markedly [4,6,13–16]. The need for immediate processing of samples, the variety of in-house modifications of the method and the subjective nature of quantification limit the use of the antigenemia test in the clinical practice [4,17,18]. To resolve these difficulties, molecular assays to detect CMV DNAemia by PCR techniques and CMV mRNAemia by nucleic acid sequence-based amplification (NASBA) in peripheral blood have been developed. DNAemia levels measured by commercially available quantitative PCR assay (The Cobas Amplicor CMV Monitor Test; Roche, Indianapolis, IN, USA) have shown a good correlation with antigenemia test results and high viral loads predict CMV disease and recurrent CMV infection [19–24]. However, the usefulness of DNAemia test for guidance of pre-emptive therapy has not been widely studied in HTRs and LTRs. The presence of CMV pp67 mRNAemia detected by NASBA indicates active viral replication and is a marker for active CMV infection [18]. Although some studies have found the pp67 mRNAemia test to be less sensitive than the DNAemia and antigenemia tests, others suggest that NASBA is a useful method in the surveillance of CMV and guidance of pre-emptive therapy [15,19,25–27].

While modern treatment and prophylaxis strategies have undoubtedly declined the morbidity associated to CMV infections, the optimal tests and relevant thresholds for guidance of antiviral therapy still remain to be determined. In this prospective study, we compared the CMV-antigenemia, CMV DNAemia (PCR) and CMV pp67 mRNAemia (NASBA) tests in detecting CMV infection in thoracic organ transplant recipients. Especially, the feasibility of the NASBA and the PCR tests in guiding the pre-emptive therapy was evaluated, when the CMV antigenemia test was used as the reference assay.

Patients and methods

Patients

A total of 24 thoracic organ transplant recipients operated between December 2000 and April 2003 at the Helsinki University Central Hospital were enrolled. One patient surviving <30 days postoperatively and two CMV seronegative patients receiving organ from CMV seronegative donors (R−/D−) who did not develop primary CMV infection were excluded. Thus, 21 thoracic organ transplant recipients (7 LTRs and 14 HTRs) were included. The Local Ethics Committee approved the study and an informed consent was received from each patient. Patient characteristics are summarized in Table 1. Performing lung transplantation from CMV-seropositive donor to CMV-seronegative recipient (R−/D+) was avoided. All recipients survived over 12 months.

Antithymocyte globulin (1.25 mg/kg/day) was given to five LTRs and all HTRs for three postoperative days (POD). The maintenance immunosuppressive regimen consisted of cyclosporine (200–400 ng/ml whole-blood trough level), azathioprine 1–2 mg/kg/day (eight recipients) or mycophenolate mofetil 2–3 g/day (13 recipients), and methylprednisolone starting with 1 g perioperatively and tapered down to 0.1 mg/kg/day. Rejection episodes were treated with i.v. methylprednisolone 0.5–1 g daily for 3 days.

All LTRs and one HTR (R−/D+) received anti-CMV prophylaxis. Intravenous ganciclovir 5 mg/kg b.i.d. through POD 7–21, then 5 mg/kg/day for 5 days a week through POD 22–28, continued with oral ganciclovir (1 g t.i.d./day) through POD 29–90 was given to three LTRs. Two LTRs received oral ganciclovir (1 g t.i.d./day) and another two LTRs oral valganciclovir (450–900 mg once/day) from POD 7 to 90. The HTR (R−/D+) received oral valganciclovir (dose adjusted according to renal function)

Table 1. Patient demographics.

Number of patients	21
Gender (male/female)	11/10
Age, mean (range)	43 (14–63)
Type of transplantation	
Heart	14 (66%)
Lung	5 (24%)
Heart–lung	2 (10%)
Indication for transplantation	
Cardiomyopathy*	11 (52%)
Emphysema†	4 (19%)
Other‡	6 (28%)
Pretransplant CMV-serostatus	
R+/D+	14 (67%)
R+/D−	6 (28%)
R−/D+	1 (5%)§

CMV, cytomegalovirus; R, recipient; D, donor.
*Heart transplantation.
†Lung transplantation.
‡Coronary artery disease (heart), giant-cell myocarditis (heart), sdr. Noonan (heart), bronchiectasiae (lung), pulmonary hypertension (heart–lung) and idiopathic pulmonary fibrosis (heart–lung).
§Heart transplant recipient.

through POD 7–180. The remaining 13 HTRs without CMV prophylaxis received oral acyclovir for three postoperative months to prevent other herpes virus diseases.

All patients were prospectively monitored for CMV infection for a 12-month period. During the follow-up, heparinized or EDTA-treated blood samples were collected weekly during the hospital stay, once every 2 weeks until 6 months, and monthly during 6–12 months postoperatively. Additional blood samples were drawn if CMV infection was suspected and 1–2 times a week during antiviral therapy. Blood samples for all the CMV tests studied were taken at the same time. The recipients under the supervision of other hospitals were monitored similarly and the blood samples were transported to the Transplantation Laboratory of the Helsinki University Central Hospital in <8 h. CMV antigenemia test was performed within the working day.

Demonstration of CMV using antigenemia, DNAemia (PCR) and RNAemia (NASBA) tests

The detection of CMV pp65 antigenemia in peripheral blood was based on the standard CMV pp65 antigen test [17]. Red blood cells were lyzed and cytocentrifuge preparations were made onto microscope slides. A three-layer indirect immunoperoxidase technique and a monoclonal antibody against CMV pp65 antigen (Biotest, Frankfurt, Germany) were used. CMV antigenemia in peripheral blood was quantified by calculating CMV pp65-positive polymorphonuclear leucocytes (PMNL) per 50 000 PMNL on the slide.

The Cobas Amplicor CMV Monitor Test was used for the quantification of CMV DNA in the EDTA blood samples. The test was carried out according to the manufacturer's instructions and as described in Refs. [22,23]. The lower detection limit of the assay was 400 copies/ml and the linear range 400–100 000 copies/ml of plasma.

Cytomegalovirus RNAemia was demonstrated by the NucliSens assay (BioMérieux, Boxtel, Netherlands) according to the manufacturer's instructions. The assay uses a qualitative NASBA to detect pp67 mRNA and the results are reported as positive or negative.

Diagnosis and treatment of CMV infection

Cytomegalovirus infection was defined as a positive CMV antigenemia, DNAemia (PCR) or RNAemia (NASBA) test in peripheral blood. However, during the prospective follow-up, only CMV antigenemia test was used to guide the antiviral therapy. *Symptomatic CMV infection* was diagnosed by the presence of otherwise unexplained symptoms or findings referring to viral infection (e.g. fever, thrombocytopenia and/or leukopenia) together with CMV antigenemia. The diagnosis of *CMV disease* was confirmed by the presence of characteristic intracellular inclusion bodies or CMV pp65-antigen on tissue specimens or bronchoalveolar lavage (BAL) fluid. All effort was made to confirm CMV disease (e.g. bronchoscopy with BAL was performed if CMV pneumonia was suspected). In addition, surveillance bronchoscopies with BAL and transbronchial lung biopsies were performed to LTRs in every 1–3 months.

Our clinical experience at Helsinki University Central Hospital is that if left untreated, even a low antigenemia level in LTR tends to increase and may lead to CMV disease. Thus, pre-emptive antiviral therapy was initiated with antigenemia level of ≥2 pp65-positive leukocytes in the LTRs. In HTRs, pre-emptive therapy was initiated if ≥10 pp65-positive leukocytes were detected. HTRs with antigenemia level from 5 to 9 pp65-positive leukocytes received antiviral therapy immediately or the assay was controlled in 1 week and therapy was initiated if increasing antigenemia was detected. All patients with symptomatic CMV infection, CMV disease or concomitant CMV antigenemia and antirejection treatment received antiviral therapy regardless of the antigenemia level (rescue therapy).

Intravenous ganciclovir (5 mg/kg b.i.d.) or peroral valganciclovir (900 mg b.i.d.) was used as antiviral therapy for CMV infection. Treatment was continued until the CMV antigenemia test was negative and for a minimum of 14 days. Secondary anti-CMV prophylaxis with oral ganciclovir (3 g/day) or valganciclovir (450–900 mg/day) after successfully treated CMV infection was used at the discretion of the doctor.

Statistical analysis

The Mann–Whitney *U*-test was used to compare means of the peak DNAemia levels in CMV infections with or without antiviral therapy. Days to the first positive CMV test results and to the first CMV infection in recipients with and without prophylaxis were compared using the Kaplan–Meier method, and differences between the groups were analyzed by the log-rank test. The Pearson's chi-squared test was used to analyze differences in proportions of blood samples, while CMV infections positive by different assays were tested by the Fisher's exact test. The number of CMV pp65 antigen-positive leukocytes and CMV DNAemia level were compared using Spearman's rank correlation test. A statistical significance was accepted for $P < 0.05$. Receiver-operating characteristic (ROC) curves and ROC plot analysis were performed to determine optimal threshold CMV DNAemia levels for the initiation of pre-emptive antiviral therapy when different CMV antigenemia cut-off levels were used as a reference.

Results

Altogether 448 blood samples from 21 recipients were received. Two (0.4%), eight (1.8%) and 25 (5.6%) of them were not applicable or valid for antigenemia test, PCR and NASBA, respectively. In addition, because of the long geographic distances, 28 blood samples could not be collected as the follow-up protocol warranted.

A total of 46 CMV infections occurred in the study population and 28 (61%) of these required antiviral treatment. Seventeen (81%) of the recipients received one to three courses of antiviral therapy. Twenty-six infections were treated with ganciclovir or valganciclovir and two with a combination of ganciclovir and low-dose foscarnet (60 mg/kg/day) because of unsatisfactory response to valganciclovir or ganciclovir alone. The dose of the antiviral drugs was reduced because of the impaired renal function in six cases. One asymptomatic HTR with a peak antigenemia level of 24 pp65-positive leukocytes was left untreated by the discretion of the physician and another HTR followed-up in a distant center received antiviral therapy to the sixth CMV infection although the peak antigenemia level was only 2 pp65-positive leukocytes. The former was considered as an infection requiring antiviral therapy and the latter was not. All the other CMV infections were treated or left untreated by the criteria described above. Four patients received antirejection therapy and two of these developed concomitant CMV-infection.

Three LTRs had CMV disease (pneumonia). Seven additional CMV infections were associated with otherwise unexplained symptoms [fever (four), leukopenia (two) and a flu-like syndrome with thrombocytopenia (one)]. Thus, 10/46 (22%) of the CMV infections were symptomatic. All CMV infections resolved either spontaneously or with antiviral therapy.

Detection of CMV infection and response to antiviral agents

Cytomegalovirus antigenemia, DNAemia and RNAemia were detected in 21/21 (100%), 20/21 (95%) and 13/21 (62%) of the recipients, respectively. The only recipient who did not develop CMV DNAemia had a low antigenemia level of 1 pp65-positive leukocytes. The turning point of the blood samples exhibiting antigenemia, DNAemia and RNAemia after transplantation is presented in Fig. 1. The first CMV infection after transplantation was detected by antigenemia test alone, antigenemia test + PCR and antigenemia test + PCR + NASBA at the same time in 9/21 (43%), 7/21 (33%) and 4/21 (19%) of the patients, respectively. One recipient had positive antigenemia test at 23 days after the transplantation, while PCR was negative and NASBA gave an invalid test result.

Cytomegalovirus infections detected by antigenemia test, PCR and NASBA are presented in Table 2. The only CMV infection missed by the antigenemia test manifested as a single NASBA-positive and PCR-negative blood sample resolving without antiviral therapy. All the PCR-positive infections were also detected by the antigenemia test. CMV DNAemia was not detected in 12 (27%) of all the CMV antigenemias. However, all these antigenemias manifested as a low antigenemia level of ≤5 pp65-positive

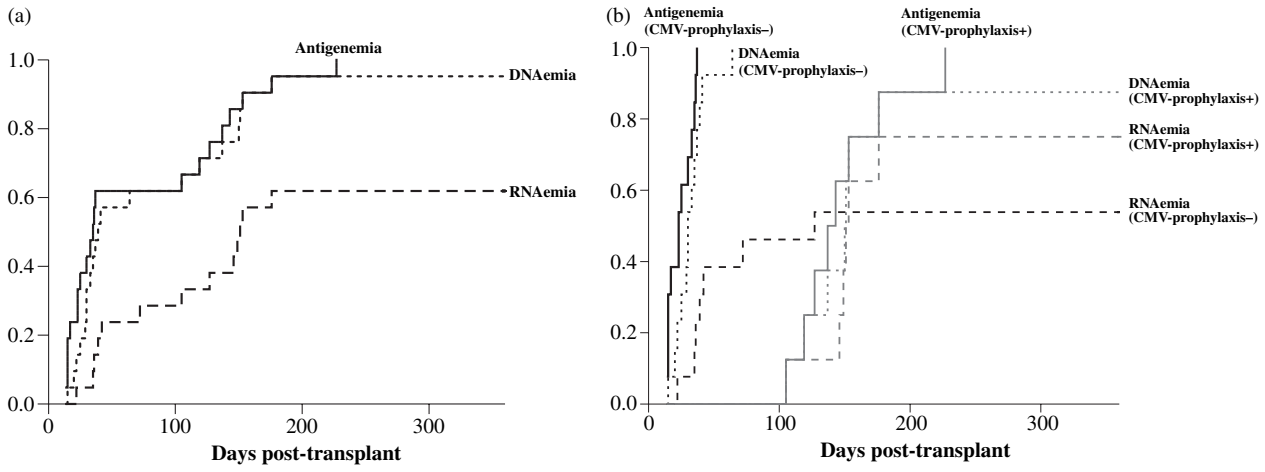


Figure 1 Log-rank curves showing the timing of the first detection of antigenemia, DNAemia and RNAemia after transplantation in all the recipients (a) and in patients with and without CMV prophylaxis (b). Antigenemia vs. RNAemia ($P < 0.001$), DNAemia vs. mRNAemia ($P < 0.01$) and antigenemia vs. DNAemia (not significant) in all patients (a). The first detection of CMV infection (defined as a positive result in either one of the assays) was significantly delayed in patients with CMV prophylaxis ($P < 0.0001$). The differences between the appearance of antigenemia, DNAemia and RNAemia were not significant in recipients with CMV prophylaxis (b).

Table 2. Cytomegalovirus infections detected by CMV antigenemia, DNAemia (PCR) and RNAemia (NASBA) tests. CMV infection is defined as a positive result in any of the tests.

	CMV-infections		
	Antiviral therapy required	No antiviral therapy required	Total
Antigenemia			
Negative	0 (0%)*	1 (6%)	1 (2%)
Positive	28 (100%)*	17 (94%)	45 (98%)
Peak antigenemia†	15 (2–2030)	2 (0–7)	
DNAemia (PCR)			
Negative	2 (7%)	11 (61%)	13 (28%)
Positive	26 (93%)	7 (39%)	33 (72%)
Positive at the initiation of therapy	26 (93%)		
Peak DNAemia‡	4510 (401 to >100 000)	1650 (798–3880)§	
RNAemia (NASBA)			
Negative	11 (39%)	15 (83%)	26 (57%)
Positive	17 (61%)	3 (17%)	20 (43%)
Positive at the initiation of therapy	12/26 (46%)¶		
Total	28	18	46

*Antiviral therapy was initiated based on the results of the antigenemia test.
†Median number of pp65-antigen-positive leukocytes (range).
‡Median number of copies/ml (range).
§$P < 0.05$ (no antiviral therapy required versus antiviral therapy required).
¶Valid blood samples for NASBA were not available in two cases at the initiation of therapy.

leukocytes and only two required antiviral therapy. One of these occurred in a LTR with peak antigenemia level of 5 pp65-positive leukocytes and the other in a HTR with a low but persistent antigenemia level of 2 pp65-positive leukocytes and concomitant leukopenia considered to be because of CMV infection (symptomatic CMV infection). NASBA detected 43% of all CMV infections and 61% of the antigenemias requiring antiviral therapy. The PCR test detected CMV antigenemias requiring antiviral therapy more frequently than the NASBA test ($P = 0.01$). CMV infections requiring antiviral therapy were first detected by the antigenemia test in 12/28 (43%), by PCR in 1/28 (3%) and by both tests in 15/28 (54%) of the cases. However, CMV DNAemia was detected before or at the initiation of therapy in all PCR-positive CMV infections treated with antiviral agents. There was only one case when the PCR test detected CMV infection earlier than the antigenemia test. In that case, the antigenemia test turned positive 12 days later, which was before antiviral therapy was initiated. NASBA was positive at the initiation of therapy in 12 cases.

During antiviral therapy, antigenemia, DNAemia and RNAemia resolved in a median of 13 (range 5–109), 24 (range 4–116) and 17 (range 7–102) days, respectively. At the end of the therapy, PCR was still positive in seven cases. Recurrent antigenemia developed at a median of 30 days (range 20–75 days) in six of these cases, but only one of the episodes required antiviral therapy. DNAemia subsided in 18–22 days in six infections, but one DNAemia lasted up to 102 days and resolved then spontaneously. RNAemia was detected in two cases at the end of the therapy, but these resolved in 4 and 18 days after the cessation of therapy.

The first CMV infection occurred at a median of 137 days (range 105–225 days) in recipients receiving prophylaxis, which was later than in patients without antiviral prophylaxis (at a median of 23 days; range 14–37 days) ($P < 0.0001$) (Fig. 1). There was one breakthrough CMV infection in a HTR (R−/D+) receiving low-dose valganciclovir prophylaxis. The dose (450 mg every other day) had been tapered down because of an impaired renal function.

Comparison of CMV antigenemia, DNAemia and RNAemia in blood samples

Cytomegalovirus was detected more frequently in blood samples by the antigenemia test (145/446; 33%) and PCR (125/440; 28%) than by NASBA (40/423; 9%) ($P < 0.0001$).

A statistically significant correlation between the number of CMV pp65-positive leukocytes and CMV DNAemia level was found ($r = 0.69$, $P < 0.0001$). There were 46 samples positive by the antigenemia test, but under

Table 3. Sensitivity, spesificity and PPV of the different DNAemia levels (PCR) and RNAemia (NASBA) test results using antigenemia test as the reference. Blood samples collected during antiviral therapy are excluded.

	Threshold levels of CMV antigenemia		
	≥2 pp65-positive leukocytes	≥5 pp65-positive leukocytes	≥10 pp65-positive leukocytes
Threshold levels of DNAemia			
>400 copies/ml			
Sensitivity (%)	75.9	91.3	100
Specificity (%)	92.7	87.2	85.7
PPV (%)	66.1	33.9	24.2
≥1000 copies/ml			
Sensitivity (%)	61.1	87.0	100
Specificity (%)	95.5	91.9	90.5
PPV (%)	71.7	43.5	32.6
≥5000 copies/ml			
Sensitivity (%)	24.1	47.8	66.7
Specificity (%)	100	99.4	99.1
PPV (%)	100	84.6	76.9
Optimal DNAemia level*	400 copies/ml	850 copies/ml	1250 copies/ml
Sensitivity (%)	75.9	91.3	100
Specificity (%)	92.7	91.3	91.5
PPV (%)	66.1	42.9	34.9
RNAemia (NASBA)			
Sensitivity (%)	25.9	43.5	56.3
Specificity (%)	99.6	98.4	98.1
PPV (%)	93.3	66.7	60.0

*Optimal DNAemia levels from the ROC curves were chosen as the point nearest to the top-left corner in order to achieve the maximal sum of sensitivity and specificity.

the detection limit of the PCR test. All these samples represented low-level antigenemia from one to 5 pp65-positive leukocytes and only a single pp65-positive leukocyte was detected in 32/46 (70%) of the samples. Four (9%) of these antigenemia test-positive/PCR-negative samples were taken during antiviral therapy. PCR was not available in three samples positive by the antigenemia test. The antigenemia test was negative in 29 samples, which showed CMV DNAemia (median of 1240 copies/ml, range 439–4490). Seventeen (59%) of these samples were taken during antiviral therapy.

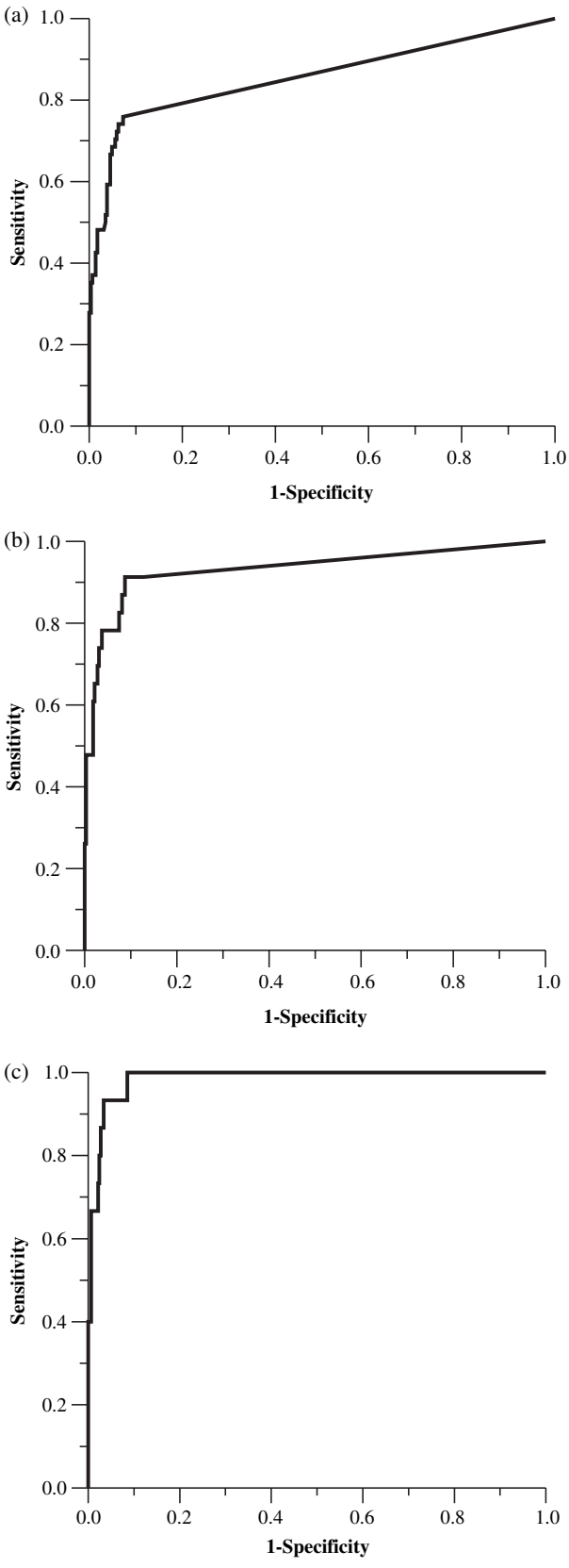
Nucleic acid sequence-based amplification was positive in 34/137 (24.8%) and 35/120 (29%) of the samples positive by antigenemia test and PCR, respectively. NASBA was not available in eight of the antigenemia-positive samples and five of the PCR-positive samples.

Sensitivity, specificity and positive predictive value (PPV) for different DNAemia levels and NASBA results using the antigenemia test as the reference standard are presented in the Table 3. In order to define the relevant CMV DNAemia levels corresponding to the antigenemia levels used for the initiation of pre-emptive antiviral therapy, ROC plot analysis was performed. Optimal DNAemia levels from the ROC curves were chosen as the point nearest to the top-left corner to achieve maximal sum of sensitivity and specificity. ROC curves for CMV DNAemia using CMV antigenemia threshold levels of 2, 5 and 10 pp65-positive leukocytes/50 000 PMNLs as the reference are shown in Fig. 2. Blood samples collected during antiviral therapy ($n = 96$) were excluded from ROC curve analysis.

Discussion

In this prospective study of 21 thoracic organ transplant recipients monitored for the presence of CMV during 12 months, we compared CMV pp65-antigenemia, DNAemia (Cobas Amplicor PCR Monitor) and pp67-mRNAemia (NASBA) tests in detecting CMV infection and the usefulness of the NASBA and the PCR test in guiding pre-emptive therapy using the antigenemia test as the reference.

The antigenemia and PCR tests turned out to be more sensitive than NASBA in our study population. Blood samples were more frequently positive by the antigenemia and PCR tests than by NASBA and the sensitivity of NASBA was low (from 25.9% to 56.3%) in detecting CMV antigenemia level of 2–10 pp65-positive leukocytes. The inability to use NASBA in guiding antiviral therapy was demonstrated by the fact that 39% of the CMV antigenemia episodes requiring antiviral therapy were not detected by NASBA. The detection of CMV by NASBA was also



delayed when compared with the other two CMV tests studied, although the difference was statistically significant only in patients without CMV prophylaxis. Our results are in concordance with most of the previous studies showing that NASBA is less sensitive than PCR and antigenemia tests [19,25,27]. However, Gerna *et al.* [15] concluded in their recent study that antigenemia test could be replaced by NASBA in the guidance of pre-emptive therapy in thoracic organ transplant recipients. A higher threshold level for the initiation of pre-emptive therapy (100 pp65-antigen-positive leukocytes/200 000 PMNLs) was used compared with the present study. In our material, the sensitivity of NASBA rose up to 80.0% (specificity 96.6%) when the test was compared with the similar antigenemia-level of 25 or more pp65-antigen-positive leukocytes/50 000 PMNLs (data not shown).

All the episodes of CMV DNAemia were also demonstrated by the antigenemia test, while 12 (27%) of the antigenemia episodes were PCR-negative. CMV was also detected more frequently in blood samples by the antigenemia test (33% vs. 28%). Thus, the antigenemia test was found to be slightly more sensitive than the PCR assay. Some studies have suggested the Cobas PCR test or the antigenemia test to be more sensitive than the other, while others have found similar sensitivities for both tests [20–23,28]. These discrepancies are probably because of different patient groups studied and some in-house variability in performing the antigenemia test. Most of the earlier studies comparing Cobas Amplicor PCR and the pp65-antigenemia tests have not used pre-emptive treatment strategies, which make the comparison of the previous and the present study difficult. Nevertheless, the differences between the antigenemia and the PCR test were not considered to be of major clinical significance in our material as all the antigenemia episodes not detected by PCR manifested as low-level antigenemia (≤5 pp65-antigen-positive leukocytes), only two of the CMV infections requiring antiviral therapy were PCR-negative and DNAemia was present until the initiation of therapy in all the PCR-positive cases. Recently, many real-time PCR assays based on LightCycler or Taqman technologies have been developed and are shown to be even more sensitive than the Cobas Amplicor PCR test and the

Figure 2 Receiver-operating characteristic curves for CMV DNAemia levels (PCR) using different cut-off levels of antigenemia as the reference standard. (a) ROC curve using a cut-off level of 2-positive leukocytes/50 000 PMNLs as the reference. Area under the curve (AUC) = 0.856 (95% CI: 0.786–0.926). (b) ROC curve using a cut-off level of 5-positive leukocytes/50 000 PMNLs as the reference. AUC = 0.932 (95% CI: 0.861–1.000). (c) ROC curve using a cut-off level of 10-positive leucocytes/50 000 PMNLs as the reference. AUC = 0.986 (95% CI: 0.972–0.999).

pp65-antigenemia test [20,23,29,30]. Yakushiji *et al.* concluded in their study on stem cell transplant recipients that real-time PCR and the pp65-antigenemia test could equally be used for the early detection of CMV. Importantly, pre-emptive treatment strategy with a cut-off level of 3 pp65-antigen-positive leukocytes/50 000 PMNLs was used in that study [29]. These newer PCR assays with a lower detection level for CMV DNA could serve as a good alternative for guiding the pre-emptive therapy also in high-risk thoracic organ transplant recipients where low-level antigenemia (<5 pp65-positive leukocytes) has been traditionally used for the initiation of pre-emptive therapy.

Cytomegalovirus DNAemia levels measured by The Cobas Amplicor PCR assay showed a good overall correlation with the pp65-antigenemia test results in general, which is in line with previous reports [19,20,23]. However, in order to evaluate the clinical usefulness of this correlation, we performed ROC curve analysis to estimate cut-off levels for CMV DNAemia corresponding to the antigenemia levels used for the initiation of pre-emptive therapy. Optimal cut-off levels of CMV DNAemia to achieve maximal combined sensitivity and specificity were 850 and 1250 copies/ml for the antigenemia levels of 5 and 10 pp65-positive leukocytes, respectively. The sensitivities and specificities with these DNAemia thresholds were over 90%, but the PPVs were low (43% and 35%). Thus, using the above-mentioned cut-off levels for the initiation of pre-emptive therapy, all of the CMV antigenemias over 10 pp65-positive leukocytes and a great majority of antigenemias over 5 pp65-positive leukocytes are treated, but if the antiviral therapy is initiated with DNAemia levels from *c.* 1000 to 5000 copies/ml, some of the CMV infections might actually represent an antigenemia under 5–10 pp65-positive leukocytes. The sensitivity of any positive PCR result for an antigenemia level of 2 or more pp65-positive leukocytes was only 75.9%. If this lowest antigenemia level is used for the initiation of pre-emptive therapy, perhaps the best alternative to the pp65-antigenemia test is one of the real-time PCR assays discussed above. The better overall usefulness of the Cobas Amplicor PCR test to detect antigenemia levels of 5 and 10 pp65-positive leukocytes when compared with the lower antigenemia level of 2 pp65-positive leukocytes was also shown by the increase in area under the ROC curves.

Of interest, 81% of the patients studied actually received antiviral therapy during the first postoperative year. Furthermore, CMV infection usually developed soon after the cessation of the CMV prophylaxis. This raises a question about the reasonable length of the antiviral prophylaxis especially when a low threshold for the initiation of the pre-emptive therapy is used. In our institution, we have recently expanded the length of the CMV prophylaxis up to 6–12 months in LTRs.

The actual usefulness of the different CMV assays studied in the present study depends on whether low-level antigenemia is considered significant and warrants pre-emptive therapy. A wide variation of the cut-off levels for the initiation of pre-emptive therapy (usually equal to 2–25 pp65-antigen-positive leukocytes/50 000 PMNLs) between transplant centers, different patient populations and clinical studies exists [4,6,15,31,32]. In our material, there were 17 episodes of spontaneously resolving low-level antigenemia of which DNAemia and RNAemia were present in only 41% and 12% of the cases, respectively. One could argue that using the antigenemia test in the surveillance of CMV with low thresholds for pre-emptive therapy might lead to unnecessary controlling of the assay and too aggressive treatment of CMV antigenemia increasing the costs of the pre-emptive treatment strategy and the incidence of the side-effects of antiviral drugs. On the other hand, in addition to the short-term morbidity and mortality of CMV infection, activation of the virus is also associated to acute and chronic allograft injury (e.g. acute rejection, transplant coronary artery disease and obliterative bronchiolitis) [33–36]. Some of the recent reports suggest that even asymptomatic CMV antigenemia or DNAemia could lead to chronic allograft dysfunction, but an unanswered question is if blocking all CMV replication (low-level antigenemia or DNAemia) could prevent this [33–36]. Future prospective studies are needed to finally answer the question: what is the optimal threshold for the initiation of pre-emptive therapy in thoracic organ transplant recipients?

We acknowledge some limitations of the present study. Firstly, there were some differences in the prevention and treatment of CMV infections between the HTRs and the LTRs (prophylaxis and thresholds for pre-emptive therapy). This may weaken the clinical relevance of the conclusions made, when considered for each transplant group separately. However, we were able to compare the two other CMV tests to the antigenemia test in general by reporting the results of the three CMV tests in each CMV infection and by calculating the sensitivities and specificities for different cut-off levels of antigenemia. Secondly, some of the blood samples could not be retrieved to our laboratory in time to be valid for the CMV assays. Long geographical distances is one of the major problems in collecting samples for the CMV tests and using pre-emptive treatment strategy [3,37]. This is especially true in sparsely populated countries such as Finland with only one transplant center. Thus, CMV tests that could be reliably performed also in distant centers (e.g. based on commercially available PCR) are urgently needed.

To conclude, in thoracic organ transplant recipients, the pp65-antigenemia test was somewhat more sensitive than the Cobas Amplicor PCR test, but the differences of these two assays were not considered to be of major

clinical significance. The Cobas PCR assay is as good as the antigenemia test in the guidance of pre-emptive therapy when thresholds over 5 pp65-antigen-positive leukocytes/50 000 PMNLs are used for the initiation of therapy. In contrast, the low sensitivity of NASBA limits its usefulness in the surveillance of CMV and guidance of antiviral therapy, when cut-off levels of 2 to 10 pp65-antigen-positive leukocytes/50 000 PMNLs are used as the reference.

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