Jpn. J. Infect. Dis., 54, 12-16, 2001

Original Article

Evaluation of the AMPLICOR CMV, COBAS AMPLICOR CMV Monitor and Antigenemia Assay for Cytomegalovirus Disease

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(Received September 12, 2000. Accepted January 31, 2001)

SUMMARY: The AMPLICOR CMV (qualitative DNA assay by PCR), COBAS AMPLICOR CMV Monitor (quantitative DNA assay by PCR), and antigenemia assay were tested for their ability to diagnose cytomegalovirus (CMV) infection in 115 immunocompromised patients. The AMPLICOR qualitative assay and the antigenemia assay were positive for all nine patients with a clinical diagnosis of CMV disease. The AMPLICOR quantitative assay was negative for one of the nine patients. In 106 patients without CMV disease, the AMPLICOR qualitative test was positive in 22, the quantitative test was positive in 23, and the antigenemia test was positive in 55 patients. The AMPLICOR qualitative and quantitative assays had specificities of 79% and 78% in patients without CMV disease, while that of the antigenemia assay was 48%. Diagnostic efficiencies were 79% for the AMPLICOR qualitative assay, 69% for the AMPLICOR quantitative assay, and 48% for the antigenemia assay. All three tests yielded positive results before, or at the same time as, the onset of CMV disease in most cases, which suggests they can be used to predict disease before the onset of symptoms. During antiviral treatment, test results tended to decrease quantitatively and finally became negative; negative results were followed by remission of symptoms. This suggests that the AMPLICOR quantitative assay and the antigenemia assay could be useful for monitoring therapeutic efficacy. The AMPLICOR qualitative and quantitative assays, as well as the antigenemia assay were considered effective for all of the following: diagnosing CMV disease, predicting the onset of disease, and evaluating the effectiveness of antiviral chemotherapy. The antigenemia assay was at times difficult to perform in the case of severely neutropenic patients, whereas the AMPLICOR assays could be used in such cases.

INTRODUCTION

Cytomegalovirus (CMV) infection may cause serious disease in immunocompromised patients. Antigenemia testing and polymerase chain reaction (PCR) are now considered promising methods of diagnosing of CMV (1-3). The antigenemia assay has been recommended with several guidelines for bone marrow transplant patients (4,5). However, some reports have suggested that both methods may be too sensitive for diagnosing CMV infection, predicting disease onset, and monitoring the clinical course of disease (6).

The AMPLICOR qualitative and quantitative assays were developed by Roche Molecular Systems, Inc. (Pleasanton, Calif., USA) (7). It has been reported that the sensitivity of the AMPLICOR qualitative assay could be adjusted by establishment of a suitable cut-off point (8). In order to determine the sensitivity and specificity of these tests and to compare their performance to that of the antigenemia assay, we conducted a clinical study in patients who underwent bone marrow transplantation (BMT) or kidney transplantation (KT) and in patients with acquired immunodeficiency syndrome (AIDS).

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MATEIRALS AND METHODS

Patients and samples: A total of 115 patients were registered as follows: BMT: 54 patients hospitalized for BMT. KT: 39 patients hospitalized for KT. AIDS: 22 patients with CD4 lymphocyte counts of less than 100/μl. Informed consent

was obtained from all patients. Blood samples were collected from patients with BMT and KT at weekly intervals for 12-16 weeks, starting the week before transplantation. Blood samples from AIDS patients were collected at weekly intervals for 12-16 weeks. Eight patients with symptoms such as fever or cough were followed further at a physician's request.

Blood samples were kept at 2-8°C during transport to the laboratory. Serum was separated and tested by PCR on the same day the blood was collected. Similarly, white blood cells (WBC) were isolated from whole blood and tested in the antigenemia assay on the day of collection.

AMPLICOR CMV: This is a qualitative PCR test for the detection of CMV DNA in serum and was performed according to the manufacturer's instructions. Briefly, 50 μl serum was mixed with 500 μl of extraction reagent and incubated at 100°C for 30 min. The mixture (50 μl) was transferred into the PCR tube containing all components necessary for amplification, plus an internal control (IC). The IC was a non-infectious plasmid with primer binding sequences identical to and signal sequences distinct from the target DNA. The IC was added to permit the identification of processed specimens containing substances that may have interfered with PCR amplification. Amplification was performed on a GeneAmp PCR system 9600 thermal cycler manufactured by Applied Biosystems (Foster, Calif., USA). After amplification, nucleotide sequences were hybridized to oligonucleotide-coated microwell plates. The probe-bound amplified products were detected colorimetrically with an avidin-horseradish peroxidase conjugate and 3,3'5,5'-tetramethylbenzidine (TMB). Absorbance was measured at 450 nm on a NovaPath MICROPLATE READER manufactured by Bio-Rad Laboratories (Hercules, Calif., USA). Samples with an absorbance value of ≥0.35 were considered positive. Samples with an absorbance value of <0.35 and IC with an absorbance of ≥0.35 were considered negative. When the absorbance for both CMV and the IC were below 0.35, the results were considered invalid. In such cases, it is generally recommended that another aliquot of the original specimen be processed, or that a new specimen be collected. The limit of detection of this assay was 1000 copies/ml.

COBAS AMPLICOR CMV monitor: This quantitative PCR test for CMV DNA in serum was performed according to the manufacturer's instructions. Two hundred microliters of serum was mixed with 600 μl of lysis reagent containing a known number of quantitation standard (QS) DNA molecules. The DNA was precipitated with isopropanol, washed with 70% ethanol, and suspended in the Monitor Specimen Diluent. Fifty microliters of processed samples were added to 50 μl of the master mix for amplification. Amplification and detection were automatically performed with a COBAS AMPLICOR Analyzer manufactured by Roche Molecular Systems, Inc.

The COBAS AMPLICOR Analyzer calculates the CMV DNA levels in the test specimens by comparing the CMV signal to the QS signal for each specimen. Results were expressed as number of copies/ml. The COBAS AMPLICOR Analyzer reports the result as invalid for any sample with a QS optical density outside of an acceptable range, which is automatically programmed into the Analyzer. The limit of detection of this assay is 400 copies/ml.

Antigenemia test: This assay was performed with a CMV Kogen "Mitsubishi" kit, manufactured by Yuka Medias Co., Ltd. (Ami, Ibaraki). Assays were conducted according to the manufacturer's instructions (9). A 4-ml sample of EDTA - anticoagulated venous blood was mixed with 1 ml dextran solution. After sedimentation for 15 min at 30°C, the supernatant was collected and centrifuged at 150 G for 6 min. The red blood cells were lysed with 3 ml of erythrolysis solution for 5 min on ice.

The pellet was washed, centrifuged, and resuspended at a cell concentration of 1.5×10^6/ml. One hundred microliters of suspension was spotted onto a slide with a cytocentrifuge, and the suspension was then dried and fixed. The slides were stained by an immunofluorescence assay with a monoclonal antibody against CMV pp65 antigen. The slides for each specimen were prepared in duplicate. The presence of positive cells/1.5×10^5 WBC was considered as a positive result. The results are calculated by taking the average of two slides.

Criteria for clinical diagnosis of CMV disease: The criteria for clinical diagnosis of CMV pneumonia, enteritis, retinitis, systemic infection, hepatitis, and other CMV diseases were determined by a committee that relied primarily on criteria previously reported by Ljungman and Plotkin (10), as shown in Table 1. Patients were classified with systemic infection when they presented with symptoms suspicious of CMV infection and when they tested positive for CMV antigen or CMV DNA by in-house testing.

Period of study: This study began in September 1998 and was completed in August 1999.

RESULTS

Patients: Of the 115 patients, 9 were diagnosed with CMV disease (Table 2). Four patients were diagnosed with pneumonia, 2 with retinitis, 2 with gastroduodenal ulcer, 1 with hepatitis, and 1 with systemic disease; 1 patient presented with pneumonia and retinitis. The remaining 106 patients were free of CMV disease throughout the study.

Table 1. Criteria for diagnosis of CMV disease

CMV Disease	Clinical Findings
CMV Pneumonia	① Hypoxia ② Chest X ray ③ Pneumonia symptoms ④ Positive CMV by BAL[1] or biopsy
CMV Enteritis	① Nausea vomiting abdominal pain ② Melena ③ Clinical symptoms such as peptic ulcer ④ Histology of CMV by biopsy
CMV Retinitis	① Typical ophthalmological finding
CMV Systemic Infection	① Fever ② General fatigue ③ Leukocytopenia ④ Thrombocytopenia ⑤ Detection of CMV antigen or CMV DNA in blood
CMV Hepatitis	① Abnormal liver function (elevation of AST[2], ALT[3]) ② Histological detection of CMV ③ exclusion of other viral hepatitis
Others	① Encephalitis ② Nephritis ③ Pancreatitis ④ Organ symptom and detection of CMV in that organ

[1] BAL: Broncho-alveolar lavage [2] AST: Aspartate aminotransferase [3] ALT: Alanine aminotransferase

Table 2. Patients with CMV disease

Case Number	Patients	CMV Disease
1	AIDS	CMV Pneumonia
2	AIDS	CMV Retinitis
3	AIDS	Esophageal ulcer
4	AIDS	CMV Pneumonia
5	Kidney Transplantation	CMV Pneumonia
6	Kidney Transplantation	CMV Enteritis
7	Kidney Transplantation	CMV Systemic Infection
8	Bone Marrow Transplantation	CMV Retinitis, CMV Pneumonia
9	Bone Marrow Transplantation	CMV Hepatitis

Sensitivity for CMV disease: The sensitivity, specificity, and diagnostic efficiency of the respective tests are summarized in Table 3. Both the AMPLICOR qualitative assay and the antigenemia assay yielded positive results for at least one blood sample for each of 9 patients with CMV disease. However, the AMPLICOR quantitative assay results were negative in 1 patient (case 3) among these 9 patients. This patient was human immunodeficiency virus (HIV)-positive, had an esophageal ulcer, and was diagnosed with CMV retinitis after the end of the observation period. No treatment was administered during the observation period. The AMPLICOR qualitative assay was positive only once during the observation period. The antigenemia assay was positive from the second week, but the number of positive cells was consistently less than $10/1.5 \times 10^5$ WBC during weeks 2-12.

The sensitivities of the AMPLICOR qualitative, AMPLICOR quantitative, and antigenemia assays for CMV disease was 100%, 89%, and 100%, respectively.

Specificity for CMV disease: Among the 106 patients without CMV disease, the AMPLICOR qualitative test was positive in 22, the AMPLICOR quantitative test was positive in 23, and the antigenemia assay was positive in 55 patients.

The respective specificities for CMV disease of the AMPLICOR qualitative assay, AMPLICOR quantitative assay, and antigenemia assay were 79%, 78%, and 48%.

Diagnostic efficiency: The diagnostic efficiency of each test was calculated by the following formula: sensitivity (%)/100 × specificity (%)/100. Respective efficiency percentages were 79%, 69%, and 48% for the AMPLICOR qualitative, AMPLICOR quantitative assay, and antigenemia assay.

Time course from CMV detection until disease diagnosis: The time course of CMV disease and assay results are shown in Table 4. In 9 patients, 12 episodes of CMV disease were observed. The AMPLICOR qualitative test was positive in 10 episodes. In 6 of the 10 episodes, this test was positive at least 1 week before the onset of disease. The AMPLICOR quantitative test was positive in 9 episodes. In 6 of the 9 episodes, it was positive before the onset of disease. The

Table 3. Sensitivity, specificity and diagnostics efficiency of three assays

Assay	CMV disease 9 cases		Sensitivity (%)	Non CMV disease 106 cases		Specificity (%)	Diagnostic Efficiency (%)
	Positive	Negative		Positive	Negative		
1) AMPLICOR qualitative assay	9	0	100	22	84	79	79
2) AMPLICOR quantitative assay	8	1	89	23	83	78	69
3) Antigenemia assay	9	0	100	55	51	48	48

Diagnostic efficiency was obtained as sensitivity (%)/100 × specificity (%)/100

Table 4. Time course of CMV disease (Weeks)

Case	Period of CMV Disease	Period of Antiviral Therapy	AMPLICOR qualitative		AMPLICOR quantitative		Antigenemia	
			+	−	+	−	+	−
1	1-4	1-6	1-3, 8	0, 4-6	1-3, 8	0, 4-6	1-2, 4, 8	0, 3, 5-6, 8
2	4-9	1-27	0, 2-4	1, 6-9	2	0-1, 4-9	0-6, 8	7, 9-10
	13-20		none	10-23	none	10-23	11, 13, 16, 19	15, 19, 20-22
	25-27		24-27	29	24-29	none	23-27	29
3	0-20	none	3	0-2, 4-20	none	0-20	2-20	0
4	3-5	3-7	2, 4-5	0, 1, 7	1-2, 4-5	0, 7	1-3, 5	0, 7
5	5-6	5-6	none	5-7	none	5-7	5-6	none
	8-9	8-10	8	9-10	8, 9	10	7-9	10
6	6-7	7-9	5, 7-8	0-4, 9-13	5, 7-8	0-4, 9-13	4-5, 7, 13	0-3, 8-12
7	14	13-14	13	0-12, 14	13, 14	0-12	11-13, 14	0-10
8	37-46	25-47	30-40	24-29, 41-47	30-31, 35, 38	24-25, 27, 29, 41-43, 45-47	27, 29-30, 32, 35-39	24-26, 28, 31, 34, 40-47
9	9-11	11-13	5, 10	0-4, 6-9, 11-14	5-6, 10, 11-12	0-4, 7-9, 13-14	0, 4-5, 9-10, 11	3, 6-8, 11-14

Table 5. Test results for patients without CMV disease

Number of cases	Test results		
Total 106	AMPLICOR qualitative	AMPLICOR quantitaive	Antigenemia
50	–	–	–
20	+	+	+
32	–	–	+
1	+	+	–
1	+	–	+
2	–	+	+

Table 6. Frequencies of positive on antigenemia assay

32 cases non- CMV disease group		9 cases of CMV disease group	
Frequency of positive test during observation period (times)	Number of cases (%)	Frequency of positive test during observation period (times)	Number of cases (%)
1	16 (50.0%)	1	0 (0.0%)
2	11 (34.4%)	2	0 (0.0%)
3	2 (6.3%)	3	0 (0.0%)
4	1 (3.1%)	4	3 (33.3%)
5	1 (3.1%)	5	2 (22.2%)
6	1 (3.1%)	6	0 (0.0%)
≧7	0 (0.0%)	≧7	4 (44.4%)

antigenemia assay was positive in all 12 episodes. In 8 of the 12 episodes, it was positive before the onset of disease. These findings suggest that these tests may be effective for preemptive or early diagnosis of CMV disease.

Completion of antiviral therapy and negative test results: In cases 1, 4, 5, 6, and 9, all three tests became negative after several weeks of antiviral therapy and after the clinical symptoms had also subsided. In case 8, all three tests remained positive after acyclovir and ganciclovir administration and they became negative after foscarnet administration, which induced a remission of symptoms. In case 7, the onset of CMV disease occurred just prior to the end of the observation period of 14 weeks. Thus, tests were not performed on this patient during the course of the study. In case 2, tests became negative after the first treatment; at the time of the second treatment, the AMPLICOR qualitative and quantitative assays were already negative.

Quantitative difference between true and false positive tests: Results of all three tests for patients without disease are shown in Table 5. The false positive rates were 20.8% for the AMPLICOR qualitative assay, 21.7% for the AMPLICOR quantitative assay, and 51.9% for the antigenemia test. For the AMPLICOR quantitative assay, the average copies/ml was 3,746 from 31 samples in the group with CMV disease and 1,948 from 50 samples in the group without CMV disease. The number of copies between the two groups was statistically different ($P = 0.029$).

As regard to the antigenemia assay, the average number of positive cells/1.5×10^5 WBC was 13.1 for 65 samples in the group with CMV disease and 4.5 for 150 samples in the group without CMV disease. The number of cells between the two groups was statistically different ($P = 0.001$).

Among the 32 patients who received false positive results by the antigenemia assay only, positive results occurred 1-2 times in 84% of the patients, and the number of positive cells was lower than $4/1.5 \times 10^5$ WBC cells in 92% of the positive samples. In the patients with CMV disease, positive results were observed more frequently than four times with more than five positive cells/1.5×10^5 WBC as shown in Table 6.

Other aspects: Due to leukocytopenia, the antigenemia was not tested during the 1-2 weeks after BMT in 31 of 54 patients. The AMPLICOR qualitative and quantitative assays were tested during that period. The AMPLICOR quantitative assay yielded invalid QS results several times in 6 patients. It appears that an interfering substance was present in the blood samples of these patients. Although heparin is known to interfere with PCR, we did not use heparin for blood collection.

DISCUSSION

For the diagnosis of CMV disease, the AMPLICOR qualitative assay had a sensitivity of 100%, a specificity of 79%, and a diagnostic efficiency of 79%. The AMPLICOR quantitative assay had a sensitivity of 89%, a specificity of 78%, and a diagnostic efficiency of 69%. The antigenemia assay had a sensitivity of 100%, a specificity of 48%, and a diagnostic efficiency of 48%. The AMPLICOR qualitative and quantitative assays thus appear to yield higher specificity and higher diagnostic efficiency.

Boivin et al. (11) reported that in HIV infected patients, the antigenemia assay was very sensitive (96%), yet less specific (83%) than the AMPLICOR qualitative assay (sensitivity: 65%, specificity: 92%) or a PCR test using leukocytes with a cut-off point of 690 copies /10^5 cells (sensitivity: 65%, specificity: 94%). Tong et al. (12) tested eight types of assay, including an antigenemia assay and the AMPLICOR qualitative test, in 37 kidney transplant patients. They reported that the AMPLICOR PCR produced the best results and furthermore that it predicted CMV disease 2-6 days before the onset of symptoms.

Pellegrin et al. (13) reported that the specificity of the antigenemia assay was elevated by setting the cut-off point at five positive cells. In the present study, we observed similarly high sensitivities in all three tests, as reported. When the AMPLICOR qualitative assay was performed with the cut-off point set at 0.35 absorbance, we observed similarly high

specificity. In the antigenemia assay, the result was judged positive with a positive cell /1.5 × 10^5 WBC. If a suitable cut-off point could be established, a higher grade of specificity might be obtained. For example, when a cut-off point of 5/1.5 × 10^5 was set in the case of the antigenemia assay in this study, the specificity increased from 48.1% to 77.4%.

All three tests produced positive results in 7 of 9 patients and 8 of 12 episodes. These results were obtained before, or at the same time as, the onset of CMV disease. The AMPLICOR qualitative test, the AMPLICOR quantitative test, and the antigenemia assay all produced positive results at 1.9 weeks, 1.8 weeks, and 2.4 weeks, respectively, before the onset of CMV disease. This suggests that early diagnosis may be possible. Aitken et al. (14) reported that 14 out of 52 kidney transplant patients with CMV disease tested positive by the AMPLICOR assays 12.5 days before the onset of CMV disease. In BMT patients, Boeckh et al. (15) reported that the antigenemia assay guided preemptive treatment with good timing. Einsele et al. (16) also reported that PCR monitoring reduces the severity of CMV disease as well as the duration of antiviral therapy. The Japanese Society for Hematopoietic Cell Transplantation (5) recommended the antigenemia assay in the 1997 Japanese guidelines for BMT. In the Centers for Disease Control and Prevention (CDC) guidelines for BMT in 2000, Dykewitz et al. (4) recommended monitoring patients weekly with either the antigenemia assay or a plasma PCR such as AMPLICOR for the purpose of administering rapid preemptive treatment.

The AMPLICOR qualitative and quantitative assays, as well as the antigenemia assay were considered to be useful for the diagnosis and monitoring of CMV disease in immunocompromised patients, including those with BMT, KT, and AIDS.

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