

Comparison of a rapid cytomegalovirus pp65 antigenemia assay revealed by immunofluorescence to an in-house assay revealed by immunoperoxidase for diagnosis in solid organ transplant recipient patients

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ABSTRACT

Cytomegalovirus (CMV) antigenemia is still one of the two major assays available for diagnosis and monitoring of CMV infections. A commercial rapid test recently available in Brazil for quantification of human cytomegalovirus pp65 antigenemia revealed by immunofluorescence technique was compared with the original in-house method revealed by immunoperoxidase in patients receiving solid organ transplants. Of 80 blood samples tested for CMV antigenemia, 34 (42.5%) were positive: commercial assay detected 33 (97%) and in-house assay detected 20 (58.8%) samples. The numbers of positive cells in the two assays were different, with a median of 4.5 and 12 positive cells obtained by in-house and commercial kit, respectively. Discrepancies between assays occurred in 15 specimens from patients with low-grade antigenemia (median 6 positive cells). The assay-time was reduced in approximately 50% compared to in-house methodology. In conclusion, besides comparable results obtained for both assays, the commercial antigenemia assay provides more rapid and sensitive results.

Keywords: cytomegalovirus, antigenemia assay, immunoperoxidase, immunofluorescence.

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INTRODUCTION

Cytomegalovirus (CMV) continues to be important pathogens in immunosuppressed individuals, particularly transplant recipients and patients infected with human immunodeficiency virus. In addition to variable CMV clinical presentation, in transplant recipients, CMV disease can mimic the symptoms of allograft rejection, and differentiation is crucial because intensification of immunosuppression would only aggravate CMV infection.¹

Antiviral therapy can provide significant clinical benefit and be life-saving, provided that therapy is initiated promptly. It is therefore essential to have an assay that allows the rapid and reliable diagnosis of CMV disease and monitoring response to treatment. The CMV pp65 antigenemia detect and quantifies CMV-infected leukocytes by staining CMV pp65 antigens in peripheral blood. Many studies have shown that a positive result is strongly associated with active CMV infection and disease, can be used for monitoring antiviral therapy efficacy and in resistance emergency.² However, the great limitation of this test is that it requires

approximately 6 hours and is labor-intensive to perform. Several modifications in the technical procedure have been proposed since the initial use in the 80s,³ but one-step, erythrocyte lysis without neutrophils concentration have demonstrated to save time and simplify the process.⁴ Recently available in Brazil, a commercial assay CMV Brite Turbo (IQ Products, Netherlands), with one-step erythrocyte lysis and revealed by immunofluorescence incorporated the main advantages of this procedure.

In this study, a rapid commercial Cytomegalovirus pp65 antigenemia assay, with revelation by immunofluorescence, was compared to an in-house assay, with revelation by immunoperoxidase, for detection and quantitation of CMV in clinical samples for transplant recipient patients.

MATERIAL AND METHODS

Study samples

Eighty fresh EDTA-anticoagulated blood samples from consecutive recipients of solid organ transplantation were submitted to the Albert

Einstein Hospital Microbiology Laboratory for CMV pp65 antigenemia evaluation. From each patient, 5 mL of blood was used for in-house immunoperoxidase antigenemia assay and 1 mL was used for commercial immunofluorescence antigenemia assay. Each slide was evaluated by two well-trained readers independently, and only results with up to 10% difference in number of positive cells were accepted.

In-house immunoperoxidase revealed CMV antigenemia assay

A total of 5 mL of EDTA-anticoagulated blood was mixed with 2 mL of 5% dextran solution (Amersham), and the mixture was incubated at 37° C for 15 min to allow sedimentation of erythrocytes in 45° angle inclination tube. The leukocytes-rich supernatant was mixed with 10 mL of phosphated-buffered saline (PBS). After centrifugation at 200 g for 5 min, the cell pellet was suspended in 8 mL of NH₄Cl solution (NH₄Cl [8.3 g/L], KHCO₃ [1.0 g/L], EDTA [0.03 g/L], pH 7.4) on iced water for 10 min at 4° C for rupture of the remaining erythrocytes. The cells were then washed two times in PBS and centrifuged at 200 g for 5 min. The final cell pellet was suspended in PBS and concentration was adjusted after leukocyte counts obtained in T890 cell analyzer (Coulter, USA). Cytospin slides were obtained by centrifugation of 3x10⁵ leucocytes on glass slides at 500 rpm for 5 min (Cytospin 3; Shandon, USA). After slide fixation with cold acetone for 5 min, each slide was covered with 25 µL of 1/10 diluted CMV monoclonal mouse antibody clone C10/11 (Dako Cytomation, Denmark), and incubated for 45 min at 37° C. After washing for 5 min with PBS, the slides were incubated with 25 µL of 1/100 diluted Horsesch Peroxidase conjugated goat anti-mouse IgG antibody (Dako Cytomation, Denmark). After washing for 5 min with PBS, slides were incubated with 25 µL of AEC solution (3-amino-9-ethylcarbazole solution in 0.1M acetate buffer [pH 4.9]) for 10 min at room temperature in the dark for color development. This coloration was stopped with acetate solution for 5 min, and negative leukocytes were stained with Mayer's Hematoxylin. The total numbers of positively stained leukocytes with bright red nuclei were counted by light microscopy and results expressed as the number of pp65-positive/3x10⁵ leukocytes.

Commercial immunofluorescence revealed CMV antigenemia

A total of 1 mL of EDTA-anticoagulated blood was added to 14 mL of 0.8% NH₄Cl solution in a plastic centrifuge tube and incubated at 4° C for 15 min. Afterwards, the tube was centrifuged 200 g for 3 min and cell pellet washed twice with PBS under the same conditions of centrifugation. The final cell pellet was suspended in PBS and concentration was adjusted after leukocyte counts obtained in T890 cell analyzer (Coulter, USA). Cytospin slides were obtained by centrifu-

gation of 4x10⁵ leucocytes on glass slides at 500 rpm for 5 min (Cytospin 3; Shandon, USA). The slides were air-dried, fixed with 5% formaldehyde, permeabilized with Nonidet P-40, and incubated for 20 min at 37° C with 35 µL of primary monoclonal antibody. After washing for 5 min with PBS, slides were incubated with 35 µL of secondary antibody fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin. At the end, the slides were washed with PBS and mounting glycerol and glass. The number of nuclear green fluorescence was scored under epifluorescence microscope at 40X magnification and results expressed as the number of pp65-positive/4x10⁵ leukocytes.

RESULTS

The results are shown in Table 1. Of 80 samples tested, 34 (42.5%) were positive by either or both CMV antigenemia tests. Of these, 19 samples were positive by in-house CMV antigenemia assay and commercial CMV antigenemia assay, with 20 (55.8%) and 33 (97%) samples detected in each assay, respectively. The numbers of positive cells in the two assays were different, with a median of 4.5 and 12 positive cells obtained by in-house and commercial kit, respectively.

Discrepancies between commercial assay and in-house developed assay results occurred in 15 specimens from patients with low-grade antigenemia. Only one positive sample in the in-house assay had two positive cells per 3x10⁵ leukocytes, and the remaining discordant samples had median of 6.5 positive cells per 4x10⁵ leukocytes. The difference between assays was obtained in different times to complete the assays results: approximately 6 hours for in-house assay revealed by immunoperoxidase and 3 hours for commercial assay revealed by immunofluorescence. Considering as true positive the result obtained by any of the tests, commercial CMV antigenemia and in-house assays had a sensitivity of 95% and 60%, respectively, with 82.5% of accuracy and 0.62 Kappa index. None of the samples showed counts considerable different between the two independent readers in any assay.

Table 1. Comparison of immunofluorescence and immunoperoxidase stained CMV antigenemia among 80 solid organ transplanted patients

IFI	IPO	number	cell positive count (median)
+	+	19	12/4.5
+	-	14	6.5
-	+	1	2
-	-	46	0

IFI, indirect immunofluorescence; IPO, immunoperoxidase.

DISCUSSION

The quality of the stained slides was comparable between the two methods, with low nonspecific background staining and excellent leukocyte morphology. The technical hands-on time for the commercial CMV antigenemia kit was shorter and need less blood volume sample. Thus, the availability of a commercial assay that can be completed in 3 hours is of great benefit to the laboratory, as well as to patient care. Importantly, the 3-h assay provided quantitative results equivalent to or slightly better than those of the standard test.⁵

Besides the reduced testing time, our results according to others authors indicate a good correlation between immunoperoxidase antigenemia and immunofluorescence antigenemia.^{5,6} Both assays use a mixture of C10-C11 monoclonal antibodies that react specifically with HCMV pp65 proteins giving reaction to different epitopes and improving the determination and quantitation of CMV antigenemia.⁷ Clinical significance of specimens found to be positive by only one test was not evaluated, but some discrepancies in the results produced by both tests was probably due to the more than 33% cells that were applied on slides for rapid testing, as recommended by the manufacturer. The justification for the increased number of cells is that standard dextran sedimentation for separation of leukocytes enriched for neutrophils, the cells predominantly express the CMV pp65 antigen in the peripheral blood. With direct erythrocyte lysis, there is presumably no such enrichment. Therefore, to obtain equivalent numbers of neutrophils, a greater number of cells must be examined. However, Ho *et al.*⁴ reported that differential counts of PBLs separated by dextran sedimentation versus direct erythrocyte lysis were, in fact, similar. We confirmed this finding.

Although antigenemia has been replaced by DNAemia for diagnosis and preemptive therapy of HCMV infection in some transplantation centers, pp65 antigenemia still remains the guiding assay for preemptive therapy in several other centers.⁸ A variety of sophisticated molecular tests are now available for CMV. In general, the reagents are more expensive, and special equipment and/or separate rooms are often required. Because of the time and expense involved in performing quantitative nucleic acid detection methods, single or even small numbers of samples are usually not tested. Rather, samples are batch tested once or several times per week. DNA based methods may be advantageous for reference laboratories with high test volumes and processing delays due to specimen transportation.⁹ However, in the hospital laboratory, CMV antigenemia has many advantages. None of the molecular tests can provide a quantitative result with a 2-h turnaround time. Pp65 CMV antigenemia

commercial kits have limitations: influence of time delayed sample processing on cytomegalovirus antigenemia assay results in lowest positive cell counts.¹⁰⁻¹² and impossibility of samples storage,¹³ besides the possibility that long-term storage can be achieved by keeping fixed slides at -80° C.⁹

In conclusion, the new commercial CMV antigenemia immunofluorescence revealed to have a good diagnostic value and faster assay for the detection and quantification of HCMV infection in solid organ transplanted patients.

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