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Comparison of Six Methods for the Detection of Antibody to Cytomegalovirus

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Five commercial assays were compared to a standardized complement fixation (CF) test for the detection of antibody to cytomegalovirus. Two hundred and thirty serum specimens were analyzed. In addition, nine pairs of acute- and convalescent-phase sera were tested by two of the commercial assays. The assays were compared as to sensitivity, specificity, and positive and negative predictive value, as well as incidence of false-positive and -negative results. Samples which did not agree in all the assays were retested and tested with an indirect fluorescent-antibody assay. Of 228 specimens, 103 (45.2%) were positive by CF. Of the 230 samples, 2 (0.9%) were inconclusive by CF and readable in the other assays. Of the 230 specimens, 97 (42.2%) were positive by an enzyme immunoassay (EIA; Litton Bionetics), 100 (43.5%) were positive by a second EIA (Abbott Laboratories), 104 (45.2%) were positive by a third EIA (M. A. Bioproducts). One hundred and eight (47.0%) were positive by indirect hemagglutination (IHA; Cetus Corporation), and 110 (47.8%) were positive by latex agglutination (LA; Hynson, Westcott and Dunning). Sensitivity and specificity were similar with all the assays (93 to 100%). The greater numbers of positive results by IHA and LA were confirmed by repeat CF testing at <1:8 dilution, and by indirect fluorescent-antibody assay. Acute- and convalescent-phase serum pairs showed a significant rise in antibody titer when tested by anticomplement immunofluorescence, IHA, and LA. There was good agreement among the assays, with LA having the highest sensitivity.

Cytomegalovirus (CMV) infections can be asymptomatic or have a variety of clinical symptoms, with patients presenting with an infectious mononucleosis syndrome or hepatitis, which vary depending on several factors. Concurrent infections, drug therapy, age, and immune status all play a major role in the severity of infections due to CMV. Persons at risk for acquired immunodeficiency syndrome have increased prevalence of CMV infection, but its role in the syndrome and especially Kaposi's sarcoma remains unclear.

Primary infections occur in serologically negative individuals after exposure to the virus. Secondary infections are a result of activation of latent infections or reinfection of a serologically positive individual (7).

CMV is a leading cause of congenital viral infections and is associated with infections after blood transfusions, renal transplants, and bone marrow transplants (2, 14, 15, 17). The CMV antibody status of the transfusion or transplant donor and recipient is an important factor in the management of patients at risk, such as low-birth-weight neonates and transplant recipients (1, 11, 13, 15, 19).

Although the presence of antibody to CMV does not indicate infectious blood or tissues, it is a marker indicating previous infection. Serologic tests for the detection and quantitation of antibodies to CMV are a valuable aid in determining clinical diagnosis (18). The presence of specific CMV antibody as evidence of past or present infection is the most valuable indirect indicator of CMV infection or a potentially infective donor. Demonstration of a significant rise in antibody titer from the acute to the convalescent phase substantiates acute infection with CMV.

Recently, several studies report results of comparisons between several serological assays for the detection of CMV antibody (3–5, 8, 9, 16). These reports indicate different methods as the test of choice for screening blood donors.

We compared five commercial assays with the standardized complement fixation (CF) assay by using samples from clinical patients and organ or bone marrow donors and recipients. In addition, two of the assays were used to detect seroconversion in nine pairs of sera previously tested for seroconversion by an anticomplement immunofluorescence assay (ACIF).

MATERIALS AND METHODS

Serum specimens. Sera were (i) 200 samples sent to the clinical laboratory and (ii) 30 samples from patients with acquired immunodeficiency syndrome from the University of California at San Francisco, (iii) 9 pairs of acute- and convalescent-phase samples from Mt. Zion Hospital, San Francisco (L. Drew). All samples were stored in capped glass tubes at −20°C.

CF. Serum samples were submitted to the microbiology laboratory of the San Francisco Department of Public Health for CMV antibody testing by the standard CF microtiter method previously described (10). Briefly, the sera were diluted in veronal-buffered saline, heat inactivated, and further diluted on microtiter plates in serial twofold dilutions. The screening dilution used was 1:8. Two units of CMV antigen (1:4 dilution of CMV strain AD-169; Flow Laboratories, Inc., McLean, Va.) and 2 U of guinea pig complement (M. A. Bioproducts, Walkersville, Md.) were added, and the plates were incubated overnight at 4°C. After the plates and reagents equilibrated to room temperature, an indicator system consisting of equal parts of 2 U of hemolysin (rabbit anti-sheep erythrocyte stromata serum in 50% glycerin; M. A. Bioproducts) and a 1.4% sheep blood cell suspension in VBS (sterile sheep blood in modified Alsever solution; Microbiological Media, Concord, Calif.) was added. The plates were sealed, vibrated, and incubated at 36°C for 18 to 21 min. After incubation, the plates were stored at 4°C for 1 h to allow for settling of the cells, and the

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degree of hemolysis in the supernatant fluid was read visually. The CMV antibody titer was the highest serum dilution demonstrating no more than 25% hemolysis (≥75% CF). All appropriate controls were included in each run.

ACIF. The nuclear ACIF was used as the standard method for determining acute CMV infection with paired samples. Samples were submitted to and tested at Mt. Zion Hospital and Medical Center, San Francisco, Calif. The ACIF procedure uses isolated nuclei from CMV-infected human fibroblasts as described previously (12). Briefly, isolated nuclei were fixed on glass slides in acetone at room temperature for 10 min. Heat-inactivated serum (56°C for 30 min) was added to slides and incubated at 37°C for 60 min under a moist cover. Slides were washed for 5 min in phosphate-buffered saline. Guinea pig complement (M. A. Bioproducts) was added and incubated at 37°C for 30 min under a moist cover. Slides were washed as described above. Fluorescein isothiocyanate-conjugated goat anti-guinea pig complement C3 (B_1C-B_1A) (Cappel Laboratories, Cochranville, Pa.) was added and incubated at 37°C for 30 min under a moist cover. Slides were washed as described above and air dried. Fluorescence was examined without cover slips under a Zeiss epifluorescence microscope. Nuclear fluorescence was graded as 0, ±, 1+, 2+, or 3+. Antibody titer was determined as the highest serum dilution producing 1+ specific nuclear fluorescence. Uninfected control nuclei were included in each run as were positive and negative control sera.

EIA. Three enzyme immunoassays (EIA) were evaluated. The CMV Total AB EIA (Abbott Laboratories, North Chicago, Ill.) uses a 1:21 dilution of serum. Serum was incubated with the solid-phase bead coated with CMV antigen (AD 169) for 60 min at 40°C. Beads were washed three times with deionized water with the Pentawash II (Abbott). Horseradish peroxidase-conjugated goat anti-human immunoglobulin (Ig) was added to each bead and incubated for 30 min at 40°C. Beads were again washed and transferred to plastic tubes (12 by 75 mm). *O*-Phenylenediamine substrate was added to each tube and incubated for 30 min at 22°C. Sulfuric acid (1 N) was added to each tube. Absorbance was read on a Quantum II photometer (Abbott). A cutoff value of the mean absorbance of negative controls plus 0.075 was calculated. Samples with absorbance greater than 10% above the cutoff were positive. Samples less than 10% of the cutoff were negative. Samples within 10% of the cutoff value were equivocal and were retested.

The CMV RQ Bio-Enzabead (Litton Bionetics, Kensington, Md.) uses a 1:50 dilution of serum. All reagents were prewarmed at 37°C. Serum was incubated with two solid-phase, plastic-coated ferrous beads, one coated with CMV antigen (AD 169) and the second coated without CMV, for 10 min at 37°C. Beads were washed 12 times in water–0.05% Tween 20 with a magnetic transfer device. Beads were transferred to a plate containing peroxidase-conjugated goat anti-human IgG (heavy and light chain specific) and incubated for 10 min at 37°C. Beads were washed as before and transferred to a plate containing 0.03% 2,2′-azino-di(3-ethyl)benzthiazoline sulfonate and incubated for 10 min at 37°C. Sodium fluoride (1.25%) was added to each well and mixed. Absorbance was read at 690 nm on an LBI-300 enzyme-linked immunosorbent assay (ELISA) reader (Litton). A calibration curve was drawn with absorbance versus ELISA values provided by the manufacturer for six calibrators. Absorbances of samples from patients were converted to ELISA values from the calibration curve. An ELISA value <0.085 from the antigen bead was considered negative. The ELISA value from the antigen and control beads was used to calculate a delta value and a ratio. The ELISA value of the antigen bead minus the control bead equals delta. The ELISA value of the antigen bead divided by that of the control bead equals the ratio. A delta value of ≥0.025 and a ratio of ≥0.028 were considered positive. A delta value of 0.021 to 0.024 and a ratio of 0.023 to 0.027 were equivocal and were retested. Delta <0.021 and ratio <0.023 were negative.

The Cytomegelisa test (M. A. Bioproducts) used a 1:26 dilution of serum. Serum was placed into 2 wells of a 96-well tray and incubated for 45 min at 22°C in a humidified chamber. One well was coated with CMV antigen (AD 169), and the second well was coated with a suspension without CMV. Sera were decanted, and the plate was washed with phosphate-buffered saline–0.05% Tween 20 for 3 min. Washing was repeated twice after the initial wash. Excess buffer was removed by blotting. Alkaline phosphatase-conjugated rabbit anti-human IgG (heavy and light chain specific) was added to each well and incubated for 45 min at 22°C in a humidified chamber. Conjugate was decanted and the plate was washed as described above. *p*-Nitrophenyl phosphate substrate was added to each well and incubated for 45 min at 22°C. NaOH (1 N) was added to each well and mixed. The plates were read at 405 nm on a Titertek Multiskan (Flow Laboratories). The absorbance from the control antigen well was subtracted from that of the CMV antigen well. Three calibrators supplied were used to construct a calibration curve by linear regression. The regression used calibrator absorbance values versus Cytomegelisa values provided by the manufacturer. A correlation coefficient ≥0.95 is necessary for an acceptable test. Two control sera need to be within manufacturer-defined limits for an acceptable test. Absorbance was converted to Cytomegelisa values with the calibration curve. Values ≤0.22 were negative, those between 0.23 and 0.24 were equivocal, and those ≥0.25 were positive. Equivocal samples were retested.

IFA. An indirect fluorescent-antibody test (IFA) was used on selected samples. The CMV antibody IFA (Electro-Nucleonics, Inc., Columbia, Md.) uses a 1:8 dilution for IgM. For IgG, a 1:16 dilution of serum was placed in a well containing a mixture of uninfected and CMV-infected human foreskin fibroblasts and incubated for 30 min at 22°C under a moist cover. For IgM, the first incubation was 60 min at 22°C under a moist cover. Slides were washed with three 5-min washes in phosphate-buffered saline. Slides were air dried. Fluorescein isothiocyanate-conjugated goat anti-human IgG (heavy and light chain specific) or fluorescein isothiocyanate-conjugated goat anti-human IgM (heavy chain specific) was added to each well and incubated for 30 min at 22°C under a moist cover. Slides were washed as described above and air dried. Cover slips were placed on slides with buffered glycerol filling the interface. Fluorescence was read on a Zeiss epifluorescence microscope. Fluorescent intranuclear inclusions in infected cells were considered positive.

IHA. An indirect hemagglutination assay (IHA) was evaluated. The CMV IHA (Cetus Corp., Emeryville, Calif.) uses a 1:4 dilution of serum. Samples were pipetted into 2 wells each of a 96-well V-bottom plate. One well contained CMV antigen (AD 169)-coated human O-erythrocytes. A second well contained noncoated erythrocytes. The plates were covered with plate sealers, and the reactants were mixed. The plates were incubated at 22°C on a moist towel. Cell patterns were read on a plate viewer with a concave magnifying mirror at 90 min, 4 h, and 18 h. Patterns were interpreted in relation to negative and low and high positive

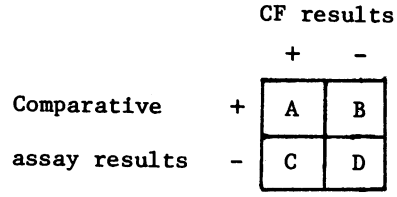

FIG. 1. Percent sensitivity = A/(A + C) × 100. Percent specificity = D/(B + D) × 100. Positive predictive value = A/(A + B) × 100. Negative predictive value = D/(C + D) × 100. False-positive rate = B/(B + D) × 100. False-negative rate = C/(A + C) × 100.

controls. Any sample in which the control wells showed a dispersed mat was considered invalid and was repeated. Negative samples showed a compact button with CMV-coated cells. Positive samples exhibited dispersed cell mats with CMV-coated cells. Samples which were positive at the 1:4 dilution were repeated with dilutions from 1:4 through 1:8,192. The endpoint was the reciprocal of the highest dilution showing a dispersed mat of CMV-coated cells.

LA. A passive latex agglutination (LA) test was evaluated. The CMV Scan (Hynson, Westcott and Dunning, Inc., Baltimore, Md.) uses undiluted serum. Serum was placed onto a test card and spread with a plastic stirrer to fill the scribed circle. The suspension of CMV antigen (AD 169)-coated latex particles was mixed on a vortex mixer just before use. One drop of the latex suspension was added to each circle with serum. The card was rotated three to four times by hand and then placed on a horizontal rotator. Cards were incubated for 8 min at 22°C under a humidifying cover while rotating at 100 rpm. The latex patterns were read under a high-intensity incandescent lamp. Samples showing any agglutination were considered to be positive. Positive samples were retested undiluted through dilutions of 1:8,192. The endpoint was the reciprocal of the highest dilution showing agglutination of the latex suspension.

Analysis of results. All testing was carried out without knowledge of previous results. The CF test was used as the reference method. Discrepant samples were retested with the CF test. Samples which were <1:8 by CF but positive by one or more of the other assays were retested by CF at a dilution of 1:4. The IFA was run on any sample which did not agree in all the assays. IFA was used as the confirmatory test, since IgM-specific testing was desired. Percent sensitivity, specificity, positive and negative predictive value, and the incidence of false-positive and -negative results were calculated as described in Fig. 1, which was adapted from Griner et al. (6).

RESULTS

Table 1 lists the results as total number positive or negative compared with CF. Of the 230 sera tested, all were used in the comparison with the CF test. Forty-five percent (103 of 228) of the sera were positive by CF, and two samples (0.9%) were inconclusive by CF. One of these samples showed a nonspecific hemolysis reaction to control antigen in the CF and was negative in all the other assays. The second was anticomplementary and positive in the other assays. All of the assays had some discrepant results when compared with CF for discriminating positive versus negative results.

Table 2 summarizes the performance characteristics of all of the assays. Percent sensitivity, specificity, false-positive and -negative rates, and positive and negative predictive values are shown. These results were calculated as described in Materials and Methods with a 1:8 screening dilution in CF as the standard test. The data from Table 2 can be used to select the test best suited for laboratory use. High sensitivity is a requirement of a screening test. By this criterion, the LA test had the highest percent sensitivity. The negative preditive value was also the highest with the LA test. Since the CF screen missed low-level positive samples which were detected by the LA and IHA, the statistics show a high percentage of false-positive results and a low predictive value for both the LA and IHA.

TABLE 1. CMV seroreactivity of study samples

Assay method	Screening dilution	Assay result	No. of samples tested by CF as: +	No. of samples tested by CF as: −	Total no. of samples (%)
Abbott EIA	1:21	+	96	3	100 (43.5)
		−	7	122	130 (56.5)
Litton EIA	1:50	+	96	0	97 (42.4)[a]
		−	7	125	132 (57.6)
M. A. Bioproducts EIA	1:26	+	96	7	104 (45.2)
		−	7	118	126 (54.8)
Cetus IHA	1:4	+	98	9	108 (47.0)
		−	5	116	122 (53.0)
H. W. & D.[b] LA	Undiluted	+	100	9	110 (47.8)
		−	3	116	120 (52.2)

[a] Since one sample showed increased binding to the negative control bead, $n = 229$.
[b] Hynson, Westcott and Dunning, Inc.

Table 3 lists those samples which gave discordant values in one or more of the assays. Included are the repeat CF and IFA results on selected samples. In five cases, samples which were <8 by CF and positive by one or more of the other assays were positive by CF when tested at 1:4. Eight samples were negative at 1:4 in the CF, whereas five others were anticomplementary at 1:4. The IFA results were positive in all but two discordant samples. One sample had a nonspecific reaction in CF and was negative in all of the other assays. The second IFA-negative sample was anticomplementary in CF and positive in all of the others. One sample, which was positive by IHA and LA only, showed only IgM by IFA.

Table 4 lists results of paired samples submitted for diagnosis of acute CMV infection. ACIF was the reference method. Both the IHA and LA detected significant rises in antibody titers. A fourfold rise in the titer of sample 4 was not detected by IHA.

DISCUSSION

Documentation of infection with CMV is often clinically important. Serological testing becomes the method of choice, since routine culture of the virus is impractical. The method of choice used should be efficient and technically easy to perform. Ideally, the assay would be highly sensitive and have a low incidence of false-negative results.

CF is an established method for detection of antibody to CMV. The procedure is time consuming and requires considerable expertise to perform. In addition, screening at a 1:8 dilution may miss some low positive sera, whereas screening at less than 1:8 is impractical because of nonspecific reac-

TABLE 2. Performance characteristics of assays

Assay	Sensitivity (%)	Specificity (%)	False result rate (%)		Predictive value (%)	
			Positive	Negative	Positive	Negative
Abbott EIA	93	98	2	7	97	95
Litton EIA	93	100	0	7	100	95
M. A. Bioproducts EIA	93	94	6	7	93	94
Cetus IHA	95	93	7	5	92	96
H. W. & D.[a] LA	97	93	7	3	92	98

[a] Hynson, Westcott and Dunning, Inc.

tions and the anticomplement nature of many samples. It is also known that some antibodies do not fix complement. The IFA test, which was used to confirm seroreactivity of discordant samples, is also time consuming when many samples are tested. This procedure requires considerable expertise in interpreting staining patterns by fluorescence microscopy.

Recently, commercial assays have made CMV testing easier to perform. The choice of the assay is based on clinical application and performance characteristics.

In our study, the IHA and LA tests were highly sensitive. Specimens which were positive by these two assays when tested at low dilutions and negative by EIA were positive by IFA. Also, no interfering autoantibodies could be detected in these samples. All three EIA procedures detected fewer positive sera than did either the IHA or LA. In addition, the

TABLE 3. Results of discordant samples

Sample no.	Reactivity when tested by:						Reactivity on confirmatory testing by:	
	CF	Abbott EIA	Litton EIA	M. A. Bioproducts EIA	Cetus IHA	H. W. & D.[a] LA	CF[b]	IFA for IgG[c]
1	+	+	+	−	−	+	ND[d]	+
2	NS[e]	−		−	−	−	ND	−
3	AC[f]	+	+	+	+	+	ND	−
4	−	−	−	−	+	+	−	−
5	+	−	+	+	+	+	ND	+
6	+	+	+	−	+	+	ND	+
7	+	−	+	+	+	+	ND	+
8	+	−	+	+	+	+	ND	+
9	+	−	+	+	+	+	ND	+
10	−	−	−	−	+	+	−	+
11	+	+	−	−	−	+	ND	+
12	+	+	−	−	+	+	ND	+
13	+	+	−	+	+	−	ND	+
14	+	+	−	−	−	+	ND	+
15	−	−	−	−	+	+	−	+
16	−	−	−	+	+	+	−	+
17	−	−	−	+	+	+	+	+
18	−	−	−	+	+	−	AC	+
19	−	−	−	−	+	+	+	+
20	−	−	−	−	+	+	−	+
21	+	−	−	−	−	−	ND	+
22	−	+	−	−	−	−	AC	+
23	−	+	−	−	−	−	AC	+
24	−	+	−	−	−	−	−	+
25	−	−	−	+	−	−	AC	+
26	−	−	−	+	−	−	+	+
27	−	−	−	+	−	−	AC	+
28	−	−	−	+	−	−	+	+
29	−	−	NS	+	−	−	+	+
30	−	−	−	−	−	+	−	+
31	−	−	−	−	−	+	−	+
32	−	−	−	−	+	−	−	+

[a] Hynson, Westcott and Dunning, Inc.
[b] CF repeat done at 1:4.
[c] IFA testing = if samples were positive for IgG, IgM testing was not done. Samples 2 and 3 were negative when tested for IgM; sample 4 was positive for IgM.
[d] ND, Not done.
[e] NS, Nonspecific reaction.
[f] AC, Anticomplementary in CF.

TABLE 4. Seroreactivity[a] of acute- and convalescent-phase samples

Sample no.	Phase of infection[b]	ACIF	Cetus IHA	H. W. & D.[c] LA
1	A	<8	<4	–
	C	256	128	256
2	A	<8	<4	–
	C	256	16	64
3	A	<8	<4	–
	C	1,024	64	128
4	A	<8	<4	–
	C	2,048	8	16
5	A	<8	<4	–
	C	2,048	32	64
6	A	<8	<4	–
	C	1,024	128	128
7	A	<8	<4	–
	C	512	32	8
8	A	<8	<4	–
	C	1,024	256	256
9	A	16	32	2
	C	512	2,048	512

[a] Results are expressed as the reciprocal of the highest dilution showing reactivity.
[b] A, Acute; C, convalescent.
[c] –, Negative when tested on undiluted serum.

incidence of false-negative results was higher with EIA than with IHA or LA.

The Abbott EIA is not designed for detection of acute infection with acute- and convalescent-phase serum pairs. This eliminates the use of this assay in a laboratory setting in which serological verification of acute CMV is needed. The Abbott EIA also detected only 10 samples as positive out of the 32 discrepant samples. The Litton EIA is designed to detect acute infection with paired sera. Since the lowest number of positive sera were detected by the Litton EIA and the lowest number of discrepant samples were detected as positive, paired sera were not tested by this method. The M. A. Bioproducts EIA is designed to detect acute infection with paired sera. However, paired serum samples were not tested with the M. A. Bioproducts EIA, since fewer positive sera were detected among the discrepant samples than were detected with IHA or LA.

Some of the discrepancies in discriminating positive versus negative results among all the assays may be attributed to the screening dilution used. Although we did repeat negative CF samples at lower than 1:8 dilutions, we did not retest negative specimens with any of the commercial assays at dilutions less than those recommended by the manufacturer. If one ignores differences in sensitivity due to the methodology and only considers the screening dilution, the commercial assays can be ranked in the following order based on number of specimens detected as positive and the screening dilution. The highest number of positive sera was detected by the LA test, which uses undiluted serum. The IHA, using a 1:4 dilution of serum, detected the next highest number of positive sera. The M. A. Bioproducts EIA, which uses a 1:26 dilution, was next. The Abbott EIA, at a 1:21 dilution, detected the next highest number of positive sera. The Litton EIA, which uses 1:50, the highest dilution, detected the fewest positive sera.

The IHA test, although comparable in performance to the LA test, is more difficult to read. We felt that the reading of positive versus negative results at 1:4 was straightforward, although determination of endpoints was more difficult. Significant variation in endpoints can arise with IHA when they are read by different technologists. Most specimens tested by IHA gave stable endpoints when read at 90 min through 18 h. Greater than 1 dilution difference was observed in approximately 6% (6 of 108 positive sera) when the IHA endpoint was determined at 90 min and at 4 h. In these 6 instances, the endpoint was less at 4 h than at 90 min. A greater than 1 dilution difference was not observed in any specimens when readings were made at 4 and 18 h.

All endpoints were easy to read with the LA assay. Also, intertechnologist variation in reading endpoints was quite low. However, determining the titers of positive specimens can be time consuming and tedious.

All five commercial assays were similarly priced when the screening dilution was used. Both the IHA and LA are considerably more expensive than EIA when samples are titrated to endpoint.

Technically, the LA test is the easiest to perform. For our purposes, the LA test is the most applicable to a clinical immunology laboratory where there is a need for many different technologists to perform the test. The high level of sensitivity, ability to detect acute infection with paired sera, and technical ease of performance are advantages of the LA procedure.

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