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Comparison of Commercially Available Enzyme Immunoassay with Traditional Serological Tests for Detection of Antibodies to *Coccidioides immitis*

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A newly released commercially available enzyme-linked immunosorbent assay (ELISA) was evaluated for its ability to detect immunoglobulin M (IgM) and IgG antibodies against the tube precipitin and complement fixation (CF) antigens of *Coccidioides immitis*. The ELISA was compared with more traditional diagnostic assays, CF, latex agglutination (LA), and immunodiffusion (ID). When the IgM-specific portion of the ELISA was compared with LA, there was an agreement of 81.8%, a specificity of 75.0%, and a sensitivity of 84.6%. For the determination of the presence of IgG antibodies, the results of the IgG-specific part of the ELISA were compared with the combined results of ID and CF. After resolution of discrepant results, there was an agreement of 95.6%, a specificity of 98.3%, and a sensitivity of 92.6%. When the results of the IgG- and IgM-specific portions of the ELISA combined were compared with the results of the three traditional assays (CF, LA, and ID) there was an agreement of 96.7%, a specificity of 98.5%, and a sensitivity of 94.8%. The ELISA proved to be a reliable assay for the detection of antibodies against the tube precipitin and CF antigens and did not suffer from the objectivity required to interpret the results of the traditional assays and anticomplement interference associated with the traditional assays.

Coccidioides immitis is a dimorphic fungus that grows as a mold in the soil. Its distribution is limited to the Western Hemisphere between California and Argentina. In North America, the region where *C. immitis* is endemic includes the southwestern United States from California to Texas and occurs as far north as parts of Nevada and Utah and extends south into Mexico (1).

Coccidioidomycosis presents a diagnostic challenge to physicians and laboratory technicians. Lesions produced by *C. immitis* may be difficult to distinguish from the lesions resulting from other fungal diseases, tuberculous lesions, or neoplasms (benign and malignant). Symptoms are often unremarkable and can mimic various pneumonias, sarcoidosis, cancer, and other maladies (1, 4). It can be difficult to demonstrate the presence of the organisms histologically and to isolate the organisms in culture even after repeated attempts.

Frequently, serologic results are the only evidence available that can be used to suggest a preliminary diagnosis. A positive serologic result would indicate the need for more definitive diagnostic techniques such as culture or biopsy to confirm the diagnosis. Serologic diagnosis is generally based on the detection of antibodies to two main *Coccidioides* antigens, the tube precipitin (TP) and the complement fixation (CF) antigens. The antibody response to the TP antigen has been shown to be primarily an immunoglobulin M (IgM) antibody response, while the CF antigen is more associated with an IgG antibody response (1, 4–6). In our laboratory we use the CF test as an initial screen to detect antibodies against *C. immitis*. This partially purified antigen is obtained from mycelial-phase culture filtrates of the organism and primarily contains CF antigen. This method detects both IgG and IgM antibodies directed against *C. immitis* but does not distinguish between the two. We use two other assays, latex agglutination (LA) and immunodiffusion (ID), in our laboratory to identify *C. immitis* antibodies. The LA test detects agglutinating antibodies that are of sufficiently large size to bridge between TP antigen-coated latex particles. Ig's other than IgM (e.g., IgG, IgA, and IgD) are not as effective at bridging between the sensitized particles, and therefore, positive reactions by LA are mostly associated with IgM antibodies. LA is a sensitive and rapid screening test, but it has a high false-positive rate (up to 15%), so that confirmation of the results of LA by other tests is recommended (2). It is also recommended that all specimens with positive LA results be tested for rheumatoid factor (RF) to rule out potential false-positive reactions. Another method used to reduce false-positive reactions caused by RF in IgM serology is to remove the IgG from the specimen. This prevents the specific IgG antibodies present in the specimen from binding to the antigen, presenting a site for the anti-IgG IgM RF to bind. IgG-absorbent diluents and columns have been shown to be very effective in clearing IgG while leaving IgM intact (3).

For the detection of IgG antibodies more indicative of later-phase convalescence or past infection, the ID assay is used. The test is based on the principles of double diffusion in which antibodies and soluble antigens are placed into separate wells cut in agarose plates. Visible lines of precipitate are formed in the agarose when the antigens and antibodies have combined in the proper relative concentrations, and the results of the reaction are read relative to the reactions of known control antigens and antibodies. Since IgG molecules are much smaller in size and can diffuse more quickly and easily than IgM, the precipitate bands formed after a 24-h incubation at room temperature are usually of the IgG class. In the present studies, we evaluated the performance of a newly released commercially available enzyme-linked immunosorbent assay (ELISA) and compared the results of the ELISA with those of the tradi-

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tional CF, LA, and ID serologic tests for the detection of *C. immitis* antibodies.

MATERIALS AND METHODS

Clinical samples. A total of 111 specimens submitted to our laboratory for *Coccidioides* antibody testing and 15 random blood bank samples were included in the study. These samples were selectively picked from nearly 1,000 samples over a 4-month period (April to July 1993). Extra samples with known positive reactivities, samples with discrepant results among the traditional assays, and samples with anticomplement activity by CF were included in the panel. Therefore, the panel does not represent a normal sample population but was selected to include a variety of specimens with positive, negative, and discrepant results on the basis of traditional tests for *Coccidioides* antibodies (CF, ID, and LA). Of these 126 samples, 112 were serum and 14 were cerebrospinal fluid. Since these samples were submitted from other laboratories and physician's offices, clinical information was not available. Even though an attempt was made to contact the referring laboratory or the individual physician, it was impossible for us to obtain sufficient useful clinical data for inclusion in the study. In most cases the laboratory personnel or physician could not give us a definitive diagnosis. All samples were shipped to the laboratory on ice and were stored at 4°C for no more than 4 days while the CF, LA, and ID tests were conducted. Samples were then stored at −20°C in a nondefrosting freezer for up to 4 months until they were thawed and tested by ELISA within a 24-h period. All samples with discrepant results were then retested by all methods in the following 4 days.

LA procedure. LA was performed according to the instructions of the manufacturer (Immuno-Mycologics, Inc., Norman, Okla.). Serum samples were heat inactivated at 56°C for 30 min. The latex particles provided by the manufacturer were coated primarily with TP antigens. Samples were considered positive for *C. immitis* antibodies when agglutination (clumping) greater than that of the negative control was observed. All positive samples were also tested for RF to rule out potential false-positive reactions.

ELISA procedure. For the ELISA the manufacturer's product insert was followed (Meridian Diagnostics, Inc., Cincinnati, Ohio). A 1:441 dilution was made for serum samples, and a 1:21 dilution was made for cerebrospinal fluid samples. The microwells provided by the manufacturer were coated with a purified mixture of TP and CF antigens. The IgG- and IgM-specific assays use the same sample dilution and were run identically, with the exception of the specific IgG or IgM horseradish peroxidase conjugates. All samples with positive results by the IgM-specific assay were tested for the presence of RFs to rule out false-positive reactions. Four controls, two positive and two negative, were included in both the IgG- and IgM-specific assays. The entire assay, including dilutions, can be performed in less than 2 h. This includes two 30-min incubations and one 5-min incubation. The microplate was read at dual wavelengths of 450 and 630 nm, and optical density (OD) values of ≥0.200 were considered positive, ODs of ≥0.150 but <0.200 were considered indeterminate, and ODs of <0.150 were considered negative.

ID. ID was performed on Cleargel plates with a *C. immitis* mycelial-phase culture filtrate containing both TP and CF antigens according to the manufacturer's instructions (Immuno-Mycologics, Inc.). Plates were read on a Hyperion viewer with a magnifier (Behring Diagnostics, San Diego, Calif.), and the results were reported as follows: (i) negative when only the control precipitin bands were present, with no bands observed between the well containing the patient specimen and the well containing the antigen, and (ii) positive when the control precipitin bands formed bands of identity with the patient specimen precipitin bands.

CF. Specimens were heat inactivated for 30 min at 56°C and were diluted 1:2 by adding 0.2 ml of specimen to 0.2 ml of CF-saline (0.865% NaCl, 0.01% $MgSO_4$, 0.004% CaCl [wt/vol]). By using U-bottom disposable microtiter plates (ICN Flow Laboratories, Costa Mesa, Calif.), 0.025 ml of CF-saline was added to enough wells to test each serum sample plus known positive and negative controls. A twofold serial dilution was made with 0.025-ml microdiluters, discarding 0.025 ml at the end of each row. For each serum sample a dilution series was made for both the antigen and the anticomplement control wells. Next, 0.025 ml of CF antigen (Immuno-Mycologics, Inc.) was added to each test well, and 0.025 ml of CF-saline was added to each anticomplement control well. This was followed by the addition of 0.05 ml of 2 U of guinea pig complement (BioWhittaker, Inc. Walkersville, Md.) to each well, and the plate was incubated for 15 to 18 h at 4 to 8°C. A 1% suspension of sensitized sheep erythrocytes (Immuno-Mycologics, Inc.) was made by mixing equal volumes of 2 U of hemolysin (BioWhittaker, Inc.) and 2% sheep erythrocytes. This mixture was incubated at room temperature for 15 min, and then 0.05 ml was added to each well of a microtiter plate. The plates were placed in a 37°C water bath for 30 min. An end point titer was then determined as that of the well before the first dilution which demonstrated 100% lysis. A titer of 1:2 was considered positive for the presence of antibodies against *C. immitis* if no anticomplement activity was seen. If anticomplement activity was seen in the control wells without antigen, the titer in the wells containing antigen had to be fourfold or greater for the specimen to still be considered positive.

Statistical analysis. For calculating agreement, specificity, and sensitivity, the following formulas were used: *A* is a specimen with a positive result by both ELISA and the traditional assays. *B* is a specimen with a positive result by ELISA but negative results by the traditional assays. *C* is a specimen with a negative result by ELISA but positive results by the traditional assays. *D* is a specimen with negative results by both types of assays. Agreement was equal to $(A + D)/(A + B + C + D)$, sensitivity was equal to $A/(A + C)$, specificity was equal to $D/(B + D)$, the positive predictive value was equal to $A/(A + B)$, and the negative predictive value was equal to $C/(C + D)$.

RESULTS

Comparison of ELISA results with those of CF, LA, and ID. Since the LA test claims to detect mostly IgM antibodies, the IgM-specific part of the ELISA was compared with LA. Of the 126 samples in the study, 14 were cerebrospinal fluid samples and were not included in these statistics because the LA tests for IgM antibodies in serum only. There was an agreement of 81.8%, a sensitivity of 75.0%, and a specificity of 84.6%, with positive and negative predictive values of 66.7 and 89.2%, respectively. According to the LA results, 12 specimens were false positive by ELISA (24 specimens were true positive by both assays) and 8 specimens were false negative by ELISA (66 specimens were true negative by both assays). Two specimens had indeterminate results by ELISA (not included in the calculations), and both of the specimens tested positive by LA. The specific discrepant results are given in Table 1.

For IgG antibody detection, the results of the antibody-specific part of the ELISA were compared with those of CF and ID since these traditional methods primarily detect IgG antibodies. Of the 126 specimens tested by CF, there was sufficient anticomplement activity in 29 samples to prevent determination of a positive or a negative result. Because of this difficulty with CF and for comparison reasons, the results of the CF and ID assays were combined. When these combined results were compared with those of the IgG-specific ELISA, results for 12 samples were not in agreement; 1 sample was negative by both CF and ID and positive by ELISA, while the remaining 11 samples were positive by one method and negative by the other two. In an attempt to resolve these discrepancies, the following criteria were established. (i) A sample was considered false positive by ELISA if it was negative by CF and ID but positive by the IgG-specific ELISA. (ii) A sample false negative by ELISA was positive by both CF and ID and negative or equivocal by the IgG-specific ELISA. After this resolution of discrepant results, there was an agreement of 95.6%, a specificity of 98.3%, and a sensitivity of 92.6%, with positive and negative predictive values of 98.0 and 93.7%, respectively. According to the resolved results, one sample was false positive by ELISA (50 samples were true positive) and four samples were false negative by ELISA (59 samples were true negative). Three samples (samples 62, 74, and 109, Table 1) had anticomplement activities on CF, and the results for those samples remained unresolved.

To determine the overall performance characteristics of the assays, the combined results of the IgG- and IgM-specific ELISAs were compared with the combined results of the three traditional assays (CF, LA, and ID). For this final analysis the following criteria were established: (i) a sample was considered false positive by ELISA if it was negative by all three traditional assays (CF, ID, and LA) but positive by either or both of the IgG- and IgM-specific ELISAs, and (ii) a sample false negative by ELISA was positive by at least two of the three traditional assays and negative by both the IgG- and IgM-specific ELISAs. This method of comparison gave the highest correlation with the ELISA, giving an agreement of 96.7%, a specificity of 98.5%, and a sensitivity of 94.8%. The positive and negative predictive values were 98.2 and 95.5%, respectively. One sample was false positive by the ELISA (55 samples were true positive), and 3 samples were false negative by the

TABLE 1. Comparison of samples with discrepant results by ELISA and traditional assays

Sample no.	OD by ELISA[a]		CF titer (AC titer)[b]	LA	ID	Key for discrepant results[c]
	IgM	IgG				
2	0.361	2.874	64 (4)	NEG	POS	M
6	0.222	0.006	8	NEG	NEG	M
7	0.015	0.304	NEG	NEG	NEG	G, C
8	0.281	0.240	2	NEG	NEG	M
13	0.004	0.828	4 (2)	POS	POS	M
16	0.203	0.236	2	NEG	NEG	M
19	0.175	0.004	NEG	POS	NEG	M
20	0.005	0.004	NEG	POS	NEG	M
25	0.052	0.194	4	NEG	POS	G, C
28	0.003	0.007	2	NEG	POS	G, C
31	0.005	0.003	2	POS	POS	M, G, C
34	0.184	2.168	8	POS	POS	M
36	0.007	3.424	32 (32)	POS	POS	M
62	0.006	0.222	4 (2)	NEG	NEG	UNR
63	0.070	1.001	8 (2)	POS	POS	M
68	0.008	2.659	16	POS	POS	M
74	0.480	0.006	4 (2)	NEG	NEG	M, UNR
78	0.005	2.763	32 (4)	POS	POS	M
81	0.824	2.771	8	NEG	POS	M
82	0.741	2.612	8	NEG	POS	M
83	0.494	0.835	2	NEG	NEG	M
84	0.235	0.007	4	NEG	NEG	M
93	0.670	0.465	4	NEG	POS	M
97	1.002	0.040	2	POS	POS	G
99	0.880	0.409	4	NEG	NEG	M
102	0.022	0.830	4	POS	NEG	M
109	0.202	0.834	8 (8)	NEG	NEG	M, UNR

[a] A negative result was an OD of <0.150, an indeterminate result was an OD of ≥0.150 but <0.200, and a positive result was an OD of ≥0.200.
[b] AC, anticomplement activity.
[c] M, discrepant result by IgM-specific ELISA; G, discrepant result by IgG-specific ELISA; UNR, unresolved by IgG-specific ELISA because of anticomplement activity; C, combined result was discrepant.

ELISA (64 samples were true negative). Data for the four samples with discrepant results by the three traditional assays combined (samples 7, 25, 28, and 31) are given in Table 1.

Reproducibility and cross-reactivity of ELISA. Upon retesting of the samples with discrepant results by ELISA, an average interassay coefficient of variation of 11.7% for the positive ODs and 32.6% for the negative ODs was calculated. The results for all samples with discrepant results remained the same after retesting with the exception of sample 49, which changed from an indeterminate (OD, 0.190) to a positive (OD, 0.232) result. Further testing of this sample gave average ODs which fell in the indeterminate range.

Seven samples which were positive by CF for other fungal antibodies were tested by ELISA to check for the cross-reactivity of the assay. All seven samples had antibody directed against a *Histoplasma* yeast with the following titers: 1:8 (n = 3), 1:16 (n = 3), 1:32 (n = 1), and 1:512 (n = 1). Four of these samples had fungal antibodies directed against *Histoplasma* mycelia with titers of 1:8 (n = 1), 1:16 (n = 2), and 1:128 (n = 1). The seven samples had negative CF results for antibodies directed against *Blastomyces* and *Aspergillus* species. All seven samples tested negative for both IgG and IgM antibodies by the ELISA. Results of tests with this limited panel therefore show no cross-reactivity by the ELISA with antibodies to other fungal pathogens.

Blood bank specimens. The 15 serum samples randomly obtained from a blood bank were from donors living in or near Salt Lake City, Utah, an area with a very low incidence of *C. immitis* infection. All 15 samples tested negative for *C. immitis* by the IgG- and IgM-specific ELISAs, CF, LA, and ID, and therefore showed no serologic evidence of previous exposure to *C. immitis.*

DISCUSSION

When the IgM-specific part of the ELISA was compared with LA, there were 22 discrepant samples; 2 were indeterminate, 8 were false negative, and 12 were false positive by ELISA. When the eight samples testing false negative were examined, one sample (sample 20, Table 1) was also negative by CF, the IgG-specific ELISA, and ID and would therefore appear to actually be false positive by LA. Five of the other samples testing false negative by ELISA (samples 13, 36, 63, 68, and 78) were negative by the IgM-specific ELISA but were highly positive by the IgG-specific ELISA (average OD, 2.135). Three of these samples were positive by CF, and all five samples were positive by ID. This indicates either that LA is more sensitive than the IgM-specific ELISA in picking up early IgM antibody or, more likely, that the high IgG antibody levels caused the agglutination and the reaction was not due to IgM antibodies. Samples 31 and 102 may have been truly false negative by the IgM-specific ELISA because the results of CF and LA for both samples were positive. Of the 12 samples with false-positive results by ELISA, 10 samples (samples 2, 6, 8, 16, 81, 82, 83, 84, 93, and 99) were probably truly positive because these samples were positive by both the IgM-specific ELISA and CF. The other two samples (samples 74 and 109) were positive by the IgM-specific ELISA and had anticomplement activity by the CF test, and the results were therefore inconclusive. These data indicate that even though the LA test is designed to detect mostly IgM, high IgG titers may give positive results. For these reasons, the ELISA may be a more sensitive and specific method for IgM antibody detection.

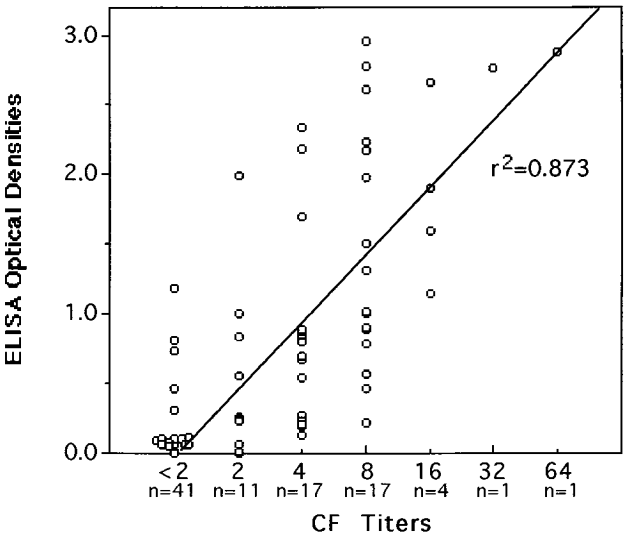

FIG. 1. Correlation of *Coccidioides* CF titers with ELISA OD values.

When the results of the IgG-specific part of the ELISA were compared with the resolved ID and CF results, five samples had discrepant results. The four samples with false-negative results by ELISA (samples 25, 28, 31, and 97, Table 1) were tested by CF and ID for antibodies to other organisms that cause systemic mycoses (*Histoplasma* sp. strain Y, *Histoplasma* sp. strain M, and *Blastomyces* and *Aspergillus* species) and found to be negative. Their positivities by the traditional assays therefore could not be attributed to cross-reactivity. There was also one sample with a false-positive result by ELISA which could not be further resolved without clinical data, which was unobtainable for the patient who provided the sample.

Because CF titers equal to or greater than 1:16 have historically been interpreted as a preliminary indication of extrapulmonary dissemination, CF titers in samples from patients were plotted against the respective ELISA data to see if they correlated (Fig. 1). The resulting correlation coefficient of 0.873 showed a reasonable agreement between OD values and CF titers. More studies with clinical data need to be carried out to determine if the amount of antibody detected by ELISA correlates with the stage or progression of disease. Of the original 126 samples, 29 samples had anticomplement activity by CF, so no result could be determined, and therefore, they were not included in the plot. Of these 29 samples, the results of ELISA, LA, and ID for 27 samples were in agreement (6 samples were positive and 21 were negative). This is a distinct advantage of the ELISA because anticomplement activity does not interfere with antibody detection and reportable results can be obtained.

The best overall correlation was achieved when the combined ELISA results were compared with the combined results of the traditional assays. Of the four samples with discrepant results, one (sample 7, Table 1) was false positive by ELISA and three (samples 25, 28, and 31) were false negative by ELISA and negative for antibodies to organisms that cause other mycoses.

Multiple samples from a patient were submitted to our laboratory. Samples from this patient were drawn on three dates over a period of 23 days, with the first and second samples drawn 10 days apart and the second and third samples drawn 13 days apart. The patient was both positive for both IgG and IgM antibodies by ELISA and showed declining IgM OD values (0.824, 0.741, and 0.670) and increasing IgG OD values (2.612, 2.771, and 3.039) over the 23-day period. The CF titers were 1:8 for the first two samples, but they dropped to 1:4 for the third sample. These data suggest that the OD values obtained by the ELISA can be used to follow the stage or progression of the disease, but a more extensive study is required.

Aside from performance, there were many other advantages to the ELISA. Results are determined objectively with a spectrophotometer. Few indeterminate results were reported by ELISA, and it did not suffer from indeterminate results because of anticomplement activity. Both TP and CF antigens are present in the ELISA, and antibodies in both serum and cerebrospinal fluid are detected. The ELISA is also the only test investigated that uses specific anti-IgG and anti-IgM conjugates to give a definitive IgG or IgM result. The test can also be run in less than 2 h, allowing for results to be reported on the same day that the sample is obtained. A disadvantage of the ELISA is that since the same serum dilution is used for both IgG and IgM testing, there is no IgG absorbent in the diluent, and samples with IgM-positive results must still be tested for RF. Also, the ELISA is approved by the U.S. Food and Drug Administration only for semiquantitative use, and therefore, results can only be reported as positive, negative, or equivocal. Initial studies indicate, however, that there is some correlation between ELISA OD values and both CF titers and antibody levels in the patient.

The CF test has the advantage of an end point titer and can be used for monitoring antibody levels in both the serum and cerebrospinal fluid of a patient. Disadvantages are the need to heat inactivate the specimen, the need for overnight incubation and anticomplementary masking of reactions, and the subjectivity involved in the reading of the test results. The CF test primarily detects antibody to the CF antigen and does not distinguish between IgG- or IgM-positive results.

The LA test has the advantage of being rapid and simple, and it is not affected by anticomplement activity. It primarily detects IgM antibody to the TP antigen, but it does not give a definitive answer because high titers of IgG antibodies can also cause agglutination. The disadvantages of LA are the subjectivity involved in the reading of the results, the test detects antibodies in serum only, and the need to heat inactivate the specimens, which requires an additional 30 min.

The advantages of the ID test include the facts that antibodies can be detected in both serum and cerebrospinal fluid, there is no interference with anticomplement activity, and both TP and CF antigens can be used. The overnight incubation time, the subjectivity involved in the reading of the results, and the inability of the test to give a definitive IgG or IgM response are disadvantages.

We have found the ELISA to be a reproducible, sensitive, and specific assay for the detection of IgG and IgM antibodies directed against the TP and CF antigens of *C. immitis*. Additionally, the ELISA did not suffer from objectivity in determining the results and anticomplement interference associated with the traditional TP, CF, and ID assays.

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