Comparison of Immunodiffusion and Complement Fixation Tests in the Diagnosis of Histoplasmosis

DAVID S. BAUMAN* AND COY D. SMITH

Division of Mycological Diseases, Department of Community Medicine, College of Medicine, University of Kentucky, Lexington, Kentucky 40506

Received for publication 16 April 1975

The immunodiffusion and complement fixation tests for histoplasmosis were compared on 2,090 initial sera from 70 proven cases of histoplasmosis, 177 routine chest hospital patients, and 1,843 coal miners from outside the major endemic area for histoplasmosis. The complement fixation test using histoplasmin as antigen detected antibodies in the sera of 72.8% of the 70 proven cases, while the complement fixation test using yeast antigen detected antibodies in 94.3% of the case sera, and the immunodiffusion test using histoplasmin antigen detected antibodies in 90% of the sera. The tests were also compared for sensitivity, specificity, and ease of performance.

The sequence of events which usually results in the diagnosis of histoplasmosis are as follows. (i) The patient develops symptoms such as cough, fever, and malaise and seeks medical attention; (ii) the physician performs a physical examination and orders a chest X ray; (iii) abnormalities on the chest X ray prompt further investigations such as skin tests, acid-fast stains of sputum smears, and fungal serologies; (iv) after finding a positive skin test and serology, the physician orders sputum cultures to confirm the diagnosis. This sequence of events often takes from 1 to 3 months before specific therapy can be initiated.

The complement fixation (CF) test has long been the main-stay test for reference fungal serology laboratories (1, 4). The chief disadvantage of the CF test has been the lengthy and intricate standardization, which makes the test unsuitable for low-volume laboratories.

Laboratories in private clinics and in small and medium size hospitals usually do not run CF tests for fungal antibodies for a number of reasons. Primarily, maintenance of technical competence is impossible with low volumes of test requests, and the costs of equipment and reagents are prohibitive with the expected request volume. The solution for laboratories in this category is to send the patient specimens to a reference laboratory and this results in a delay of from 1 to 4 weeks before the serological titers are available to prompt the next step in the diagnostic process.

Heiner (2) demonstrated the usefulness of the immunodiffusion (ID) test for the diagnosis of histoplasmosis in pediatric patients. Further, the ID test for histoplasmosis, although slightly

less sensitive than the CF test, is very specific (if properly controlled and standardized), simple to perform, and inexpensive. The ID test can be used in any type institution, the results are available within 24 h and, if positive, provide considerable savings of time to patients and physicians in initiating cultures to confirm the diagnosis. If the ID test is negative and the serum has been sent to a reference laboratory for CF tests, no additional time lag will have been incurred.

The present communication compares the results of the ID and CF tests on three population groups: proven cases of histoplasmosis, routine chest hospital patients, and coal miners from outside the histoplasmosis endemic area. The objective of the study was to determine whether or not the ID test would offer advantages in the routine clinical laboratory for immediate (24 h) serological evidence of histoplasmosis while awaiting the return of CF test results from a reference fungal serology laboratory.

MATERIALS AND METHODS

Study populations. Initial serum samples from 2,090 individuals were examined for CF and precipitating antibodies against *Histoplasma capsulatum*. Two hundred forty-seven of the samples were from patients in chest hospitals in the state of Kentucky. Seventy of the patients subsequently had the diagnosis of active histoplasmosis confirmed by culture and/or histopathology. One hundred seventy-seven of the patients have not been shown to have active histoplasmosis as of this time.

One thousand eight hundred forty-three of the sera were from coal miners from northern West Virginia and southern Pennsylvania. None of these individuals had signs or symptoms of histoplasmosis

at the time of testing. The histoplasmin skin test reactor rate to this population was 19.4%.

The histoplasmosis patient population (70) consisted of 14 females and 56 males and had a mean age of 48.6 years. The majority of these individuals (58 patients) were shown to have pulmonary cavities. The first serum sample from each of the chest hospital patients was used for test comparison to establish the diagnostic value of each of the tests.

Serologic tests. The antigens used in the CF test were HKC-43 histoplasmin (CF-M, optimum dilution 1:32) and yeast-phase *H. capsulatum* A-811 (CF-Y, optimum dilution 1:32). The optimum dilution of each antigen was determined by box titration against a minimum of four proven case sera with titers ranging from 1:8 to 1:128. The CF tests were performed by the standard Laboratory Branch micro-complement fixation test (5) using microtiter equipment (Cook Engineering, Inc.).

The antigen used in the ID test consisted of HKC-43 histoplasmin concentrated to $1/10$ the original histoplasmin (1 g of Sephadex/5 ml of antigen). The ID test was performed in commercial immunodiffusion plates (Immunoplate, pattern D, Hyland Laboratories) with the antigen in the center well of each series and the control serum and patient sera in the peripheral wells. The sera were arranged in the wells so that a control serum well (containing known H and M precipitating antibodies) was adjacent to each patient serum well; in this way each series of peripheral wells contained two control serum wells and three patient serum wells (Fig. 1). The plates were incubated for 24 h at room temperature in a moist chamber and were read in a beam of

high-intensity light with the plates held at a 45° angle to the central ray of the beam, and the eye of the reader at a 90° angle to the beam and outside the light path so that the reader only perceived the reflected light from the precipitin bands.

Sera which formed precipitin bands having identity arcs with the H and M or M bands of the control serum were reported as positive. Sera which formed bands other than H and M, against components of histoplasmin, were investigated with additional antigens such as blastomycin, aspergillin, and coccidioidin in an attempt to discover the correct diagnosis (to be reported in a later communication).

RESULTS

Table 1 compares the CF-M and CF-Y test results on the initial sera from 70 proven cases of histoplasmosis. Three patients (4.3%) were serologically negative to both antigens in the CF test, whereas 20 patients (28.6%) had a negative serology in one of the tests (19 with CF-M, 1 with CF-Y). Twenty-five of the 47 sera, positive in both tests, had higher titers in the CF-Y test, 10 had higher titers in the CF-M test, and 12 sera had equal titers in the tests.

Tables 2 and 3 compare the ID test results on the 70 histoplasmosis patients with the CF-M and CF-Y tests, respectively. Seven of the sera (10%) did not show evidence of precipitating antibodies against histoplasmin. Twenty-two sera (31%) were negative in the CF-M test and four sera (6%) were negative in the CF-Y test. When the ID and CF-M test results were compared (Table 2), five sera from proven cases (7%) were negative in both tests. Comparison of the ID and CF-Y test results (Table 3) showed that all of the proven case sera were positive in at least one of the tests (four positive in ID only, seven positive in CF-Y only, and 59 positive in both tests).

Tables 4 and 5 compare the ID test results on the admission sera from 177 patients in chest disease hospitals (proven histoplasmosis cases were excluded) against the CF-M and CF-Y test results. Eight patients' sera (4.5%) were positive in all three tests, five (2.8%) were positive

FIG. 1. Immunodiffusion pattern illustrating the H and M bands produced between control serum (wells 1 and 3), proven case serum (well 5), and histoplasmin (well 6). Two sera from skin test-negative coal miners (wells 2 and 4) did not produce bands.

TABLE 1. Comparison of CF-M and CF-Y test results on sera from 70 proven cases of histoplasmosis

CF-Y titer	No. with CF-M titer of:					
	0	8	16	32	64	128
0	3	0	0	1	0	0
8	7	6	1	1	0	0
16	7	7	1	5	1	0
32	1	5	5	4	1	1
64	2	2	1	1	1	0
128	2	1	0	1	0	0
256	0	0	1	0	1	0

TABLE 2. Comparison of ID and CF-M test results on sera from 70 proven cases of histoplasmosis

CF-M titer	Immunodiffusion		
	Negative	M band	H and M band
0	5	16	1
8	2	14	5
16	0	4	5
32	0	9	4
64	0	1	3
128	0	0	1

TABLE 3. Comparison of ID and CF-Y test results on sera from 70 proven cases of histoplasmosis

CF-Y titer	Immunodiffusion		
	Negative	M band	H and M band
0	0	4	0
8	2	10	3
16	3	13	5
32	0	10	7
64	2	2	3
128	0	4	0
256	0	1	1

in the ID test only, one was positive in only the CF-M test, and 20 (11.3%) were positive in the CF-Y test only. These patients are currently being investigated to determine which, if any, have active histoplasmosis.

In the population group composed of 1,843 coal miners from outside the endemic area for histoplasmosis, 90 of the sera (4.9%) were positive in more than one of the serological tests (Tables 6 and 7). Five sera were positive in the ID test only, 12 (0.7%) were positive in the CF-Y test only, and none of the sera were positive in the CF-M test only.

The agreement between the CF-M and CF-Y test results on sera from 70 proven cases was 71.4% (3 negative in both tests, 47 positive in both tests). The overall agreement between CF-M and CF-Y on all sera (2,090) was 93.2% (1,883 negative in both tests, 66 positive in both tests).

The agreement between the ID and CF-M test results with proven case sera was 72.9% (5 negative in both tests, 46 positive in both tests). The overall agreement between ID and CF-M was 94.7% (1,916 negative in both tests, 64 positive in both tests).

The agreement between the ID and CF-Y tests with proven case sera was 84.3% (0 negative in both tests, 59 positive in both tests). The overall agreement between ID and CF-Y was 97.5% (1,880 negative in both tests, 157 positive in both tests).

DISCUSSION

The two most meaningful parameters by which a laboratory test can be evaluated for its diagnostic benefit, are sensitivity and specificity. Sensitivity is measured by the number of true cases that are correctly classified by the test and specificity is measured by the number of noncases or cases of other diseases that are incorrectly classified as true cases. In the present study the sensitivity of the ID test, in correctly classifying proven cases, was 90% (63/70). The sensitivity of the CF-M test was 68% (48/70) and for the CF-Y test the sensitivity was 94% (66/70).

Of those individuals tested in the study, the 1,843 coal miners provide the best population for estimating the specificity of the three tests. One major drawback of this population was that, even though the miners lived outside the major histoplasmosis endemic area (skin test reactor rate 19.4%), some might have been infected with *H. capsulatum* at the time they were tested. If we make the assumption, correctly or incorrectly, that this population was free from active histoplasmosis, then the specificities for each of the tests are: 94.2% (1,736/1,843) for the ID test, 99.5% (1,833/1,843) for CF-M, and 94.5% (1,741/1,843) for CF-Y.

Other experience in this laboratory with the

TABLE 4. Comparison of ID and CF-M test results on routine admission sera from 177 chest hospital patients

CF-M titer	Immunodiffusion		
	Negative	M band	H and M band
0	163	5	0
8	1	6	2
16	0	0	0
32	0	0	0
64	0	0	0
128	0	0	0

TABLE 5. Comparison of ID and CF-Y test results on routine admission sera from 177 chest hospital patients

CF-Y titer	Immunodiffusion		
	Negative	M band	H and M band
0	144	5	0
8	20	5	1
16	0	1	0
32	0	0	1
64	0	0	0
128	0	0	0

TABLE 6. Comparison of ID and CF-M test results on sera from 1,843 coal miners

CF-M titer	Immunodiffusion		
	Negative	M band	H and M band
0	1,748	81	4
8	0	7	3
16	0	0	0
32	0	0	0
64	0	0	0

ID test has shown that sera from blastomycosis, coccidioidomycosis, and aspergillosis cases frequently contain precipitating antibodies against components of histoplasmin; however, the precipitin bands formed by these sera are easily distinguished from the H and M bands of histoplasmosis case sera since they fail to form identity arcs with the H and M control bands. By running further ID tests against blastomycin, aspergillin, and coccidioidin along with appropriate control sera, the correct antigen can usually be matched against the patient serum to provide presumptive evidence for the correct diagnosis.

The most attractive feature of the ID test is its simplicity. With properly standardized reagents, antigen, control serum, and diffusion medium, the test is highly reliable.

The most sensitive combination of tests for correctly identifying proven cases of histoplasmosis was the ID and CF-Y tests. None of the cases were missed by this combination, whereas three cases were missed with CF-M and CF-Y in combination and five were missed with ID and CF-M in combination.

The ID test alone was negative with seven of the 70 sera from proven histoplasmosis cases. Those seven sera were positive in the CF-Y test; however, four of the case sera were negative in the CF-Y test, but positive in the ID test.

Kaufman and Clark (3) recently reported a similar comparison of the ID and CF tests for the serodiagnosis of coccidioidomycosis. In their study, the sensitivity (calculated from the published data) of the ID test was 79% (41/52), whereas the sensitivity of the CF test was 80% (40/50, two sera were anticomplementary). Although the calculations for specificity could not be made from the data, the CF test was subject to cross-reactions whereas the ID test showed no cross-reactions.

TABLE 7. Comparison of ID and CF-Y test results on sera from 1,843 coal miners

CF-Y titer	Immunodiffusion		
	Negative	M band	H and M band
0	1,736	5	0
8	12	81	1
16	0	2	5
32	0	0	1
64	0	0	0

The CF-Y test remains the most sensitive single serological test for the presumptive diagnosis of histoplasmosis; however, due to the technical difficulties associated with the standardization and performance of the CF procedure, this test should be reserved for reference laboratories and large hospitals whose volume of test requests justifies the expense of maintaining technical competence. For those laboratories unable to justify performing the CF test, the ID test is available for screening all sera for precipitating antibodies. The sera should also be submitted to a reference laboratory for CF titers. In this way, the most sensitive combination of serodiagnostic procedures for histoplasmosis is available to all patients, regardless of the clinical setting. The ID results can be available in 24 h, with the CF results to follow, providing confirmation and back-up for the ID test as well as quantitation of the serum antibody content. The ID test is more specific than the CF test and provides assistance in determining the etiological agent for those patients whose sera react to more than one fungal antigen.

LITERATURE CITED

1. Buechner, H. A., J. H. Seabury, C. C. Campbell, L. K. George, L. Kaufman, and W. Kaplan. 1973. The current status of serologic, immunologic and skin tests in the diagnosis of pulmonary mycoses. *Chest* 63: 259-270.
2. Heiner, D. C. 1958. Diagnosis of histoplasmosis using precipitin reactions in agar gel. *Pediatrics* 22:616-627.
3. Kaufman, L., and M. J. Clark. 1974. Value of the concomitant use of complement fixation and immunodiffusion tests in the diagnosis of coccidioidomycosis. *Appl. Microbiol.* 28:641-643.
4. Salvin, S. B. 1947. Complement fixation studies in experimental histoplasmosis. *Proc. Soc. Exp. Biol. Med.* 66:342-345.
5. U. S. Public Health Service. 1965. Standardized diagnostic complement fixation method and adaptation to micro test. U. S. Public Health Service Publ. no. 1228.