

Evaluation of a Microtiter Latex Agglutination Test for Histoplasmosis

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Experiments were designed to evaluate a Microtiter latex agglutination (Micro-LA) test, as a serological aid in the diagnosis of histoplasmosis, and to compare this test with the conventional microtiter-complement fixation (CF) test for histoplasmosis. Sera tested were from cases of acute and chronic pulmonary and disseminated histoplasmosis, as well as from individuals not having histoplasmosis. Ninety-seven percent of the cases of acute pulmonary histoplasmosis had positive Micro-LA tests, whereas 91% had positive CF tests. Ninety-six percent of the patients having chronic pulmonary histoplasmosis showed positive Micro-LA tests and 91% had positive CF tests. In contrast, 64% of the cases of disseminated histoplasmosis had positive Micro-LA tests, whereas 82% had positive CF tests. None of these differences was statistically significant. Although there were no significant differences in complement fixing and agglutinating antibody cross-reactivity with *Blastomyces* antigens, more patients demonstrated CF titers than Micro-LA titers. Sera from patients with acute and chronic histoplasmosis showed higher Micro-LA titers than CF titers, whereas sera from cases of disseminated histoplasmosis showed higher CF titers. Histoplasmin skin testing has less of a boosting effect on agglutinating antibodies than on CF antibodies to histoplasmin. Anticomplementary sera can be used in the Micro-LA test. This test is simple to perform, and results can be obtained in 2 to 4 hr.

Investigators have shown the latex agglutination (LA) test to be a valuable tool in the diagnosis of various diseases. Latex particle agglutination testing has been used in the detection of rheumatoid factor (5, 17, 26), lupus erythematosus (4), and leptospirosis (11). Hipp, et al. (10) described a latex slide agglutination test for *Aspergillus* antibodies. Philp et al. (18) applied the latex particle precipitation test in the diagnosis of thyroid disease, and Morris, et al. (14) used it to demonstrate the presence of antibody against *Entamoeba histolytica*.

Bloomfield and co-workers (2) and Goodman et al. (7) detected *Cryptococcus neoformans* antigen in body fluids by coating the latex particles with immune rabbit globulin antibodies to *C. neoformans* antigen. Newman, et al. (15) devised a similar test system as an aid in detecting *Haemophilus influenzae* antigen in the diagnosis of *H. influenzae* meningitis.

The LA test for histoplasmosis evolved from the collodion agglutination test used by Saslaw

and Campbell (22) to screen for histoplasmosis. Carlisle and Saslaw (3) first introduced the use of polystyrene latex particles as a carrier for histoplasmin in 1958. Their results were comparable to those obtained with the collodion agglutination test. The LA test was found to be superior to the collodion agglutination test and less complex to perform (9). These tests were macro methods, i.e., tube agglutination tests using sizeable amounts of serum, antigen, and latex particles.

The purpose of this report is to describe a Microtiter LA (Micro-LA) test for histoplasmosis using micro amounts of serum, antigen, and latex particles and to compare its diagnostic capabilities with the established micro-complement fixation (CF) test for histoplasmosis.

MATERIALS AND METHODS

Antigens used were histoplasmin HKC-43, *Histoplasma capsulatum* whole yeast cells, blastomycin KCB-26, and *Blastomyces dermatitidis* whole yeast cells—all prepared in our Kansas City laboratories.

Although histoplasmin and blastomycin were the only antigen preparations used in the Micro-LA test, *H. capsulatum* and *B. dermatitidis* whole yeast cells, in addition to histoplasmin, were used routinely in our CF testing and were, therefore, included in this comparative study. The only antigen we used to test for cross-reacting CF antibodies to *B. dermatitidis* was *B. dermatitidis* whole yeast cells. The histoplasmin and blastomycin were analyzed for protein by the method of Lowry et al. (12) by using bovine serum albumin as a standard.

Human sera from proven cases on record at the Center for Disease Control, Kansas City, Kansas, were available for serological testing. These sera were from acute pulmonary, chronic pulmonary, and disseminated cases of histoplasmosis. A diagnosis of acute histoplasmosis was based on clinical symptoms, chest roentgenograms, serological and skin tests, and, in many cases, the epidemiologic relationship of the patient to the infecting organism. Only sera taken from the patient within 3 months of the date of onset were tested. The cases of chronic pulmonary histoplasmosis showed chronic cavitary pulmonary lesions as indicated by chest roentgenograms, and *H. capsulatum* was isolated from their sputum. Patients with disseminated histoplasmosis had metastasis of the fungus from the lungs to other tissues. The diagnosis was established by isolation or visualization of *H. capsulatum* in tissues other than lung.

Negative sera were from 41 patients who had at least five negative cultures for *H. capsulatum* and other pathogenic fungi and who had no record of ever having had histoplasmosis. Additional sera were obtained from experimentally infected dogs (beagles), New Zealand white rabbits, and a burro immunized to *H. capsulatum* yeast antigen. All sera were either preserved with Merthiolate at a final concentration of 1:10,000 or frozen at -15°C .

Dogs included in the study were infected intratracheally with 130,000 viable particles of the mycelial form of *H. capsulatum*. All dogs developed clinical evidence of histoplasmosis. After 15 weeks, the dogs were skin tested with 0.1 ml of undiluted histoplasmin HKC-43. Average weekly Micro-LA and CF titers of the 10 dogs were determined.

The 50% endpoint CF test described by Mayer et al. (13), modified in 1959 by the Serology Research Laboratory at the Center for Disease Control, Atlanta, Georgia, and further adapted to the Microtiter system here in Kansas City, was used to test for CF antibodies to *H. capsulatum* and *B. dermatitidis*.

Polystyrene latex particles ($0.796\ \mu\text{m}$) were obtained from the Dow Chemical Co. A stock suspension was prepared from the original suspension containing 10% solids by diluting one volume of this material with nine volumes of distilled water. The stock latex suspension (1:100 dilution) was stored at 4°C and diluted in borate-buffered saline, pH 8.2, prior to use.

The antigen-latex conjugate was prepared the day before the test was to be run. Test concentrations of latex particles were sensitized with equal volumes of predetermined dilutions of either histoplasmin or

blastomycin. A control was prepared by adding to the latex dilution an equal volume of borate-buffered saline instead of dilutions of histoplasmin or blastomycin.

Serial dilutions of 0.05 ml of positive serum were made in Microtiter "U" plates (Cooke Engineering Co.). Fresh borate-buffered saline was used as the diluent. The initial dilution of serum was 1:16; control dilutions were carried out to a dilution of 1:64, and 0.05 ml of sensitized latex particles was added to the serially diluted serum. Nonsensitized particles were added to the serum controls. The reactants were mixed on a Thomas shaking apparatus and incubated at 37°C . After incubation, the reactants were centrifuged and read with the aid of a mirror.

Positive agglutination reactions appear as an "irregular" ring of agglutinated particles (Fig. 1). Negative reactions may appear either as a suspension of latex particles or as a button of latex particles in the bottom of the well.

The following parameters were considered: the optimal dilution of histoplasmin and of latex particles, the optimal temperature, the time of incubation, and the speed and duration of centrifugation.

RESULTS

Optimal test conditions. The initial concentration of latex particles and dilutions of histoplasmin and blastomycin were derived empirically. With regard to nonspecific agglutination and distinctness of the agglutination pattern, an initial dilution of latex particles of 1:2000 proved to be optimal. When this concentration of latex was further diluted 1:100, its optical density was 0.10 at 650 nm in a Bausch & Lomb Spectronic 20 colorimeter. Histoplasmin dilutions of 1:200 to 1:600 and blastomycin dilutions of 1:100 to 1:400 gave maximum antibody titers to histoplasmin and blastomycin in the four species of mammals tested. Diminished titers were obtained with higher or lower dilutions of these antigens.

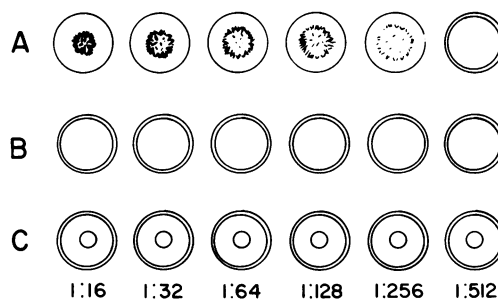


FIG. 1. Positive and negative latex agglutination reactions. Positive reactions appear as an "irregular" ring of agglutinated particles (A). Negative reactions appear as a clear suspension of latex particles (B) or as a button of nonagglutinated latex particles in the bottom of the well (C).

Throughout this study, a histoplasmin dilution of 1:300 (5.7 μg of protein per ml) and a blastomycin dilution of 1:200 (12.6 μg of protein per ml) were used. After all reactants were added, the final dilution of latex particles was 1:8000, the final dilution of histoplasmin was 1:1200, and the final dilution of blastomycin was 1:800.

Optimal temperature and time of incubation was 37 C for 1 hr. Agglutination titers were lower at incubation times of less than 1 hr at temperatures of 25 and 37 C. Control readings were higher at incubation times longer than 1 hr.

An International centrifuge, model V, with a 6-inch radius horizontal head was used for centrifugation. Centrifugation at 1,500 rev/min for 5 min proved to be optimal. At higher speeds we were not able to distinguish between control and positive reactions. At lower speeds, and for 5 min or less, we could not easily detect positive reactions.

Repeatability of histoplasmin Micro-LA test. The titers of positive sera from four dogs, in tests performed on each of four days, are presented in Table 1. Slight differences were seen only in the controls. Titers of sera from individuals with acute pulmonary histoplasmosis (primary infection) are presented in Table 2. When these sera were titered 3 weeks later, in no case was there more than a one-fold difference in titer.

Sensitivity and specificity of the LA test. The average Micro-LA and CF titers for the 10 *Histoplasma*-infected dogs are presented in

TABLE 1. Repeatability and cross-reactivity of histoplasmin Microtiter-latex agglutination tests using positive dog sera

Date of test	Specimen	Control titer	Histoplasmin titer
4/10/70	Dog sera 1	16	256
	2	32	256
	3	16	256
	4	16	512
4/13/70	Dog sera 1	32	256
	2	32	256
	3	16	256
	4	16	512
4/14/70	Dog sera 1	32	256
	2	32	256
	3	16	256
	4	16	512
4/15/70	Dog sera 1	32	256
	2	16	256
	3	16	256
	4	16	512

TABLE 2. Repeatability and cross-reactivity of histoplasmin Microtiter-latex agglutination tests using positive sera from human cases of acute pulmonary histoplasmosis

Positive human serum	Reciprocal of titer					
	August 4, 1970			August 25, 1970		
	Control	Hm ^a	Bm ^b	Control	Hm ^a	Bm ^b
V.C.	0	256	32	0	256	32
H.D.	0	64	0	0	64	0
D.D.	0	64	0	0	64	0
R.D.	0	256	0	0	256	0
R.F.	0	128	32	0	256	32
G.G.	0	256	0	0	256	0
H.G.	0	32	0	0	32	0
K.H.	0	64	0	0	64	0
F.H.	0	256	0	0	256	0
D.H.	0	512	0	0	256	0
L.H.	0	512	0	0	256	0
T.L.	0	256	0	0	128	0
F.M.	0	32	0	0	32	0
D.P.	0	32	0	0	32	0
E.P.	0	256	0	0	128	0
B.R.	0	32	64	0	64	64
L.R.	0	128	0	0	128	0
J.W.	32	128	0	32	128	32
H.Y.	0	64	0	0	64	0

^a Histoplasmin, 1:300 dilution.

^b Blastomycin, 1:200 dilution.

Fig. 2. Micro-LA titers were higher than CF titers, especially in the early stages of infection. After 92 weeks, only five dogs remained in the study; the other five had been sacrificed. The agglutination titers of the five remaining dogs had dropped to zero, whereas CF titers persisted.

Two weeks after skin testing with histoplasmin, there was more than a fourfold increase in the average CF titer. The slight increase in the average Micro-LA titer was due entirely to a threefold rise in the titer of a single dog. The remaining nine dogs all showed a decrease in titer.

Control titers of 1:16 and 1:32 were consistently found when dog sera were tested. Also, human sera that had been stored frozen for five years or more, when diluted 1:16 or 1:32, would frequently agglutinate nonsensitized latex particles. In this study, a titer of 1:16 was considered indicative of antibodies to histoplasmin in the patient's serum when the corresponding control reaction was negative. When a patient had a control titer of 1:32, an antibody titer of 1:32 was not diagnostic.

Only 2.4% of the patients who had no evi-

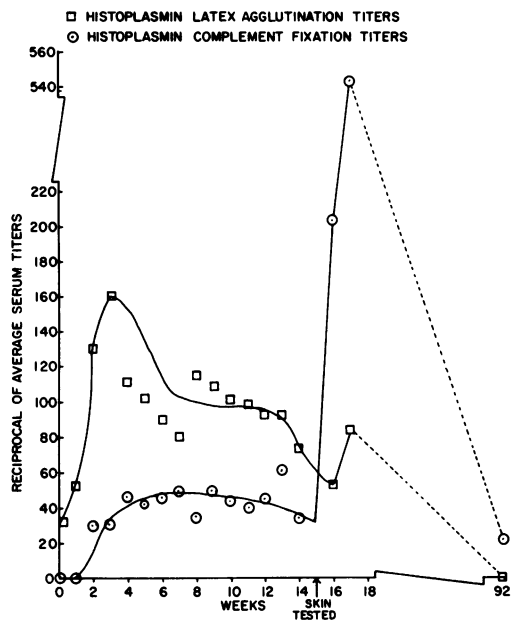


FIG. 2. Comparison of the histoplasmin latex agglutination (LA) test and the complement fixation (CF) test using the sera from 10 dogs experimentally infected with *Histoplasma capsulatum*. Note the increase in the CF titers after the dogs were skin tested at the end of week 15. The increase in the average LA titers was due to a threefold rise in the titer of a single dog. After 92 weeks, agglutination titers dropped to zero, whereas CF titers persisted.

dence of histoplasmosis showed Micro-LA titers of histoplasmin, whereas 12.2% of these patients showed CF titers.

To assess the value of the Micro-LA test as a clinical aid in the diagnosis of histoplasmosis, sera from acute, chronic, and disseminated cases were tested. Thirty-one out of 32 sera (97%) diagnosed as from acute pulmonary cases of histoplasmosis had positive Micro-LA titers (Table 3), 91% of these patients were positive by the CF test. There was no significant difference, however, in the reactivity of the tests. The Micro-LA test showed less, although not significantly less, cross-reactivity with *Blastomyces* antigens.

More patients with chronic pulmonary histoplasmosis were positive by the Micro-LA test (96%) than by the CF test (91%) (Table 4). Although the Micro-LA test was more reactive than the CF test it was, at the same time, more specific. The Micro-LA test showed less, although not significantly less, cross-reactivity with *Blastomyces* antigens.

There was no significant difference in the reactivity of the two tests when sera from dis-

TABLE 3. Comparison of the complement fixation and latex agglutination tests in cases of acute pulmonary histoplasmosis

Test and antigen	Patients tested (no.)	Patients positive	
		No.	%
Complement fixation			
Histoplasmin	32	12	38
<i>Histoplasma</i> yeast	32	29	91
<i>Histoplasma</i> yeast or histoplasmin, or both	32	29	91
<i>Blastomyces</i> yeast	32	7	22
Latex agglutination			
Histoplasmin	32	31	97
Blastomycin	32	4	12

TABLE 4. Comparison of the complement fixation and latex agglutination tests in the diagnosis of chronic pulmonary histoplasmosis

Test and antigen	Patients tested (no.)	Patients positive	
		No.	%
Complement fixation			
Histoplasmin	70	51	73
<i>Histoplasma</i> yeast	70	64	91
<i>Histoplasma</i> yeast or histoplasmin, or both	70	64	91
<i>Blastomyces</i> yeast	70	33	47
Latex agglutination			
Histoplasmin	70	67	96
Blastomycin	70	25	36

seminated cases were titered; however, more patients exhibited CF antibodies than agglutinating antibodies (Table 5). Nine out of 11 patients (82%) had positive CF tests as compared with 7 of 11 patients (64%) positive by the Micro-LA test. As in acute and chronic cases, the Micro-LA test showed less, but not significantly less, cross-reactivity with *Blastomyces* antigens.

Micro-LA titers were generally higher than CF titers when sera from acute and chronic cases were tested. Of the sera from acute pulmonary cases, positive by both the CF and Micro-LA tests, 69% showed higher Micro-LA titers (Table 6); 10% of these sera had equal CF and Micro-LA titers. Of the sera from patients having chronic pulmonary histoplasmosis that were positive by both tests, 90% showed higher Micro-LA titers; 4% of these sera showed equal CF and LA titers. However, in testing sera from disseminated cases of histoplasmosis, 89% of the sera that were positive by both tests showed higher CF titers; 11%

TABLE 5. Comparison of the complement fixation and latex agglutination tests in the diagnosis of disseminated histoplasmosis

Test and antigen	Patients tested (no.)	Patients positive	
		No.	%
Complement fixation			
Histoplasmin	11	5	45
<i>Histoplasma</i> yeast	11	9	82
<i>Histoplasma</i> yeast or histoplasmin, or both	11	9	82
<i>Blastomyces</i> yeast	11	5	45
Latex agglutination			
Histoplasmin	11	7	64
Blastomycin	11	2	18

TABLE 6. Complement fixation and latex agglutination titers of sera from cases of acute pulmonary histoplasmosis

Patient	Latex agglutination Hm ^a	Complement fixation		Patient	Latex agglutination Hm ^a	Complement fixation	
		Hm	Hy ^b			Hm	Hy ^b
A.A.	256	0	16	R.M.	0	8	32
V.C.	256	32	64	F.M.	32	0	128
P.D.	32	0	0	W.N.	512	64	128
H.D.	64	0	64	I.N.	64	32	16
D.D.	64	16	32	D.P.	32	0	0
R.D.	256	16	64	E.P.	256	32	64
R.F.	128	0	32	B.R.	32	0	64
G.G.	256	16	64	L.R.	128	0	32
H.G.	32	32	256	P.R.	128	16	32
K.H.	64	0	32	C.S.	128	0	64
F.H.	256	64	128	W.S.	32	0	32
D.H.	512	0	128	H.T.	128	0	64
R.H.	128	0	64	J.W.	128	0	32
L.H.	512	0	32	L.W.	64	0	32
T.L.	256	0	0	R.W.	32	0	32
M.L.	32	0	128	H.Y.	64	0	128

^a Histoplasmin; expressed as reciprocal of titer.

^b *Histoplasma* yeast.

showed equal CF and Micro-LA titers. No acute or chronic patient was negative by both the CF and Micro-LA tests; one patient with disseminated histoplasmosis was negative by both tests.

The value of the LA test as an aid in the diagnosis of histoplasmosis is further evidenced in that seven sera from two patients were anti-complementary and thus unsatisfactory for CF testing. Four of these sera from a single patient showed positive LA titers.

DISCUSSION

Because of the several-week delay necessary

to obtain cultural proof of histoplasmosis, serological tests are important in arriving at a tentative diagnosis. The first serologic test to come into wide use for the diagnosis of histoplasmosis was the CF test. More recent investigations (1, 3, 9, 24, 25) have shown the LA test to be an important aid in the diagnosis of this respiratory fungus infection.

Our results show that the Microtiter adaptation of the histoplasmin LA test can be employed successfully to demonstrate agglutinins to histoplasmin. A wide range of antigen and latex dilutions is allowable, but the optimal temperature and time of incubation and the optimal speed and duration of centrifugation are more critical.

The LA test has certain advantages over the CF test. The LA test is simple to perform. Results can be obtained in 2 to 4 hr. In contrast, the CF test is complex, consisting of a hemolytic system, nonhemolytic system, complement, and the diluent; in addition, it requires close control and experienced personnel. Most often, sera must be sent to a reference laboratory for testing, thus causing considerable delay in obtaining results. Because LA titers fall to normal rapidly after acute disease, whereas CF titers may remain elevated for years, the LA test is more indicative of active histoplasmosis (1) (Fig. 2).

Some sera are anti-complementary and cannot be used for CF testing but can be tested for agglutinins by the LA test. However, rheumatoid factor present in the serum from some patients with rheumatoid arthritis may interfere with the LA test (26).

The serological reactivity of a patient with histoplasmosis varies depending on his immunological responsiveness at any one time during the course of his disease. Antibodies of varying sensitivities, specificities, and concentrations are produced depending on prior infection, the clinical type of histoplasmosis, the duration of the disease, and the effect of therapy (6). Agglutinins appear earlier than CF antibodies in the course of infection, but disappear sooner (21; Fig. 2). It is possible that complement fixing and agglutinating antibodies, or both, may be present in concentrations that are undetectable by the most sensitive of tests. Therefore, it is important to have a serological test, or tests, as sensitive and specific as possible to take into account such variation in immunological reactivity.

Researchers have reported certain serological tests to be superior to others as diagnostic aids. Hill and Campbell (9) found the latex agglutination reactions to be consistently

stronger and more sensitive than CF reactions when sera from primary pulmonary cases of histoplasmosis were tested. However, Schubert and Wiggins (25) found that the CF test, with both histoplasmin and yeast phase antigen, gave higher titers and was more sensitive than the LA test. Both Hill and Campbell (9) and Schubert and Wiggins (25) reported that the CF test was more sensitive than the LA test in diagnosing chronic cases of histoplasmosis. We found the sensitivity and specificity of the Micro-LA and the CF tests varied with the clinical type of histoplasmosis. Although not statistically significant, a higher incidence of positive LA tests than positive CF tests was found in testing patients with acute, as well as chronic, histoplasmosis (Table 3 and 4). In addition, Micro-LA titers were generally higher than CF titers. Even though the Micro-LA test was more reactive than the CF test with sera from acute and chronic cases, it also showed less, although not significantly less, cross-reactivity with *Blastomyces* antigens. There was no significant difference in the reactivity of the two tests when titrating sera from disseminated cases; however, more patients exhibited CF antibodies than agglutinating antibodies (Table 5), and in all but one patient CF titers were higher than Micro-LA titers. As in acute and chronic cases, the Micro-LA test showed less, but not significantly less, cross-reactivity with *Blastomyces* antigens.

Hill and Campbell (9) found by using the tube LA test that only 45% of 35 chronic cases of histoplasmosis had titers. We found, by using the micro method, that 96% of 70 chronic patients showed agglutinin titers (Table 4). The lower concentration of antigen used in the micro method may make it possible to detect lower concentrations of certain serum agglutinins.

A single histoplasmin skin test has been shown to change CF titers from negative to positive in some subjects who were known skin reactors to histoplasmin (8, 16). Prior and Saslaw (19) showed that repeated skin testing can yield "false positive" reactions. Salvin et al. (20) found that repeated histoplasmin skin testing boosted CF titers when histoplasmin, but not whole yeast cells, was used as the CF antigen. Saslaw and Campbell (23) further demonstrated that repeated histoplasmin skin testing has a greater effect in boosting titers of complement-fixing antibodies than of agglutinating antibodies. We also demonstrated this when we skin tested experimentally infected dogs with histoplasmin (Fig. 2). Two weeks after a single skin test, CF titers were much

greater than Micro-LA titers, whereas Micro-LA titers previously had been slightly higher.

Only in testing sera from disseminated cases of histoplasmosis did the Micro-LA test show less reactivity than the CF test, and in no clinical case was the Micro-LA test less specific than the CF test. All 102 acute and chronic patients we tested had either positive CF or Micro-LA tests. Only one out of 11 cases of disseminated histoplasmosis was negative by both tests.

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