Detection and Identification of Diagnostic 
Histoplasma capsulatum Precipitates by Counterelectrophoresis

BRUCE KLEGER1 AND LEO KAUFMAN

Department of Parasitology and Laboratory Practice, School of Public Health, University of North Carolina, Chapel Hill, North Carolina 27514, and the Center for Disease Control, Atlanta, Georgia 30333

Received for publication 9 April 1973

Studies were carried out to develop and evaluate a counterelectrophoresis (CEP) technique for the rapid and specific identification of the diagnostically important histoplasmosis H and M precipitin bands. Well-defined and centrally located precipitin bands were produced by using a discontinuous buffer system and a gel matrix composed of agarose and ionagar no. 2. A template was devised which allowed the selective identification of the H and M precipitins. Comparative evaluations were performed with the microimmunodiffusion (ID) and complement fixation tests. In 52 sera from persons with histoplasmosis, either the H or M precipitin, or both, were identified in 42 (81%) of the cases with the CEP technique and in 43 (83%) with the ID test. With sera from 28 persons with heterologous diseases, the CEP technique, like the ID test, failed to react. The specificity of the CEP technique was dependent upon the use of the identity template. The CEP technique is recommended for routine use in laboratories testing moderate numbers of sera. It provides accurate and reproducible results within 90 min, in contrast to the ID test, which requires 18 to 24 h.

Counterelectrophoresis (CEP) provides the laboratory with a rapid means of detecting antigens and antibodies (1, 2, 3, 5, 8, 12). Although its greatest use has been in the detection of Australia antigen, it has also been applied recently to the serodiagnosis of systemic myotic infections (4, 14).

In 1971, we initiated a study to determine the applicability of the CEP technique to the serodiagnosis of histoplasmosis. Our goals were threefold: (i) to define the optimal conditions for the rapid formation of centrally located, well-defined, or distinct H and M precipitin bands, (ii) to design an identity template that would permit the specific identification of the diagnostically important H and M precipitin bands, and (iii) to compare the sensitivity and specificity of the optimal CEP technique incorporating the selected identity template with that of the conventional complement fixation (CF) and immunodiffusion (ID) tests.

During this investigation, Gordon et al. (4) reported on the application of the CEP technique to the serodiagnosis of histoplasmosis and other myotic infections. By using sera from eight persons suspected of having histoplasmosis, Gordon and his co-workers demonstrated the potential of the technique to rapidly detect Histoplasma capsulatum precipitins.

The development and evaluation of a CEP technique that permits the simultaneous detection and identification of centrally located and well-defined H and M precipitin bands are described in this paper.

This is part of a dissertation submitted by B.K. in partial fulfillment of the requirements for the Ph.D. degree at the University of North Carolina.)

MATERIALS AND METHODS

CF and ID tests. CF tests were performed according to previously published procedures (13) by using Blastomyces dermatitidis ground yeast-form antigen (Center for Disease Control [CDC] lot 24), coccidioidin (CDC lot 1), H. capsulatum whole yeast cells (CDC lot 45), and histoplasmin (CDC lot 13). ID tests were performed according to published procedures (10) with B. dermatitidis yeast-form filtrate antigen (CDC lot 10), coccidioidin (CDC lot 1), and histoplasmin (CDC lot 8). This lot of histoplasmin was prepared by pooling the mycelial culture filtrates of eight isolates of H. capsulatum grown individually for 6 months at 25 C in Smith asparagine medium.

1 Present address: Bureau of Laboratories, Pennsylvania Department of Health, Philadelphia, Pennsylvania 19150.
Thimerosol was added to a final concentration of 1:5,000. After 1 week at 25°C, the *H. capsulatum* filtrate pool was filtered through a membrane filter (0.45 μm pore size; Millipore Corp.) and then concentrated to one-tenth of its original volume by use of an Amicon ultrafiltration system. This lot of histoplasmin was also used in the CEP test.

Sera. Sera from patients with culturally proven cases of aspergillosis, blastomycosis, coccidioidomycosis, and histoplasmosis were obtained from the Fungus Immunology Section’s serum collection at the CDC. The control serum (CDC lot 39) was obtained from the CDC’s Biological Reagents Section.

**Counterelectrophoresis test.** Projector slide cover glasses (3.25 by 4 in; approximately 8.2 by 10 cm) were precoated with molten 1% agarose (Industrie Biologique Francaise S. A.) in distilled water by using a cotton swab and dried at 37°C. A portion (10 ml) of an equal mixture of 0.85% agarose and 0.85% ionagar no. 2, dissolved in 0.01 M Veronal buffer, pH 7.2, was then applied. If necessary, plates can be stored in a humidity chamber (Grafar, Detroit, Mich.) at room temperature for 1 week. A 75-well pattern, consisting of three parallel columns of paired wells, was cut (Fig. 1). Each column contained 13 serum wells and 12 antigen wells. Wells were 5 mm in diameter. Serum wells were separated by 1 mm from edge to edge, as were the antigen wells. Each antigen well was 3 mm from each of two serum wells. The wells were cut through a plastic template with a beveled metal core. The agar plugs were removed by suction.

Serum to be tested for antibody was placed in the anodic wells of each pair, and histoplasmin was placed in the cathodic wells. A control serum containing H and M antibodies was placed in the wells adjacent to the serum to be tested. Wells were filled once with capillary hematocrit tubes to the level of the agar (0.025 ml). Electrophoresis was performed in a model EP-1 Meloy electrophoresis unit (Meloy Laboratories, Springfield, Va.) with 0.05 M Veronal buffer, pH 7.2, in each chamber. Telfa wicks (Telfa Non-Adherent Strips, 2.5 by 4 in, Kendall Hospital Products Div., Chicago, Ill.) were used to connect the slides to the buffer. A constant current of 25 mA was applied across the narrow dimension of the slide for 90 min. Electrophoresis was performed at room temperature. Slides were read against a dark background with indirect light. The results were recorded and the slides were stained with thiazine red R, according to the procedure of Palmer et al. (11). No reactions were seen in the stained slide that had not been observed in the unstained slide; however, stained slides were more easily photographed.

**RESULTS**

**Optimal test conditions.** Preliminary studies with the combination of variables most widely used in the detection of Australia antigen—0.85% agarose prepared in 0.05 M Veronal buffer, pH 8.2, with the same buffer used in the electrode chambers—did not permit the development of well-defined and centrally located histoplasmosis precipitins. The M band was diffuse, and the H band was just adjacent to, or even overlapped, the serum well.

Studies were therefore carried out to determine the optimal conditions necessary to achieve our first objective, the formation of centrally located and well-defined H and M precipitin bands. The variables tested are outlined in Table 1. Agarose, at a concentration of 0.85%, was dissolved in one of four buffers. The buffer used in the electrode chambers was 0.05 M Veronal, pH 7.2, in all instances. Well-defined H and M bands, with the H band overlapping the serum well, were formed in agarose dissolved in 0.01 M Veronal buffer.

Slides that had been precoated with 1% agarose in distilled water were coated with 10 ml of any one or a combination of several agarases at a concentration of 0.85% in 0.01 M Veronal buffer, pH 7.2. In all instances, 0.05 M Veronal buffer, pH 7.2, was used in the electrode chambers. An equal mixture of agarose and ionagar no. 2 resulted in centered, intense precipitin bands, and the number of precipitin bands was consistent with the number expected.

An equal mixture of agarose and ionagar no. 2 was dissolved in 0.01 M Veronal buffer, pH 7.2, and one of three buffers was used in the electrode chambers (Table 1). The use of 0.05 M Veronal buffer, pH 7.2, in the electrode chambers resulted in well-centered, intense, and well-defined precipitin bands. Results with 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.9, were comparable with those obtained with 0.05 M Veronal, pH 7.2, but the use of two different buffer systems, Veronal and Tris, added a degree of complexity that was deemed undesirable.

**Fig. 1. Counterelectrophoresis identity template.** Wells are 5 mm in diameter. Serum wells are 1 mm from edge to edge, as are antigen wells. Every antigen well is 3 mm from each of two serum wells. ×0.33.
Electrophoresis for 60 min resulted in well-centered, moderately intense, but ill-defined H and M precipitin bands. After 90 min of electrophoresis, the H and M precipitin bands were well-centered, intense, and well-defined. Electrophoresis for 120 min did not further intensify precipitin formation.

**Development of an identity template.** Only two of the six precipitating antibodies to histoplasmin described by Heiner (6), the H and M antibodies, are considered to be of diagnostic importance. Consequently, one of the objectives of this study was to develop a template that would permit the specific identification of the precipitin bands formed by the interaction of H and M antibodies with histoplasmin and their differentiation from precipitin bands resulting from the interaction of other antibodies with histoplasmin.

When a template was designed so that the serum wells were 3 mm apart and the antigen well was equidistant (5 mm) from two serum wells, high electrophoretic mobility of the H antigen seemed to cause it to migrate between the serum wells, resulting in minimal interaction with the H antibody. Therefore, we decided to place the serum wells as close together as possible to obtain maximal interaction between antigen and antibody.

The template that was designed is shown in Fig. 1. Serum wells 5 mm in diameter were placed 1 mm apart and antigen wells 5 mm in diameter were placed so that they were 3 mm from each of two serum wells. Results obtained by use of this template with control serum containing H and M antibodies and test sera placed in alternate wells are shown in Fig. 2. Preliminary evaluations with this identity template indicated the procedure to be of potential diagnostic value. The results were very similar to those obtained by use of the ID test. Sera with M antibody, as determined with the ID test, produced a line of identity with the M precipitin band when reacted with histoplasmin in the CEP technique; sera with both H and M antibodies produced two lines of identity with the control serum containing H and M antibodies, and sera from patients with culturally proven histoplasmosis that were negative in the ID test were negative or produced lines of nonidentity with either the H or M precipitin lines (Fig. 2, wells no. 23, 25, 30). The diagnostic importance of these unidentified lines observed with the CEP technique remains undetermined.

High magnifications of the patterns of identity and nonidentity are shown in Fig. 3 and 4. Reactions of identity for both the H and M precipitin bands were clearly visible and dis-
Fig. 2. Reactions of identity by counterelectrophoresis with control serum known to contain H and M antibodies in alternating wells 1-13, 14-26, and 27-39, and sera from persons with culturally proven histoplasmosis (×1.3). Stained with thiazine red R. Ag, histoplasmin wells; serum, serum wells. Wells no. 2, 10, and 12 contained serum having only the M antibody as determined with the ID test; wells no. 4, 6, and 8 contained serum having both the H and M antibody. Wells no. 15, 17, 19, and 21 contained serum having only the M antibody as determined by the ID test. Wells No. 23 and 25 contained sera that demonstrated no precipitin lines in the ID test. The precipitin line seen in the counterelectrophoresis technique with these sera (no. 23 and 25) was not identical with either the H or M precipitin lines. Wells no. 28, 32, 34, and 36 contained serum having only the M antibody as determined by the ID test. Well no. 30 contained serum that was negative in the ID test; once again, a line of nonidentity was produced. Well no. 38 contained serum having both the H and M antibodies, as determined in the ID test.

Fig. 3. Reactions of identity by counterelectrophoresis with control serum known to contain H and M antibodies (wells 2, 4, and 6) and sera from persons with culturally proven histoplasmosis (wells 1, 3 and 5). Stained with thiazine red R.

tinct (Fig. 3). Figure 4 illustrates both a reaction of identity for the M precipitin and a reaction of nonidentity.

Sensitivity of the CEP test. The sensitivity of the CEP test was compared to that of the conventional tests most widely used in the serodiagnosis of histoplasmosis with sera from 52 persons with proven histoplasmosis (Table 2). The CF test, with histoplasmin and yeast-form antigens of H. capsulatum, detected complement-fixing antibodies in 46 (88%) of the 52 sera. Five of the sera were anticomplementary and were considered negative. Note the extreme cross-reactivity of the histoplasmosis case sera in the CF test; 28 (54%) of 52 of the sera from persons with proven histoplasmosis reacted with the B. dermatitidis antigen. Some of the heterologous titers were identical with or higher than the homologous titers.

In the ID test with histoplasmin antigen, antibodies were detected in 43 (83%) of 52 cases, and in the CEP test, antibodies were detected in 42 (81%) of the 52 histoplasmosis cases. A serum was considered positive when it contained a precipitin band identical with that of the H or M precipitins, or both (Fig. 2). None of the 52 histoplasmosis case sera reacted in ID tests with B. dermatitidis or coccidioidin precipitins. In one instance (serum 20), the CEP test
from persons with aspergillosis (A1 and A3) reacted with histoplasmin in the CEP test to produce lines of nonidentity. These lines are of unknown diagnostic value, and the sera were considered negative.

**DISCUSSION**

Gordon et al. (4) reported the development of a CEP technique for histoplasmosis. Their CEP technique was basically similar to the one used by Prince and Burke (12) for the detection of Australia antigen. In this technique, a continuous buffer system (0.05 M Veronal, pH 8.8) and a matrix composed solely of agarose were used.

A study of the factors affecting the CEP technique revealed that the combination of factors optimal for the detection of Australia antigen (3, 11, 12, 15) was not optimal for the serodiagnosis of histoplasmosis.

Our study (Table 1) demonstrated that an effective CEP test required the use of a buffer system discontinuous with respect to molarity—0.05 M Veronal, pH 7.2 to 7.4, used in the electrode chambers and 0.01 M Veronal, pH 7.2 to 7.4, used to suspend the matrix. The discontinuous buffer system was complemented by use of a matrix composed of equal parts of 0.85% agar and ionagar no. 2 and by electrophoresis for 90 min. This optimal combination of factors allowed the formation of well-defined, intense, and well-centered H and M precipitins.

Alter et al. (1) described a CEP test template for the identification of the precipitates formed by the interaction of Australia antigen and antibody. However, this development has remained relatively unnoticed. In a recent publication, Schmidt and Lennette (16) stated that the ID test must be used to confirm the identity of positive reactions obtained with the CEP test. This was necessary because the CEP test without an identity pattern may produce false-positive reactions (1). A template which permits the identification of precipitates formed with the CEP technique allows the simultaneous detection and identification of precipitating antigen-antibody.

The need for reference precipitin bands to determine lines of identity in the ID test is well established in the serodiagnosis of mycotic infections. Although Gordon et al. (4) demonstrated lines of identity for the M precipitin bands, they did not succeed in demonstrating an identity pattern for the H precipitin bands. In contrast, the CEP technique and identity template developed in this study permit the demonstration of lines of identity for both the H and M precipitin bands (Fig. 1 and Table 1).
Contrary to our expectations, there was little
difference in sensitivity between the ID and
CEP tests in detecting precipitating *H. capsulatum*
antibodies in sera from 52 persons with
proven histoplasmosis.

The lack of increased sensitivity of the CEP
test for *H. capsulatum* antibodies, when com-
pared with the ID test, was surprising in the
light of the work that has been done with
Australia antigen. In studies with Australia
antigen, Holper and Jambazian (7) found the
CEP test to be eight times more sensitive than
the ID test, Schmidt et al. (15) found it four-to
sixteenfold more sensitive, and Alter et al. (1)
found it 21 times more sensitive. Theoretically,
one might expect the CEP test to be more
sensitive than the ID test, since in the former
the reactants are forced toward each other in
one direction and in the latter the reactants
merely diffuse radially in all directions from the
point of origin. However, the lack of increased
sensitivity of the CEP test in the serodiagnosis
of histoplasmosis, when compared with the ID
test, is in agreement with the findings of Gordon
et al. (4). These workers found that two sera
from suspected cases of histoplasmosis, which
had CF titers of 1:4 and 1:16 with the *H.
capsulatum* yeast-form antigen and were nega-
tive in the ID test, were also negative by the
CEP test.

In accord with previous observations (9), the
results of this study confirm the high degree of
nonspecificity of the CF test for histoplasmosis.
In contrast to the CF test, the CEP test, like the
ID test, was 100% specific. Demonstration of the
specificity of the CEP test was dependent on the
use of the identity template with a control
serum. That such a control was mandatory for
specific diagnosis of histoplasmosis was indi-
cated by the fact that three sera from persons
with mycotic diseases other than histoplasmosis
produced precipitin bands not identical with
either the H or M precipitin bands.

The CEP test for the serodiagnosis of histo-

| Serum no. | CF titer* | ID bands* | CEP bands* | Serum no. | CF titer* | ID bands* | CEP bands* |
|-----------|-----------|-----------|------------|-----------|-----------|-----------|------------|
| 1         | 8         | 32        | 0          | M         | 0         | 16        | 8          | 0          | 0          |
| 2         | 64        | 64        | 16         | M         | M         | M         | 28         | 128        | 0          | 0          | 0          |
| 3         | 32        | 64        | 16         | 0          | M         | M         | 29         | 64         | 32         | 0          | M          | M          |
| 4         | 0         | 32        | 8          | 0          | M         | M         | 30         | 32         | 8          | 0          | M          | M          |
| 5         | 0         | 32        | 8          | 0          | M         | M         | 31         | 16         | 8          | 0          | M          | M          |
| 6         | 0         | 8         | 8          | 0          | M         | M         | 32         | 32         | 16         | 0          | M          | M          |
| 7         | 0         | 8         | 16         | 0          | M         | M         | 33         | 32         | 8          | 0          | H          | H          |
| 8         | 0         | 8         | 8          | 0          | M         | M         | 34         | 16         | 32         | 64         | 32         | 0          | 0          |
| 9         | 32        | 128       | 8          | 0          | H/M       | H/M       | 35         | 8          | 256        | 8          | 0          | M          |
| 10        | 64        | 128       | 0          | 0          | M         | M         | 36         | 8          | 32         | 0          | 0          | M          |
| 11        | 32        | 64        | 0          | 0          | H/M       | H/M       | 37         | 32         | 128        | 8          | 0          | H/M        |
| 12        | 32        | 32        | 32         | 8          | M         | M         | 38         | 8          | 16         | 0          | 0          | M          |
| 13        | 32        | 64        | 32         | 8          | M         | M         | 39         | 32         | 32         | 0          | H/M        |
| 14        | 64        | 64        | 32         | 8          | M         | M         | 40         | 64         | 128        | 256        | 0          | M          |
| 15        | 0         | 16        | 0          | 0          | M         | M         | 41         | 8          | 16         | 0          | 0          | M          |
| 16        | 128       | 256       | 0          | 0          | M         | M         | 42         | 0          | 16         | 0          | 0          | M          |
| 17        | 128       | 512       | 0          | 0          | M         | M         | 43         | 0          | 16         | 8          | 0          | M          |
| 18        | 0         | 8         | 0          | 0          | M         | M         | 44         | AC         | 0          | 0          | 0          | M          |
| 19        | 0         | 8         | 0          | 0          | M         | M         | 45         | 8          | 16         | 32         | 0          | 0          | 0          |
| 20        | AC        | 0         | 0          | 0          | M         | M         | 46         | 8          | 16         | 0          | 0          | M          | 0          |
| 21        | AC        | 0         | 0          | 0          | M         | M         | 47         | AC         | 0          | 0          | 0          | M          |
| 22        | 0         | 32        | 16         | 0          | 0*        | 48         | 8          | 16         | 8          | 0          | 0          | 0          |
| 23        | 0         | 16        | 8          | 0          | 0*        | 49         | 32         | 8          | 0          | 0          | M          | M          |
| 24        | 32        | 32        | 0          | 0          | M         | M         | 50         | AC         | 0          | 0          | 0          | M          |
| 25        | 0         | 0         | 0          | 0          | 0*        | 51         | 0          | 16         | 0          | 0          | M          | M          |
| 26        | 512       | 256       | 32         | 0          | H/M       | H/M       | 52         | 8          | 32         | 8          | 0          | M          |

* Abbreviations: Hi, histoplasmin; Y, Yeast form of *H. capsulatum*; B, *B. dermatitidis* yeast-form antigen; C, coccidioidin; AC, anticomplementary.

None of the sera tested were positive in the ID test with *B. dermatitidis* or *Coccidioides immitis* antigens.

0, Negative reaction; 0*, precipitin band observed but not identical with H or M.
plasmosis combines the simplicity, sensitivity, and specificity of the ID test with the added dimension of rapidity. Results are obtained with the CEP test within 90 min, whereas the ID test takes 18 h or longer. The CEP test for histoplasmosis is a valuable adjunctive procedure in interpreting the cross-reactions so often encountered with the CF test and is also useful in testing anticomplementary sera. Use of the CF test combined with the CEP test can contribute greatly to the accurate and rapid diagnosis of histoplasmosis and to its proper treatment.

### ACKNOWLEDGMENTS

Training was provided by the Laboratory Practice Training Program which is supported by a training grant (1 DO4 AH 01126) from the Bureau of Health Manpower Education, National Institutes of Health, and a research program project grant (CC 00006) from the Center for Disease Control. B.K. was the recipient of a full-time out-service training grant from the Division of Professional Education, Bureau of Educational Activities, Commonwealth of Pennsylvania Department of Health. The laboratory research was performed in the Laboratory Division's Mycology Branch, Center for Disease Control, under the supervision of L.K.

### LITERATURE CITED

1. Alter, H. J., P. V. Holland, and R. H. Purcell. 1971. Counter-electrophoresis for detection of hepatitis-associated antigen: methodology and comparison with gel diffusion and complement fixation. J. Lab. Clin. Med. 77:1000–1010.

2. Edwards, E. A., P. M. Muehl, and R. O. Pecknighal. 1972. Diagnosis of bacterial meningitis by counterimmunoelectrophoresis. J. Lab. Clin. Med. 80:449–454.

3. Gocke, D. J., and C. Howe. 1970. Rapid detection of Australia antigen by counterimmunoelectrophoresis. J. Immunol. 104:1031–1032.

4. Gordon, M. A., R. E. Almy, C. H. Greene, and J. W. Fenton. 1971. Diagnostic mycology by immunoelectrophoresis: a general, rapid, and sensitive microtechnic. Amer. J. Clin. Pathol. 56:471–474.

5. Greenwood, B. M., H. C. Whittle, and O. Dominic-Rajkovic. 1971. Counter-current immunoelectrophoresis in the diagnosis of meningococcal infections. Lancet 2:519–521.

6. Heiner, D. C. 1958. Diagnosis of histoplasmosis using precipitin reactions in agar gel. Pediatrics 22:616–627.

7. Holper, J. C., and A. Jambazian. 1971. Comparative sensitivity of complement fixation, counterimmunooelectrophoresis, radial, and double diffusion for detection of Australia antigen. Transfusion 11:157–161.

8. Jameson, J. E. 1968. Rapid and sensitive test for the diagnosis of farmer’s lung using immunooxymophoresis. J. Clin. Pathol. 21:376–382.

9. Kaufman, L. 1970. Serodiagnosis of fungal diseases, p. 386-394. In J. E. Blair, E. H. Lennette, and J. P. Truant (ed.), Manual of clinical microbiology. American Society for Microbiology, Bethesda, Maryland.

10. Manual of standardized serodiagnostic procedures for systemic mycoses. Part I. Agar immunodiffusion tests, 1972. Pan American Health Organization Scientific Publication no. RD 11/9.

11. Palmer, D. F., J. J. Cavallaro, and R. H. Galt. 1971. Laboratory detection of Australia antigen. Immunology series no. 4, procedural guide. Center for Disease Control, Atlanta, Georgia.

12. Prince, A. M., and K. Burke. 1970. Serum hepatitis antigen (SH): rapid detection by high voltage immunoelectrooxygenophoresis. Science 169:593–595.

13. Public Health Service. 1965. Standardized diagnostic
complement fixation method and adaptation to micro-
test. U.S. Public Health Serv. Publ. no. 1228. Washing-
ton, D.C.

14. Remington, J. S., J. D. Gaines, and M. A. Gilmer. 1972. Demonstration of Candida precipitins in human sera by counterimmuonelectrophoresis. Lancet 1:413.

15. Schmidt, N. J., P. S. Gee, and E. H. Lennette. 1971. Relative sensitivity of gel diffusion, complement fixa-
tion and immunoimmunolectrophoresis tests for detection of hepatitis-associated antigen and antibody. Appl. Microbiol. 22:165-170.

16. Schmidt, N. J., and E. H. Lennette. 1972. Evaluation of various antisera and gels for detection of hepatitis-associated antigen by immunodiagnosis and immuno-
electroosmophoresis tests. Amer. J. Clin. Pathol. 58:317-325.