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TECHNICAL MANUSCRIPT 368

AGAR GEL PRECIPITIN-INHIBITION TECHNIQUE FOR HISTOPLASMOSIS ANTIBODY DETERMINATIONS

John G. Ray, Jr,

MARCH 1967

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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TECHNICAL MANUSCRIPT 368

AGAR GEL PRECIPITIN-INHIBITION TECHNIQUE FOR HISTOPLASMOSIS ANTIBODY DETERMINATIONS

John G. Ray, Jr.

Medical Investigation Division
MEDICAL SCIENCES LABORATORY

Project 1B622401A072 March 1967

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

The agar gel precipitin-inhibition techniques of Ray, Kadull and Shay have been modified to detect Histoplasma capsulatum antibodies in sera from human clinical cases and experimental animals infected with this organism. With the modified procedure antibody was detected more consistently and reliably than with the direct agar gel diffusion test and titers were comparable to those attained by complement-fixation techniques.

I. INTRODUCTION

The complement-fixation (CF) test is the standard serological procedure for diagnosis of histoplasmosis. However, the agar gel precipitin test has recently gained recognition as a useful serological procedure in this application.

Heiner,[1] Schubert, Lynch, and Ajello,[2] and Goldin and McMillen[3] have advocated macromethod and micromethod agar gel serological tests for histoplasmosis. These direct agar gel diffusion methods were compared with the complement fixation method by the above authors. More recently, Kaufman, Brandt, and McLaughlin[4] compared them with the fluorescent antibody (FA) inhibition test, which resulted in advocacy of the agar gel and FA procedures, especially in anticomplementary (AC) sera suspected of containing antibodies to Histoplasma capsulatum.

Klite[5] indicated that the agar gel test was more reactive and diagnostically more useful than the CF test in human histoplasmosis. He reemphasized, however, the concomitant use of the CF and the agar gel precipitin tests because of the variable human antibody response to various antigens of H. capsulatum; this supported an earlier report concerning the CF test by Campbell and Binkley.[6]

Unfortunately, neither the direct diffusion agar gel nor the FA serological procedure could detect comparable titrations of the tested sera when compared with the CF test. Moreover, CF titers of low magnitude (1:8 or 1:16) were negative by the FA and agar gel techniques. This condition is similar to that observed by Ray and Kadull[7,8] and Ray and Shay[9] in soluble antigen-antibody systems of anthrax and C-reactive protein, respectively, and prompted investigation with the agar gel precipitin-inhibition (AGPI) technique into this serological system.

II. MATERIALS AND METHODS

A. PREPARATION OF AGAR DIFFUSION TEST PLATES AND READING LAMP

The medium test plates and agar diffusion reading lamp were prepared as described by Ray and Kadull.[7]

B. *H. CAPSULATUM* TEST ANTIGENS

The antigen used in this proposed serological AGPI test was obtained from the Communicable Disease Center (CDC), Atlanta, Georgia, as their histoplasmin (agar gel) precipitin lots 2 and 3, catalog number 77-1002, which corresponds to the fivefold concentration of histoplasmin employed by Schubert, Lynch, and Ajello.[2]

C. *H. CAPSULATUM* TEST ANTISERUM

The test antiserum used routinely in the AGPI test was lots 13 or 22 human control histoplasmosis serum obtained from CDC.

D. BOX TITRATION OF THE HISTOPLASMOSIS ANTIGEN-ANTIBODY SYSTEM

A box titration of the histoplasmosis antigen versus the histoplasmosis antiserum was performed in agar diffusion plates. Five serial twofold dilutions of the antigen (0.5 ml) and of the antiserum (0.2 ml) were prepared in physiological saline. To each dilution, an equivalent volume of physiological saline was added to give a final dilution range of 1:2 to 1:32.

The antigen dilutions were added sequentially and in duplicate to the two outer rows of wells (approximately 0.07 ml volume per well) so that one row exactly duplicated the opposite row in serial dilutions of antigen. This was performed in five different agar diffusion plates, one for each antiserum dilution. The center row of reservoirs (approximately 0.025 ml volume per well) was filled with one of the prepared antiserum dilutions, one plate for each dilution. Plates were incubated at room temperature, 23 to 27 C, for 24 hours, after which the observed antigen-antibody precipitin reaction was read, aided by the previously described visual apparatus; the final reading occurred at 48 hours.

The antigen-antibody end point was determined as that combination of the highest dilutions of antigen and antibody that produced a visible line of precipitate in 48 hours. This initial titration in the agar gel plates was essential to the establishment of maximum sensitivity and consistent reproducibility in subsequent tests with unknown suspect sera. The end point reading was arbitrarily assumed to represent a minimal reacting dilution of histoplasmin antigen (MRD_a) and a minimal reacting dilution of antibody to histoplasmosis (MRD_s) (Tables 1 and 2). The MRD_a of lots 2 and 3 histoplasmin (agar gel) precipitin antigen were established as a 1:2 and 1:4 dilution, respectively.

TABLE 1. HISTOPLASMOSIS ANTIGEN-ANTIBODY AGAR GEL BOX TITRATION[a]

Lot 13 CDC Human Control Dilution	Lot 2 Precipitin Antigen Dilution 1:2	1:4	1:8	1:16	1:32
1:2	+	±	-	-	-
1:4	+	±	-	-	-
1:8	±	-	-	-	-
1:16	±	-	-	-	-
1:32	-	-	-	-	-

a. MRD_a = 1:2; MRD_s = 1:4.

TABLE 2. HISTOPLASMOSIS ANTIGEN-ANTIBODY AGAR GEL BOX TITRATION[a]

Lot 22 CDC Human Control Dilution	Lot 3 Precipitin Antigen Dilution 1:2	1:4	1:8	1:16	1:32
1:2	+	+	±	-	-
1:4	+	+	±	-	-
1:8	+	±	-	-	-
1:16	±	-	-	-	-
1:32	-	-	-	-	-

a. MRD_a = 1:4; MRD_s = 1:4.

E. SERUM TITRATIONS

The inhibition, or indirect, method was used in titrating unknown serum specimens. Serial twofold dilutions of 0.2 ml of unknown serum were made in physiological saline. To each dilution, 0.2 ml of the previously box-titered antigen MRD_a was added; the final mixture thus contained one-half MRD_a plus unknown serum dilutions ranging from 1:2 to 1:32. The mixtures were hand-shaken for 10 seconds and incubated in a 37 C water bath for 30 minutes to permit antigen-antibody binding to proceed to completion.

Each of the wells in the center row of agar diffusion plates was filled with a MRD_s of the positive histoplasmosis human control serum, and the plates were incubated at room temperature, 23 to 27 C, for 1 hour. Conclusions of incubation periods for the plates and for the antigen-antibody binding mixture were timed to coincide and permit immediate completion of the final step.

Outer rows of wells were filled sequentially and in duplicate with the incubated antigen-serum dilution mixtures. Thus, each well in one outer row contained the same mixture as the corresponding well in the opposite outer row. The end point, or titer, of an unknown positive serum was the dilution that completely inhibited the formation of a visible line of precipitate with the box-titered MRD_a and MRD_s in the agar gel plates (Fig. 1).

Controls subjected to the same test procedure consisted of dilutions of the test antigen in saline and combinations of the predetermined MRD_a of antigen with negative and positive histoplasmosis sera.

F. AGAR-PLATE PRECIPITIN TEST FOR HISTOPLASMOSIS

The direct diffusion agar-plate precipitin test for histoplasmosis of Schubert, Lynch, and Ajello[3] was performed in the same agar gel plates as previously described for use in the AGPI test. This allowed similar conditions of diffusion for the Histoplasma antigen and comparative evaluation of antibody reactions.

G. CF TEST FOR HISTOPLASMOSIS

When the CF test was performed on serum specimens, the procedure used was that described by Smith et al.[10] The overnight fixation procedure and 100% fixation end point were employed in these CF determinations.

The Histoplasma mycelial and yeast phase antigens and the positive human CF control serum were obtained from the Diagnostic Reagents Section, Communicable Disease Center, Atlanta, Georgia, and were from lots 23, 31, and 22, respectively.

III. RESULTS

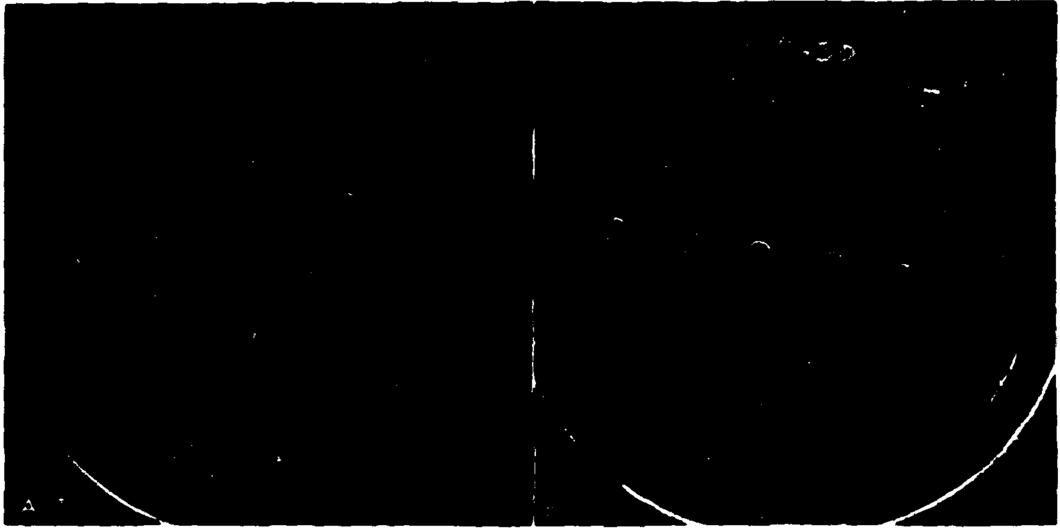
The AGPI test sensitivity was initially determined on serial sera from two human cases of histoplasmosis and a positive human CF control serum (Table 3, Fig. 2). In addition, positive human CF control sera from cases of coccidioidomycosis and blastomycosis were used as negative controls. All sera from histoplasmosis patients were positive in the agar-plate precipitin test of Schubert et al.,[2] whereas sera from cases of coccidiomycosis and blastomycosis cases were negative.

To obtain titers, it was necessary first to perform a box titration of the antigen-antibody system. Serial dilutions of precipitin antigen (lot 2) were reacted in agar gel plates with serial dilutions of human positive histoplasmosis CF control serum (lot 13). These dilutions were added to each agar gel plate simultaneously and incubated at room temperature for an observation period of 48 hours. Additional incubation did not enhance the precipitin line formation and the results were recorded at this time as shown in Table 1. Thus, the derivation of the minimal reacting dilution (MRD) of each reactant was obtained and could be applied to the titration of the positive histoplasmosis human sera in the AGPI technique. The line of precipitate, however, was relatively close to the antiserum or antibody well; to obtain a reaction equidistant between these antigen and antibody wells, it was necessary to prediffuse the MRD_s 1 hour prior to adding the unknown serum - MRD_a mixture for the test.

Using the lot 2 - lot 13 antigen-antibody MRD system (Table 1), titers of the tested serum samples were obtained as shown in Table 3 under AGPI Titer Lot 2. To ascertain the degree of reproducibility of this antigen-antibody system in the AGPI technique, lot 3 and lot 22 of histoplasmosis precipitin antigen and human positive CF control serum, respectively, were obtained and a similar box titration was performed; final results are shown in Table 2. Employing the MRD of lot 3 and lot 22 in the AGPI technique, the same sera were assayed, with results depicted in Table 3 under AGPI Titers Lot 3. This indicated the degree of sensitivity and reproducibility of this test antigen-antibody system in histoplasmosis serology compared with different lots of precipitin antigen and positive control serum. Additionally, the AGPI test provided a titration end point of each histoplasmosis-positive human serum specimen, which was not obtained by the agar-plate precipitin test.

Figure 1. Serum Titrations by Indirect Technique.
A. Negative serum: Note undulating lines of visible precipitate between center and outer rows of wells, indicating absence of binding of MRD_a.
B. Positive serum: Note absence of a visible line of precipitate between the first, second, third, and fourth outer wells and the center row of wells, indicating complete binding of the MRD_a; end point is 1:256 dilution of serum.

Figure 2. Direct Diffusion Agar-Plate Precipitin Test for Histoplasmosis. Note lines of precipitate between center and outer wells. Numbers correspond to those serum specimens reported in Table 3. Results were recorded after 48 hours' incubation at room temperature.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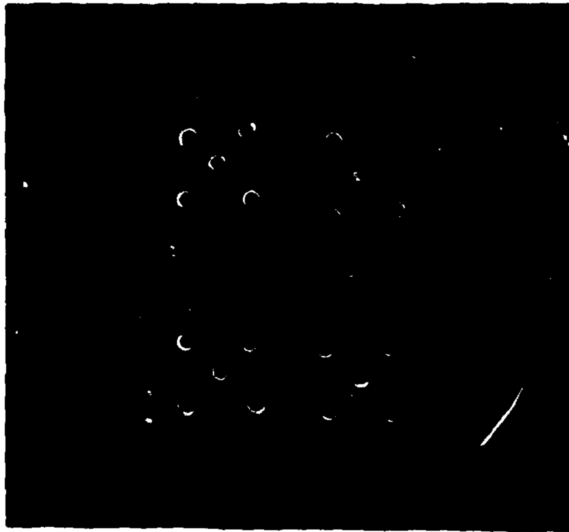

TABLE 3. SEROLOGICAL COMPARISON OF HUMAN AND ANIMAL HISTOPLASMOSIS SERUM SPECIMENS

Fig. 2 Reference	Serum	Source CF Titer[a/] Mycelial	Source CF Titer[a/] Yeast	FDSL[b/] CF Titer Mycelial	FDSL[b/] CF Titer Yeast	AGPI Titer Lot 2	AGPI Titer Lot 3	Agar-Plate Precipitin
c	Pos. Coccy. Human serum	Histo. Neg.	Histo. Neg	Histo. Neg	Histo. Neg	Neg	Neg	Neg
c	Pos. Blasto. Human serum	Neg	Neg	Neg	Neg	Neg	Neg	Neg
1	O.G.	16	16	20	5	8	16	+
2	V.O.	256	64	40	40	512	1024	+
3	H.Y.[d/]	N.T.[e/]	512	320	320	512	512	+
4	H.M.[d/]	128	N.T.	40	80	256	512	+
5	F.T.-1	32	32	10	40	32	32	+
6	F.T.-2	N.A.	N.A.[c/]	5	40	32	32	+
7	F.T.-3	N.A.	N.A.	5	40	32	32	+
8	F.T.-4	32	32	10	40	32	64	+
9	F.T.-5	16	16	10	80	32	32	+
10	F.H.-1	16	16	5	10	16	32	+
11	F.H.-2	16	16	5	10	32	32	+
12	F.H.-3	N.A.	N.A.	5	20	64	64	+
13	F.H.-4	N.A.	N.A.	5	20	32	64	+
14	F.H.-5	N.A.	N.A.	5	20	64	64	+

a. All titers are reported as reciprocal titers.
b. FDSL = Fort Detrick Serological Laboratory.
c. Not available from hospital records.
d. Rabbit sera inoculated with *Histoplasma* yeast phase (HY) and mycelial phase (HM) antigens.
e. Not tested.

The CF test was performed on these same serum specimens (Table 3), using the H. capsulatum mycelial and yeast phase antigenic determinants. It is interesting to note that the titers of these sera by the Fort Detrick Serological Laboratory (FDSL) 100% CF method correlated with the serum source CF titers and that each serum had titers to antigens of both phases of H. capsulatum. However, the 100% FDSL method evidenced a greater variation in titer response to either antigenic phase of H. capsulatum compared with the source CF titer results. Generally, the AGPI titers exceeded those attained by either CF test result.

Recently, three human sera obtained from Dr. Furcolow's laboratory through the courtesy of Mr. John Converse were studied (sera number 1, 2, and 3, Table 4). These were aberrant sera in that sera 1 and 2 were not positive by the agar-plate method of Schubert et al.[a] nor by the FDSL CF technique; serum 3 showed AC activity by the latter test. In addition, a human control serum (lot 22) was included as an aberrant serum because it was negative by the FDSL CF technique. These sera, in addition to control sera from the previous experiment (Table 3, sera 2, 3, 4, and 5), were diffused in the agar gel plate against the mycelial phase, yeast phase, and precipitin (agar gel) antigens of H. capsulatum. The developing reactions were observed after 24, 48, 72, 96, 120, and 144 hours at room temperature incubation. Table 4 depicts the results of these serum reactions with the respective antigens after 72 hours' incubation, as well as their titers by the source and FDSL CF techniques. Figure 3 shows the precipitin reaction. It is significant that serum 7 (Fig. 3) reacted with the precipitin agar-plate antigen with four lines of precipitate, two lines of precipitate with the mycelial phase antigen, and one diffuse line of precipitate with the yeast phase antigen of H. capsulatum. Additionally, this same serum reacted with serum 5 irrespective of the antigen wells and indicated the presence of antihuman serum antibodies in this serum.

After 144 hours' incubation at room temperature, sera 1, 2, 3, and 4 had developed an extra line of precipitate close to the agar gel precipitin test antigen well (Fig. 4) that was not observed at the same reactive time with sera 5, 6, 7, and 8. This indicates a complexity of antigen-antibody reactions in agar gel to the various tested Histoplasma standard antigens and will necessitate a further investigation of these systems to classify the observed lines.

Figure 3. Serum Precipitin Reaction for Histoplasmosis after 72 Hours.
A. Aberrant sera 1, 2, 3, and 4 (Table 4) diffused in agar gel against the yeast phase (YP), histoplasmin (agar gel) precipitin (P), and mycelial phase (M) antigens.
B. Typical sera 5, 6, 7, and 8 (Table 4) diffused in agar gel against the same antigen sequence.

Figure 4. Serum Precipitin Reaction for Histoplasmosis after 144 Hours.
A. Aberrant sera 1, 2, 3, and 4 (Table 4) diffused in agar gel against the yeast phase (YP), histoplasmin (agar gel) precipitin (P), and mycelial phase (M) antigens.
B. Typical sera 5, 6, 7, and 8 (Table 4) diffused in agar gel against the same antigen sequence.

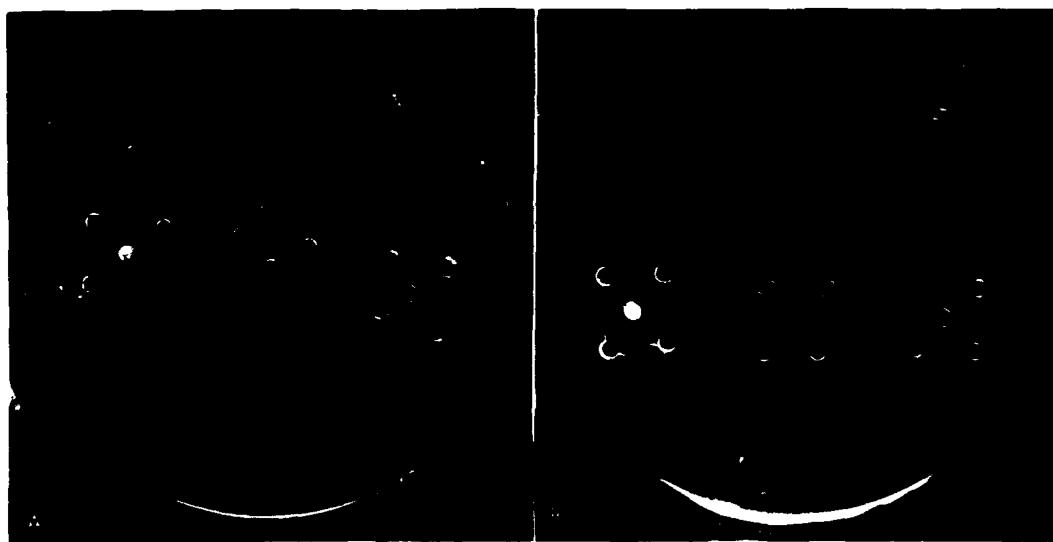

TABLE 4. ABERRANT HISTOPLASMOSIS SERUM

Fig. 3 Reference	Serum	Source CF Titer[a/]		FDSL CF Titer		AGPI Titer		Agar-Plate Precipitin Test[b/]		
		Mycelial	Yeast	Mycelial	Yeast	Lot 2	Lot 3	MP	P_pt	YP
1	Blaine[c/]	Neg	32	Neg	Neg	2	2	-	-	-
2	Scott[c/]	64	Neg	10	Neg	32	32	+	-	+
3	Land[c/]	32	64	AC[d/]	AC	32	32	-	+	-
4	Lot 22[c/] HHC	16	64	Neg	Neg	128	64	-	+	-
5	F.T.-1 1-20-65	32	32	10	40	32	32	-	+	-
6	V.O.	256	64	40	40	512	1024	-	+	-
7	H.Y.	N.T.[e/]	512	320	320	512	512	+	+	+
8	H.M.	128	N.T.	40	80	256	512	-	+	-

a. All titers are reported as reciprocal titers.
b. MP, P_pt, and YP = mycelial phase, precipitin, and yeast phase antigens.
c. Aberrant.
d. Anticomplementary serum.
e. Not tested.

IV. DISCUSSION

The agar gel test for histoplasmosis described by Schubert, Lynch, and Ajello[2] and later evaluated with other serological tests for clinical diagnosis of this disease by Schubert and Wiggins[11] correlated 90 to 100% with the mycelial phase histoplasmin CF results. Unfortunately, serum titration end points were not obtained by the Schubert et al. agar gel test.

Our results indicate that the AGPI procedure can determine titers in positive Histoplasma sera similar to those attained by the CF method. Additionally, serum specimens that are AC by the CF procedure can be titrated in the AGPI test because this test does not depend on complement for its activity. Thus, the AGPI test eliminated the necessity for standardization of complement and the use of a sheep-cell indicator system.

The AGPI procedure, compared with the agar-plate direct diffusion test for detection of Histoplasma antibodies, shows a greater sensitivity because low-titered positive sera may not be detected by the direct method. Probably this is due to insufficient antibody in the serum specimen to precipitate enough antigen to form a visible precipitin line by the direct diffusion method.

The advocated AGPI procedure derives its sensitivity in determining Histoplasma antibodies from inhibition of the MRD of a soluble, diffusible antigen-antibody system that is predetermined by a box titration of the reacting reagents in agar gel plates. Variations in batch lots of the H. capsulatum antigen or in the antibody content of any known positive serum are minimized by this box titration.

These results also indicate the necessity for a definitive study of the various antigen-antibody systems of Histoplasma in agar gel. This is necessary to determine the role played by the mycelial phase, yeast phase, and precipitin serological antigens in the serodiagnosis of histoplasmosis.

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13. ABSTRACT

The agar gel precipitin-inhibition techniques of Ray, Kadull and Shay have been modified to detect Histoplasma capsulatum antibodies in sera from human clinical cases and experimental animals infected with this organism. With the modified procedure antibody was detected more consistently and reliably than with the direct agar gel diffusion test and titers were comparable to those attained by complement-fixation techniques.

14. Key Words

*Agar gel precipitin-inhibition technique
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