comparison of

COMPLEMENT FIXATION TESTS

for

coccidioidomycosis

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THE VALUE of serologic tests in the diagnosis and prognosis of coccidioidomycosis has been established in long-term studies at the University of California School of Public Health (*1*, *2*). Prognostically, a serum titer greater than 1:16 in the quantitative complement fixation test has indicated increased likelihood of dissemination of the infection. Complement-fixing titers have exceeded this "critical" level in serum samples from three-fifths of more than 700 patients with disseminated disease and from five-sixths of approximately 300 patients with extensive disseminations other than meningitis. On the other hand, in serum samples from more than 3,000 patients with primary nondisseminated disease, complement-fixing titers exceeding 1:16 have been observed in less than one-tenth.

These diagnostic and prognostic values have been determined by a single complement fixation technique. Not all laboratories, however, routinely use the same procedure. Since optimal utilization of serologic tests for coccidioidomycosis will be favored if laboratories can use, without modification, the techniques in which they are skilled (*3*), it is important to determine whether other complement fixation techniques are diagnostically applicable. In particular, it is important to learn whether they alter the prognostic use of the critical titer.

Therefore, in 1948 a study was initiated to evaluate the consistency of five techniques of complement fixation. A corollary purpose was to determine the applicability of a positive control serum standardized to approximate the

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titer critical for dissemination. Collaborating with the University of California School of Public Health in this study were the Rocky Mountain Laboratory of the Public Health Service, the Army Medical Service Graduate School (now the Walter Reed Army Institute of Research), and the Fort Miley Veterans Administration Hospital. The investigations were sponsored by the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, which is supported by the Office of the Surgeon General, Department of the Army.

Method of the Study

The serum samples tested were specimens sent to the University of California School of Public Health for serologic tests for coccidioidomycosis. After an initial test, appropriate specimens of sufficient volume were divided into five equal parts. Three were sent as unknowns to the collaborating laboratories and two were retained.

Serums of persons without coccidioidal disease and of patients having various clinical forms of the infection were included in each set of unknowns. Using a 1:8 dilution of lot 47–54 of the previously described antigen (*1*), each collaborating laboratory performed quantitative complement fixation tests by the method it routinely uses. The University of California tested the specimens a second time with the original technique of binding for 2 hours at 37° C. and simultaneously with 18-hour 4° C. binding. The five methods are outlined in table 1. The collaborating laboratories returned the results to the University of California for tabulation. Only specimens with four plus (++++) values were considered positive.

The study was continued for 4 years, during which time a total of 508 human serum samples were submitted to each collaborator. These were derived from a group of 18,500 specimens (*2*) with serologic patterns similar, except for slightly higher titers in all varieties of the infection, to those of the 21,000 specimens described in the initial report (*1*). Unfortunately, many tubes were broken or the serums became anticomplementary during transit. Consequently, the numbers actually tested by the different methods varied. However, a total of 206 specimens were tested by all five methods.

Specificity

Forty of the 508 specimens were from individuals known not to have coccidioidal infection. Seventeen were satisfactorily tested by all five methods. Only a single specimen was reported as positive by one laboratory (titer, 1:256).

Comparison of Titers

The comparison of titers obtained with the various techniques of complement fixation is based on analysis of the 206 serum samples tested by all five methods.

Table 1. Methods used by collaborating laboratories in performing complement fixation tests for coccidioidomycosis

Laboratory	Number of units of complement	Binding		Hemolytic system		
		Time (hours)	Temperature (degrees centigrade)	Time (hours)	Temperature (degrees centigrade)	End point (percent)
University of California [1]	2	2	37	1	37	100
University of California	2	18	4	1	37	100
Rocky Mountain Laboratory	2	1	37	1	37	100
Fort Miley Veterans Administration Hospital	4	18	4	½	37	50
Army Medical Service Graduate School	3	18	4	½	37	50

[1] The method also of the initial test, references 1 and 2.

Table 2. Factors of comparison and percentage agreement among five methods of coccidioidal complement fixation

Method	Factor of comparison	Percentage agreement
U. of C. 2-hr. 37° C. with original U. of C. 2-hr. 37° C.	Same	94
U. of C. 2-hr. 37° C. with U. of C. 18-hr. 4° C.	2-fold increase.	93
U. of C. 2-hr. 37° C. with RML 1-hr. 37° C.	2-fold increase.	88
U. of C. 2-hr. 37° C. with Fort Miley 18-hr. 4° C.	2-fold increase.	76
U. of C. 2-hr. 37° C. with Army 18-hr. 4° C.	5-fold increase.	88
U. of C. 18-hr. 4° C. with Fort Miley 18-hr. 4° C.	Same	80
U. of C. 18-hr. 4° C. with Army 18-hr. 4° C.	2½-fold increase.	88

The results of the original test and those of the repeat test at the University of California using the 2-hour 37° C. technique show agreement of 94 percent when the usual allowance is made for a variation of one serial dilution. However, even this difference demonstrates the importance of repeating the complement fixation test of a previous specimen concurrently with a new specimen. Otherwise, prognostically significant changes may be wrongly inferred.

Comparison of the simultaneous tests with 2-hour 37° C. binding and with 18-hour 4° C. binding at the University of California indicates a consistent shift one serial dilution higher in the latter. Again there is agreement of 94 percent within a one-serial-dilution variation.

The 1-hour 37° C. technique of the Rocky Mountain Laboratory also produces titers one serial dilution higher than those of the University of California's 2-hour 37° C. method. For these two methods, agreement is 88 percent when a variation of one serial dilution is allowed.

The method of the Fort Miley Veterans Administration Hospital, in which 18-hour 4° C. binding and four 50-percent units of complement are used, results in slightly greater scattering of titers than the 2-hour 37° C. method. A relatively large number of specimens found positive by other methods were negative by the Fort Miley technique. Nevertheless, agreement at one serial dilution higher than the University of California's values is 76 percent.

Use of three 50 percent units of complement and an overnight period at 4° C. for fixation, the method of the Army Medical Service Graduate School, results in titers 5 times higher than the University of California's two 100-percent units of complement and 2-hour 37° C. binding. Agreement between these two techniques is 88 percent.

Since the Fort Miley and the Army techniques both include 18-hour icebox binding, the results obtained with these two methods have been compared with those obtained with similar

Table 3. Percentage distribution of maximal complement-fixing titers in serums of patients with primary coccidioidal infection: comparative results of six tests

Maximal titer of complement fixation, other than Army	U. of C. 2-hr. 37° C., concurrent series (percent of 1,365 patients)	U. of C. 2-hr. 37° C. (percent of 109 patients)	U. of C. 18-hr. 4° C. (percent of 142 patients)	RML (percent of 103 patients)	Fort Miley (percent of 84 patients)	Maximal titer of complement fixation, Army	Army (percent of 112 patients)
2	42	46	13	26	27	5	18
4	23	22	27	29	26	10	23
8	16	14	28	15	23	20	30
16	10	11	15	10	10	40	13
32	7	2	13	10	8	80	9
64	1	3	1	4	3	160	5
128	1	1	2	2	2	320	1
256	(0. 3)	1	1	4	1	640	1
<32	**91**	93	83	80	86	<80	84
<64	98	**95**	**96**	**90**	**94**	<160	**93**

Table 4. Percentage distribution of maximal complement-fixing titers in serums of patients with coccidioidal pulmonary residuals (with and without cavities): comparative results of six tests

Maximal titer of complement fixation, other than Army	U. of C. 2-hr. 37° C., concurrent series (percent of 384 patients)	U. of C. 2-hr. 37° C. (percent of 47 patients)	U. of C. 18-hr. 4° C. (percent of 48 patients)	RML (percent of 40 patients)	Fort Miley (percent of 31 patients)	Maximal titer of complement fixation, Army	Army (percent of 41 patients)
2	56	49	35	38	39	5	10
4	25	26	23	18	29	10	22
8	10	13	21	10	20	20	27
16	6	6	10	22	6	40	19
32	3	6	9	8	6	80	17
64	0	0	2	2	0	160	2
128	0	0	0	2	0	320	3
256	0	0	0	0	0	640	0
<32	**97**	**94**	89	88	94	<80	85
<64	100	100	**98**	**96**	**100**	<160	**97**

fixation at the University of California. The latter, as one recalls, yields titers one serial dilution higher than does the standard 2-hour 37° binding. Also, the titers at Fort Miley are one serial dilution higher than those of the University of California's standard technique. Thus, it would be expected that the Fort Miley test and the University of California's overnight test would produce titers of the same level. This assumption has been found to be correct, with an agreement of 80 percent. By the same reasoning, results of the Army test should correlate with the results of the University of California overnight, icebox binding at a 2½-fold increase in titer. This, too, has been found to be true, with the excellent agreement of 88 percent.

The factors of comparison for the various techniques, together with the degree of agreement, are summarized in table 2.

Comparison of Prognostic Applications

As previously noted, experience at the University of California with the 2-hour 37° C. technique of binding complement has indicated that 1:16 is the titer critical for disseminated coccidioidal infection. The present study has shown that the results of four other techniques are consistently comparable with the results of the 2-hour 37° C. procedure. By use of the factors of comparison given in table 2, the "equivalent" critical titers for these four techniques are estimated to be as follows:

University of California 18-hour 4° C	1:32
Rocky Mountain Laboratory 1-hour 37° C	1:32
Fort Miley 18-hour 4° C	1:32
Army 18-hour 4° C	1:80

The maximal titers of complement fixation in the serums from individual patients with primary coccidioidal infection are compared in table 3. The numbers tested by the different methods vary because of unsatisfactory (broken or anticomplementary) specimens. If multiple specimens from the same patient are included in the comparisons, only the maximal titer is indicated. Also shown in this table are the results of the entire series of specimens from patients with primary infection (designated "concurrent series") from which the samples for the comparisons were obtained. The cumulative percentage distributions at the respective critical titers indicate that these levels consistently include between 90 and 96 percent of the patients' serums.

Correlation among the five techniques for fixing complement is close also for patients with coccidioidal pulmonary residuals (with or without cavities), as shown in table 4. The previously reported studies revealed that serums of patients with this form of the infection fix complement at titers even lower than do those of patients with primary infection. In the present study, from 94 to 100 percent of the

patients' serums are no higher than the equivalent critical titers.

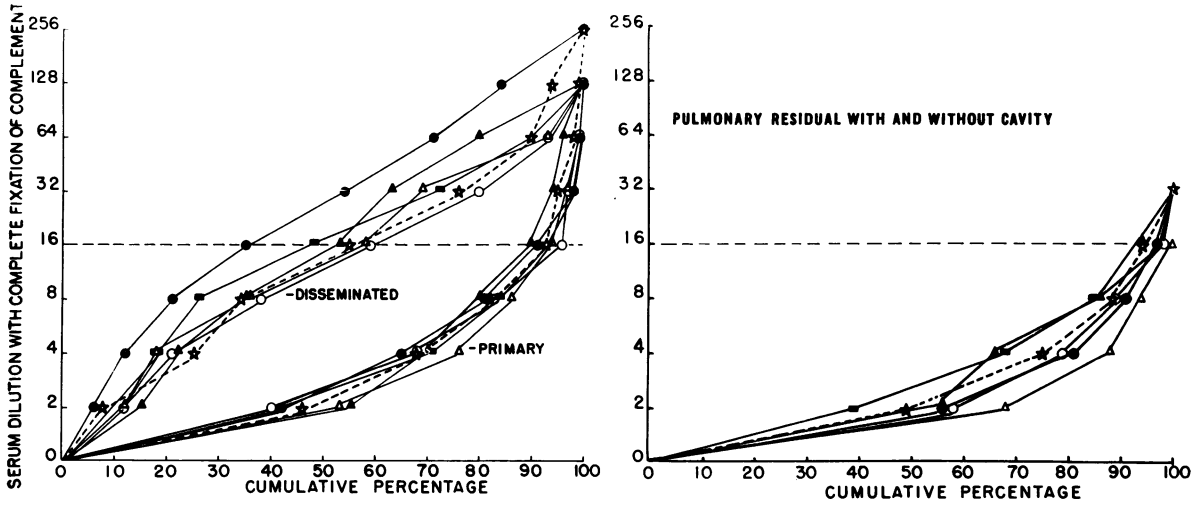
The characteristically high titers of complement fixation in serums of patients with disseminated disease are shown in table 5. The critical titers are exceeded in from 41 to 52 percent of the patients' serums.

The accompanying illustration shows the

Table 5. Percentage distribution of maximal complement-fixing titers in serums of patients with disseminated coccidioidal infection: comparative results of six tests

Maximal titer of complement fixation, other than Army	U. of C. 2-hr. 37° C. concurrent series (percent of 300 patients)	U. of C. 2-hr. 37° C. (percent of 66 patients)	U. of C. 18-hr. 4° C. (percent of 67 patients)	RML (percent of 60 patients)	Fort Miley (percent of 55 patients)	Maximal titer of complement fixation, Army	Army (percent of 50 patients)
2	6	8	3	10	7	5	2
4	6	17	9	5	5	10	4
8	9	9	9	7	6	20	12
16	14	21	17	13	18	40	8
32	19	21	21	18	22	80	22
64	17	14	21	10	11	160	24
128	13	6	13	17	24	320	18
256	16	4	7	20	7	640	10
<32	**35**	**55**	38	35	36	<80	26
<64	54		**59**	**53**	**58**	<160	**48**

Cumulative percentage distributions of maximal complement-fixing titers for three types of coccidioidal infection: results of four methods adjusted to equivalence with the University of California 2-hour 37° technique.



		Number of patients		
		Primary	*Dissemi-nated*	*Pulmonary residual*
●——●	U. of C. 2-hr. 37° C., concurrent series	1,365	300	384
☆------☆	U. of C. 2-hr. 37° C., comparative series	109	66	47
○——○	U. of C. 18-hr. 4° C., comparative series	142	67	48
▲——▲	RML 1-hr. 37° C., comparative series	103	60	40
△——△	Fort Miley 18-hr. 4° C., comparative series	84	55	31
■——■	Army 18-hr. 4° C., comparative series	112	50	41

cumulative percentage distributions of the maximal titers for the three categories of patients when the results of the four other methods are adjusted to equivalence with the results of the University of California 2-hour 37° C. procedure. In each category, all the distribution curves are remarkably similar. This finding further demonstrates that results obtained by a variety of complement fixation techniques are comparable, provided an index of equivalence can be devised. The contrast in the patterns of the curves in the nondisseminated and the disseminated infections also can be seen in this illustration.

For patients with disseminated disease, the titers of the comparative tests are not quite as high as those of the total concurrent series. A possible reason for this discrepancy is that specimens for the comparative study frequently have been selected from patients with earlier positive specimens. Although only the maximal titer is selected for a single patient, such repeat specimens characteristically come from less severely ill patients. Unfortunately, the volume of serums sent us from seriously ill patients who die of fulminating disease has been too small to permit use of these serums in the comparative tests. Thus the comparative series is weighted by patients with less severe disease whose serums have lower titers.

Standard Positive Control Serum

Laboratories performing complement fixation tests for coccidioidomycosis require a positive control serum. One feasible method for comparing the results of different techniques would be to have this positive control standardized at the critical level. Accordingly, Cutter Laboratories was supplied with suspensions of multiple strains of *Coccidioides immitis* in the mycelial phase. A series of horses was infected intravenously. After precipitin and complement-fixing antibodies had developed, the serums were harvested and pooled. Various dilutions were made with normal horse serum and, together with normal horse serums, sent out under fictitious names as part of the comparative testing of human serums.

The results obtained with the five complement fixation techniques are summarized in table 6. Regardless of the method employed, the pooled horse serums diluted 1:4 approximate the critical titer. Each of the five sets of normal horse serums has been negative. Thus the objective of a standardized positive control serum appears to have been achieved. Bieberdorf (*4*), recognizing the necessity of a positive control serum for coccidioidal complement fixation, has proposed the use of lyophilized serum from infected rabbits. Use of the positive control serum in combination with the critical titer to provide an index of equivalence, as discussed above, appears more desirable. Also, a larger volume of appropriately standardized positive serum can be obtained from the horse.

Although Bieberdorf discusses the relative merits of the specificity of coccidioidal rabbit serum with antigens of *Histoplasma* and *Blastomyces*, we believe that specificity in a serum control is not a primary consideration. The control is to be used in tests with coccidioidin as the only antigen. Thus the results will relate only to the titers of the serums that are tested with this substance. Campbell and

Table 6. Titers obtained on various dilutions of horse serums by collaborating laboratories

Dilution of serum	U. of C. 2-hr. 37° C.	U. of C. 18-hr. 4° C.	RML	Fort Miley	Army
	Range of titers				
Undiluted	32–256	64–256	128–256	64–256	640–1280
2	16–64	32–128	32–128	32–64	160–640
4	**8–32**	**32–128**	**16–32**	**32**	**80**
8	4–16	8–16	2–16	2–8	40–80
16	2–8	4–8	4–16	4	20
32	0–4	2–4	0–4	0	10
64	2	4	2	------	10
128	0	2	2	------	5
256	0	0	0	0	0
	Modal titer				
Undiluted	64	128	128	128	320
2	32	64	32	64	160
4	**16**	**32**	**32**	**32**	**80**
8	8	16	16	8	40
16	4	8	8	4	20
32	2	4	4	2	10
64	0	2	2	0	5
128	0	0	0	0	0
256	0	0	0	0	0

Salvin have both found markedly strong cross reactions in the horse serums when they use antigens of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. However, these findings relate to the lack of specificity of these two heterologous antigens. The testing of human serums is a wholly different matter. In these complement fixation tests coccidioidin appears to have a relatively high degree of specificity. Nevertheless, as discussed earlier (*2*, *5*), cross reactions in human serums should be kept in mind.

To investigate further the practicality of the general use of complement fixation tests for coccidioidomycosis, a second study with eight additional collaborating laboratories is now under way. Only 2 of the 13 complement fixation methods are alike. Thus this study is investigating not only the variations of complement fixation results which may be encountered but also the degree of consistency and reproducibility of the comparative values and whether the control horse serum can be successfully lyophilized at a standardized critical titer.

Summary

A study of five different techniques for performing quantitative complement fixation tests for coccidioidomycosis by four laboratories has revealed indexes of comparability. When these factors of equivalence are applied to the results of the complement fixation tests in various clinical types of coccidioidal infection, the titers follow the characteristic distribution curves of nondisseminated and disseminated infections irrespective of the technique used. The consistency of the results indicates that the quantitative complement fixation tests have general prognostic as well as diagnostic applicability. An appropriately diluted serum from infected horses appears to set a "critical titer" above which dissemination is frequent and at and below which it is infrequent by whatever method is used. The investigation is now being extended to a larger number of laboratories.

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DOCUMENTATION NOTE

Seven tables presenting comparative results of the five methods of complement fixation have been deposited as document No. 5358 with the American Documentation Institute Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A photoprint copy may be obtained by remitting $1.25; a 35-mm. microfilm copy by remitting $1.25. Cite document number. Advance payment is required. Make checks or money orders payable to Chief, Photoduplication Service, Library of Congress.