Comparison of Methods for Coccidioidomycosis
Complement Fixation

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A Laboratory Branch Task Force of the National Communicable Disease Center has proposed a standardized complement fixation procedure (LBCF) and an adaptation of this to microtitration techniques (MT) as uniform methods for performing complement fixation (CF) tests. A common procedure should make CF results from one laboratory more comparable to another. In addition, it would be preferable if the common procedure reproduced the titer levels of a testing procedure which is to be replaced, particularly when valid clinical interpretations have been derived from the latter. Replicated sets of sera were tested by the LBCF, MT, and the standard Smith CF procedure for coccidioidomycosis. Results with all three procedures were highly reproducible within an acceptable one-tube variation of a twofold dilution series, but the frequency of one-tube variations was greater with the MT method than with the other two. There was no statistical difference in the titers obtained with the Smith and LBCF procedures, but there was a significant difference when the MT results were compared to those with the Smith method. The LBCF method should be acceptable as a standardized and uniform CF procedure for coccidioidomycosis, subject to comparative testing between different laboratories.

The immunological tests for coccidioidomycosis, as developed and evaluated for clinical interpretations by Smith and his colleagues (9, 11), have been among the most useful and practical laboratory procedures employed for an infectious disease. The quantitative complement fixation (CF) test has been particularly valuable. Under appropriate circumstances, it can serve as a diagnostic aid, as a measure of the severity and extent of disease, or as an objective guide for clinical management of the patient. Analysis of the results obtained in Smith's laboratory indicated that CF titers generally paralleled the extent and severity of the disease, except for cases with meningitis, and that the "critical titer" was 1:16; i.e., 95% of specimens from patients with uncomplicated pulmonary disease yielded titers of 1:16 or lower, whereas 83% of specimens from patients with extensive, non-meningeal, extrapulmonary disease exceeded this critical titer. Increasing confidence in the reliability of these interpretations has been founded on the continued accuracy and reproducibility of the CF titers when using the procedure of Smith. Unfortunately, however, comparative tests on the same specimens and with the same batch of antigen (coccidioidin) by four laboratories using five different procedures for CF revealed that the critical titer varied according to the laboratory and the procedure employed, even though the correlation between high titers and severity of disease was retained (10). As a consequence, most physicians have been compelled to send specimens only to those few laboratories which use the procedure of Smith for CF tests and to accept a delay in receiving the results.

Since the titers of CF tests for coccidioidomycosis are so valuable, it is advantageous for the physician to know the results of such tests as early as possible. Recently, coccidioidin antigen has become available, and an increasing number of laboratories are performing CF tests for coccidioidomycosis. Although it is imperative that the validity of the "critical titer" interpretation should be preserved, it is appropriate also that consideration be given to a uniform testing procedure. Kaufman (7) and others (12) have pointed out that a rigidly controlled procedure could yield reproducible results among different laboratories. The Laboratory Branch Complement Fixation (LBCF) method had been developed and evaluated for this purpose by a Task Force of the National Communicable Disease Center, pointing out that, "A standardized complement fixation (CF) procedure, useful with all diagnostic CF antigens, has long been needed.
Until such a procedure is established, CF results from one laboratory cannot be compared with those of another" (12).

For the past eleven years we have used the procedure of Smith for CF tests for coccidioidomycosis, but it appeared to us that the time had come to consider replacing this procedure with one which would have more general acceptance. In this report, we compare the results obtained with three CF procedures—the LBCF method, the adaptation of the LBCF method to the micro-titer (MT) technique, and the Smith procedure as used in our laboratory. We hoped one of these proposed substitute methods would not change the critical titer, and our results indicate that this change does not occur with the full volume LBCF procedure.

MATERIALS AND METHODS

The procedure used for the full volume LBCF and the adaptation of the LBCF to the MT technique were those recommended by the Laboratory Task Force (12). The titers obtained with these two procedures were compared to results with the second method of Smith in which the original 2-hr incubation at 37°C for binding of complement is replaced by overnight incubation at 5°C. This has raised the "critical titer" from 1:16 to 1:32 in Smith's laboratory (11) and in ours, based on results of testing more than 16,000 specimens. The overnight incubation procedure was adopted by us originally on the recommendation of Smith as being more sensitive than incubation at 37°C for 2 hr and less likely to yield false-negative results with sera of low titer.

The solution of antigen (coccidioidin) used in all tests was prepared as described earlier from a pool of filtrates obtained from shake cultures of 24 strains of Coccidioides immitis grown in a dialyzed yeast extract-glucose broth (3, 5). The pooled filtrate was concentrated to one-tenth of the original volume by ultrafiltration behind a 4% collodion membrane and then washed by the same procedure with phosphate-buffered saline (pH 7.2) containing thimerosal at a final concentration of 1:10,000. The optimal dilution of this coccidioidin was determined for each CF procedure by preliminary titration of dilutions of the antigen against a reference serum. This was repeated several times with a narrowing range of antigen dilutions, and, with this lot of coccidioidin, the final optimal titer of antigen was the same for all three procedures; i.e., a 1:300 dilution of stock antigen. The optimal dilution of the coccidioidin was confirmed by testing a battery of 10 specimens with known titers.

Serum specimens were obtained from the clinical laboratory of this hospital. These had been screened routinely for reactions to coccidioidin in both agar gel immunodiffusion and latex particle agglutination tests (6), and positive specimens had been confirmed with the quantitative CF test of Smith. To permit valid statistical comparisons of the three CF procedures, the following "blind" protocol was adopted. Twenty specimens in each of two sets were coded according to a table of random numbers by a person other than the one performing the test. After all three CF procedures were completed and the results were recorded, the same 20 specimens in each set were coded with new numbers and the tests were repeated. This protocol permitted an evaluation of reproducibility for each method as well as a comparison among the three procedures. In addition, a series of consecutive specimens which were positive in at least one of the screening tests for coccidioidomycosis were selected for routine testing of each specimen by all three CF procedures.

The results were evaluated for statistical significance by two methods. The chi square (X²) value was determined with the NcNemar test for the significance of changes among two sets of responses for the same specimens (8). A difference of proportions test (w'), as a measure of association between any two procedures when compared to a standard procedure, was calculated by a modification of the method of Chase (1, 2). Since the theory and derivation of this modification will be published separately by A.J.G. and M.H.), the formula is reproduced here:

\[
    w' = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\left(\hat{p}_1(1 - \hat{p}_1) - (\hat{p}^2 - \hat{p}^2_0)\right)/N}}
\]

where \( \hat{p}_1 \) is the observed proportion of matching results for one procedure (i.e., LBCF) compared to the standard, \( \hat{p}_2 \) is the observed proportion of matching results for the second procedure (i.e., MT), \( \hat{p}_s \) is the combined observed proportion of matching results for both procedures, \( \hat{p} \) is the observed proportion of matching results for all three procedures, and \( N \) is the number of specimens tested. The two-sided critical values of \( w' \) for the normal distribution with mean zero and variance unity would be \( \pm 1.64 \) with \( \alpha = 0.10 \) for rejecting the hypothesis that the two procedures are equivalent as substitutes for the standard procedure, \( \pm 1.96 \) with \( \alpha = 0.05 \), and \( \pm 2.58 \) with \( \alpha = 0.01 \), where \( \alpha \) is the probability of type I error.

RESULTS

Among the specimens selected for use in the "blind" protocol, four or five known negative sera were included in each set to avoid a potential bias in the readings which might have been introduced if the reader expected all specimens to yield positive results. The CF titers of these positive specimens ranged from 1:2 to 1:128 as determined earlier by the Smith procedure. Reproducibility for each CF procedure was measured by comparing the results obtained with the replicated sets of coded specimens. Each procedure was highly reproducible within the generally accepted limit of a one-tube difference in a twofold dilution series (Table 1, 2), but it was obvious that the frequency of this one-tube
Table 1. Results obtained with three complement fixation procedures done on each specimen of the coded and replicated sets of sera

| Original titers | Titers on repeat tests |
|----------------|------------------------|
|                | S  | LBFC | MT | S  | LBFC | MT |
| 32             | 32 | 32   | 32 | 64 | 32   | 32 |
| 2              | 2  | 2    | 2  | 2  | 2    | 2  |
| 0              | 0  | 0    | 0  | 0  | 0    | 0  |
| 32             | 32 | 32   | 32 | 32 | 32   | 32 |
| 128            | 64 | 128  | 128| 128| 64   | 64 |
| 0              | 0  | 0    | 0  | 0  | 0    | 0  |
| 64             | 32 | 32   | 32 | 64 | 64   | 64 |
| 0              | 0  | 0    | 0  | 0  | 0    | 0  |
| 32             | 32 | 32   | 32 | 64 | 64   | 64 |
| 8              | 8  | 8    | 8  | 8  | 8    | 8  |
| 0              | 0  | 0    | 0  | 0  | 0    | 0  |
| 8              | 8  | 8    | 8  | 8  | 8    | 8  |
| 4              | 2  | 2    | 2  | 4  | 2    | 4  |
| 4              | 4  | 4    | 4  | 4  | 4    | 4  |

a Anticomplementary specimens not included. Abbreviations are: S, procedure of Smith; LBFC, Laboratory Branch Task Force procedure; and MT, microtiter adaptation of LBFC.

b Titers recorded as the reciprocal.

variation was greater for the MT procedure than for the other two. Since the objective of this study was to evaluate the LBFC and MT procedures as possible substitutes for the Smith method, the results with each (i.e., LBFC and MT) were compared separately to results obtained on the same specimens with the Smith method. The combined data for all comparisons of results with the replicated sets of coded specimens are presented in Table 3. The frequency of specimens with a titer deviation of one-tube or more was only slightly greater for the MT method than for the LBFC procedure, but the MT method yielded results exceeding the one-tube deviation tolerance in four instances. Neverthe-
TABLE 4. Frequency distribution of titers on consecutive specimens: comparison of LBCF and Smith procedures

| Smith titer | LBCF titer |
|------------|-----------|
|            | 0 2 4 8 16 32 64 128 256 512 1024 2048 |
| 0          | 16       |
| 2          | 12 10 1  |
| 4          | 2 7 11 1 |
| 8          | 1 9 19   |
| 16         | 9 13 4 1 |
| 32         | 1 7 18 4 |
| 64         | 4 12 2 3 |
| 128        | 3 5 2   |
| 256        | 1 2     |
| 512        |         |

- See footnote a, Table 1.
- Titer recorded as the reciprocal.

TABLE 5. Frequency distribution of titers on consecutive specimens: comparison of MT and Smith procedures

| Smith titer | MT titer |
|------------|----------|
|            | 0 2 4 8 16 32 64 128 256 512 1024 2048 |
| 0          | 16       |
| 2          | 11 11 1  |
| 4          | 3 8 10  |
| 8          | 1 2 17 9 |
| 16         | 1 10 12 3 |
| 32         | 3 14 10 |
| 64         | 13 5    |
| 128        | 1 4 2 1 |
| 256        | 1 1     |
| 512        |         |

- See footnote a, Table 1.
- Titer recorded as the reciprocal.

procedures as substitutes for the Smith method with the assumption that the Smith test yielded the true results. The statistically significant values (Table 6) indicated that the null hypothesis (i.e., there was no difference between the LBCF and MT tests as substitutes for the Smith test) should be rejected with a high level of confidence, and that the LBCF method was superior to the MT as a substitute for the standard Smith procedure. Another approach to interpretation of these results is to ask what is the probability of a difference in titer occurring with the LBCF and MT procedures, respectively, when compared to titers obtained with the Smith procedure on the same specimen. At a 99% confidence interval, the probability of obtaining titers within one tube of that with the Smith procedure is 0.977 ± 0.029 for the LBCF procedure and 0.925 ± 0.052 for

TABLE 6. Comparison of results obtained with three complement fixation procedures done on consecutive specimens to determine whether the LBCF and MT methods were equivalent as substitutes for the standard Smith method

| Tests compared | No. of specimens with Titers equal | Titer different | Exact matching | One-tube tolerance |
|----------------|-----------------------------------|----------------|---------------|--------------------|
| LBCF to S      | 108                               | 61             | 3             | 19.3 4.44          |
| MT to S        | 75                                | 85             | 11            | 5.8 2.74          |

- See footnote a, Table 1.
- For exact matching, *x* = 69/173; for 1-tube tolerance, *x* = 158/173.
the MT procedure, or between 95 and 100% of the specimens tested by the LBCF procedure and between 87 and 98% of the specimens tested by the MT method.

**DISCUSSION**

We do support the objective of the Laboratory Branch Task Force in attempting to establish a uniform standardized CF procedure as the initial step toward obtaining comparable results from different laboratories. It must be recognized, however, that substitution of this new standardized procedure for one which has been yielding results of a practical clinical value must be accompanied by evidence that either the results are equivalent or that they are associated by a constant interpretable relationship. In the study reported here, we have compared the proposed LBCF procedure and its adaptation to the microtitration technique with the Smith CF procedure which has been used routinely as the standard serological test for coccidioidomycosis. The results show that substitution of the full volume LBCF procedure for the Smith method does not effect any significant change in the serum titers obtained. Each method has a high level of reproducibility within an acceptable one-tube deviation in a series of twofold dilutions, and there is no statistically significant difference between results of the two tests. These equivalent results were obtained, even though the comparison was made on the assumption that the results by the Smith CF procedure were the true ones and that differences occurring with the LBCF method were deviations.

The second proposed method, the MT adaptation of the LBCF technique, was less satisfactory as a substitute for the Smith procedure. Although the titers obtained with the MT technique were highly reproducible within a one-tube error tolerance, the frequency of this one-tube variation was greater than with the Smith and LBCF procedures. We suspected that the microtiter dilution loops might be erratic in transferring 0.025 ml directly from undiluted serum to make the initial 1:2 dilution used in serological tests for coccidioidomycosis. One additional set of 20 coded serum specimens was tested, but the initial 1:2 dilution was done in test tubes by pipetting before making transfers with the microtiter dilution loops. Statistical analysis of these limited results showed that, under these conditions, the MT technique was not significantly inferior to the LBCF method as a substitute for the Smith procedure. We must emphasize that it is most important to begin the serum dilutions at 1:2 in the CF test for coccidioidomycosis. Our experience supports the reports by Smith and his colleagues that a positive reaction even at a 1:2 dilution may be significant, especially as an aid in the diagnosis of early acute disease and as a diagnostic test on spinal fluid from a meningitis case.

The results reported here might be interpreted as indicating that the Smith CF procedure is more reproducible than the other two methods. This may be true in our laboratory but would not necessarily be so in a comparison made among several laboratories. In fact, it is quite likely that the LBCF procedure would prove to be the more reliable technique in such a comparison, for the reagents used are controlled and standardized by more exact and more objective determinations than in the Smith CF method. Protocols for a cooperative study involving several laboratories have been initiated.

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**LITERATURE CITED**

1. Chase, G. R. 1968. On the efficiency of matched pairs in Bernoulli trials. Biometrika 55:365–369.
2. Dixon, W. J., and F. J. Massey. 1969. Introduction to statistical analysis, p. 249–250. McGraw-Hill Book Co., New York.
3. Huppert, M., and J. W. Bailey. 1963. Immunodiffusion as a screening test for coccidioidomycosis serology. Sabouraudia 2:284–291.
4. Huppert, M., and J. W. Bailey. 1965. The use of immunodiffusion tests in coccidioidomycosis. Amer. J. Clin. Pathol. 44:364–368.
5. Huppert, M., and D. Pappagianis. 1963. Growth of Coccidioides immitis in shake culture, p. 13–16. In Methodology manual. American Thoracic Society, New York.
6. Huppert, M., E. T. Peterson, S. H. Sun, P. A. Chitjian, and W. Derrevere. 1968. Evaluation of a latex particle agglutination test for coccidioidomycosis. Amer. J. Clin. Pathol. 49:96–102.
7. Kaufman, L. 1966. Serology of systemic fungus diseases. Pub. Health. Rep. 81:177–185.
8. Siegel, S. 1956. Nonparametric statistics, p. 63–67. McGraw-Hill Book Co., New York.
9. Smith, C. E., M. T. Saito, R. R. Beard, R. McF. Kepp, R. W. Clark, and B. U. Eddie. 1950. Serological tests in the diagnosis and prognosis of coccidioidomycosis. Amer. J. Hyg. 52:1–21.
10. Smith, C. E., M. T. Saito, C. C. Campbell, G. B. Hill, S. Saslaw, S. B. Salvin, J. E. Fenton, and M. A. Krupp. 1957. Comparison of complement fixation tests for coccidioidomycosis. Pub. Health Rep. 72:888–894.
11. Smith, C. E., M. T. Saito, and S. A. Simons. 1956. Pattern of 39,500 serologic tests in coccidioidomycosis. J. Amer. Med. Ass. 160:546–552.
12. U.S. Public Health Service. 1965. Standardized diagnostic, complement fixation method and adaptation to micro test, p. 1–34. PHS Publ. no. 1228, U.S. Government Printing Office, Washington, D.C.