Comparison of Immunodiffusion Techniques with Standard Complement Fixation Assay for Quantitation of Coccidioidal Antibodies

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Quantitative immunodiffusion (QID) and complement fixation (CF) methods were compared for their agreement in detecting coccidioidal antibodies. For these studies, we assayed 719 sera from 181 patients with coccidioidomycosis. Over 60% of the specimens had CF results of 1:2 to 1:256. A total of 43 patients had five or more specimens obtained over periods of between 1 and 8 years. The QID method, as originally performed, agreed within a twofold dilution of the CF titer in 191 of 267 sera (71.5%). Modification of QID by repeated filling of the antigen and serum wells improved agreement to 84.7% (383 of 452 sera). The degree of CF titer change in patients over time periods was more closely matched by the modified than by the original QID method. Discrepancies between the CF and QID methods appeared not to be due to a subpopulation of patients. QID measurement of coccidioidal antibodies may be a useful substitute for the CF assay in certain clinical laboratories.

Complement-fixing antibodies for coccidioidal antigens have been found to be a specific finding in patients with coccidioidomycosis. The quantitative complement fixation (CF) method, originally described by Smith (8), has provided both diagnostic and prognostic information about patients and is considered by many to represent the most successful application of serological techniques to clinical mycology. However, this method is relatively difficult to perform in a standard fashion. Reproducible results depend on meticulous control of several test variables including sheep erythrocytes, anti-sheep erythrocyte hemolysin, complement, and the condition of the serum from the patient. Because of the complexity of the assay, its use generally has been restricted to testing numerous specimens by a few reference laboratories, most of which are geographically within the endemic regions. This may provide a delay in the return of the results when specimens are submitted to reference laboratories, particularly from outside endemic areas.

The Ouchterlony double-diffusion technique, as described by Huppert et al. (2) and Kaufman (4), can detect complement-fixing type antibodies with certain antigen preparations from the mycelial phase of Coccidioides immitis. Their qualitative techniques were specific and more sensitive than those of the CF method. Because the immunodiffusion assay is reliable, its application has become an established and commercially available means for screening specimens for the qualitative presence of CF antibody.

Quantitative measurement of CF antibodies by the immunodiffusion method previously reported by Hampson and Larwood (1) and Huppert et al. (3) would be potentially beneficial. Huppert reported that a rough correlation could be demonstrated between the two methods but raised concern that fourfold or greater discrepancies frequently occurred on individual specimens.

Since 1971, a quantitative immunodiffusion (QID) assay has been used at the Tucson Veterans Administration Medical Center to test selected clinical specimens, portions of which were also sent to the reference laboratory of one of the authors (D.P.) for the 2-h binding CF assay. In 1977, the QID was modified by the repeated addition of antigen and serum dilutions (recharging) to those wells which produced no precipitation lines. The comparison of results from these QID methods with CF results forms the basis of this report.

MATERIALS AND METHODS

Clinical specimens. Sera were received by the Clinical Microbiology Laboratory of the Tucson Veterans Administration Medical Center for coccidioidal serology. All specimens were assayed by the qualitative immunodiffusion technique (2). Positive sera were
selected for quantitative evaluation. When the patient was known to have coccidioidomycosis or when specially requested by the referring physician, specimens with qualitative negative immunodiffusion were evaluated further.

**Antigen preparations.** Antigen preparations were culture filtrates of the mycelial phase of *C. immitis* grown in yeast dialysate (2). prepared and standardized for the immunodiffusion test. Those used in the QID assay were kindly provided by Milton Huppert (Veterans Administration Medical Center, San Antonio, Tex.). Two antigen lots were used during the test period. Reactivity of each sample of antigen was tested with reference sera of known titers immediately upon receipt and before use. Antigens were stored at 5°C. Sterile antigen preparations remain stable under refrigeration indefinitely.

**Immunodiffusion assay.** The QID tests were performed by modifications of the method described by Huppert et al. (3). A 1.5% agar (purified agar; BBL Microbiology Systems, Cockeysville, Md.) was prepared in 99 ml of 0.85% sodium chloride. To this was added 1 ml of 0.67 mM Sørensen’s phosphate buffer (pH 7.4) and 10 ml of merthiolate (1:1,000) diluted with sterile distilled water to a final concentration of 1:10,000. Freshly prepared agar was then dispensed in 5-ml amounts into plastic dishes (50 by 9 mm) with tightly sealing lids (Falcon Plastics, Oxnard, Calif.). The poured agar plates were stored in a sealed moist chamber at 5°C for at least 2 h but no more than 7 days before use.

A hexagonally shaped, seven-well pattern consisting of a central well and six equally spaced peripheral wells, 4.5 mm in diameter, was cut and aspirated from each agar plate. The peripheral wells were spaced 4 mm from the center well. Quality assurance was achieved by testing each batch of agar against reference sera and previously prepared batches of agar.

Two diametrically opposed peripheral wells were filled with positive reference serum with capillary pipettes. Well capacity was ca. 0.025 ml. Each remaining peripheral well was filled with serial twofold saline dilution of the qualitative positive test serum. After a 2-h prediffusion period, the center well was filled with coccidiodial antigen. Plates were then incubated in a moist chamber at room temperature. In the original method, if no precipitation line was evident after 1 day, the serum dilution wells only were rechallenged a single time and the plate was reincubated and examined daily for the next 2 days before recording the final result.

In 1977 we modified our procedure so that the nonreactive dilutions of the test serum and the antigen were both refiled at 24 and 48 h. The titer was recorded as the greatest test serum dilution producing a precipitation line of identity or bend in the line of precipitation between the antigen and serum dilution wells (Fig. 1) after 72 h of incubation. Occasionally, two or more lines of identity may be seen (Fig. 1). The antigen filtrate known to contain numerous antigens reacts because of its broader spectrum (7).

**CF assay.** The CF assay was that of Smith et al. (8) and represented a Kolmer quantitative method in which 0.25 ml of each serial dilution of serum, 0.25 ml of antigen, and 0.5 ml containing 2 U of complement were used. Although greater sensitivity of the CF method could be achieved by overnight complement binding at 4°C (9), the original 2-h incubation at 37°C was retained because of greater experience with clinical correlation and faster results. In this method, the endpoint (no hemolysis) was recorded as 4+. A 3+ reaction (ca. 25% hemolysis) was regarded as equivocal at the first (1:2) dilution. This method differs from the Centers for Disease Control Laboratory Branch Complement Fixation test in that the latter utilizes 0.05 ml of five 50% hemolytic complement units of complement and incubation for 15 to 18 h at 4°C and that 30% or less hemolysis is regarded as positive (6).

**Data assembly.** The following information was collected for each serum sample: the date of sample collection, anticomplementarity or contamination as noted in the CF assay, and the QID and CF results (the highest titer which produced 3+ or more CF). Frequently, a previous specimen was available to be tested in parallel with the current patient serum by the CF assay and the change (Δ) in the titers was recorded as ΔCF (log base 2). When a ΔCF result from a previous specimen was available, the geometric difference between the current and corresponding previous QID results was also recorded (ΔQID). Results in which the CF and the ΔCF were both negative (i.e., a negative CF result was obtained for both of a pair of sera) were not incorporated into subsequent analyses since their inclusion would falsely overestimate agreement between the ΔCF and ΔQID. Computer tabulation and displays assisted in the descriptive analysis. The chi-square method was used in statistical tests.

**RESULTS**

Between 1 May 1971 and 30 June 1980, 886 serum samples from 181 patients were evaluated for coccidioidal antibodies by both the CF and a QID method. Of these sera, 72 anticomplementary sera and 1 contaminated serum were deleted from analysis.

The original QID method (refilling serum wells once only) was utilized from 1 May 1971 to 30 December 1976. The modified QID method

![FIG. 1. QID plate demonstrating reactivity up to a dilution of 1:16. This serum would have to be further diluted to determine the endpoint titer. The double lines of precipitation present are often seen in sera of disseminated patients.](http://jcm.asm.org/Downloaded from http://jcm.asm.org on September 26, 2017 by guest)
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(repeated filling of both serum and antigen wells) was performed from January 1977 to June 1980 to assay 452 sera from 108 patients. Of the 452 sera tested by the modified QID method, 290 samples had previous observation values for comparison.

Comparison of absolute QID and CF results. To define a representative portion of time for each QID method used, we arbitrarily chose to compare the time period, composed of quarters in which five or more sera were tested, immediately preceding the change of method and of equal duration (42 months) to the period in which the modified QID was performed (Fig. 2). During this period (July 1972 to December 1976), 267 sera from 92 patients were assayed. Of the 267 sera tested by the original QID method, 143 samples had previous observations.

In comparing the results of either QID method to those of the CF for all 719 sera (Fig. 3), agreement within ±1 twofold dilution was 71.5% by the original method and 84.7% by the modified method (P < 0.001). The improved agreement of the modified QID method to the CF method appeared to be related to a more even distribution above and below the reference result (Fig. 4). Sera for which the CF result was 1:2 to 1:32 constituted over 60% of the total sera assayed. Agreement within one dilution at various CF results ranged from 59 to 96% for the original method and from 83 to 95% for the modified method. It is apparent that at these dilutions the modified QID method maintained closer and more even agreement with the CF titer than the original method.

Comparison of ΔQID and ΔCF results. ΔQID
results for the original and modified techniques are shown for the corresponding ΔCF in Fig. 5. The average ΔQID agreed within one dilution of the ΔCF in 78.6% of the samples assayed by the original method, which was similar to 82.2% agreement by the modified method. Furthermore, results from both methods appeared to be evenly distributed above and below the reference result.

Since clinical significance is often ascribed to
rising or falling antibody titers, we examined discrepancies in the direction of change between the ΔCF and the ΔQID results (Table 1). Changes in opposite directions were considered major discrepancies. These changes occurred in 11.4% (17 of 143) of the sera with the original QID method and 7.9% (17 of 290) of those assayed by the modified QID (P < 0.05).

**Analysis of variability between the QID and CF methods.** The mean differences (log base 2) of the paired absolute CF value minus the absolute QID value of 43 patients each with five or more samples (555 sera) (Fig. 6) show a normal distribution clustered around a mean of zero. Normal distribution indicates that the overall small variability was not due to a subpopulation or group of patients whose CF and QID results were extremely discordant.

Similarly, the mean difference between the ΔCF and ΔQID for 30 patients each with five or more specimens with previous observations (386 sera) was also normally distributed, with the majority of patients clustered around zero change. Only one patient or 1.3% of the sera had a mean difference in the CF minus the QID of greater than one dilution. Repeated CF tests usually gave the same results but were routinely performed to control for occasional variations of more than one serial dilution.

Variability of the QID test was assessed by replicate testing (Table 2). Overall exact agreement was 89.2%; 100% agreement occurred within the accepted one dilution variance. Samples of the specimens had been held for 3 months to 2 years at −20 to −70°C for use as controls. Variability in the QID test has not been shown to occur with such storage or in CF results with sera stored at −15°C for 2 years.

**DISCUSSION**

Although previous reports indicate that the immunodiffusion technique is sensitive, specific, and simple to perform for the qualitative detection of human coccidioidal complement-fixing antibodies (2, 5), quantitative correlation of the QID and CF tests is not well documented. Huppert et al. (3) initially found unacceptable QID agreement in their study of 349 positive CF sera in which ca. 80% of the sera agreed within one twofold dilution of the CF result. Later, in a study of 79 known cases of coccidioidomycosis (I. Krasnow and M. Huppert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, F54, p. 127), 87% agreement within one twofold dilution between QID and CF tests was described. Recently, Wood et al. (10) evaluated 133 sera or cerebrospinal fluid specimens of which 57 were positive by either the CF technique or the QID method, and four were positive only by the QID method. Overall agreement within one twofold dilution was 82% (50 of 61). Our study, which evaluated 267 sera by the original QID method, yielded slightly less (72%) overall agreement than the study by Huppert et al. We evaluated sera under prevailing clinical laboratory conditions with various microbiologists performing and interpreting the assays throughout the study periods. For this reason, our estimate of agreement may more closely reflect results that other laboratories might expect.

At CF dilutions of 1:2 through 1:32, ca. 76% of the original QID method results agreed within one twofold dilution. The modified QID agreement improved to 88% for the same dilutions.
Both QID methods tended to underestimate CF values, as did the results from the study by Huppert et al. However, underestimation was less with the modified QID than with the original method, and this difference appears to account for the improved agreement of the newer method.

Comparisons of the ΔCF and ΔQID have not been previously reported, although changes in titer often are used in the management of coccidioidal infections (8, 9). The ΔQID for the original method agreed within one dilution (log base 2) of the ΔCF in 78.6% of the sera, but the ΔQID for the modified method increased to 82.2% agreement. Discrepancies between the ΔCF and ΔQID results in the direction of change were reduced from ca. 12% of the sera by the original QID method to 8% by the modified QID method.

A variation of one serial dilution in the titer of an individual serum can be observed in both modified QID and CF tests and could account for the variability seen between the two methods. Furthermore, our studies demonstrate that the mean discrepancies between the two tests for individual patients were very similar. This would suggest that the discrepancy was not due to a few patients who consistently demonstrated large discrepancies. Properly conducted, therefore, the modified QID provides serological information comparable to that of the CF test.

The choice of which method (CF or modified QID) to use in a clinical laboratory depends on several factors. Both procedures are dependent on the consistent quality of antigen. The CF test, considered a reference method for 30 years, is faster but more complex and expensive (10). The modified QID method is simpler and cheaper to perform but requires 72 h for completion. This completion time, however, may be shorter than the total time required to obtain results if the specimen must be transported to a reference laboratory for the CF test. With these considerations in mind, certain laboratories may find the modified QID method useful to measure coccidioidal antibodies.

**TABLE 2. Repeated test results of positive sera by the modified QID method**

<table>
<thead>
<tr>
<th>Reference serum no.</th>
<th>Expected titer determined by CF</th>
<th>No. of replicates</th>
<th>No. of sera with QID titer of:</th>
<th>% Exact agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:4</td>
<td>1:8</td>
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<tr>
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</table>

*The testing of 83 samples of sera from seven patients was performed over periods ranging from 3 months to 2 years.*

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**LITERATURE CITED**