

Indirect Fluorescent-Antibody and Quantitative Agar-Gel Immunodiffusion Tests for the Serological Diagnosis of Paracoccidioidomycosis

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The value of various serological tests in the diagnosis of paracoccidioidomycosis was studied. Quantitative agar-gel immunodiffusion and indirect immunofluorescent tests were performed, and the results were compared with those of complement fixation and qualitative agar-gel procedures. The quantitative immunodiffusion procedure was found to serve as the simplest and safest quantitative test that could be performed for evaluation purposes, whereas the indirect fluorescent-antibody test gave nonspecific reactions and, as such, proved unsuitable.

Serological procedures play an important role in both diagnosis and prognosis of patients with paracoccidioidomycosis. In our laboratory for the past 7 years, we have been using two different tests, namely, quantitative complement fixation (CF) and qualitative agar-gel immunodiffusion (ID). When these tests are used in a combined fashion, over 95.0% of the cases can be initially diagnosed, and the response to treatment can be evaluated quite closely (8, 9).

The tests mentioned, however, have their limitations. The CF test, for example, requires a well-equipped laboratory and specially trained personnel. Physicians working in regional hospitals must mail the patients' sera to the Central Laboratory, and, owing to the fact that many specimens are improperly preserved and handled, they are reported back as "anti-complementary." This means loss of time and discouragement both to the physician and to the laboratory performing the tests. The ID test, although much easier to perform at the level of small laboratories, is usually carried out as a qualitative test and has mainly a diagnostic value. These limitations prompted us to try other methods which could, eventually, be used as substitutes for the time-honored and complex CF test. Because of encouraging reports obtained with indirect fluorescent-antibody (IFA) tests and with quantitative agar-gel immunodiffusion (QID) techniques in other deep-seated mycoses (1-3, 6), these tests were

evaluated in paracoccidioidomycosis.

MATERIALS AND METHODS

Sera. A number of serum samples were obtained from patients with proven mycotic disorders as follows: paracoccidioidomycosis, 74; histoplasmosis, 50; and coccidioidomycosis, 50. (Histoplasmosis and coccidioidomycosis specimens were kindly furnished by L. Kaufman, Fungus Immunology Unit, Mycology Section, Center for Disease Control, Atlanta, Ga.) Samples were also obtained from 50 patients with tuberculosis and from 50 control subjects (blood donors). Blood specimens were allowed to clot, the serum was separated, and aqueous Merthiolate was added to a final concentration of 1:10,000. The specimens were all kept at -20 C for periods ranging from 1 week to 4 years. Prior to their use in the serological tests, required amounts of sera were inactivated at 56 C for 30 min in a water bath.

Antigens. Only antigens derived from *Paracoccidioides brasiliensis* were employed. They included: (i) a pooled, concentrated yeast-form culture filtrate already described (7, 10), which was used in the CF, ID, and QID tests, and (ii) an individual yeast cell suspension utilized in the IFA technique. The latter was prepared from strain C 81, by use of 5- to 7-day-old cultures grown on tubes of Kelley's medium (5). Growth in each tube was harvested with 10.0-ml amounts of sterile saline containing 0.1% formaldehyde [E. Merck, A. G., Fomaldehyde solution, G. R. (35.0%)] and was transferred to a 50.0-ml vial containing 40 to 60 large glass beads. To assure proper distribution of the killing agent, the suspension was kept at -4 C for 18 hr with frequent manual shaking. The beads were discarded, and the cells were then centrifuged in the cold (National Refriger-

ated Centrifuge, model PR 2) at $1,400 \times g$ for 20 min at -5 C ; the supernatant fluid was discarded. The sedimented cells were washed twice and finally adjusted to tube no. 1 of the McFarland nephelometric scale with phosphate-buffered saline, pH 7.2. This antigen was employed the same day it was prepared. New acid-clean microscopic slides were prepared by placing one drop of the antigen into each of four circles previously inscribed in the glass. Slides were air-dried, heat-fixed, and stored in desiccator jars at -20 C for periods up to 4 months. Prior to use, each slide was again gently heat-fixed. With young cultures (5 to 7 days old), the cell suspension gave preparations containing 10 to 20 cells per microscopic field (10 times magnification), a concentration that exhibited good adherence to the glass.

Tests. Groups of 20 sera representing proven patients with the various diseases and controls were processed simultaneously. The CF and the ID tests were performed according to methods already described (8, 9). The QID test used the same agar-gel slides and the same well pattern as the ID technique. This pattern consisted of a central well located 9 mm from the surrounding wells, which were six in number. Each well received 0.15 ml of the required reagent: antigen for the center well and serum dilutions for the peripheral wells. Serial twofold dilutions (1:8 to 1:128) were prepared in fresh normal saline and were placed on the slides; in the case of the paracoccidiodomycosis sera, the undiluted nontitrated sample served also as the positive control. The antigen well was filled immediately after distribution of the serum dilutions. The slides were incubated in moist chambers at room temperature ($22 \pm 4\text{ C}$) for 3 days, with daily readings. To define the titer of some specimens, dilutions over 1:128 were prepared on a later occasion and a new test was set up. Titers are expressed as reciprocal of the dilution.

For the IFA test, the following steps were undertaken. Serial twofold serum dilutions were prepared in phosphate-buffered saline, pH 7.2. Dilutions ranged from 1:8 to 1:1,024. One drop of each dilution was laid on top of the engraved circles containing the yeast cells, care being taken not to let dilutions run over the slide. Two diamond pencil-labeled slides were used for each serum. Slides were incubated at room temperature, in a moist chamber for 30 min, washed off with phosphate-buffered saline, pH 7.2, and rinsed with fresh saline for 10 min. A drop of fluorescein isothiocyanate-conjugated rabbit anti-human globulin [Bacto-FA human globulin antiglobulin (rabbit), Difco] was then added. This reagent was diluted in phosphate-buffered saline of pH 7.2 at the optimal concentration, 1:80, as determined by a previous chess-board titration with a known system (*Treponema pallidum* and syphilitic serum, fluorescent treponemal antibody absorption test). Incubation, washing, and drying were repeated as described. The preparations were mounted in a drop of glycerol-saline (nine parts glycerol to one part phosphate-buffered saline, pH 7.2). Slides were examined within the next 3 hr by use of a Leitz Ortholux fluorescent microscope equipped with an H

BO 200 mercury vapor bulb, housed in a Leitz unit and with a dark-field condenser. The filter combination consisted of a primary BG 12 (3 mm) filter and an ocular K 630 filter. Slides were read with the $10\times$ lens, but equivocal staining was confirmed by using a larger magnification ($40\times$). The highest dilution of serum giving a complete ring of intense fluorescence (4+) around the cells was taken as the titer of specimen. Observation of the gradual fading of fluorescence (3+ to 1+) as the serum dilution increased also contributed to determination of the titer. When the serum titer exceeded 1:1,024, additional serum dilutions were prepared. The titers are expressed as the reciprocal of the dilutions.

Because of the small quantities of sera available from histoplasmosis and coccidiodomycosis patients, the CF test was not run with these specimens. However, they were processed by the remaining techniques. Sera from tuberculosis patients, from blood donors, and from paracoccidiodomycosis patients were tested by the four methods. Because 108 of the old paracoccidiodomycosis specimens were found to be anticomplementary on retesting, the original titer, obtained at time of bleeding, was the one utilized. The ID test was run again, within 2 weeks of the QID and the IFA tests.

RESULTS

A total of 274 subjects were examined, but, as more than one serum sample was available from some of the paracoccidiodomycosis patients, a total of 360 specimens were studied.

Sera from control subjects gave negative reactions in both the CF and the ID tests. CF tests were also negative with the tuberculosis patients' specimens, but one of such patients exhibited a reactive ID test.

The results obtained with the IFA test (Table 1) show that 161 sera gave negative results; the remainder proved to be reactive. The nonreactive specimens were distributed as follows: 41 of the 50 control subjects, 46 of the 50 tuberculosis patients, 33 of the 50 histoplasmosis patients, 39 of the 50 coccidiodomycosis patients, and 2 of the 74 paracoccidiodomycosis patients. The reactions exhibited by controls and by tuberculosis patients were, with one exception, low titered (not over 8). Among the histoplasmosis-coccidiodomycosis sera, titers did not exceed 32. On the other hand, 80.0% (129 of 160) of the paracoccidiodomycosis sera exhibited titers of 32 and above. Statistical analysis between controls (blood donors and tuberculosis patients) and paracoccidiodomycosis patients showed that at titers greater than 16 there was a significant difference ($P = 0.001$). Comparison between the histoplasmosis-coccidiodomycosis group and paracoccidiodomycosis patients also showed a significant difference ($P = 0.001$)

above the 32 titer. Hence, it appears that the 64 titer would separate the specific from the nonspecific reactions.

In the QID test (Table 2), 206 of the 360 samples were nonreactive. This test was more specific than the IFA test, as none of the control sera and only one of the histoplasmosis-coccidioidomycosis sera gave precipitin bands. Sera from patients with paracoccidioidomycosis showed 95.6% (153 of 160) positive reactions. Among these, 36.2% (54 of 153) reacted only when undiluted; 16.3% (25 of 153), at a dilution of 8; 21.5% (33 of 153), at a dilution of 16; 17.0% (26 of 153), at a dilution of 32; and 9.8% (15 of 153), at a dilution 64 or above. The tuberculosis patient who gave a reactive test (line of identity with a proven serum) did so only with the undiluted serum. As expected, the ID and the QID tests correlated in every instance. Whenever there were precipitin bands in the qualitative test, they were corroborated by the quantitative procedure.

Titers obtained with paracoccidioidomycosis

sera in the three quantitative tests were compared. Table 3 shows that only 20 of the 147 specimens with CF titers (13 samples were anticomplementary) gave identical CF and IFA titers; 38 gave higher and 76 gave lower CF titers. The two tests were not comparable in 13 cases; 11 of these showed nonreactive CF but reactive IFA tests, and 2 exhibited the reverse pattern. Comparison of the CF titers with those of the QID test shows that the former test gave higher titers with 124 specimens, lower titers with only 3, and equal titers with 12. In eight cases results were not comparable, as one test turned out to be positive while the other was negative.

Among the 13 anticomplementary specimens, 11 were reactive in both the IFA and QID tests; 2 were reactive only in the IFA procedure. With these specimens, IFA titers were higher than 32 and QID titers varied from undiluted to 32.

The sera from proven paracoccidioidomycosis patients which were nonreactive in the IFA test were also nonreactive in the CF

TABLE 1. Indirect fluorescent-antibody test: titers of 360 sera in the presence of *Paracoccidioides brasiliensis* yeast cells

Group	No. of subjects	No. of sera	No. of sera nonreactive ^a	No. of sera reactive							
				Undiluted	1:8	1:16	1:32	1:64	1:128	1:256	1:512 and over
Control subjects	50	50	41	8	1	—	—	—	—	—	—
Tuberculosis patients	50	50	46	1	2	—	—	—	1 ^b	—	—
Histoplasmosis patients	50	50	33	8	3	4	2	—	—	—	—
Coccidioidomycosis patients	50	50	39	7	3	—	1	—	—	—	—
Paracoccidioidomycosis patients	74	160	2	4	10	15	24	23	33	22	27
Totals	274	360	161	28	19	19	27	23	34	22	27

^a Undiluted serum.

^b Patient who also gave a reactive immunodiffusion test.

TABLE 2. Quantitative agar-gel immunodiffusion test: titers of 360 sera in the presence of *Paracoccidioides brasiliensis* yeast cell culture filtrate

Group	No. of subjects	No. of sera	No. of nonreactive ^a	No. of sera reactive						
				Undiluted	1:8	1:16	1:32	1:64	1:128	1:256 and over
Control subjects	50	50	50	—	—	—	—	—	—	—
Tuberculosis patients	50	50	49	1 ^b	—	—	—	—	—	—
Histoplasmosis patients	50	50	50	—	—	—	—	—	—	—
Coccidioidomycosis patients	50	50	50	—	—	—	—	—	—	—
Paracoccidioidomycosis patients	74	160	7	54	25	33	26	6	4	5
Totals	274	360	206	55	25	33	26	6	4	5

^a Undiluted serum.

^b Patient who also gave a reactive indirect immunofluorescence test.

TABLE 3. *Paracoccidioidomycosis* sera: comparisons of titers obtained with complement-fixation (CF), quantitative agar-gel immunodiffusion (QID), and indirect immunofluorescent-antibody (IFA) tests

Tests compared ^a	Tests comparable				Test not comparable		
	No. (and %) of sera with CF titers			No. (and %) reactive in both tests	No. (and %) of sera with		No. (and %) reactive in one test
	Higher	Lower	Equal		CF -, other +	CF +, other -	
CF to IFA	38 (25.8)	76 (51.6)	20 (13.6)	134 (91.1)	11 (7.4)	2 (1.3)	13 (8.8)
CF to QID	124 (84.3)	3 (2.0)	12 (8.1)	139 (94.5)	6 (4.0)	2 (1.3)	8 (15.4)

^a The number of sera tested was 147; 13 anticomplementary sera were not tabulated.

test, but had precipitin bands with the undiluted serum. These samples had been taken 4 and 6 years after diagnosis, and all patients had had previous antifungal treatment.

The service that each test renders to diagnosis and follow-up is exemplified by five patients (Table 4) who were followed for 3 years. Most of the time and for any of the quantitative tests, the initial higher titers have a tendency to diminish with time. Also, lower titers are obtained with the QID test. Patient E. P. is an interesting case, as his sera were practically negative in all tests with the exception of the IFA test, which gave titers of 1,024 at time of diagnosis and of 256 at the end of the 3 years.

Table 5 correlates the results obtained with the four tests and with the 147 paracoccidioidomycosis sera. It can be seen that all of the tests were simultaneously reactive in 128 cases and nonreactive in 1, for an overall agreement of 87.7%. Eleven sera were nonreactive in the CF test but reactive in the remaining procedures.

Six sera were reactive only in the IFA test; one was reactive in both the CF and the IFA tests but nonreactive in the ID and QID tests. The IFA test was more sensitive than CF, ID, or QID tests, but, as shown above, it was also less specific. Briefly, in 12.2% (18 of 147) of the sera there was no agreement among the tests.

DISCUSSION

The findings of this study indicate that both the IFA and the QID tests can be used in certain instances for the rapid and accurate detection of *P. brasiliensis* antibodies.

The IFA test appears to be more sensitive but less specific than the conventional tests (CF and ID). Only two (1.2%) of the paracoccidioidomycosis sera gave negative reactions, and 65.6% of these specimens exhibited anti-

TABLE 4. *Paracoccidioidomycosis* sera: comparison of the results obtained with four serological tests as exemplified by five patients followed for 3 years

Patient	Test ^a	Serum at time of diagnosis	Titers (reciprocal of dilutions) of sera after			
			6 months	12 months	24 months	36 months
L.D.	CF	1,024	256	256	256	256
	ID	+ ^b	+	+	+	+
	QID	64	32	32	32	8
	IFA	256	128	64	64	16
C.T.	CF	256	16	8	N ^c	N
	ID	+	+	+	N	N
	QID	8	Und. ^d	Und.	N	N
	IFA	256	512	512	128	8
P.N.R.	CF	32	AC ^e	64	32	16
	ID	+	+	+	+	+
	QID	8	8	8	8	Und.
	IFA	32	64	32	32	16
P.B.	CF	256	128	128	128	32
	ID	+	+	+	+	+
	QID	16	8	16	Und.	Und.
	IFA	128	512	156	156	128
E.P.	CF	N	N	8	N	N
	ID	N	N	N	N	N
	QID	N	N	N	N	N
	IFA	1,024	156	512	156	156

^a CF = complement fixation; ID = agar-gel immunodiffusion; QID = quantitative agar-gel immunodiffusion; IFA = indirect fluorescent-antibody test.

^b One or more precipitin bands.

^c Nonreactive.

^d Reactive only with the undiluted serum.

^e Anticomplementary.

body titers above the 32 dilution. Cross-reactions were, as expected, noticed with the sera from patients with either histoplasmosis or

TABLE 5. *Paracoccidioidomycosis* sera: correlation among results of complement-fixation (CF), agar-gel immunodiffusion (ID), quantitative agar-gel immunodiffusion (QID), and indirect fluorescent-antibody (IFA) tests

Test	No. of sera	Correlation (%)
CF positive, ID and QID positive, IFA positive	128	87.0
CF negative, ID and QID negative, IFA negative	1	0.6
CF negative, ID and QID positive, IFA positive	11	7.4
CF negative, ID and QID negative, IFA positive	6	4.0
CF positive, ID and QID negative, IFA positive	1	0.6
Totals	147 ^a	100.0

^a Thirteen anticomplementary sera were not tabulated.

coccidioidomycosis (34.0 and 21.0%, respectively), but these were primarily of low order, titers not exceeding the 32 dilution. Lack of specificity was demonstrated by the reactivity of the control sera (blood donors and tuberculosis patients), which reacted in 8 to 18.0% of the cases. Here again, titers were low and not above the 8 dilution. It was not ascertained by other methods (skin testing, for example) whether the cross-reactivity of the latter group was due to previous contacts with specific (*P. brasiliensis*) or nonspecific (*Histoplasma capsulatum*, *Coccidioides immitis*) fungal agents.

The IFA titer of 64 was found to be limiting between controls, patients with other mycoses, and patients with paracoccidioidomycosis, with significant differences among the groups. Although titers below 64 do not exclude paracoccidioidomycosis (53 sera from 25 patients had titers below this range), higher titers confirm a specific clinical infection.

We are aware of the fact that the anticomplementary nature of the stored paracoccidioidomycosis sera precludes the precise correlation among titers given by the various tests. However, some considerations were attempted.

Comparing CF with IFA tests, one finds that there is an overall correlation of 91.1%, but when titers are compared such correlation is of a much lower order, 13.6%. CF titers were lower than the IFA titers in 51.6% and higher in 25.8% of the sera. Also, lack of correlation was apparent in 8.8%. All of these figures indicate that, although the IFA test could be used in special cases (anticomplementary sera, for

example), the lack of specificity limits its application.

The QID test was almost completely specific, as only one of the 200 serum samples from controls and from patients not having paracoccidioidomycosis reacted in the test. Although such specificity is indeed remarkable, previous studies (9) have shown that a reduced number of proven histoplasmosis patients react with *P. brasiliensis* antigen in the ID test. Nonetheless, a positive ID test, or better still a positive QID test, constitutes a solid diagnostic basis and offers more reliable results than the IFA test, or even the CF test. The QID test was reactive in 95.6% of the paracoccidioidomycosis sera; titers were generally low, with approximately one-third of the sera (54 of 160) reacting only when undiluted, another third (58 of 160) in the 8 to 16 dilutions, and the rest (41 to 160) in higher dilutions. The seven nonreactive serum samples had been obtained from patients already treated who had been diagnosed 1 to 3 years previously. In the CF test, these sera were negative, anticomplementary, or showed low titers.

When the CF and the QID tests are compared, one finds that both agree 94.5% of the time, but again comparison by titers shows a low correlation, with only 8.1% agreement. CF titers were consistently higher (84.3%) than QID titers. There was no correlation in 5.4% of the cases. The lower titers with the QID test in paracoccidioidomycosis have already been confirmed by R. Negroni (Ph.D. thesis, Univ. of Buenos Aires, Buenos Aires, Argentina, 1968). Negroni also pointed out that the QID test was the simplest of the quantitative techniques available. The QID test has a definite place in the serological diagnosis of paracoccidioidomycosis, and more laboratories should undertake it. As the QID titers were generally low, it is suggested that serum dilutions be prepared at a closer range (1:2; 1:4; 1:8, etc.), so that the standard one-tube error accepted for all dilution procedures will be overcome and the real change in titers will be obtained.

Mention should be made of the tuberculous patient who had an IFA titer of 128, a reactive (undiluted serum) QID test, and a negative CF test. Although our attempts to isolate a fungal agent from this patient were unsuccessful, it is still clinically and mycologically possible that he might have had a mycotic disorder associated with his proven tuberculosis. Unluckily, the patient left the hospital before a final agreement concerning the fungal nature of his complaints could be reached, and thus further

serological and cultural evaluations were hindered.

With the paracoccidiodomycosis sera, the battery of tests employed showed that results were simultaneously reactive or nonreactive in 87% of the tests. In other words, any one of the four tests will allow diagnosis in such a number of patients. The CF test will detect antibodies in 0.6% of sera negative by other tests; the QID-ID combinations will allow 7.4% more cases to be detected, a fact already noted (9). The IFA test will detect antibodies, not always specific ones, in 12.0% of sera giving negative results in other tests.

Previous studies (9) indicated that clinical improvement is accompanied by a decrease in CF titers up to a point where no further decreases occur ("serological scar"). Also, in some patients, during the course of treatment, all precipitin bands disappear. Results of the QID test tend to parallel those of CF tests; that is to say, a drop in titers occurred during the time of observation, suggesting healing of the active lesions. However, we consider the number of patients who have been followed with the QID technique insufficient to permit generalizations, and we believe that each case should be evaluated separately.

It is our belief that three of the tests have their place and particular employment. It is suggested that the CF test be considered the standard, reference procedure for the follow-up of all patients; the ID test should be used as the screening test for all laboratories, especially small ones; and the QID test would serve as the simplest quantitative test that can be performed for evaluation purposes. The IFA test has a limited role and is not recommended.

The alternate or combined use of these methods should allow not only correct and

accurate diagnosis but also a thorough evaluation of the patients' response to treatment.

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