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Characterization of the Precipitin Bands Detected in the Immunodiffusion Test for Paracoccidioidomycosis

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In order to characterize the precipitin bands detected in the immunodiffusion test for paracoccidioidomycosis, a study was undertaken in 54 patients with the disease. On the basis of the pattern of known control sera, the three commonly observed lines of precipitate were designated as 1, 2, and 3 according to their location in the immunodiffusion plate. At time of diagnosis, 28 of the patients exhibited all three bands, 16 gave two bands, and 10 showed only one precipitin line. Over 50 of the sera with three bands had high complement fixation titers (above 1:512), whereas those with one band exhibited lower titers. A similar picture was obtained with the quantitative agar-gel techniques, where titers of 1:64 and above were more commonly observed in sera with three precipitin lines. Follow-up studies carried out in 18 patients revealed that band 3 disappeared first, followed by band 2, and, finally, by band 1. At the end of 2 to 3 years, 85.7% of the patients had lost band 3, 75% band 2, and only 27.7% band 1. Cross-reactions with histoplasmin were found in eight patients who gave the M precipitin line with this antigen. It was found that the latter band and our paracoccidioidin band 3 fused, producing lines of identity. Bands 1 and 2 were specific. The implications of these findings are discussed.

In earlier publications, we have analyzed the characteristics of the immunodiffusion test for the serological diagnosis of paracoccidioidomycosis (9, 11, 12). It has been shown that a yeast-phase antigen from *Paracoccidioides brasiliensis* produced positive reactions in 95% of the cases (6, 11). One or two of the precipitin lines demonstrable at the time of diagnosis were gradually lost in the course of time. The antigen used was specific, and no precipitin bands were detected in patients with other mycoses (6). It was observed, however, that some patients with proven paracoccidioidomycosis reacted to the immunodiffusion test with histoplasmin (11).

A more detailed study aimed towards standardization of the technique was deemed necessary. The purpose of the present report is to characterize the precipitin bands, determining their frequency and tendency to disappear. Also, the relationship between the cross-reacting histoplasmin band and the newly characterized paracoccidiodin bands was investigated in an effort to select those which appear to be specific.

MATERIALS AND METHODS

Blood was obtained under fasting conditions from 54 newly diagnosed paracoccidioidomycosis patients. The serum was removed, preserved with merthiolate 1:10,000, divided into 1.0-ml portions, and frozen for periods up to 3 years. Before testing, the required number of vials were thawed, and the serum was utilized the same day. A total of 140 serum specimens were available for the study. Serological follow-ups were possible in 18 patients, blood specimens being collected at the time of diagnosis, at 6 months, and 1, 2, and sometimes 3 years after establishment of the diagnosis. During the follow-up period, all patients received treatment with sulfa drugs or amphotericin B or both.

The complement fixation (CF) test and the agar-gel immunodiffusion techniques were performed according to standard methods (9, 14). For the latter, both qualitative and quantitative procedures were utilized (12). The basic immunodiffusion pattern consisted of a central well surrounded by six lateral reservoirs; when cross-reactions were being investigated, the designs proposed by Huppert (5) were utilized.

A single batch of the yeast paracoccidioidin, prepared according to techniques previously described, was utilized (13). The histoplasmin was obtained from the Center for Disease Control (CDC), Atlanta, Ga. Both antigens were utilized in the study of all serum specimens.

For characterization purposes, six sera known to produce three precipitin bands with paracoccidioidin were pooled and used as a control. Subsequently, all sera were compared with this standard. The precipitin lines were designated as 1, 2, and 3, according to their

position. Band 1 was formed closer to the antigen depot, band 3 was located closer to the serum well, and band 2 occupied an intermediate position (Fig. 1). The cross-reacting band was checked against a serum from CDC known to produce both the H and the M but not the C, histoplasmin bands.

RESULTS

Serum samples obtained from each patient at the time of diagnosis revealed the simultaneous presence of bands 1, 2, and 3 in 28 of the 54 patients (51.8%). Among these, there were two who produced an additional 4th line. Sixteen patients had two precipitin bands (29.6%), whereas 10 produced only one line (18.5%). In total, and at the time of diagnosis, 46 patients (85.1%) showed band 1, 45 (83.3%) band 2, and 35 (64.8%) band 3. Table 1 presents the data.

The presence of the various precipitin lines was compared with the results of the other serological tests. It was found that the sera exhibiting the three precipitin bands were also those with the highest CF titers (Table 2). Seventeen of the 28 specimens (60.7%) in this category had CF titers above 1:512. Over half of the specimens (9/16) with two bands had CF titers in the 1:64 to 1:256 range. A sizeable proportion (5/10) of the specimens with just one band exhibited lower CF titers, between 1:8 and 1:32.

Similar findings were obtained when the results of the quantitative immunodiffusion test were compared with the number of precipitin bands (Table 3). Twenty-two of the 28 sera with three bands gave immunodiffusion titers of 1:16 and above. Although a number of sera with two bands also had titers in this range, most of these specimens (9/16) exhibited lower titers. This was also true for the patients giving one precipitin band.

In the follow-up studies, one group of patients was followed for 2 years and another for 3 (Table 4). Bands 1, 2, and 3 in combination were present in 14 of the 18 patients. Six months after treatment, one patient had lost bands 2 and 3; four patients lost band 3, whereas the remaining nine maintained the initial pattern of precipitin lines. After one year, band 3 had disappeared from 5 more patients; in one pa-

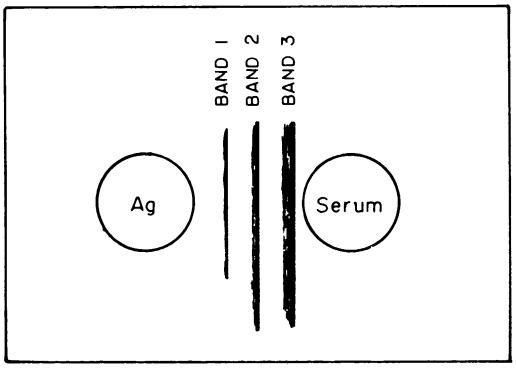

FIG. 1. *Location of the paracoccidioidin precipitin bands in the immunodiffusion test.*

TABLE 1. *Type of precipitin bands found at time of diagnosis in 54 patients with paracoccidioidomycosis*

Types of precipitin bands	No. of patients	% Positive
1, 2, 3	28[a]	51.8
1, 2	9	
1, 3	4	29.6
2, 3	3	
1	5	
2	5	18.5
Total	54	100.0

[a] Two of these patients gave a 4th line of precipitate.

TABLE 2. *Comparison of types of precipitin bands with complement fixation titers in 54 patients with paracoccidioidomycosis*

CF titers	Precipitin bands					
	1, 2, 3		1, 2; 1, 3; or 2, 3		1 or 2	
	No.	%	No.	%	No.	%
Negative[a]	2	7.1	1	6.2	1	10.0
1:8-1:32	3	10.7	1	6.2	5	50.0
1:64-1:256	6	21.4	9	56.2	3	30.0
>1:512	17	60.7	5	31.2	1	10.0
Total	28	100.0	16	100.0	10	100.0

[a] No fixation at the 1:8 dilution.

TABLE 3. *Comparison of types of precipitin bands with quantitative immunodiffusion titers in 54 patients with paracoccidioidomycosis*

Immunodiffusion titers	Precipitin bands					
	1, 2, 3		1, 2; 1, 3; or 2, 3		1 or 2	
	No.	%	No.	%	No.	%
Undiluted-1:8	6	21.4	9	56.2	7	77.7
1:16-1:32	11	39.2	4	25.0	3	30.3
>1:64	11	39.2	3	18.7	0	
Total	28	100.0	16	100.0	10	100.0

tient, band 2 also disappeared, but band 1 continued to be present in 17 patients. From here on, bands 2 and 3 were lost by most of the remaining patients; band 1, however, disappeared only from four cases.

TABLE 4. *Serological follow-up of patients with paracoccidioidomycosis and fate of the precipitin bands*

Patient no.	Precipitin bands at time of testing				
	Time of diagnosis	6 months	1 year	2 years	3 years
1	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
2	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, -[a]
3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, -	1, - -
4	1, 2, 3	1, 2, 3	1, 2, -	1, - -	1, - -
5	1, 2, 3	1, 2, 3	1, 2, -	1, - -	1, - -
6	1, 2, 3	1, - -	1, - -	1, - -	1, - -
7	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3
8	1, 2	1, 2	1, 2	1, -	1, -
9	1	1	-	-	-
10	1	1	1	-	ND[b]
11	1, 2, 3	1, 2, -	1, 2, -	1, - -	ND
12	1, 2, 3	1, 2, -	1, 2, -	1, - -	ND
13	1, 2, 3	1, 2, -	1, 2, -	-	ND
14	1, 2, 3	1, 2, -	1, - -	-	ND
15	1, 2, 3	1, 2, 3	1, 2, -	1, - -	ND
16	1, 2,	1, 2	1, - -	-	ND
17	1, 2, 3	1, 2, 3	1, 2, -	1, 2, -	ND
18	1, 2, 3	1, 2, 3	1, 2, -	1, - -	ND

[a] Precipitin line was lost (shown by dash).
[b] ND, not done.

During the period of observation, band 3 was lost by 12 of the 14 patients (85.6%), band 2 disappeared from 12 of the 16 patients (75.0%), and band 1 was lost only by five of the 18 patients (27.7%). The sequence of events is illustrated in Fig. 2 and 3.

Among the patients studied, there were eight (14.8%) whose sera also produced a precipitin band with histoplasmin (M band). In six patients this cross-reactivity was already present in the first serum sample examined, and, in one case, it persisted for the 3 years of the study. The remaining two patients acquired the cross-reactivity during the first 6 months of observation. Three of the patients also exhibited complement-fixing antibodies with the *Histoplasma capsulatum* antigen employed (Table 5). In general, these patients had high CF titers with paracoccidioidin, but the quantitative immunodiffusion titers were not always elevated. All, however, had the three precipitin bands when the cross-reaction was detected. It must be stated that none of these patients had been given a skin test with histoplasmin.

Sera from these patients were compared in the same agar-gel plate with 2 homologous antigen-antibody systems, namely, *P. brasiliensis* and *H. capsulatum*. It was observed that paracoccidioidin band 3 and histoplasmin band M fused, producing lines of identity (Fig. 4). Bands 1 and 2 did not cross-react with the soluble *H. capsulatum* antigen employed in the test (Fig. 5).

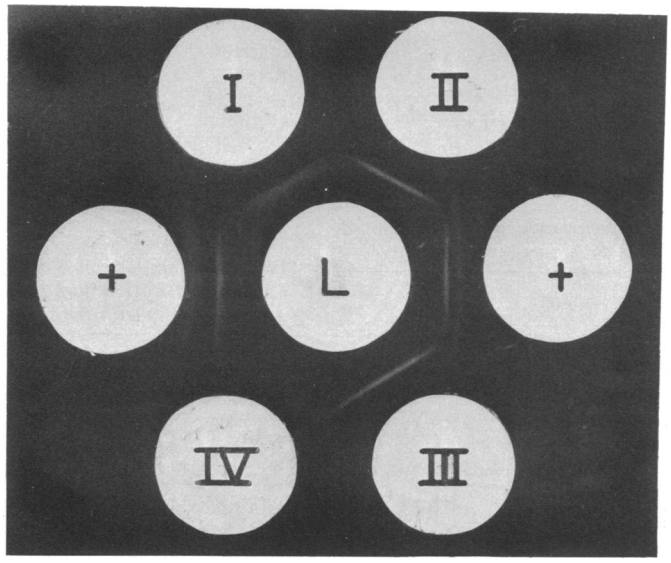

FIG. 2. *Patient 10, follow-up. Central well has paracoccidioidin; peripheral wells have: + (left), control sera with bands 2 and 3; + (right), control serum with bands 1 and 2. Wells I, II, III, and IV have patient's sera taken at diagnosis, 6 months, 1 and 2 years after treatment, respectively. In well IV note that band 1 has disappeared.*

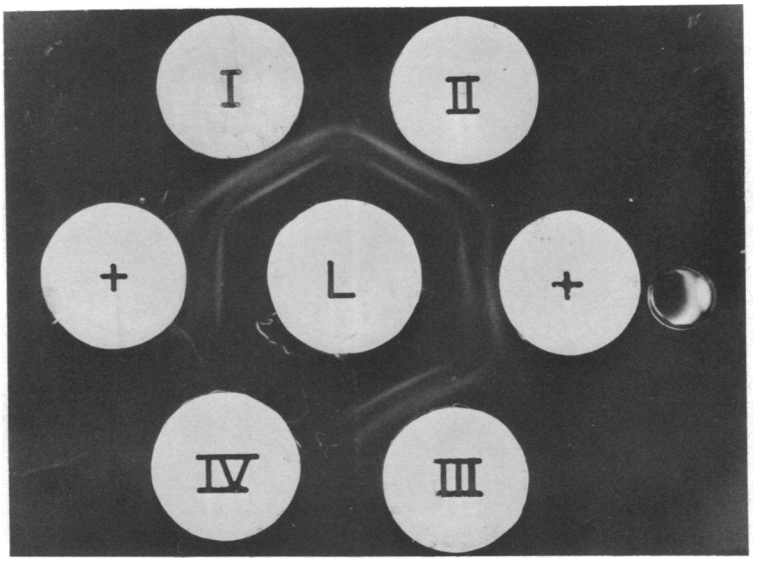

FIG. 3. *Patient 3, follow-up. Central well has paracoccidioidin; peripheral wells have: + (left), control serum with bands 1, 2, 3; + (right), control serum with bands 2, 3. Wells I, II, III, and IV have patient's sera taken at diagnosis, 6 months, 1 and 3 years after treatment, respectively. In well IV note weak band 1 and absence of other bands.*

TABLE 5. *Results of serological tests in eight patients with paracoccidioidomycosis who reacted with both paracoccidioidin and histoplasmin*

Patient no.	Results of serological tests					
	Complement fixation[a]		Immunodiffusion			
	P. brasiliensis	*H. capsulatum*	*P. brasiliensis*		*H. capsulatum* bands	
			No. of bands	Titer[a]	H	M
2	4.096	64	3	64	–	+
3	2.048	N[b]	3	8	–	+
4	1.024	8	3	128	–	+
11	512	N	3	Undiluted	–	+
13	128	N	3	32	–	+
14	128	N	3	8	–	+
15	256	8	3	128	–	+

[a] Reciprocal of the titer.
[b] No fixation at the 1:8 dilution.

DISCUSSION

With the characterization of the precipitin lines formed by patients with paracoccidioidomycosis, the results of the immunodiffusion test can be better interpreted. In a new patient the simultaneous presence of bands 1 and 2, or even of band 1 alone, allows a presumptive diagnosis. Band 3, because of its cross-reactivity, should be interpreted judiciously, and patients exhibiting only this line of precipitate should be subjected to other mycological studies before establishing a diagnosis.

In the patient undergoing treatment, one may expect a reduction in the number of precipitin bands. Band 3 should be lost in 1 to 2 years and band 2 in 2 to 3 years. Band 1 will probably be detectable for extended periods of time, even in the presence of substantial clinical improvement. Mention should be made of two of our patients (Table 4, no. 1, 7) who, in over 3 years of careful follow-up and treatment, did not change their immunodiffusion patterns nor their CF titers ranging from 1:216 to 1:1024. Both patients exhibited pulmonary paracoccidioidomycosis but improved clinically and radiologically and had negative sputum cultures. If the serological reactivity indicates an active infection, these patients would not be considered cured in spite of the improvement noticed; perhaps their immune cellular mechanisms were powerful enough to cope with the persistent activity of the fungus, thus hindering the progress of the mycotic process.

The value of the agar-gel immunodiffusion techniques in the diagnosis of various pulmonary mycoses is well established (2). In histoplasmosis and coccidioidomycosis, for instance, the precipitin bands have been properly characterized. In coccidioidomycosis, the F band, present in virtually all patients, is specific for

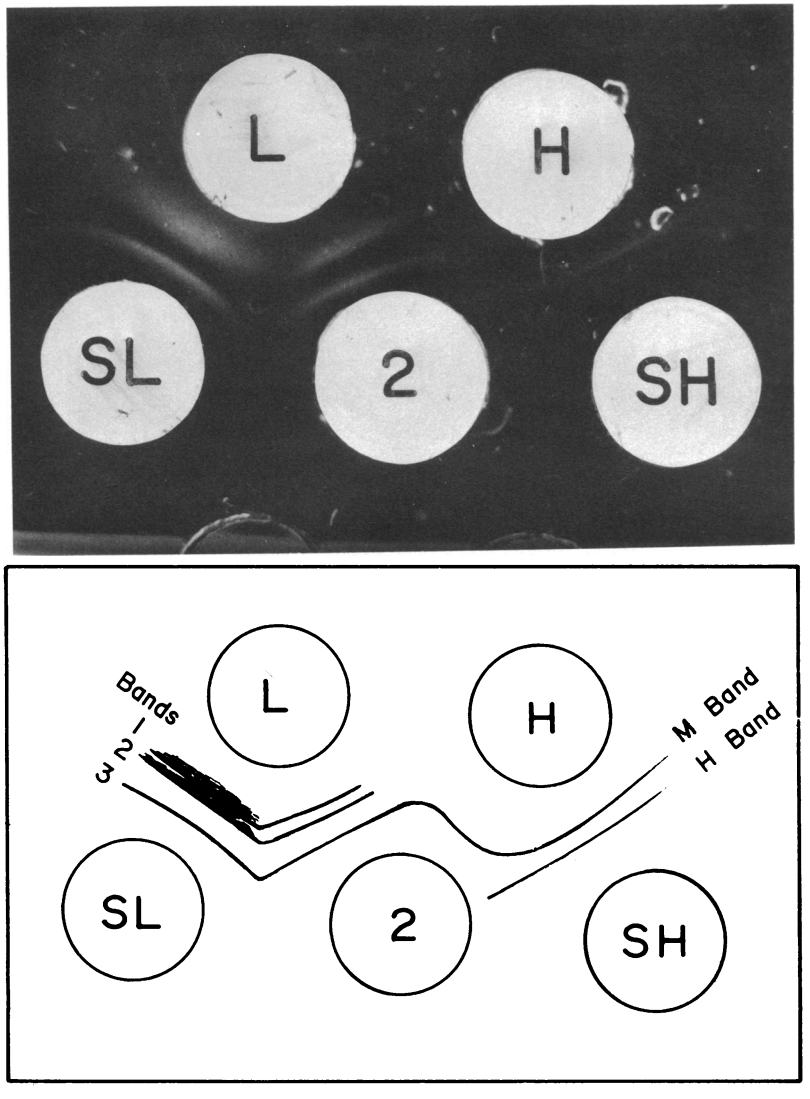

FIG. 4. *Comparison of cross-reacting serum from a patient with paracoccidioidomycosis with standardized reference patterns for paracoccidioidomycosis and histoplasmosis. Upper wells: L, paracoccidioidin; H, histoplasmin. Lower wells: SL, paracoccidioidomycosis antiserum; SH, histoplasmosis antiserum; 2, cross-reacting serum. In diagrammatical presentation below, note fusion of paracoccidioidin band 3 with histoplasmin band M.*

this mycotic disorder (5). In histoplasmosis, the H band and, to some extent, the M band are considered specific, and their joint presence is diagnostically significant (2, 4). The contrary is the case with band C which cross-reacts with antigens from *Coccidiodes immitis* (4).

The results of the present study indicate that some patients with paracoccidioidomycosis, who have not been skin-tested with histoplasmin, can react with this antigen, producing the M band. The fact that this band and our paracoccidioidomycosis band 3 fuse to produce a line of identity is of interest, as there are possibilities for an erroneous diagnosis. If only histoplasmin is utilized in the immunodiffusion test, some patients with paracoccidioidomycosis might be misdiagnosed. If paracoccidiodin is also employed, the presence of various precipitin lines with this antigen would clarify the picture, because cross-reactions with histoplasmin are apparently present only in patients exhibiting paracoccidiodin bands 1, 2, and 3. It is rec-

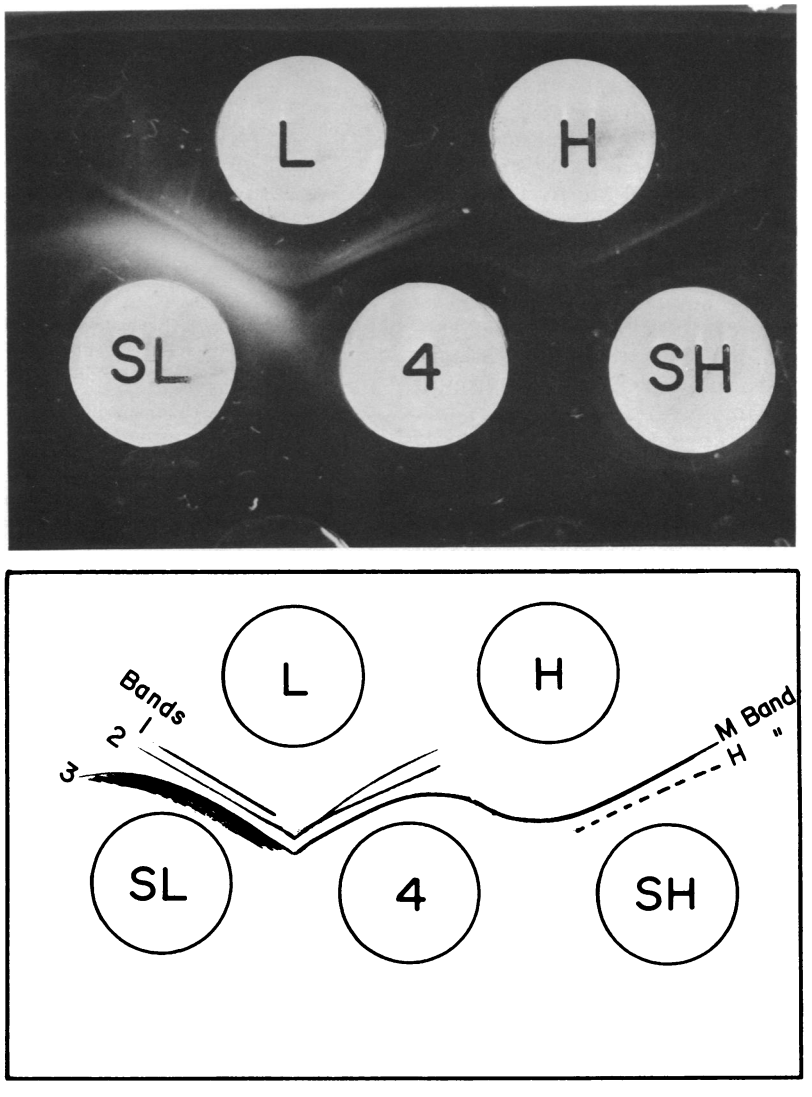

FIG. 5. *Nonidentity of paracoccidioidin bands 1 and 2 and identity of band 3 with histoplasmin band. Upper wells contain paracoccidioidin (left) and histoplasmin (right). Lower wells: SL, paracoccidioidomycosis antiserum; SH, histoplasmosis antiserum; 4, cross-reacting serum. In diagrammatical presentation below, note fusion of histoplasmin M band with paracoccidioidin band 3.*

ommended that laboratories processing samples from patients living in areas endemic for histoplasmosis and paracoccidioidomycosis make use of antigens derived from both etiologic agents.

As concerns the cross-reacting band, one is puzzled by the fact that 35 patients produced band 3, but only eight cross-reacted with histoplasmin. If band 3 is identical to band M, why did the other 27 patients fail to react? A possible explanation would be that a relatively large quantity of the respective antibody is required to produce a distinct M band, whereas lesser quantities are sufficient to form band 3. Perhaps an answer could be obtained by employing a more sensitive indicator such as the precipitation inhibition immunodiffusion technique.

Using immunoelectrophoretic techniques and antigens identical to the ones employed in the present study, various precipitin arcs (A, B, C, D, E) have been shown to occur in paracoccidioidomycosis (10). It is of interest that the A band described in the latter study is detected with equal frequency and for the same long periods of time as is band 1. Yarzábal (15),

working with different antigenic preparations but also employing immunoelectrophoresis, found 25 arcs of precipitation; an arc, designated E and characterized by its cathodic migration, was considered completely specific, as it was absent from the sera of patients with other mycoses. We think that arc E is identical to the previously described arc A. Thus, it is possible that arc A, which appears to have the characteristics of band 1, may also be identical to Yarzábal's specific arc E. More recently, and by means of the immunoelectroosmophoresis-immunodiffusion test (3), the presence of specific precipitin lines located in the cathodic zone have been described, thus confirming Yarzábal's (15) findings.

Although immunoelectrophoresis usually gives more precise results than the immunodiffusion test, the former is not easy to perform and requires complex equipment. Consequently, its use is limited to specialized laboratories. The immunodiffusion test, on the other hand, is an extremely simple technique which can be adapted to the clinical laboratory.

It is expected that the results of the present study will strengthen the value of the immunodiffusion test in the diagnosis of paracoccidioidomycosis, especially now that more laboratories are starting to perform the test (1, 7, 8). It is also hoped that in the future "reference reagents, tested for potency and specifity, (5)" can be made available through international agencies so that results from various laboratories can be compared.

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