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In Vitro Susceptibility Testing of *Paracoccidioides brasiliensis* to Sulfonamides

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A total of 60 clinical isolates of *Paracoccidioides brasiliensis* were tested for susceptibility to sulfadiazine and sulfadimethoxyne by the agar dilution technique. A modification of the Mueller-Hinton medium was devised which gave good growth of the yeast form. The minimum inhibitory concentrations for only 51.6% of the isolates were in the range of the recommended blood serum concentration (50 μg/ml). For 6 to 8% of the isolates, the minimum inhibitory concentrations were above 200 μg of both sulfadiazine and sulfadimethoxyne per ml. A significant decrease in susceptibility was demonstrated for one isolate obtained from a patient relapsing during sulfonamide therapy.

Sulfonamides, both rapid and slow-acting compounds, have been the antimicrobial agents most frequently employed in the treatment of paracoccidioidomycosis. Results of extensive trials have indicated that adequate responses are obtained in approximately 70.0% of the patients subjected to continuous and prolonged therapy (3, 10-12). Clinical observations have also revealed that relapses occur in approximately 10% of the cases, presumably because of fungal resistance acquired during the course of inadequate treatment. Some patients, however, appear refractory to sulfonamides from the beginning of sulfonamide therapy, suggesting infection with a sulfonamide-resistant strain (7, 14).

In spite of these observations, there has been no survey of the in vitro susceptibility of the causative agent, *Paracoccidioides brasiliensis*, to sulfonamides. Earlier studies either employed complex culture media, which could have antagonized the effect of the drug, or made use of the fungus mycelial phase, which is different from the phase found in tissues, that is, the yeast phase of this dimorphic fungus (5, 8, 15). Consequently, the results of such studies have not clarified whether resistance can be present de novo or be acquired. One of the difficulties which may have deterred such studies is the difficulty of obtaining adequate growth of the yeast phase in media appropriate for susceptibility testing within a period of time compatible with drug activity (21, 22).

This report describes the procedure developed for sulfonamide susceptibility testing and presents data on the susceptibility pattern of 60 different isolates of *P. brasiliensis* to sulfadiazine and sulfadimethoxyne.

MATERIALS AND METHODS

Fungi. Sixty-five isolates of *P. brasiliensis* were used in this study. All were from clinical sources (Colombian patients) and represented the original isolate at time of diagnosis, with the exception of three which were obtained during relapses. The stock cultures were kept in Sabouraud glucose agar at approximately 25°C. When needed, they were converted to the yeast phase by subculturing in the special media described below and incubated at 36°C. Identity confirmation was obtained by demonstration of dimorphism and by the characteristic microscopical appearance, multiple budding yeast cells, in cultures incubated at 36°C.

Culture media. It was necessary to find a medium which would not antagonize sulfonamides and which, at the same time, could promote abundant growth of *P. brasiliensis*. Earlier attempts to culture the fungus in the conventional Mueller-Hinton medium (BBL Microbiology Systems, Cockeysville, Md.) used for sulfonamide susceptibility testing of bacteria, proved unsuccessful (4, 22). A series of experiments was performed by enriching such a medium with various chemicals known to promote growth in the fungus (1, 17). A satisfactory formulation was obtained when the Mueller-Hinton medium was supplemented with 10 g of glucose, 5 g of ammonium sulfate, and 10 mg of thiamine (Sigma Chemical Co., St. Louis, Mo.) per liter. This modified medium (MMH) was used throughout the experiments, both as a liquid preparation or as a solid medium upon the addition of 15 g of agar per liter (granulated agar, BBL Microbiology Systems).

MMH without thiamine was autoclaved at 15 lb/in^2 for 10 min and kept at 50°C in a water bath. The appropriate amounts of thiamine were then added. For this, a stock solution of thiamine at 50 mg/100 ml in distilled water was prepared, sterilized by filtration (0.45-μm pads, Millipore Corp., Bedford, Mass.), and kept at −20°C in 10-ml amounts.

This medium plus the sulfonamides (see below) was

gently but thoroughly mixed, poured into sterile standard 60-mm petri dishes, and allowed to harden at room temperature. It was then checked for sterility by overnight incubation at 36°C. The medium was utilized within 24 h of preparation.

Antimicrobial agents. Reference preparations of sulfadiazine and sulfadimethoxyne as representatives of rapid and slow acting sulfonamides, respectively, were purchased from U.S. Pharmacopeia Conventions, Inc. (Rockville, Md.). They were used as specified by the manufacturer, including heating of sulfadimethoxyne at 105°C for 3 h. Specified amounts of both drugs were solubilized in 2.0-ml quantities of sterile warm water plus a minimal amount of 10% NaOH (22), added dropwise.

Agar dilution method. Dilutions of antifungal agents in sterile distilled water were prepared at 10 times the concentrations required in the final test. Serial twofold dilutions ranging from 125 to 2,000 μg of each drug per ml were used. The appropriate amount of MMH was prepared in screw-capped bottles, autoclaved, and kept in the water bath as previously indicated. After addition of the thiamine, 1 volume of the corresponding antimicrobial agent was added to each 9 volumes of MMH agar. This was swirled and poured into the plates. Control plates without sulfonamides were also prepared (19, 22). In some cases, sulfonamide concentrations above 200 μg/ml were also employed.

Preparation of inoculum and testing. Before susceptibility testing, each isolate was inoculated on a MMH agar slant and incubated at 36°C for 7 to 10 days to adapt the fungus to the new medium. When this was obtained, accelerated growth was promoted by repeated subculturing each 2 days. Usually two to three such passages were sufficient. For the test, a 3-day-old culture with abundant growth was selected. The growth was removed, free of agar, by means of a platinum spade and suspended in 10.0 ml of MMH broth. The suspension was mixed by mechanical agitation (mixer model S8220, Scientific Products, Evanston, Ill.) at fast speed for 2 min. The homogeneous suspension was adjusted to McFarland no. 8 turbidity standard. On preliminary testing a 1:100 dilution of this standard had yielded mean viable counts of 150 colony-forming units per plate.

Direct viability testing was performed by the dye exclusion test (6). Suspensions employed always had over 90% viable cells. Before their use, plates were incubated at 36°C for 1 h to obtain a dry surface. They were then inoculated with 0.1 ml of the standardized suspension; this amount was delivered from the tip of a sterile calibrated pipette on six different points to avoid flooding. The inoculum was then spread on the whole surface. The plates were allowed to absorb the incoulum for 1 h and were then inverted for further incubation (5 days) at 36°C. Each strain was tested against five dilutions each of sulfadiazine and sulfadimethoxyne. Duplicate control plates were processed in a similar manner.

The minimal inhibitory concentration was defined as the lowest concentration of drug giving 80 to 90% reduction in surface growth of the fungus in comparison with control plates (4, 22).

RESULTS

The modified Mueller-Hinton agar proved satisfactory for the growth of *P. brasiliensis* yeast phase. From the 65 isolates originally available, 60 (92.3%) grew profusely on MMH, producing visible colonies in 3 to 5 days of incubation at 36°C. This allowed us to use young active cells for in vitro susceptibility testing. Experiments were performed with the 60 strains showing adequate growth on the MMH medium.

The number of isolates of *P. brasiliensis* inhibited by each of the sulfonamides used is shown in Table 1. Analyses of these data revealed no significant differences between the inhibitory activity of the rapid and the slow-acting compounds. We found that only 31 of the 60 strains (51.6%) were inhibited by 50 μg of either drug per ml. Higher drug concentrations proved inhibitory for over 75% of the isolates. However, 6.7 and 8.4% of such isolates remained resistant to 200 μg of sulfadiazine and sulfadimethoxyne per ml, respectively.

As stated previously, all the isolates studied with the exception of three had been obtained at the time of the patient's initial diagnosis. Presumably, no previous sulfonamide therapy had been given to such patients. The three strains obtained at time of a relapse came from three different patients. For two, both the initial and the relapse isolates were available. Comparative studies (Table 2) revealed only slight changes in susceptibility in patient 2 but marked increased resistance in patient 1. The remaining relapse isolate (patient 3) was not completely inhibited by concentrations of 100 and 200 μg/ml. Unfortunately the original isolate for this case was not viable at time of testing. Figures 1 and 2 show the growth of both isolates from patient 1.

Five of the strains showing no diminished growth at 200 μg/ml were serially tested in the presence of 300, 400, and 600 μg/ml. The results indicate that 300 μg/ml represented the minimum inhibitory concentrations for all the isolates.

TABLE 1. *In vitro susceptibility of 60 isolates of P. brasiliensis to sulfonamides*

Sulfonamide concn (μg/ml)	Isolates with minimal inhibitory concn			
	Sulfadiazine		Sulfadimethoxyne	
	No.	%	No.	%
200	56	93.3	55	91.6
100	46	76.6	47	78.3
50	31	51.6	31	51.6
25	15	25.0	14	23.3
12.5	9	15.0	6	10.0

TABLE 2. *Comparative susceptibility of P. brasiliensis original and relapse isolates to sulfadiazine*

Patient	*P. brasiliensis* isolated (yr)	% Reduction of fungal growth at sulfadiazine concn (μg/ml):					
		None	12.5	25	50	100	200
1							
	Original (1966)	0	70	90	90[a]	100	100
	Relapse (1975)	0	0	0	50	50	70[b]
2							
	Original (1974)	0	0	50	90[a]	100	100
	Relapse (1976)	0	0	0	70	90	90[a]

[a] Minimum inhibitory concentration.
[b] Minimum inhibitory concentration of 300 μg/ml.

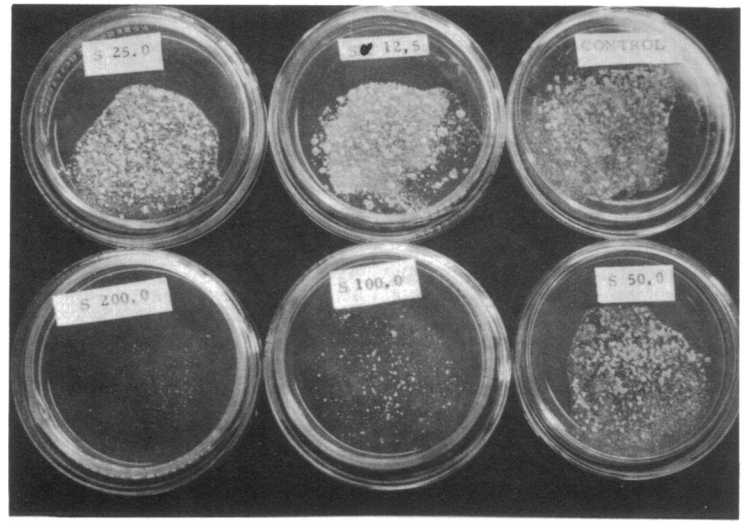

FIG. 1. *In vitro* susceptibility of *P. brasiliensis to sulfonamides. Isolate from patient 1 at time of initial diagnosis. MMH agar and sulfadiazine concentrations ranging from 6.25 to 200 μg/ml.*

DISCUSSION

The results of this study indicate that it is possible to determine the susceptibility of *P. brasiliensis* to sulfonamides on a regular basis. The simplicity and availability of the required culture medium (MMH) would make in vitro testing feasible for most myocology centers, especially for those located in the endemic paracoccidioidomycosis area.

In vitro testing of sulfonamides by the agar dilution procedure has proved satisfactory for bacteria, but endpoints must be taken on the basis of sudden, sharp (80%) diminution of growth. The carryover of sulfonamide antagonists in the inoculum may allow some growth even in the highest drug concentration (22). Our data cannot be compared with that in earlier reports (5, 8, 15), either because former testing was done with the mycelial phase or because complex culture media were used. The choice of medium is critical, since many are unsuitable because of their content of *p*-aminobenzoic acid or other sulfonamide antagonists (2, 9, 22). The modified Mueller-Hinton medium used in our experiments adds no antagonists to the classic formulation and has the advantage of supporting good growth of over 90% of the *P. brasiliensis* isolates.

Shortly after introduction of sulfonamide therapy, it was suggested that sulfonamide blood concentrations of 50 μg/ml were sufficient to control paracoccidioidomycosis (13). The classical therapeutic scheme, 3,000 to 6,000 mg of sulfadiazine or 500 mg of sulfamethoxypyridazine per day, produces such concentrations (10, 11). We studied antifungal concentrations encompassing the blood levels attainable in patients (20). It was surprising to observe that the "effective" recommended concentration (50 μg/ml) was able to inhibit only 51.6% of the isolates studied. In addition, blood concentrations not usually attainable after ordinary therapeutic doses (200 μg/ml) did not prove inhibitory for

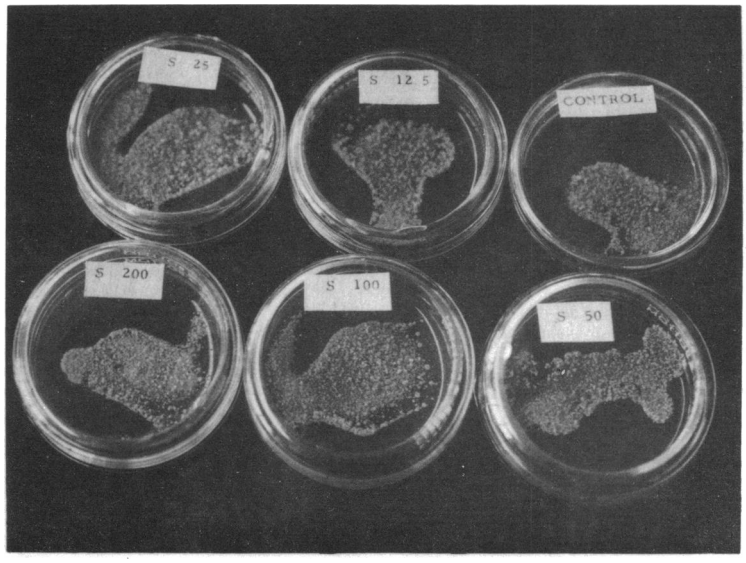

FIG. 2. *Isolate from patient 1 after a relapse of sulfonamide therapy. Culture conditions as above. Note growth even in the presence of 200 μg/ml.*

all isolates; 6 to 8% of isolates grew abundantly in the presence of such high sulfonamide levels. Mackinnon, using adequate culture medium but prolonged 30°C incubation, recorded only partial inhibition of his 6 isolates with 150 μg of sulfadiazine per ml (8). It is tempting to postulate a correlation between the in vitro findings and the recorded proportion of patients refractory to sulfonamide treatment from the start of therapy (7, 8, 14, 16, 18).

Because the majority of the isolates tested were obtained before therapy, it becomes apparent that *P. brasiliensis* is not homogeneously susceptible to sulfonamides and that de novo resistance can be found in a small, albeit important, number of isolates. By "resistance" we imply strains not susceptible to attainable drug levels, 100 to 150 μg/ml (20).

As illustrated by the difference in susceptibility of the original and the relapse isolates obtained from one patient, resistance can be acquired. This may explain therapy failures in patients who had initially responded but became refractory shortly afterwards or when control of a relapse in a previously treated patient proved unsuccessful (7, 14).

There was no difference in the susceptibility of the isolates to either one of the compounds used in this study; this finding corroborates clinical observations. It has been shown that there is no difference in therapeutic activity of various sulfonamides and that a refractory case does not improve by the change of agent (7).

In paracoccidioidomycosis, successful therapeutic outcome has been shown to depend on the continued prolonged use (3 to 5 years) of sulfonamides. These agents are fungistatic and require cooperation of the patient's immune defenses to achieve a cure (10, 11). Consequently, the results of in vitro testing furnish only ancillary evidence, which explains both lack of response to appropriate sulfonamide dosing and relapses after faulty treatment schedules. However, our findings provide evidence of variation of susceptibility among isolates and acquisition of resistance after inadequate therapy. Such evidence should be taken into consideration when sulfonamide therapy is being considered for a patient, be it a new case or one relapsing after previous treatment.

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