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Susceptibility of *Prototheca* Species to Antifungal Agents

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Twenty isolates of *Prototheca filamenta, Prototheca moriformis, Prototheca stagnora, Prototheca wickerhamii,* and *Prototheca zopfii* were tested for in vitro susceptibility to five commonly used antifungal agents: amphotericin B, 5-fluorocytosine, griseofulvin, miconazole, and nystatin. The results revealed resistance to griseofulvin of all the *Prototheca* isolates tested and an inhibitory effect on *P. filamenta* by high 5-fluorocytosine concentrations (minimal inhibitory concentration [MIC] = 12.5 to 100 μg/ml; minimal fungicidal or algacidal concentration [MFC/MAC] = 50 to 100 μg/ml). *P. filamenta* isolates were also susceptible to miconazole (MIC = 0.1 to 0.5 μg/ml, MFC/MAC = 0.5 to 1 μg/ml); isolates of the other *Prototheca* species varied in regard to miconazole activity from susceptible to resistant (MIC = 1 − >100 μg/ml, MFC/MAC = 5 − >100 μg/ml). The *Prototheca* isolates revealed an in vitro susceptibility to the polyene antifungal agents, amphotericin B, and nystatin (MIC = 0.09 to 3.12 μg/ml and 0.19 to 12.5 μg/ml, respectively; MFC/MAC = 0.19 to 25 μg/ml and 0.75 to 25 μg/ml, respectively).

Prototheca species are microscopic, achloric, heterotrophic organisms, reproducing asexually by cleavage to form endospores (4). Some investigators think that these organisms are related to fungi (3); others believe that they are achloric algae (13); still others believe that these organisms hold an intermediate position (1).

Members of the genus *Prototheca* are known to cause systemic and cutaneous disease in dogs, deer, and cattle (2, 19, 21). Since 1964 cases of cutaneous or disseminated protothecosis in humans have also been reported (5, 6, 10, 12, 15, 22).

The chemotherapy of protothecosis, generally carried out on an empirical basis (19), is usually reported to be unsatisfactory (15). Extensive studies on the in vitro susceptibility of *Prototheca* species have not been reported. We therefore thought that it would be advisable to test the in vitro activity of antifungal agents against *Prototheca* isolates. The results of that study are presented in this report.

MATERIALS AND METHODS

Test cultures. Twenty test cultures from the culture collection of the Mycology Division of the Center for Disease Control (Table 1) were used. They were isolates of the species *Prototheca filamenta, Prototheca moriformis* (designated as such for this study but considered by Sudman and Kaplan [20] as a synonym of *Prototheca zopfii*), *Prototheca stagnora, Prototheca wickerhamii,* and *Prototheca zopfii.* The test cultures were subcultured and maintained on an antibiotic-free Sabouraud's dextrose agar.

Antifungal agents. Five antifungal agents were used: amphotericin B and nystatin, both products of E. R. Squibb & Sons, New York, N.Y.; 5-fluorocytosine (5-FC), supplied by Hoffmann-La Roche, Inc., Nutley, N.J.; griseofulvin, obtained from United States Pharmacopeia Co. Inc., Rockville, Md.; and miconazole (miconazole nitrate salt), supplied by Janssen R & D, Inc., New Brunswick, N.J.

Susceptibility testing with 5-FC. The minimal inhibitory concentration (MIC) and minimal fungicidal or algacidal concentration (MFC/MAC) were determined by using the methods described by Shadomy (17) and as used in a previous study (16).

A liquid synthetic medium, composed of yeast nitrogen base (Difco Laboratories, Detroit, Mich.) supplemented with asparagine and dextrose, was used as the test medium. The testing system consisted of twofold serial dilutions of 5-FC, ranging from 100 to 0.05 μg/ml, in 1 ml of yeast nitrogen base. The inoculum of the cultures to be tested consisted of 0.05 ml of 10^5 to 3.5×10^5 organisms per ml of suspension. The test cultures were incubated for 48 h at 25 C, and the lowest 5-FC concentration inhibiting visible growth was considered the MIC. The tubes showing no visible growth and the MIC tubes were then plated on Sabouraud's agar and incubated for 48 h; the lowest 5-FC concentration in yeast nitrogen base that inhibited growth on the Sabouraud plates was considered the MFC/MAC. A *Saccharomyces cerevisiae* isolate, obtained from S. Shadomy, Virginia Commonwealth University, Richmond, Va., was used as the control culture.

Susceptibility testing with amphotericin B and nystatin. The amphotericin B and nystatin suscepti-

TABLE I. *Source of Prototheca isolates tested*

Species	Accession no.	Source
P. filamenta	B-1273	Skin; Ohio
	B-1568	Foot; Missouri
	B-1569	Bone marrow; Missouri
	B-1570	Missouri
	B-1658	Dr. Pore; West Virginia
P. moriformis (synonym of *P. zopfii*)	B-1266	NRRL[a] strain Y-6864
	B-1692	Algae collection; Indiana
P. stagnora	B-1277	Waste stabilization pond; Ohio
P. wickerhamii	B-1269	Soil; Illinois
	B-1275	Skin; Africa
	B-1278	Lavatory drain pipe; Illinois
	B-1280	Human intestine; Illinois
	B-1651	Dog; Minnesota
	B-2224	Cat; Georgia
P. zopfii	MCC-378	Raw sewage; California
	MCC-379	Raw sewage; California
	MCC-380	Raw sewage; California
	B-1272	Bovine mastitis; Ohio
	B-1270	Bovine mastitis; Ohio
	B-1413	Venezuela

[a] NRRL, National Regional Research Laboratory.

bility tests were performed by a broth dilution method based on the procedure described by Shadomy and Espinel-Ingroff (18). Amphotericin B and nystatin stock solutions were made up in dimethylsulfoxide (Me_2SO) and kept protected from light. The test system consisted of twofold dilutions (from 100 to 0.05 μg/ml) of amphotericin B and nystatin in 1 ml of antibiotic medium (M-20, Difco), inoculated with 0.05 ml of 10^5 to 3.5×10^5 organisms per ml of suspension, and incubated at 25 C for 48 h. The MIC and MFC/MAC were determined as described for 5-FC testing. *S. cerevisiae* was used as the control culture.

Susceptibility testing with miconazole. Susceptibility testing with miconazole was also carried out by the tube dilution method. A miconazole stock solution was made up in ethanol at a concentration of 10 mg/ml. The tests were performed in Sabouraud broth (1 ml/tube) by using miconazole concentrations starting from 100 μg/ml down to 0.1 μg/ml. The inocula of the test cultures, incubation temperature, incubation period, control culture, and determination of MIC and MFC/MAC were the same as described for 5-FC, amphotericin B, and nystatin testing.

Susceptibility testing with griseofulvin. Griseofulvin was dissolved in acetone at a concentration of 2 mg/ml. Susceptibility was determined using agar plates containing agar incorporated with griseofulvin in concentrations starting from 50 μg of medium per ml down to 0.9 μg of medium per ml (twofold dilutions). The inocula of the test cultures, prepared as previously described, were inoculated on Sabouraud-griseofulvin agar plates; these were incubated at 25 C for 72 h. *Trichophyton mentagrophytes* ATCC 28068 was used as the control culture. By this method the activity of griseofulvin could be established only in regard to its minimal cidal concentration (MFC/MAC).

RESULTS

Griseofulvin susceptibility. The in vitro susceptibility of the 20 *Prototheca* isolates to the five tested antifungal agents is summarized in Table 2. Griseofulvin had no inhibitory effect on the *Prototheca* cultures, since all of them grew on Sabouraud agar plates containing 50 μg of griseofulvin per ml; the control culture of *T. mentagrophytes*, however, was affected at a 6.25 μg of griseofulvin per ml concentration.

5-FC susceptibility. The activity of 5-FC on *Prototheca* species had a selective pattern; it did not affect isolates of *P. zopfii*, *P. wickerhamii*, *P. stagnora*, or *P. moriformis* but was effective on four of five *P. filamenta* isolates tested (Table 2). Even the *P. filamenta* that were affected by 5-FC, however, were inhibited only at high concentrations, namely, MIC levels ranging from 12.5 to 25 μg/ml and MFC/MAC levels ranging from 50 to 100 μg/ml, respectively, as compared to the control culture of *S. cerevisiae* whose MIC and MFC/MAC were 0.38 and 1.56 μg/ml, respectively.

Amphotericin B and nystatin susceptibility. All the *Prototheca* isolates were affected by both of the polyene compounds tested, amphotericin B and nystatin (Table 2). In the case of

four isolates (three *P. zopfii* cultures and one *P. wickerhamii*), however, high concentrations of amphotericin B (12.5 or 25 μg/ml) were required to produce a cidal effect. Furthermore, as revealed by the data in Table 3, which summarizes the minimal and maximal sensitivity values of *Prototheca* species, the *P. zopfii* isolates apparently were less sensitive to amphotericin B than to members of the other species tested.

TABLE 2. *In vitro susceptibility[a] of Prototheca sp. to amphotericin B, 5-FC, griseofulvin, miconazole, and nystatin*

Species	Amphotericin B		5-FC		Griseofulvin		Miconazole		Nystatin	
	MIC[a]	MFC/MAC[a]	MIC	MFC/MAC	MIC	MFC/MAC	MIC	MFC/MAC	MIC	MFC/MAC
P. filamenta										
B-1273	0.75	1.56	25.0	50.0		>50.0	0.1	0.5	1.56	3.12
B-1568	0.75	0.75	25.0	50.0		>50.0	0.1	0.5	3.12	3.12
B-1569	0.75	1.56	25.0	100.0		>50.0	0.5	1.0	0.75	0.75
B-1570	0.19	0.19	>100.0	>100.0		>50.0	0.5	0.5	0.38	0.75
B-1658	0.38	0.38	12.5	50.0		>50.0	0.5	0.5	0.75	1.56
P. moriformis										
B-1266	0.75	0.75	>100.0	>100.0		>50.0	0.5	1.0	1.56	3.12
B-1692	0.38	0.38	>100.0	>100.0		>50.0	1.0	1.0	1.56	3.12
P. stagnora										
B-1277	0.19	1.56	>100.0	>100.0		>50.0	100.0	100.0	3.12	12.5
P. wickerhamii										
B-1269	0.09	0.19	>100.0	>100.0		>50.0	>100.0	>100.0	3.12	3.12
B-1275	0.38	0.38	>100.0	>100.0		>50.0	10.0	50.0	0.75	1.56
B-1278	0.19	0.38	>100.0	>100.0		>50.0	100.0	100.0	0.75	1.56
B-1280	1.56	3.12	>100.0	>100.0		>50.0	10.0	50.0	0.75	0.75
B-1651	1.56	12.5	>100.0	>100.0		>50.0	50.0	50.0	3.12	3.12
B-2224	0.19	0.38	>100.0	>100.0		>50.0	>100.0	>100.0	3.12	3.12
P. zopfii										
MCC-378	0.75	1.56	>100.0	>100.0		>50.0	10.0	50.0	0.75	1.56
MCC-379	0.75	12.5	>100.0	>100.0		>50.0	10.0	25.0	1.56	3.12
MCC-380	0.75	3.12	>100.0	>100.0		>50.0	1.0	5.0	1.56	3.12
B-1270	3.12	25.0	>100.0	>100.0		>50.0	10.0	>100.0	1.56	3.12
B-1272	0.19	0.75	>100.0	>100.0		>50.0	10.0	>100.0	0.75	0.75
B-1413	3.12	25.0	>100.0	>100.0		>50.0	10.0	>100.0	12.5	25.0
Controls										
S. cerevisiae	0.38	1.56	0.38	1.56			7.5	10.0	0.75	1.56
T. mentagrophytes (ATCC 28068)						6.25				

[a] Expressed as micrograms per milliliter.

TABLE 3. *Minimal and maximal susceptibility of Prototheca sp. to amphotericin B, miconazole, and nystatin*

Species	No. of isolates tested	Amphotericin B		Miconazole		Nystatin	
		MIC (μg/ml)	MFC/MAC (μg/ml)	MIC (μg/ml)	MFC/MAC (μg/ml)	MIC (μg/ml)	MFC/MAC (μg/ml)
P. filamenta	5	0.19–0.75	0.19–1.56	0.1–0.5	0.5–1.0	0.38–3.12	0.75–3.12
P. moriformis (synonym of *P. zopfii*)	2	0.38–0.75	0.38–0.75	0.5–1.0	1.0–1.0	1.56–1.56	3.12–3.12
P. stagnora	1	0.19	1.56	100.0	100.0	3.12	12.5
P. wickerhamii	6	0.09–1.56	0.19–3.12	10.0–100	50.0–>100	0.75–3.12	0.75–3.12
P. zopfii	6	0.19–3.12	0.75–25.0	1.0–10.0	5.0–>100	0.75–12.5	0.75–25.0

Judging from both the MIC and the MFC/MAC values, the *Prototheca* isolates were generally less susceptible to nystatin than to amphotericin B (Tables 2 and 3). On the other hand, three of four *P. zopfii* isolates with high MFC/MAC values for amphotericin B showed significantly lower MFC/MAC values for nystatin (Table 2).

Miconazole susceptibility. The results obtained by the miconazole susceptibility testing of *Prototheca* isolates (Tables 2 and 3) showed that miconazole had an inhibitory effect. However, there was some variation in regard to its activity among members of the same species, some being susceptible and others resistant (*P. zopfii* and *P. wickerhamii* isolates, Table 2). In addition, there were also differences in the sensitivity among the different *Prototheca* species (Table 3). Furthermore, although miconazole was inhibitory (MIC) to all the *P. zopfii* isolates tested, three of the isolates showed resistance to its cidal action (MFC/MAC) (Table 2).

DISCUSSION

The species *Prototheca* were divided by Arnold and Ahearn (1) on the basis of physiological characteristics into five species: *P. filamenta, P. moriformis, P. stagnora, P. wickerhamii,* and *P. zopfii,* with a number of strains having various designations such as *Prototheca segbwema* (isolated by Davies and Wilkinson [6] from human cutaneous protothecosis), which these investigators considered synonymous with *P. zopfii*. Based on antigenic properties Sudman and Kaplan (20) recognized only four species in the genus *Prototheca,* with *P. moriformis* being synonymous with *P. zopfii*. As noted, in this study we used the term *P. moriformis* for the two strains B-1266 and B-1692 (see Results), since they were designated as such when referred to the Center for Disease Control Mycology Culture collection. They should, however, be considered synonymous with *P. zopfii*.

Arnold and Ahearn (1) described *P. filamenta* as a new species. However, it was found to be different from other *Prototheca* species, and its classification within the genus *Prototheca* has been questioned (9). The antifungal susceptibility pattern of *P. filamenta,* as found in this study, is indeed different from the other *Prototheca* species. The *P. filamenta* cultures were the only *Prototheca* isolates affected at all by 5-FC; they were also very susceptible to miconazole, as compared to the susceptibility of isolates of other *Prototheca* species.

Miconazole is an imidazole nitrate substance that is inhibitory to dermatophytes and yeasts and reported to be useful in the topical treatment of dermatological and gynecological infections (7, 8, 14, 15). It has also been reported to have an in vitro activity on the etiological agents of certain deep mycoses (11, 15). The data obtained in this study in regard to miconazole activity show that some isolates of *P. zopfii* and *P. wickerhamii,* the two *Prototheca* species known to cause disease (19), are affected by it and that other isolates are resistant to it. It seems important, therefore, to test more clinical *Prototheca* isolates in regard to their susceptibility to miconazole before considering its topical use in cases of cutaneous protothecosis.

As judged by the data from this study, the two polyene antimycotics amphotericin B and nystatin are apparently the antimicrobial agents most effective in vitro against the *Prototheca* species.

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LITERATURE CITED

1. **Arnold, P., and D. G. Ahearn.** 1972. The systematics of the genus *Prototheca* with a description of a new species *P. filamenta*. Mycologia 64:265–275.
2. **Carlton, W. W., and L. Austin.** 1973. Ocular protothecosis in a dog. Vet. Pathol. 10:274–280.
3. **Ciferri, O.** 1956. Thiamine deficiency of *Prototheca,* a yeast-like achloric alga. Nature (London) 178:1475–1476.
4. **Cooke, W. B.** 1968. Studies in the genus *Prototheca*. II. Taxonomy. J. Elisha Mitchell Sci. Sco. 84:217–220.
5. **Cox, E. G., J. D. Wilson, and P. Brown.** 1974. Protothecosis: a case of disseminated algae infection. Lancet 2:379–382.
6. **Davis, R. R., and J. L. Wilkinson.** 1964. Human protothecosis: supplementary studies. Ann. Trop. Med. Parasitol. 61:112–115.
7. **Gentles, J. C., G. R. Jones, and D. T. Roberts.** 1975. Efficacy of miconazole in the topical treatment of tinea pedis in sportsmen. Br. J. Dermatol. 93:79–84.
8. **Holt, R. J.** 1974. Recent developments in antimycotic chemotherapy. Infection 2:95–107.
9. **King, D. S., and S. C. Jong.** 1975. *Sarcinosporon:* a new genus to accomodate *Trichosporon inkin* and *Prototheca filamenta*. Mycotaxon 3:84–94.
10. **Klintworth, G. K., B. F. Fetter, and M. S. Nielsen, Jr.** 1968. Protothecosis, an algal infection; report of a case in man. J. Med. Microbiol. 1:211–216.
11. **Levine, H. B., D. A. Stevens, J. M. Cobb, and A. E. Gebhardt.** 1975. Miconazole in coccidioidomycosis. I. Assays of activity in mice and in vitro. J. Infect. Dis. 132:407–414.
12. **Mars, P. W., A. R. Rabson, J. J. Rippey, and L. Ajello.** 1971. Cutaneous protothecosis. Br. J. Dermol. 85(Suppl. 7):76–84.

13. **Nadakavukaren, J. M., and D. A. McCraken.** 1973. *Prototheca:* an alga or a fungus? J. Phycol. 9:113-116.
14. **Proost, J. M., F. M. Maes-Dockx, M. O. Nelis, and J. M. Van Cutsen.** 1972. Miconazole in the treatment of mycotic vulvovaginitis. Am. J. Obstet. Gynecol. 112:668-692.
15. **Rippon, J. W.** 1974. Medical mycology. The pathogenic fungi and the pathogenic actinomycetes, p. 459-464, 531-541. W. B. Saunders Co., Philadelphia.
16. **Segal, E., A. Romano, E. Eylan, and R. Stein.** 1975. Experimental and clinical studies of 5-fluorocytosine activity in *Candida* ocular infections. I. In vitro activity of 5-fluorocytosine on *Candida* species isolated from ocular infections. Chemotherapy 21:358-366.
17. **Shadomy, S.** 1969. In vitro studies with 5-fluorocytosine. Appl. Microbiol. 17:871-877.
18. **Shadomy, S., and A. Espinel-Ingroff.** 1974. Susceptibility testing of antifungal agents, p. 569-574. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology. Washington, D.C.
19. **Sudman, S. M.** 1974. Protothecosis: a critical review. Am. J. Clin. Pathol. 61:10-19.
20. **Sudman, S. M., and W. Kaplan.** 1973. Identification of the *Prototheca* species by immunofluorescence. Appl. Microbiol. 25:981-990.
21. **Van Kruiningen, M. J.** 1970. Protothecal enterocolitis in a dog. J. Am. Vet. Med. Assoc. 157:56-63.
22. **Lee, W-Sh., M. D. Lagios, and R. Leonards.** 1975. Wound infection by *Prototheca wickerhamii*, a saprophytic algapathogenic for man. J. Clin. Microbiol. 2:62-66.