Jpn. J. Med. Mycol.
Vol. 45, 101－104, 2004
ISSN 0916－4804

Original Article

Susceptibility of *Pseudallescheria boydii* and *Scedosporium apiospermum* to New Antifungal Agents

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〔Received: 26, September 2003. Accepted: 13, January 2004〕

Abstract

Hyalohyphomycoses caused by *Pseudallescheria boydii* and *Scedosporium apiospermum* have recently been on the increase. To find the appropriate treatment for this emerging disease, we examined the antifungal susceptibility of 10 isolates of *P. boydii* and 17 isolates of *S. apiospermum*, most of which were isolated from clinical specimens. When the NCCLS M38-P microdilution method was used, itraconazole showed strong antifungal activities, while amphotericin B had little efficacy. A new triazole agent, voriconazole showed a strong effect against isolates of *P. boydii* and *S. apiospermum* (MIC_{50} 0.06 μg/m*l*), whereas micafungin, a newly developed echinocandin, had little effect (MIC_{50} $>$16 μg/m*l*). There was no significant difference in the susceptibilities between *P. boydii* and *S. apiospermum* isolates against any antifungal agents. Our study suggests that voriconazole is a promising new drug in these infections, and that the same antifungal strategy can be employed in the infections by *P. boydii* and *S. apiospermum*.

Key words: voriconazole, micafungin, *P. boydii*, *S. apiospermum*, antifungal susceptibility.

Introduction

Pseudallescheria boydii (anamorph: *Scedosporium apiospermum*) is a homothallic ascomycete, which was newly recognized as a causative agent of serious disseminated fungal infections[1)]. Although most of the pathogenic fungi belong to Deuteromycota, some perfect fungi like *Cryptococcus neoformans* (teleomorph: *Filobasidiella neoformans*) and *Aspergillus nidulans* (teleomorph: *Emericella nidulans*) are also known to cause human infections. *P. boydii* (*S. apiospermum*) is another example of these pathogenic perfect fungi. As true in other perfect fungi, little has been known in this fungus about the difference between the two forms, i.e. anamorph and teleomorph, in their clinical and pathological significance.

Some promising new antifungal agents have been developed in recent years. Voriconazole is a new triazole agent which blocks the ergosterol synthesis by inhibiting fungal 14-α-sterol demethylase. Preliminary studies reported that the mean MIC (minimal inhibitory concentration) of voriconazole against *P. boydii* was lower than those of itraconazole, which has been viewed as the treatment of choice in the infections by *P. boydii* or *S. apiospermum*[2-6)]. Another newly developed antifungal agent, micafungin, is an echinocandin derivative, which inhibits 1, 3-β-D-glucan synthesis of fungi. Its activity against *P. boydii* is reported to be relatively low[7, 8)]. In these preliminary studies, however, the information on susceptibilities of *P. boydii* and *S. apiospermum* was rather limited in several aspects. For example, the number of isolates examined was relatively small to allow a definite conclusion to be reached. Another problem of these studies is that only isolates of either *P. boydii* or *S. apiospermum* were used, thereby making direct comparison of these fungi (anamorph and teleomorph) unfeasible. It is therefore not well known whether the therapeutic method should be altered in infections depending on the form of the fungus.

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Table 1. The source of isolation *Pseudallescheria boydii* and *Scedosporium apiospermum* isolates

Source	*P. boydii* (n=10)	*S. apiospermum* (n=17)
From patients		
Lung and brain tissues*	0	1
Cerebrospinal fluid	1	1
Fungus ball	1	0
Bronchoalveolar lavage	0	2
Bronchial polyp brushing	0	1
Pleural fluid	0	1
Paranasal sinus biopsy	0	3
Necrosis tissue of eye	1	1
Corneal scraping	0	2
Skin biopsy	1	0
Unspecified	5	4
Unknown	1	1

*: The patient was infected with *S. apiospermum* both in lung and brain.

For better understanding of the clinical significance of these new antifungal agents, and to find better treatment for these fungal infections, susceptibility tests using isolates of both *P. boydii* and *S. apiospermum* were carried out, and the results between the two forms compared.

Materials and methods

Isolates (Table 1): Ten isolates of *P. boydii* and seventeen isolates of *S. apiospermum* were used in this study. All but two were obtained from clinical samples in Japan or China. The remaining isolates did not carry any definite information to indicate their origins. Two isolates of *Candida albicans* were used as quality control strains (QC). All the isolates were stored and maintained in our laboratory.

Isolation of different subcultures: Because some isolates produced colonies of two different colors, the antifungal susceptibility of these fungi was compared. Each isolate was incubated on potato dextrose agar (PDA) slants at 35°C for 7 days. Conidia were collected and suspended in phosphate-buffered saline (PBS, pH 7.2). The conidia were washed and the concentrations were adjusted to 500 cells/m*l* with PBS. To make subcultures, 200 μl of the conidium suspension was spread over a PDA plate and grown at 35°C until the color of the colonies became easily recognizable. The colonies were divided into two groups depending on their color, i.e. a whitish group and a brown (dark gray) one. Then each whitish or brown colony was transferred onto a PDA slant, respectively, and incubated at 35°C for 21 days to observe their morphology and color. This procedure was repeated three times. Slide cultures were also made. Even after repeated subcultures, the colors of these subcultures remained unchanged.

Antifungal drugs: Yeast-like fungi DP Eiken trays (Eiken Chemical Company, LTD., Tokyo, Japan) were used for susceptibility testing of amphotericin B, flucytosine, fluconazole, itraconazole, miconazole and micafungin. Susceptibility testing of voriconazole was done based on the proposed standard method of the M38-P of NCCLS[9] in sterile microdilution plates. Voriconazole was provided by Pfizer Ltd. (Tokyo, Japan). Voriconazole was dissolved in dimethyl sulfoxide at a final concentration of 12.8 mg/m*l*, and stored at −85°C until used. When examined, voriconazole was serially diluted twofold with liquid RPMI 1640 medium (with L-glutamine but without sodium bicarbonate, Sigma) at twice the final concentration, which ranged from 16 μg/m*l* to 0.03 μg/m*l*, and 100 μl of each drug solution was added to the wells of sterile microdilution plates.

Inoculum preparation: After each isolate or subculture had been grown for 7 days on PDA slants at 35°C, conidia were collected and their concentrations were adjusted to 2×10^6 cells/m*l* with physiological saline. Then each suspension was diluted with RPMI 1640 to 2×10^4 cells/m*l*. The two quality control strains were cultured on PDA slants for 24 hours at 35°C and the concentrations of the conidium suspensions were also adjusted to 2×10^4 cells/m*l*.

MIC determination: One hundred microliters of each conidium suspension (2×10^4 cells/m*l*) was added to each well of the microdilution plates that contained the drugs, and the plates were incubated for 72 hours (quality control strains for 48 hours) at 35°C. The MICs of the

Table 2. Antifungal activities of seven antifungal agents aginst *P. boydii* and *S. apiospermum* isolates

Isolates	Antifungal agent	MIC range (μg/m*l*)	MIC_{50} (μg/m*l*)	MIC_{90} (μg/m*l*)
P. boydii	AMB	2- >16	4	16
(n=10)	5-FC	16- >64	>64	>64
	FLCZ	4-32	8	16
	ITCZ	0.5-2	1	2
	MCZ	≦0.06-0.5	0.125	0.5
	VCZ	≦0.03-0.25	0.06	0.125
	MCFG	16- >16	>16	>16
S. apiospermum	AMB	2- >16	4	>16
(n=17)	5-FC	8- >64	>64	>64
	FLCZ	8-64	16	32
	ITCZ	1-2	1	1
	MCZ	≦0.06-0.5	0.25	0.5
	VCZ	≦0.03-0.5	0.06	0.125
	MCFG	16- >16	>16	>16

AMB: amphotericin B, 5-FC: flucytosine, FLCZ: fluconazole
ITCZ: itraconazole, MCZ: miconazole, VCZ: voriconazole, MCFG: micafungin.

isolates tested were determined visually with the aid of a concave mirror. IC_{80} was used as MIC for most drugs, whereas IC_{100} was used for amphotericin B.

Results

Susceptibility of the isolates (Table 2): The results of the susceptibility examinations showed that most of the 10 *P. boydii* isolates and 17 *S. apiospermum* isolates were resistant to amphotericin B and flucytosine. Fluconazole, miconazole, itraconazole and voriconazole have some antifungal activities, among which voriconazole showed the lowest MIC (MIC_{50} was 0.06 μg/m*l* and MIC_{90} was 0.125 μg/m*l* for both the *P. boydii* and *S. apiospermum* isolates). In contrast, micafungin showed a high MIC against these isolates: MIC_{50}>16 μg/m*l*, MIC_{90}> 16 μg/m*l*, respectively. Taken together, voriconazole showed the strongest in vitro activity against both the isolates among the seven antifungal agents, and the MICs of each agent were essentially the same in these two forms, i.e. teleomorph and anamorph.

Isolation of different subcultures and susceptibility: When conidia of the isolates were cultured, most isolates produced colonies of single colors. However, in seven isolates (two *P. boydii* and five *S. apiospermum*) colonies showed two colors, i.e. whitish and gray colonies. The gray colonies were found to carry more conidia than the whitish ones. When the antifungal susceptibilities were examined, there was no significant difference between the MICs of the antifungal agents for these isolates; there was also no significant difference in MICs between the original isolates and subcultures, nor between these seven isolates with different colors and the other twenty isolates with the same colors.

Discussion

In *P. boydii* and *S. apiospermum* both sexual forms, i.e. teleomorph and anamorph, are frequently seen in human infections. However, our information had been very limited as to the difference of characters of these forms, particularly in virulence and antifungal susceptibility. In fact, the study made by Nielson[10] was the only one to date that had examined the difference of antifungal susceptibility, in which *P. boydii* isolates were shown to be more resistant to amphotericin B than *S. apiospermum* isolates[10]. Therefore, it has remained an unanswered question whether we should regard infections by these fungi as different entities and change our therapeutic strategies. Making the final identification as to which form is involved in the infection is time-consuming work, and it would be a serious problem if therapeutic modalities had be be altered depending on the form of the fungus. Our study disclosed that *P. boydii* and *S. apiospermum* have essentially the same susceptibilities when examined by the standard NCCLS method, and this is also true with the new antifungal agents voriconazole and micafungin. These results suggest that we do not have to change treatment methods depending on the final identification of the fungus. Our study is in strong contrast with the report by Nielson[10] as to resistance to amphotericin B. His study

was made more than 30 years ago, and hence the method he used for the MIC determination is now outdated, which might be the cause of the different results.

In previous preliminary studies, voriconazole, which appeared as a new triazole agent, was reported to have a strong fungistatic effect on *P. boydii* and *S. apiospermum*[2-6, 11]. In these studies, however, only a small number of isolates were examined, and therefore the results should be read cautiously. Thus we carried out susceptibility tests on a large scale to clarify the efficacy of voriconazole against *P. boydii* (n=10) and *S. apiospermum* (n=17). As expected, the drug showed a potent activity against the fungi, which confirmed its *in vitro* activity. As for the *in vivo* efficacy of voriconazole, only a few studies have been made in animal models or in clinical trials, and the relation between *in vitro* and *in vivo* results is not fully understood. Although the efficacy of this agent should be confirmed *in vivo*, its low MICs shown in our study suggest that voriconazole is a promising agent against this fungus.

Although information about the efficacy of micafungin against *P. boydii* and *S. apiospermum* is very limited, some previous studies reported *P. boydii* isolates are resistant to micafungin[3, 8], which was compatible with our results. It is very interesting that micafungin, an inhibitor of glucan, is not effective against *P. boydii*/*S. apiospermum* although these forms are thought to be rich in 1, 3- β-D-glucan. In fact, a very high level of 1, 3- β-D-glucan was reported in a systemic pseudallescheriasis case (personal communication). Furthermore, another glucan synthesis inhibitor, caspofungin is known to have some activity against these fungi, although its antifungal mechanism is very close to that of micafungin[12]. This may give us a clue to further clarify the antifungal mechanism of the drugs and the mechanism of resistance of the forms.

We found that the susceptibilities of the original and subcultures of the fungi to antifungal agents were essentially the same. Although the color change seen in some isolates apparently depends on the degree of sporulation, the mechanism of the diversity is not yet well understood. The results of our study in the MICs, however, suggest that these changes do not have to be considered in clinical settings, i.e. determination of MICs.

Further study is warranted to understand the clinical significance of the infection and the utility of these drugs.

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