In Vitro Antifungal-Drug Susceptibilities of Mycelial and Yeast Forms of *Penicillium marneffei* Isolates in Cambodia[▽]

Borann Sar,^{1*} Sambo Boy,¹ Chantary Keo,¹ Chan Chhaya Ngeth,² Narom Prak,²
Mich Vann,² Didier Monchy,¹ and Jean Louis Sarthou¹

Laboratory of Medical Microbiology, Unit of Clinical Testing, Institut Pasteur du Cambodge, 5 Boulevard Monivong, BP 983, Phnom Penh, Cambodia,¹ and Department of Infectious Diseases, Preah Bat Norodom Sihanouk Hospital, Phnom Penh, Cambodia²

Received 1 May 2006/Returned for modification 19 June 2006/Accepted 14 August 2006

***Penicillium marneffei* infection is an important disease among human immunodeficiency virus patients in Southeast Asia. The in vitro antifungal-drug susceptibilities of 29 clinical isolates and 5 isolates from bamboo rats collected from 2002 to 2004 were determined. The *P. marneffei* yeast form is more susceptible than the mycelial form to amphotericin B and ketoconazole, while the mycelial and yeast forms displayed similar susceptibilities to flucytosine and itraconazole. The MICs of fluconazole were higher for both mycelial and yeast forms.**

Penicillium marneffei, the only known dimorphic species in the genus *Penicillium*, was first isolated from an organ of a bamboo rat (*Rhizomyces sinensis* or *Rhizomyces pruinosus*) (2). Penicilliosis marneffei has been recognized with increasing frequency in Southeast Asia and Southern China (15, 19, 20). *P. marneffei* infection has become the third most common opportunistic infection in AIDS patients in Southeast Asia (17, 22). Penicilliosis marneffei was first reported in Cambodia in 2002 (1). We report in vitro susceptibility test results, as determined using Etest strips, for *P. marneffei* isolates from human immunodeficiency virus-infected patients who presented with disseminated *P. marneffei* infections. We include the susceptibility test results for *P. marneffei* isolates from the organs of bamboo rats. Our study is the first from Cambodia to report in vitro susceptibilities of clinical and bamboo rat isolates of *P. marneffei* to antifungal agents.

P. marneffei was isolated from 29 human immunodeficiency virus-infected patients seen at Preah Bat Norodom Sihanouk Hospital, Phnom Penh, Cambodia, from 2002 to 2004. Thirteen *P. marneffei* isolates were isolated from blood, 12 from skin biopsy specimens, and 4 from respiratory specimens. In all cases, only one specimen type was submitted to the laboratory. Bamboo rats (*Rhizomyces pruinosus*) were captured in the Monduliri province of Cambodia. Five isolates of *P. marneffei* were isolated from the lungs of 10 bamboo rats. *P. marneffei* was identified by the appearance of the fungal colonies on Sabouraud dextrose agar at 25°C and by microscopic examination (9).

One-week-old slant cultures of the mycelial form on Sabouraud dextrose agar, grown at 25°C, were flooded with deionized distilled water (10 ml/slant), and each slant was scraped with a sterile inoculation wire loop. The suspension of mycelial fragments and conidia was transferred to a glass

grinder. After the grinding, a homogenous suspension was adjusted to 1 McFarland standard (11, 14). To obtain inocula of the yeast form, brain heart infusion broth (Difco) was inoculated with a fungal suspension prepared from 7-day-old slant cultures. The inoculated brain heart infusion flasks were placed on a water bath shaker (150 rpm) and incubated at 37°C for 7 days, yielding a uniform growth of yeast cells. After incubation, the broth cultures were centrifuged at 15,000 × *g* for 20 min (4°C). After the broth was discarded, the sediment was washed three times successively with sterile deionized distilled water. The washed yeast cells were suspended in deionized distilled water and adjusted to 1 McFarland standard. Etest strips (AB Biodisk, Solna, Sweden) containing amphotericin B (AMB) (0.002 to 32 µg/ml), flucytosine (5FC) (0.002 to 32 µg/ml), fluconazole (FLC) (0.016 to 256 µg/ml), itraconazole (ITC) (0.002 to 32 µg/ml), and ketoconazole (KTC) (0.002 to 32 µg/ml) were used to determine the MICs according to the manufacturer's instructions. Susceptibility tests were carried out on RPMI 1640 (Gibco, Invitrogen Corporation, United Kingdom) supplemented with 1.5% agar and 2% glucose (12). Inoculated plates, including controls for the culture medium, were incubated at 25°C and 37°C for 72 h. The MIC was determined as the point of intersection of the elliptical inhibition zone with the scale on the E strip. *Cryptococcus neoformans* isolates from the National Collection of Microorganism Cultures (Institut Pasteur, Paris, France), IP 960.67, IP 961.67, and IP 962.67, were included to confirm the reproducibility of the results. Statistical analysis was performed with the STATA software (Stata Corp.).

The results for the *P. marneffei* isolates from patients are presented in Table 1. The MICs of AMB for the mycelial form were higher than those recorded for the yeast form (geometric mean titers, 2.994 µg/ml and 0.122 µg/ml, respectively; *P* < 0.001). The MICs of 5FC were lower for the mycelial form than for the yeast form (geometric mean titers, 0.004 µg/ml and 0.015 µg/ml, respectively; *P* < 0.001). There was essentially no difference between the MICs of FLC for mycelial and yeast forms (geometric mean titers, 69.475 µg/ml and 43.504 µg/ml,

* Corresponding author. Mailing address: Laboratory of Medical Microbiology, Unit of Clinical Testing, Institut Pasteur du Cambodge, 5 Boulevard Monivong, BP 983, Phnom Penh, Cambodia. Phone: 855-23-368-036. Fax: 855-23-426-013. E-mail: sborann@pasteur-kh.org.

[▽] Published ahead of print on 13 September 2006.

TABLE 1. In vitro susceptibilities of 29 clinical isolates of *Penicillium marneffei* to antifungal drugs, flucytosine, amphotericin B, ketoconazole, fluconazole, and itraconazole

<i>P. marneffei</i> (<i>n</i> = 29)	Antifungal agent	MIC (μ g/ml)			
		Geometric mean	Range	MIC ₅₀	MIC ₉₀
Mycelial (25°C)	Flucytosine	0.004	0.002–0.032	0.003	0.016
	Amphotericin B	2.994	0.002–32.0	4.0	24.0
	Ketoconazole	0.400	0.047–1.00	0.38	0.75
	Fluconazole	69.475	16.0–256.0	64.0	192.0
	Itraconazole	0.020	0.002–0.12	0.016	0.047
Yeast (37°C)	Flucytosine	0.015	0.002–0.019	0.012	0.094
	Amphotericin B	0.122	0.002–2.0	0.19	0.75
	Ketoconazole	0.021	0.002–0.19	0.023	0.047
	Fluconazole	43.504	1.50–256.0	32.0	128.0
	Itraconazole	0.007	0.002–0.23	0.006	0.016

respectively). The MICs of KTC were lower for the yeast form than for the mycelial form (geometric mean titers, 0.021 μ g/ml and 0.400 μ g/ml, respectively; $P < 0.001$). The MICs of ITC were similar for the mycelial and yeast forms (geometric mean titers, 0.020 μ g/ml and 0.007 μ g/ml, respectively).

The in vitro activities of the agents against the *P. marneffei* isolates from bamboo rats are presented in Table 2. The MIC geometric means titers of 5FC, AMB, KTC, FLC, and ITC for both the mycelial and yeast forms of these isolates were similar to those for the clinical isolates.

The consistent MICs of the antifungal agents against our control strains indicated that the reproducibility of our susceptibility test was excellent.

Our study demonstrates that the majority of mycelial or yeast forms of *P. marneffei* strains recovered from the patients showed greater susceptibility to 5FC and ITC. Our MIC data for 5FC and ITC are similar to those reported by Sekhon et al. (13). An in vitro study by McGinnis et al. (10) demonstrated that 30 strains of *P. marneffei* were susceptible to ITC (MICs, 0.03 to 0.5 μ g/ml). In our study, the MICs of AMB and KTC for the mycelial forms of *P. marneffei* strains were more than 10 times higher than for yeast forms. The yeast forms, with the exception of one *P. marneffei* strain from a patient, were susceptible to AMB with an MIC geometric mean titer of 0.122

μ g/ml. The majority of the isolates, from either patients or bamboo rats, in the mycelial and yeast form were resistant to FLC, with an MIC geometric mean titer of >43.504 μ g/ml. This is similar to data reported by Supparatpinyo et al. (16). Several investigators have confirmed the reliability of the Etest strips for the determination of antifungal susceptibilities (3, 12, 21).

Our finding that the isolates of *P. marneffei* demonstrate greater susceptibility to ITC in the yeast or mycelial form has been reported by others (12, 13). Hardin et al. (5), in a pharmacokinetic study of ITC, demonstrated that the plasma concentration was approximately 0.5 μ g/ml. As indicated by Sekhon et al. (13), ITC would be the drug of choice for treating penicilliosis marneffei. A study by Supparatpinyo et al. (18) indicated that long-term maintenance therapy with oral ITC at a dose of 200 mg once daily is highly effective in preventing relapse of *P. marneffei* infections. The in vivo pharmacokinetic data of Francis et al. (4) and Huang et al. (6) support our conclusion that 5FC and KTC may be effective alternate choices for the treatment of *P. marneffei* infections. Pankorn et al. (11) demonstrated that *P. marneffei* showed greater susceptibility to ITC and KTC, while AMB had only intermediate activity and FLC was least effective. Our AMB and FLC susceptibility data agree with those of Pankorn et al. (11) and are also supported by the pharmacokinetics of AMB and FLC in lung tissue, saliva, and plasma as reported by Koizumi et al. (7) and Koks et al. (8).

In Cambodia, AMB remains the agent of choice for the initial treatment of *P. marneffei* infection. This is followed after 2 or 3 weeks with oral ITC. Our in vitro results demonstrating susceptibility of the yeast phase to AMB and ITC support this protocol for the treatment of *P. marneffei* infection in Cambodia.

We are grateful to Jim McLaughlin for his constructive suggestions and helpful comments on the manuscript.

This work was supported in part by a grant from the Institut Pasteur du Cambodge, Phnom Penh, Cambodia.

REFERENCES

1. Bailloud, R., M. Sumanak, B. Sar, C. Y. Sreng, T. Ellman, D. Laureillard, and D. Monchy. 2002. Premiers cas d'infection à *Penicillium marneffei* identifiés chez l'immunodéprimé au Cambodge. *J. Mycol. Med.* 12:138–142.

TABLE 2. In vitro susceptibilities of five *Penicillium marneffei* isolates from the lungs of bamboo rats to antifungal drugs, flucytosine, Amphotericin B, ketoconazole, fluconazole, and itraconazole

<i>P. marneffei</i> (<i>n</i> = 5)	Antifungal agent	MIC (μ g/ml)	
		Geometric mean	Range
Mycelial (25°C)	Flucytosine	0.008	0.004–0.047
	Amphotericin B	4.097	0.047–32.0
	Ketoconazole	0.424	0.38–0.50
	Fluconazole	71.060	24.0–256.0
	Itraconazole	0.024	0.012–0.047
Yeast (37°C)	Flucytosine	0.073	0.032–0.25
	Amphotericin B	0.390	0.064–1.5
	Ketoconazole	0.034	0.016–0.064
	Fluconazole	91.582	32.0–256.0
	Itraconazole	0.006	0.002–0.012

2. Capponi, M., P. Sureau, and G. Sergetain. 1956. Penicilliose de *Rhizomys sinensis*. Bull. Soc. Pathol. Exot. Filiales **49**:418–421.
3. Cuenca-Estrella, M., A. Gomez-Lopez, E. Mellado, and J. L. Rodriguez-Tudela. 2005. Correlation between the procedure for antifungal susceptibility testing for *Candida* spp. of the European Committee on Antibiotic Susceptibility Testing (EUCAST) and four commercial techniques. Clin. Microbiol. Infect. **11**:486–492.
4. Francis, P., and T. J. Walsh. 1992. Evolving role of flucytosine in immunocompromised patients—new insights into safety, pharmacokinetics, and antifungal therapy. Clin. Infect. Dis. **15**:1003–1018.
5. Hardin, C. T., R. J. Graybill, R. Fetchick, R. Woestenborghs, G. M. Rinaldi, and G. J. Kuhn. 1988. Pharmacokinetics of itraconazole following oral administration to normal volunteers. Antimicrob. Agents Chemother. **32**:1310–1313.
6. Huang, Y. C., L. J. Colaizzi, H. R. Bierman, R. Woestenborghs, and J. Heykants. 1986. Pharmacokinetic and dose proportionality of ketoconazole in normal volunteers. Antimicrob. Agents Chemother. **30**:206–210.
7. Koizumi, T., K. Kubo, T. Kaneki, M. Hanaoka, T. Hayano, T. Miyahara, K. Okada, K. Fujimoto, H. Yamamoto, T. Kobayashi, and M. Sekiguchi. 1998. Pharmacokinetic evaluation of amphotericin B in lung tissue: lung lymph distribution after intravenous injection and airspace distribution after aerosolization and inhalation of amphotericin B. Antimicrob. Agents Chemother. **42**:1597–1600.
8. Koks, C. H. W., P. L. Meenhorst, M. J. X. Hillebrand, A. Bult, and J. H. Beijnen. 1996. Pharmacokinetics of fluconazole in saliva and plasma after administration of an oral suspension and capsules. Antimicrob. Agents Chemother. **40**:1935–1937.
9. Kudeken, N., K. Kawakami, and A. Saito. 1998. Different susceptibilities of yeasts and conidia of *Penicillium marneffei* to nitric oxide (NO)-mediated fungicidal activity of murine macrophages. Clin. Exp. Immunol. **112**:287–293.
10. McGinnis, M. R., N. G. Nordoff, N. S. Ryder, and G. B. Nunn. 2000. In vitro comparison of terbinafine and itraconazole against *Penicillium marneffei*. Antimicrob. Agents Chemother. **44**:1407–1408.
11. Pankorn, I., K. Thipsuvan, A. Chaiprasert, S. Danchaivijitra, R. Sutthent., and J. Jearanaisilavong. 2000. *Penicillium marneffei*: type and drug susceptibility. Mycopathologia **149**:109–115.
12. Pfaller, M. A., S. A. Messer, A. Bolmström, F. C. Odds, and J. H. Rex. 1996. Multisite reproducibility of the Etest MIC method for antifungal susceptibility testing of yeast isolates. J. Clin. Microbiol. **34**:1691–1693.
13. Sekhon, A. S., A. A. Padhye, and A. K. Garg. 1992. *In vitro* sensitivity of *Penicillium marneffei* and *Pythium insidiosum*. Eur. J. Epidemiol. **8**:427–432.
14. Sekhon, A. S., A. K. Garg, A. A. Padhye, and Z. Hamir. 1993. In vitro susceptibility of mycelial and yeast forms of *Penicillium marneffei* to amphotericin B, fluconazole, 5-fluorocytosine, and itraconazole. Eur. J. Epidemiol. **9**:553–558.
15. Supparatpinyo, K., S. Chiewchanvit, P. Hirunsri, C. Uthammachai, E. N. Kenrad, and T. Sirisanthana. 1992. *Penicillium marneffei* infection in patients infected with human immunodeficiency virus. Clin. Infect. Dis. **14**:871–874.
16. Supparatpinyo, K., E. N. Kenrad, G. M. William, J. B. Barbara, R. C. Chester, Jr., K. Chantana, and T. Sirisanthana. 1993. Response to antifungal therapy by human immunodeficiency virus-infected patients with disseminated *Penicillium marneffei* infections and *in vitro* susceptibilities of isolates from clinical specimens. Antimicrob. Agents Chemother. **37**:2407–2411.
17. Supparatpinyo, K., and T. Sirisanthana. 1994. New fungal infections in the Western Pacific. JAMA Southeast Asia **10**(Suppl.):208–209.
18. Supparatpinyo, K., J. Perriens, E. N. Kenrad, and T. Sirisanthana. 1998. A controlled trial of itraconazole to prevent relapse of *Penicillium marneffei* infection in patients infected with the human immunodeficiency virus. N. Engl. J. Med. **339**:1739–1743.
19. Tsang, D. N., P. C. Li, M. S. Tsui, Y. T. Lau, K. E. Ma, and E. K. Yeoh. 1991. *Penicillium marneffei*: another pathogen to consider in patients infected with human immunodeficiency virus. Rev. Infect. Dis. **13**:766–767.
20. Tsui, W. N., K. F. Ma., and D. N. Tsang. 1992. Disseminated *Penicillium marneffei* infection in HIV-infected subject. Histopathology **20**:287–293.
21. Vandenbossche, I., M. Vanechoutte, M. Vandevenne, T. D. Baere, and G. Verschraegen. 2002. Susceptibility testing of fluconazole by the NCCLS broth microdilution method, E-test, and disk diffusion for application in routine laboratory. J. Clin. Microbiol. **40**:918–921.
22. Walsh, T. J., and A. H. Groll. 1999. Emerging fungal pathogens: evolving challenges to immunocompromised patients for the twenty-first century. Transplant. Infect. Dis. **1**:247–261.