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IN VITRO SENSITIVITY OF *PENICILLIUM MARNEFFEI* AND *PYTHIUM INSIDIOSUM* TO VARIOUS ANTIFUNGAL AGENTS

A.S. SEKHON*, A.A. PADHYE**[1] and A.K. GARG*

*National Reference Center for Human Mycotic Diseases - Provincial Laboratory of Public Health - University of Alberta - Edmonton - Alberta T6G 2J2 - Canada.

**Mycotic Diseases Branch - Division of Bacterial and Mycotic Diseases - National Center for Infection Diseases - Centers for Disease Control Public Health Service - U.S. Department of Health and Human Services Atlanta - Georgia 30333 U.S.A.

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Ten isolates of *Penicillium marneffei* and eight of *Pythium insidiosum* were tested for their *in vitro* sensitivity to amphotericin B, hamycin (a polyene heptaene), two water-soluble analogs of amphotericin B and hamycin, namely, JAI-Amb, and JAI-hamycin, 5-fluorocytosine, fluoconazole, itraconazole, ketoconazole and miconazole. Itraconazole manifested the strongest activity against all of the 10 isolates of *P. marneffei* and would be the drug of choice in the treatment of penicilliosis due to *P. marneffei*. The polyene antibiotics amphotericin B and hamycin and their water-soluble analogs showed no appreciable activity against *P. insidiosum*. *Pytium insidiosum* isolates were sensitive to fluconazole, ketoconazole, and miconazole.

Miconazole exhibited the strongest *in vitro* activity against all of the 8 isolates of *P. insidiosum*, followed by ketoconazole.

INTRODUCTION

Penicillium marneffei, the only dimorphic species of the genus *Penicillium*, is being recognized with increasing frequency in recent years as an emerging pathogen causing systemic infections in immunocompetent (3, 6, 7, 8, 10, 11, 22, 25) as well as immunocompromised patients especially those with acquired immunodeficiency syndrome (AIDS) (1, 2, 4, 9, 12, 13, 14, 16, 19, 21, 23).

Pythium insidiosum, the etiologic agent of pythiosis, causes cutaneous, subcutaneous, and osseous infections in human and animals (5, 15, 20, 21). In humans, infections range from keratitis to cutaneous subcutaneous involvement, which may result in severe and even fatal outcome because of the predilection of *P. insidiosum* for angioinvasion (20, 21). Although penicilliosis due to *P. marneffei* and pythiosis are important emerging opportunistic mycoses in immunocompromised patients, very little information is available with respect to the sensitivity of their etiologic agents to various antifungal agents.

The present study was undertaken to determine the *in vitro* activity of amphotericin B (Bristol-Myers: Squibb Canada Inc., Montreal, P.Q.), 5-fluorocytosine (Hoffman-La Roche Ltd., Toronto, Canada), amphotericin B + 5-fluorocytosine, fluconazole (Pfizer Ltd., Sandwich, U.K.), intraconazole, ketoconazole and miconazole (Janssen Pharmaceutica Inc., Mississauga, Canada), against 10 isolates of *P. marneffei* and 8 isolates of *P. insidiosum*. During the course of this investigation, water-soluble analogs of amphotericin B and hamycin, namely, JAI-amphotericin B and JAI-hamycin (injectable and for oral use), were received through the courtesy of JAIMYCIN, Inc. Walnut Creek, California. According

[1] Corresponding author.

to Jaimycin Inc. the process utilizes currently used antibiotics and chemical therapeutants and makes them four to twenty-five times less toxic than their parent compounds while retaining their bioactivity. The two water-soluble analogs, JAI-Amb and JAI-hamycin, had only one twenty-fifth part of the parental drug. The results of this investigation are the subject of this presentation.

MATERIALS AND METHODS

Cultures: The isolation data for the 10 *P. marneffei* and 8 *P. insidiosum* isolates are summarized in Table 1. All cultures were grown on Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI) at 25° C (*P. marneffei*) and 37° C (*P. insidiosum*) for 10 days. When not in use, the mature slants were stored at 0-3° C.

Antifungal Agents: *In vitro* minimun inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) against the 10 isolates of *P. marneffei* were determined for amphotericin B (Amb), JAI-Amb, JAI-hamycin (oral and injectable), 5-fluorocytosine (5-FC), itraconazole, ketoconazole and miconazole. The *in vitro* MICs and MFCs against 8 the isolates of *P. insidiosum* were determined for Amb, 5-FC, Amb+5-FC, fluconazole, itraconazole, ketoconazole, and miconazole. Stock solutions (1 mg/ml) of JAI-Amb, JAI-hamycin, and 5-FC were prepared in sterilized distilled water. Ketoconazole was dissolved in sterile, acidic (pH 4.3) distilled water. Amb, miconazole and itraconazole were dissolved in dimethyl sulfoxide. The stock solutions wer self-sterilized by allowing them to remain at room temperatures (25° C) for 45 minutes, except for 5-FC which was membrane filter (0.45 µm) sterilized. Stock solutions were diluted in appropriate antibiotic media to give a concentration of 1000 mcg/ml (v/v) (17).

Inoculum preparation: The 10-day-old, active, conidiating slant cultures of *P. marneffei*, and 7-day-old, active, hyphal growth of each of the 8

TABLE 1. - Sources of *Penicillium marneffei* and *Pythium insidiosum* isolates.

Isolate No.	Source
P. marneffei	
PLM 504	Segretain's strain, Pasteur Institute CIP 560
PLM 689	ATCC 24100, human case, U.S.A.
PLM 1494	NRRL 6184 (Type strain)
PLM 1495	CDC B-3420, human case = 1, Thailand
PLM 1496	CDC B-3699, human case = 2, Thailand
PLM 1497	CDC B-3701, human case = 5, Thailand
PLM 1498	CDC B-3784 = ATCC 56573, human case, U.S.A.
PLM 1641	CDC B-4449 = ATCC 64101, human case, China
PLM 1642	CDC B-4450 = ATCC 64102, Bamboo rat, China
PLM 1789	Human case, AIDS patient, from Dr. Drouhet, Pasteur Institute
P. insidiosum	
PLM 2006	CDC B-4919, human, mycotic keratitis , U.S.A.
PLM 2007	CDC B-4920, cat pythiosis, North Carolina
PLM 2060	CDC B-4300, dog pythiosis, U.S.A.
PLM 2061	CDC B-4301, horse pythiosis, U.S.A.
PLM 2062	CDC B-4302, horse pythiosis, U.S.A.
PLM 2063	CDC B-4311, horse pythiosis, U.S.A.
PLM 2064	CDC B- 4313 = CBS 702.83, horse pythiosis, Netherlands
PLM 2065	CDC B-4307, human pythiosis, Thailand

P. insidiosum isolates were flooded with 10 ml of sterilized, deionized, distilled water. The growth was gently scraped and the resultant suspensions were transferred aseptically to sterile tubes. The optimal densities of the suspensions were adjusted spectrophotometrically to obtain a reading of 90% at 530 nm by adding sterilized, deionized distilled water (17, 18).

Antibiotic test media: The *In vitro* susceptibility tests were carried out using Antibiotic Medium M3 (Difco) for Amb, JAI-Amb and JAI-hamycin; yeast nitrogen broth for 5-FC; and Sabouraud dextrose broth (pH 6.3) for fluconazole, itraconazole, ketoconazole and miconazole. The standard double dilution technique was used to obtain serial dilutions of the antimycotics ranging from 100 to 0.195 mcg/ml. Each antimycotic dilution (in 1 ml quantity per tube) was inoculated with 0.1 ml of the previously standardized inoculum suspensions of *P. marneffei* and *P. insidiosum* isolates. The MICs and MFCs were determined for each antimycotic agent following the procedure of Sekhon and Funk (17) and Shadomy *et al.* (18).

TABLE 2. - *In vitro* Susceptibility of *Penicillium marneffei* to various Antimycotic Agents.

Antimycotic agent	MIC (No. isolates) mcg/ml	MFC (No. isolates) mcg/ml
Amphotericin B	< 0.195 (1) > 0.39 (2) 0.78 (6) 1.56 (1)	0.78 - 1.56 (5) 3.125 (5)
JAI-Amb (oral)	0.39 - 0.78 (4) > 50 - 100 (6)	0.78 - 3.125 (3) 1.56 (1) > 50 - 100.00 (6)
JAI-Amb (injectable)	> 0.195 - 3.125 (3) 50 - 100.000 (4) > 100.000 (3)	> 0.195 - 0.39 (2) 50 - > 100.000 (8)
JAI-hamycin (oral)	50 - 100.00 (8) > 100.00 (2)	100.00 (4) > 100.00 (6)
JAI-hamycin (injectable)	> 0.195 - 3.125 (3) 50 - 100.00 (4) > 100.00 (3)	> 0.195 - 0.39 (2) > 50.00 (1) > 100.00 (7)
5-fluorocytosine	< 0.195 (7) 0.39 (2) 0.78 (1)	< 0.195 (1) 0.78 (1) 1.56 - 3.125 (5) 6.25 - 12.5 (3)
Itraconazole	< 0.195 (10)	< 0.195 (10)
Ketoconazole	< 0.195 (9) 0.39 (1)	< 0.195 (6) 0.39 - 0.78 (4)
Miconazole	< 0.195 (10)	< 0.195 (10)

TABLE 3. - *In vitro* susceptibility of *Pythium insidiosum* isolates to various Antimycotic Agents.

Antimycotic Agent	MIC (No. isolates) mcg/ml	MFC (No. isolates) mcg/ml
Amphotericin B	50.00 (4)	50.00 (3)
	100.00 (4)	100.00 (5)
JAI-hamycin (oral)	> 100.00 (8)	> 100.00 (8)
JAI-hamycin (injectable)	> 100.00 (8)	> 100.00 (8)
5-Fluorocytosine	25 - 50 (2)	25 - 50 (2)
	> 100.00 (6)	> 100.00 (6)
Amb + 5-FC	25 - 50 (4)	25 - 50 (4)
	100.00 (3)	100.00 (1)
	> 100.00 (1)	> 100.00 (3)
Fluconazole	< 0.195 (3)	< 0.195 (3)
	3.125 (1)	3.125 (1)
	12.5 - 50 (2)	25 - 50 (2)
	100.00 (2)	100.00 (2)
Itraconazole	25 - 50 (8)	25 - 50 (8)
Ketoconazole	< 0.195 (2)	< 0.195 (2)
	12.5 - 25 (5)	12.5 - 25 (5)
	50.00 (1)	50.00 (1)
Miconazole	0.39 (3)	0.39 (3)
	0.78 (3)	0.78 (3)
	3.12 (2)	3.12 (2)

RESULTS

The results of the susceptibility tests are summarized in Table 2 for *P. marneffei* and Table 3 for *P. insidiosum*. The MICs and MFCs of the analogs JAI-Amb and JAI-hamycin varied among the isolates of *P. marneffei*. None of the 8 isolates of *P. insidiosum* was sensitive to JAI-hamycin. The MICs and MFCs against Amb varied from 50-100 mcg/ml, indicating its ineffectiveness against *P. insidiosum*.

The MICs and MFCs values for 5-FC, itraconazole, ketoconazole, and miconazole showed that itraconazole and miconazole exhibited high antifungal activity against all 10 isolates of *P. marneffei* (Table 2). Against *P. insidiosum*, the MIC values for 5-FC and Amb+5-FC varied from 25 mcg/ml to > 100 mcg/ml. The MIC and MFC values for fluconazole showed wide variations among the different isolates, ranging from 0.195 to 100 mcg/ml. Among the itraconazole, ketoconazole and miconazole, only miconazole exhibited strong activity against the *P. insidiosum* isolates (Table 3). The MFC values for the antimycotics tested were generally equal to the MICs or were higher by one or more dilutions (Tables 2, 3).

DISCUSSION

Both JAI-Amb and JAI-hamycin, the water-soluble analogs of amphotericin B and hamycin, respectively, showed fair antifungal activity *in vitro* against *P. marneffei* but not against *P. insidiosum*. Considering that both analogs contained only 1/25 of the parent drug, both analogs need to be investigated

further to determine their activity against yeasts and other pathogens. Among the other azoles tested, itraconazole exhibited the strongest activity against all 10 *P. marneffei* isolates. Recently, Van Cutsem and Van Gerven (24) showed that systemic experimental penicilliosis induced by *P. marneffei* in immunodepressed guinea pigs was rapidly cured by low dosages of itraconazole. They found good correlation between the *in vitro* and *in vivo* data and that the itraconazole treatment was free of any serious side effects. The majority of patients with AIDS, who had developed penicilliosis *marneffei* had a history of travel or stay in Southeast Asia. Most infections were treated with amphotericin B or Amb+5-FC. In many instances, the toxicity of Amb potentially became a limiting factor. The present *in vitro* data strongly support data of Van Cutsem and Van Gerven indicating that itraconazole would be the drug of choice in the treatment of penicilliosis due to *P. marneffei.*

The lack of *in vitro* activity of the polyene antibiotics (Amb and hamycin) against *P. insidiosum* was surprising. Four cases of arteritis and one of keratitis caused by *P. insidiosum* have been described in the literature (20, 21). All patients were farmers and had the thalassemia hemoglobinopathy syndrome. We are aware of three additional unpublished human cases of keratitis (2 from the United States and 1 from New Zealand) caused by *P. insidiosum.* Radical surgical removal of infected tissues and oral administration of a saturated solution of potassium iodide had been proposed empirically as the therapy of choice in the absence of *in vitro* data with currently used antifungal agents against *P. insidiosum.* The present findings showed that *P. insidiosum* isolates were susceptible to fluconazole, ketoconazole, and miconazole. Miconazole exhibited the strongest *in vitro* activity against all of the 8 isolates of *P. insidiosum* tested, and ketoconazole was the next most effective agent (Table 3).

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