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In vitro SUSCEPTIBILITY TESTING OF *Fonsecaea pedrosoi* TO ANTIFUNGALS

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SUMMARY

Based on the difficulties experienced in the treatment of chromoblastomycosis, 12 primary human isolates of *F. pedrosoi*, were tested for their *in vitro* susceptibility to various antimycotics. We adapted the recommendations of the NCCLS for yeasts and followed the indications for mold testing from other authors in order to determine their MIC's and the MLC's. It was found that a significant proportion of the isolates were resistant to 3 of the 4 antimycotics tested, as revealed by high MIC values, as follows: 33% were resistant to amphotericin B (AMB), 58.3% to 5 fluocytosine (5 FC) and 66.7% to fluconazole (FLU). Contrarywise, none of the isolates proved resistant to itraconazole (ITZ). Determination of the MLC's revealed that a larger proportion of the isolates were not killed by AMB, 5 FC (91.7%), FLU (100%) or even, ITZ (41.7%). These data indicate that it would be desirable to determine the susceptibility of *F. pedrosoi* before initiating therapy, in order to choose the more effective antifungal and avoid clinical failure.

KEYWORDS: Antifungal drugs; *Fonsecaea pedrosoi*; Susceptibility testing.

INTRODUCTION

In the humid tropical regions where chromoblastomycosis is endemic, *Fonsecaea pedrosoi* is the commonest causative agent of this disorder[9]. Treatment of the mycosis caused by this agent is unrewarding not only because the scarcity of effective antifungals and the need for prolonged periods of treatment but also, due to the difficulties found in evaluating patients living in distant rural areas [17].

Due to the above facts, it would be desirable to count with adequate techniques to determine the *in vitro* susceptibility of the causative agent to antifungal agents, previous to the instauration of therapy. This practice would avoid the use of ineffective drugs and would also permit to evaluate the changes that may arise in the agent's susceptibility during the course of prolonged treatment. Attempts to standardize these procedures for the filamentous fungi have been developed only recently[6,7,8,16] and as a consequence, the information available on their value, it still scanty.

In this report we present the *in vitro* susceptibility data for 12 *F. pedrosoi* isolates obtained at time of diagnosis from patients with chromoblastomycosis; the antifungals tested were amphotericin B, 5 fluorocytosine, fluconazole and itraconazole. For each isolate the minimal inhibitory concentration (MIC) was measured following the methods standardized for yeasts[8, 10, 14, 15] and adapted to molds according to the techniques recommended by ESPINAL-INGROFF[6] and PUJOL[16]. Attempts were made to determine the minimal lethal concentration (MLC) as done by COLOMBO et al. [4] with *Aspergillus species*.

MATERIALS AND METHODS

Isolates: 12 different isolates obtained at time of diagnosis from patients with active, untreated chromoblastomycosis and whom had been diagnosed between 1986 and 1994, were tested. All isolates were maintained by periodic subculturing in potato dextrose agar (ADP) with incubation at temperatures of 22 to 24°C [6, 13, 16]. For the quality control of the susceptibility test, we used an isolate of *Paecylomyces variotii*, ATCC 22319 [6, 7, 16].

Antifungals: The four antifungals employed were obtained in powdered form, as follows: amphotericin B (AMB) (Fungizone, Squibb, Princeton, New Jersey), 5 fluorocytosine (5 FC) (Ancobon, Roche, Sigma Chemical Co., St. Louis, Missouri), fluconazole (FLU) (Diflucan, Pfizer Pharmaceuticals, New York, New York) and itraconazole (ITZ) (Sporanox, Janssen Research Foundation, Beerse, Belgium).

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Sterile distilled water was used to dissolve AMB, 5 FC and FLU; for ITZ, polyethylene glycol (PEG) (PM 400, Fischer Scientific Co., Fair Lawn, New Jersey) was used. For complete dissolution of the latter product, heating and stirring for 1 hr in a water bath at 75°C was necessary[6, 14, 16]. The range of concentrations (in µg/ml) employed were the following: AMB, 0.03 to 16; 5 FC, 10-322, FLU 0.125 to 64, and ITZ, 0.018-10. These concentrations were those recommended by the NCCLS for the yeast macro-broth dilution test[14]. The drugs were prepared at a 10x dilution, distributed in 0.1 ml quantities in sterile polystyrene 12 x 75 mm tubes (Falcon, Beckton Dickson Laboratories, Franklin Lakes, New Jersey) and kept at minus 70°C until time of testing.

Inoculum: Stock isolates were transferred to APD tubes and grown for 7 days at 22-24°C. The inoculum was prepared by adding 5 ml of sterile distilled water to the tube; the mycelial growth was then carefully detached with the aid of a Pasteur pipette and the suspension allowed to settle for 5 minutes. The supernatant was then transferred to another sterile tube in order to analyze its contents spectophotometrically, according to the recommendations of PUJOL et al. [16]. Care was taken to vortex the suspension for 15 seconds prior to reading at 530 nm (Spectronic, Bausch & Lomb, Rochester, New York); transmittance was adjusted to 95% which is equivalent to the range 1 x 10^4 to 5 x 10^5 CFU[16]. After measurement, the suspension was diluted 1:10 in RPMI 1640 (Sigma Chemical Co., St. Louis, Missouri), so as to obtain an inoculum with approximately 1 x 10^3 to 1 x 10^4 CFU/ml.

Susceptibility Testing

1) MIC's values. Each one of the tubes containing the various antifungal concentrations received 0.9 ml of the fungal inoculum. Controls of PEG (0.9 ml of the inoculum plus 0.1 ml of PEG) and of the inoculum alone (1.0 ml) were included. *Paecylomyces variotii* ATCC 22319 was tested in parallel with the *F. pedrosoi* isolates. The series of tubes thus prepared were incubated at 22-24° C, for 96 to 120 hours [7, 16] in order to achieve abundant growth and facilitate the interpretation of the test. The tests were performed with two reading at different times, the first as soon as the control tube (isolate without drug) became positive (96 hrs) and the second, 24 hours later (120 hrs). For both triazoles and also for 5 FC, the MIC was defined as the tube exhibiting 50% growth inhibition when compared to the inoculum control thus avoiding trailing effects. For AMB, the MIC corresponded to the tube showing complete inhibition of growth [7, 18, 21].

2) MLC values. The MLC was obtained by subculturing 100 µl from the tube corresponding to the MIC as well as from the remaining tubes with higher drug concentrations, taking care of vortexing the tubes before sampling. This amount was transferred to a Sabouraud dextrose agar plate (SDA, Beckton Dickinson and Co., Cokeysville, Maryland) that was incubated at 22-24° C for 96-120 hours. CFU were then determined. For the triazoles and 5 FC, the MLC was the concentration giving ≤ 5 CFU/ml. For AMB, the MLC was achieved when there were no CFU in the plate [4].

Based in the data recorded by other authors [20], we considered as resistant those isolates that had 5 FC MIC's ≥ 25 µg/ml and ≥ 4 µg/ml for AMB. Due to the lack of information on the resistance of *F. pedrosoi* to azole compounds, we arbitrarily chose as resistant those isolates that exceeded the peak plasma levels attained by patients under treatment, as follows: for FLU 20 µg/ml[11] and for ITZ, 10 µg/ml [5].

RESULTS

For each experiment, the inoculum controls produced clearly detectable growth after the chosen incubation period, indicating that the 12 *F. pedrosoi* isolates were viable and the conditions used, appropriate for fungal growth. The studies with *P. variotii* ATCC 22319, revealed that the antifungals were active as the concentrations required to inhibit its growth, were met during testing. The MIC's values obtained with this fungus were: AMB 2 µg/ml, 5FC < 0.3 µg/ml, FLU 4 µg/ml and ITZ 0.03 µg/ml and the MLC's were 2 µg/ml, < 0.3 µg/ml, > 64 µg/ml and 0.03 µg/ml, respectively.

We found that the 95% transmittance value used to standardize the inoculum, produced a reproducible form to deliver the same quantity of fungal particles (data not shown).

The MIC's and MLC's for AMB and 5 FC, and for the azole compounds, are presented in Table 1. There were some variation in the MIC's range for the 4 antifungals; for AMB this was between 0.5 and 4 µg/ml and for 5 FC, between 10 and 161 µg/ml. FLU values varied from 8 to > 64 µg/ml while ITZ MIC's ranged from 0.018 and 0.6 µg/ml. According to the criteria chosen, 4 (33%) isolates were resistant to AMB, 7 (58.35%) to 5 FC and 8 (66.7%) to FLU. None of the 12 isolates was resistant to ITZ (Fig. 1a). Fig. 2 shows the pattern of growth of one of the *F. pedrosoi* isolates when incubated with FLU.

Concerning MLC's , the results revealed the incapacity of the antifungals, even at the highest concentrations used, to inhibit fungal growth (Table 1). Thus, 11 (91.7%) of the isolates did not achieve their MLC's with either AMB (≥ 16 µg/ml) or 5 FC (≥ 3 22 µg/ml); 12 (100%) had no MLC's for FLU (≥ 64 µg/ml) and as it concerns ITZ, 5 (41.5%) isolates were unable to attain their MLC's (≥ 10 µg/ml) (Fig. 1b).

DISCUSSION

There has been few published reports regarding antifungal susceptibility testing for filamentous fungi, specially for dematiaceous molds. A collaborative study in which differents species of filamentous fungi were tested, has been recently published but dematiaceous species were not included[7]; however, the study by PUJOL et al.[16], considered the dematiaceous fungi. CARDONA et al.[3] also attempted susceptibility testing of *F. pedrosoi*. These studies and the present report, represent an important step towards the standardization of susceptibility testing of molds, and specifically reveal that it is possible to work with the *in vitro* susceptibility of black molds.

TABLE 1
Minimal and lethal concentrations of 12 isolates of *F. pedrosoi* to various antifungals

Drug tested values (µg/ml)	Nº (%) of isolates with given values (µg/ml)	
	MIC	MLC
Amphotericin B		
0.5	3 (25)	1 (8.3)
1.0	3 (25)	-
2.0	2 (16)	-
4.0	4 (33)	-
8.0	-	-
16.0	-	2 (16.6)
> 16.0	-	9 (75)
5-fluorocytosine		
> 10	1 (8.3)	-
10	3 (25.0)	-
20	1 (8.3)	-
40	1 (8.3)	-
80	5 (41.6)	-
160	1 (8.3)	1 (8.3)
322	-	1 (8.3)
> 322	-	10 (83.3)
Fluconazole		
8	1 (8.3)	-
16	3 (25.0)	-
32	4 (33.3)	-
64	2 (16.6)	1 (8.3)
> 64	2 (16.6)	11 (91.7)
Itraconazole		
0.018	1 (8.3)	-
0.035	3 (25.0)	-
0.07	3 (25.0)	-
0.15	2 (16.6)	-
0.30	2 (16.6)	-
0.6	1 (8.3)	4 (33.3)
2.5	-	2 (16.6)
5.0	-	1 (8.3)
10	-	2 (16.6)
> 10	-	3 (25.0)

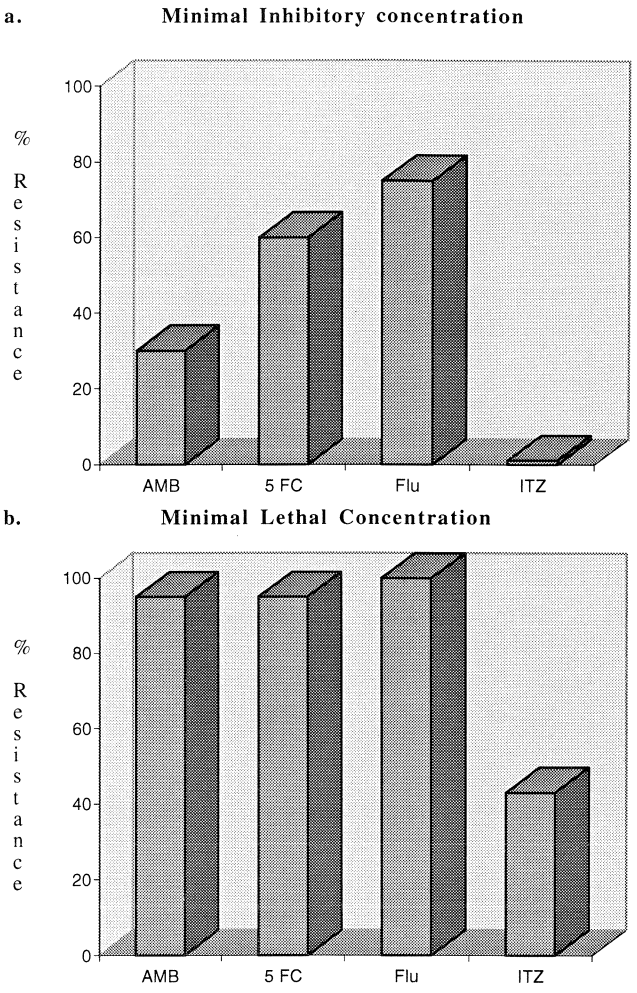

Fig.1 (a, b) - Resistence as measured by MIC's and MCL's of twelve isolates of *F. pedrosoi* against different antifungal drugs.

The results of this study indicate that *in vitro*, *F. pedrosoi* exhibits a high degree of resistance to most of the antifungals tested. Judging by the MIC's values, 4 of the 12 isolates (33%) were resistant to AMB, 7 (58.35%) to 5FC and 8 (66.7%) to FLU. The exception was ITZ which exhibit no resistance in accordance with previous data by other researchers [3,5].

The above findings were also corroborated by the MLC's obtained as 11 (91.7%) of the isolates were not killed by AMB, 5FC, 12 (100%), FLU or even, ITZ 5 (41.7%). The proportion of failures observed with the latter antifungal, correlates with the clinical experience, which shows that 10% of the chromoblastomycosis patients are not cured in spite of extended courses (20 months) of ITZ therapy [9,17].

The methodology employed in this study is commonly used for investigating the lethal effect of antimicrobial drugs[4]. The MLC results indicate that not all isolates were killed even by the most effective drug, itraconazole; this corroborates with the clinical evaluation of patients, a number of whom are not cured in spite of a higher MIC values[9,17]. The above correlation led us to consider that the methodology used appears reliable. Although the correlation between the results of susceptibility testing and clinical outcome has not been definitively demonstrated, there are several reports in candidiasis that suggest a direct relationship between the two events,[1, 2, 12, 19].

The high resistance of *F. pedrosoi* to the antifungals used indicates that it would be advantageous to test primary isolates before starting therapy. By doing so, one may avoid uneffective, costly and lenghty coursed of treatment. In view of the lack of susceptibility of this agent to the existent antimycotic drugs, it may be wiser to seriously consider the possibility of combining the best available drug, ITZ, with either AMB or 5FC in order to obtain a synergistic effect[5,9]. More studies should be conducted to determine the value of such combinations.

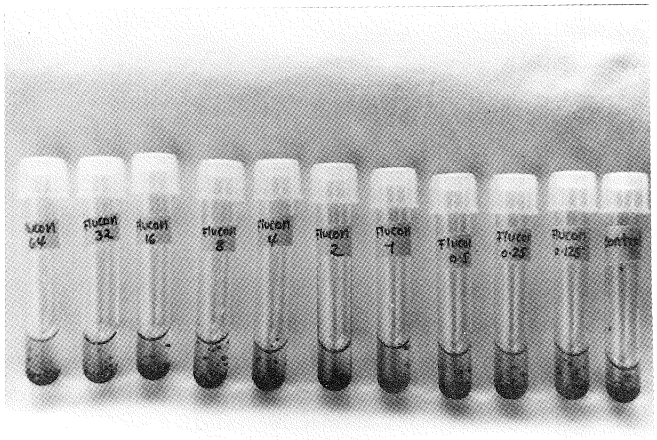

Fig. 2.- Minimum inhibitory concentration to fluconazole in one of the *F. pedrosoi* isolate. MIC: > 64 µg/ml. Range: 0.125-64 µg/ml.

RESUMO

Testes de suscetibilidade *in vitro* de *Fonsecaea pedrosoi* a antifúngicos

Baseados nas dificuldades experimentadas no sucesso do tratamento da cromoblastomicose, 12 isolamentos humanos primários do *F. pedrosoi* foram examinados para a sua suscetibilidade *in vitro* aos vários antifúngicos. Nos adaptamos às recomendações da NCCLS feitas para leveduras e, seguimos as indicações para fungos filamentosos testados dadas por outros autores para as determinações dos MIC's e dos MCLS. Nossos resultados mostraram que uma proporção significativa dos isolados testados foi resistente a três ou quatro dos antifúngicos experimentados, como revelaram os altos valores do MIC; assim, 33% dos isolados foram resistentes à anfotericina-B (AMB) 58,3% à fluorocitosina (5FC) e 66,7% ao fluconazole (FLU); pelo contrário, nenhum dos isolados examinados mostrou-se resistente ao itraconazole (ITZ). As determinações dos MLC revelaram que grande proporção dos isolados não foram mortos nem pela AMB (91,7%) 5FC (91,7%) FLU (100%) ou ainda, pelo ITZ (41,7%). Estes resultados indicam que seria muito desejável determinar a suscetibilidade do *F. pedrosoi* antes de começar o tratamento, para se poder escolher o antifúngico mais eficaz e evitar falha clínica.

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