POSTER

DEVELOPMENT OF RESISTANCE IN CANDIDA TROPICALIS : AN IN VITRO STUDY

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ABSTRAK

Kajian telah dilakukan untuk menentukan kewujudan keresistanan dalam Candida tropicalis terhadap agen antifungus selepas pendedahan kepada antifungus pada kepekatan rendah untuk jangka masa yang lama. Lima isolat klinikal Candida tropicalis telah dipilih untuk kajian ini yang sediakala resistan terhadap sama ada amphotericin B atau fluconazole. Dua isolat yang sensitif terhadap fluconazole telah didedahkan kepada 0.5 μg/ml fluconazole dalam kultur kaldu (Sabouraud dekstros) dengan pensubkulturan kerapkali sehingga 14 minggu. Tiga isolat yang sensitif terhadap amphotericin B telah didedahkan kepada 0.25 – 0.5 μg/ml amphotericin B dengan cara yang sama. Pada sela masa berlainan ujian penentuan kepekatan perencatan minimum (MIC) dilakukan terhadap isolat tadi dengan kaedah mikropencairan kaldu seperti mana disarankan oleh NCCLS (National Committee for Clinical Laboratory Standards). Hasil kajian menunjukkan peningkatan 4-kali dalam nilai MIC isolat selepas pendedahan kepada antifungus selama 10 minggu dan peningkatan ini berterusan sehingga ke akhir kajian. Isolat kawalan yang tidak didedahkan kepada antifungus tidak menunjukkan sebarang peningkatan dalam nilai MIC. Profil protein bagi semua isolat diperolehi (melalui teknik elektroforesis gel poliakrilamida sodium dodesil sulfat) sebelum dan selepas pendedahan kepada agen antifungus. Perubahan dalam profil protein (sama ada wujudnya fraksi baru atau peningkatan kepekatan) diperhatikan bagi semua isolat. Sebagai kesimpulan, pengaruhan keresistanan berlaku dalam Candida tropicalis yang didedahkan kepada agen antifungus dan kejadian ini dikaitkan dengan perubahan dalam profil protein.

ABSTRACT

A study was conducted to determine the development of resistance in Candida tropicalis against antifungal agents after long-term exposure to low drug concentrations. Five clinical isolates of Candida tropicalis were selected for this study. These isolates were found to have already shown resistance to either amphotericin B or to fluconazole. Two isolates that were sensitive to fluconazole were exposed to 0.5 μg/ml of fluconazole in a broth culture (Sabouraud dextrose broth) with frequent subculturing for up to 14 weeks. Three isolates that were sensitive to amphotericin B were exposed to 0.25 – 0.5 μg/ml of amphotericin B in the same way. At different time intervals, the MIC values of the isolates were determined by the broth microdilution test as proposed by the National Committee for Clinical Laboratory Standards (NCCLS). Our results showed a 4-fold increase in the MIC values after 10 weeks of exposure to the antifungal agent, which continued to rise until the end of the experiment. Control isolates not exposed to antifungal agents showed no increase in their MIC values. Protein profiles of all isolates (using sodium dodecyl sulphate polyacrylamide gel electrophoresis) were obtained before and after exposure to antifungal agent. A change in the protein profile (new fractions or increased concentration) was observed for all isolates. In conclusion, induction of resistance was observed in Candida tropicalis isolates exposed to antifungal agents and this occurrence correlates with a change in the protein profile.

INTRODUCTION

Candida sp. infections are the most frequently occuring opportunistic fungal infection in the immunocompromised patient. The causative species are *Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata, Candida krusei* and others (Meunier et al. 1992). Treatment of these infections is limited to the trazole drug, fluconazole and the polyene, amphotericin B. Fluconazole, however, is not effective in treating *C.krusei* and *C.glabrata* infections as these species are innately less susceptible to this antifungal agent (Wingard, 1995).

The mainstay of therapy for serious, life-threatening fungal infections is still amphotericin B. Fluconazole has been given as prophylaxis, especially to AIDS patients, due to their high risk of developing fungal infections. Long-term antifungal therapy is often necessary when systemic fungal infections occur. Studies have shown that long-term exposure to and prophylactic use of, antifungal agents have led to resistance development in

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Candida sp. (Vanden Bossche et al. 1994, Millon et al. 1994).

The development of resistance is seen primarily in *C.albicans* and to a lesser extent in *C.tropicalis* and *C.parapsilosis*. The use of fluconazole and long-term, low dose therapy with amphotericin B also appears to select for infections of less susceptible *Candida* species such as *C.krusei* and *C.glabrata* (Rex et al. 1993, Georgopapadakou et al. 1996).

Studies on the mechanisms and development of resistance have mainly focused on *C.albicans* and *C.glabrata*. In this study, we determine the development of resistance in *C.tropicalis*, *in vitro*, and observe fungal protein profiles for any related changes.

METHODOLOGY

Five clinical isolates of *Candida tropicalis* were obtained from the Institute for Medical Research, Kuala Lumpur. The MIC values of fluconazole and amphotericin B for these isolates were determined with either the broth microdilution method (National Committee for Clinical Laboratory Standards) or the commercially available Etest strip (AB Biodisk, Solna, Sweden). Interpretation of the MIC values were done using the interpretive breakpoints given in the National Committee for Clinical Laboratory Standards (NCCLS) document M27 A.

Isolates that were sensitive to fluconazole were exposed to 0.5μg/ml fluconazole and isolates that were sensitive to amphotericin B were exposed to 0.25-0.5 μg/ml amphotericin B. The concentration of the antifungal agent used was based on the MIC value for each isolate, i.e. the concentration being less than the MIC value.

The isolates were incubated in 20 ml of Sabouraud dextrose broth, containing the antifungal agent, at 37^{o}C, with frequent subculturing, as outlined in Figure 1. At different time-points throughout this period of exposure, the MIC values for the isolates were determined with the broth microdilution method. The MIC of the control strain *Candida parapsilosis* ATCC 200219, as recommended by the NCCLS, was also determined each time. A control experiment with the *C.tropicalis* isolates was also carried out in which the isolates were subcultured in the same way for the same time period, but not exposed to the antifungal agent.

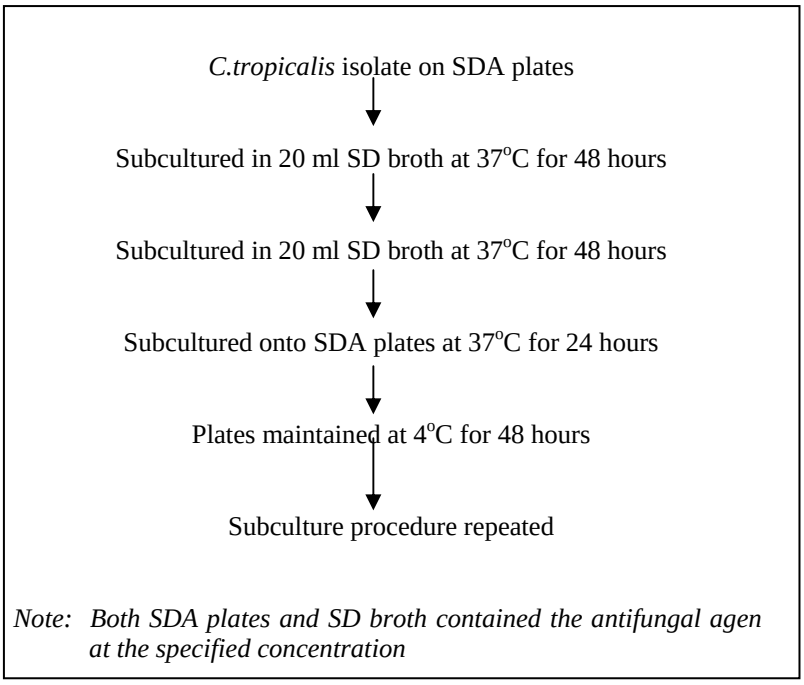

Figure 1: Method of subculture for each week throughout the experimental period

At the end of the exposure period (either 10 or 14 weeks), crude cytoplasmic protein extract of each isolate was obtained, based on the method by Manning and Mitchell (1980). Briefly, yeast cells from a 50 ml Sabouraud dextrose overnight broth culture were pelleted and washed twice in saline. The cells were resuspended in distilled water and 0.1 ml of 10 mM PMSF (phenylmethylsulfonyl fluoride) to achieve a final volume of 1 ml. Twice this volume of 0.45 mm glass beads (2ml) were added to the cell suspension and vortexed at a maximum speed for a total of 15-20 minutes to obtain adequate cell lysis.

Cells were maintained at 4°C throughout, by placing them in ice between short periods of vortexing. The suspension was centrifuged to separate cell debris from the supernatant which contained cytoplasmic proteins. This protein extract was obtained for each *C.tropicalis* isolate before and after exposure to the antifungal agent.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12.5% resolving gel and 5 % stacking gel in a discontinuous system as described by Laemmli (1970) in order to separate the proteins in each extract. Broad range protein molecular weight marker (Sigma, MO) was run in each gel. Gels were stained with Coomasie Blue and visualised to identify protein fractions.

RESULTS

The MIC values for the five *C.tropicalis* isolates at the start of the study were as shown in Table 1. Also shown in the table are the antifungal concentrations to which the isolates were exposed to in the study. The MIC values for the isolates during the period of drug exposure and at the end of this time are shown in Table 2. Also shown are the MIC values of the control isolates that were not exposed to antifungal agent, but subcultured for the same duration.

Table 1: MIC value for isolated prior to antifungal exposure

***C.tropicalis* isolate**	**MIC values (μg/ml)**		**Exposure to antifungal agent (μg/ml)**	
	Fluconazole	**Amphotericin B**	**Fluconazole**	**Amphotericin B**
1	1.0 (S)	4.0 (R)	0.5	-
2	1.0 (S)	2.0 (R)	0.5	-
3	64 (R)	1.0 (S)	-	0.5
4	128 (R)	1.0 (S)	-	0.5
5	256 (R)	0.38 (S)	-	0.25
C.parapsilosis ATCC 200219	2.0 (S)	0.5 (S)	-	-

Note: S=sensitive, R=resistant. Isolates 2 and 5 were tested with the Etest strip. The antifungal agent and concentration used for each isolate exposure are shown in the two columns on the right.

Table 2: MIC value for isolated after exposure and for control following subculture only, at different exposure periods

***C.tropicalis* isolate**	**Exposure drug**	**MIC values (μg/ml) after exposure**				
		2 weeks	**7 weeks**	**9 weeks**	**10 weeks**	**14 weeks**
1	Fluconazole	4	16	32	32 (I)	-
1 (control)	-	-	-	-	2 (S)	-
2	Fluconazole	-	-	-	8	64 (R)
2 (control)	-	-	-	-	1.0	2 (S)
3	Ampho B	1.0	2	4	4 (R)	-
3 (control)	-	-	-	-	2 (R)	-
4	Ampho B	1.0	2	4	4 (R)	-
4 (control)	-	-	-	-	1.0 (S)	-
5	Ampho B	-	-	-	1.0	8 (R)
5 (control)	-	-	-	-	0.5	1.0 (S)
C.parapsilosis ATCC 200219	-	-	-	-	2 (S) (fluconazole) 1.0 (S) (Ampho B)	-

Note: the end of the study period (10 or 14 weeks). Ampho B = amphotericin B, S = sensitive, I = intermediate, R = resistant.

Analysis of the protein extracts of the isolates and controls by SDS-PAGE revealed several differences. Isolates which had been exposed to either antifungal agent showed increased production of a 47 kiloDalton(kD) protein which was visualised as a darker, broader band on the gel. Isolate 5 which had been exposed to amphotericin B showed new protein bands of 74 and 77 kD.

DISCUSSION

In all of the isolates exposed to antifungal agent an increase in the MIC value was observed, compared to the initial value prior to exposure. For isolates exposed to fluconazole this increase was seen starting from 2 weeks after exposure and for isolates exposed to amphotericin B, increase in MIC was noted at 7 weeks into the study. The MIC values continued to rise for both antifungal agents until the end of the study. A slight increase in the MIC values for some control isolates was also observed, however this increase was much less than the increase for the test isolates.

The concentrations of antifungal agent used for exposure of isolates reflects achievable tissue concentrations of the drug in patients, although the cumulative drug effect could not be evaluated in this experiment. Studies on development of resistance have not been carried out on *C.tropicalis* although it is the second most common causative agent of candidiasis, after *C.albicans*. Hence, the results obtained in this study may be extrapolated to the patient situation. Our findings underline the need for sufficient treatment doses of antifungal agents, as a low dose may present a risk to the patient of resistance developing in the infecting *Candida* sp. This may be more pertinent when the species involved is already less susceptible to one antifungal agent (as was the case in this study).

Results from SDS-PAGE indicated an increase in the 47 kD protein in isolates exposed to antifungals. This protein is known to be a stress protein in *Candida* sp. (Matthews et al. 1988) , thus an increase in its production indicates that the presence of antifungals is a stressful situation for the organism. The new proteins (74 and 77kD) found in an isolate exposed to amphotericin B may also be other groups of stress proteins. The exact nature and identity of these proteins remain to be determined. However, these proteins may serve as a marker for resistance in *Candida* sp. Screening of more isolates from the different *Candida* sp. needs to be carried out before this can be determined.

CONCLUSION

Prolonged exposure to low doses of fluconazole and amphotericin B induces resistance in *C.tropicalis* in vitro. Isolates that were already resistant to one of these antifungal agents developed resistance to the other agent as well, when exposed to it. A change in the protein profile of the isolates exposed to antifungal agent was observed.

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