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## Susceptibility of *Candida* isolates to Amphotericin B and Imidazole: effect of CCCP on Imidazole activity

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**ABSTRACT:** Nine *Candida* strains (one *C. dubliniensis*, six *Candida albicans*, two *C. tropicalis*, and one *C. krusei*) isolated from human genital specimens were characterized based on their susceptibility to amphotericin B and imidazole (done by an adaptation of the National Clinical Laboratory Standards Broth Microdilution method). All the nine strains were susceptible to amphotericin B with minimum inhibitory concentrations varying from 0.25 to 1.0 µg/ml, and resistant to imidazole at 64 µg/ml drug concentration. Treatment of the strains with an efflux pump uncoupler (CCCP) significantly increased the killing in imidazole-treated cells, suggesting the presence of an efflux pump in imidazole resistance. These results support current evidence that amphotericin B is a more effective drug than imidazole (an azole), and that resistance of *Candida* strains to imidazole is related to the presence of efflux pump. It is suggested that CCCP effect be investigated in the resistance of *Candida* strains to other azoles.

**Keywords:** Amphotericin B, *Candida*, CCCP, efflux pump, imidazole, susceptibility.

### Introduction

*Candida* species are the most commonly isolated human opportunistic pathogens, especially in immunosuppressed individuals such as people infected with the Human Immuno-Deficiency Virus (HIV), those using in-dwelling catheters and those with prolonged chemotherapy [1, 2].

Cases of genitourinary fungal infections have increased in clinical practice [1, 2, 3]. They can lead to serious life threatening complications [4]. Early initiation of antifungal therapy is essential to reduce morbidity and mortality in patients at high risk. Such treatment strategies require rapid and specific diagnostic tests. The lack of rapid and sensitive diagnostic assays remains a limiting factor for effective antifungal therapy [5]. Accurate diagnosis begins with proper species identification. Antifungal susceptibility pattern of various *Candida* species have been studied previously [6, 7, 8]. Due to increased rates of reports of resistance of some *Candida* species to antifungal drugs, and continuous discovery of new strains of species, the determination of antifungal susceptibility pattern is a crucial part of accurate diagnosis.

This study was undertaken with the aim of providing further information on the characterization of *Candida* strains by their antifungal susceptibility pattern.

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## Materials and Methods

### *Candida* strains

Nine *Candida* isolates recovered from genital samples (high vaginal swabs and urethra swabs) at the University of Benin Teaching Hospital (UBTH), Benin City, Nigeria were maintained on Yeast Peptone Dextrose (YPD) Agar, YPDA. The nine *Candida* strains were identified at the Infectious Diseases Division of the Indian Institute of Chemical Biology (IICB), Kolkata, India, by their colony and cell morphology characteristics [9] and by their biochemical activity using the HiCandida Identification Kit (HiMedia, India). The isolates were identified as *C. dubliniensis* (isolate 1), *C. albicans* (isolates 2, 3, 5, 7, 8, and 9) and *C. tropicalis* (isolates 4, 6). Pure cultures were maintained on YPDA slants and stored at 0-4°C for further use.

### *Saccharomyces cerevisiae*

This strain used as control, was a standard laboratory strain donated by Dr. Anil Ghosh of the Department of Biotechnology, Indian Institute of Chemical Biology, Kolkata.

### Antifungal susceptibility tests

Determination of MIC: Amphotericin B (Fungizone, Invitrogen, Australia) was obtained as the 250 mg/ml stock while imidazole (1, 3-Diaza-2, 4-Cyclopentadiene) was as a standard salt (Sigma, Germany). Antibiotic Assay Medium 3 (HiMedia, India) was used. A modification of the broth macrodilution method of NCCLS [10] was employed. Each *Candida* isolate was grown overnight and diluted to  $0.5 \times 10^2$  -  $2.5 \times 10^3$  cfu/ml concentration with sterile distilled water. Amphotericin B, AMB (250 mg/ml) was dispensed in volumes equivalent to 1-64 µg/ml into tubes containing 1ml inoculation. Volume of reactants was made up to 2ml with Antibiotic Assay Medium. Tubes were in duplicates and incubated at 37°C with shaking, for 48 hours. Three sets of tubes (one without drug, the second without *Candida*) and the third with *S. cerevisiae*, served as control. Test cultures were visually observed and with a spectrophotometer (at 490nm). Minimum inhibitory concentration (MIC) was taken as lowest concentration which gave an optically clear culture.

For imidazole (IMI), cells were grown and cultures diluted as described for AMB. Imidazole was dissolved in 100% dimethylsulphide (DMSO) and serial 2-fold dilutions (1-64 µg/ml) were made with distilled water. Final concentration of DMSO in culture mixture was 0.05%. To 1ml of DMSO in a tube 1ml culture was added. Other conditions and procedures were as for AMB.

Time-Kill for AMB was done using the method of Lewis *et al.* [11]. Briefly, 1ml of 0.5 µg/ml AMB solution was added to 1ml overnight yeast peptone broth culture of *Candida* grown at 37°C under aeration (final concentration =  $0.5 \times 10^2$  -  $2.5 \times 10^3$  cfu/ml) in duplicate assay tubes. Tubes containing 1 ml of culture of each species as described above but without drug, and volume made up to 2ml with sterile distilled water, served as control. Experiment was done twice. The colony-forming unit (cfu) at predetermined time-points of 6 and 24 hours of drug treatment was determined by spreading 0.5ml of the culture on YPDA and counting the colonies after 48 hours incubation at 37°C. The MIC of AMB was taken as the lowest drug concentration that completely inhibited growth. Chi-square was used to test the difference between cfu of drug - treated cultures and control.

### Treatment with CCCP

Liquid cultures of the nine *Candida* isolates prepared as described above were diluted to a starting inoculum of  $0.5 \times 10^2$  -  $2.5 \times 10^3$  cfu/ml, and five minutes after adding 64 µg/ml of IMI as before, 5ml CCCP (carbonyl cyanide *p*-chlorophenylhydrazone) was added to a final concentration of 100mM and tubes incubated for 3 hours at 30°C under aerobic conditions. The control tubes did not contain CCCP, and all tubes were in duplicates. After incubation, the optical density of each culture was read at 490nm.

## Results

The nine *Candida* isolates were sensitive to AMB, with MIC values ranging between 0.25-1.0 µg/ml (Table 1). At 0.25-64 µg/ml IMI concentration, all the nine strains were resistant. Time-Kill results showed that all the strains had over 90% killing after 6 hours exposure to AMB (Fig. 1). There was no significant difference between killing at 6 hours and 24 hours treatment ( $p=0.05$ ). The percentage reductions in cell growth for IMI-treated cells were 3.1, 12.9, 7.9, 2.9, 1.6, 4.3, 16.6, 7.0 and 29.6 respectively, for isolates 1-9 (Fig. 2). Treatment with CCCP increased the killing rate of IMI many-fold (98.5, 71.0, 95.6, 100, 75.3, 95.1, 64.3, 45.3, and 91.0)

for isolates 1-9 respectively. The CCCP effect was most effective in isolate number 4 (100%) followed by isolate number 1 (98.5%).

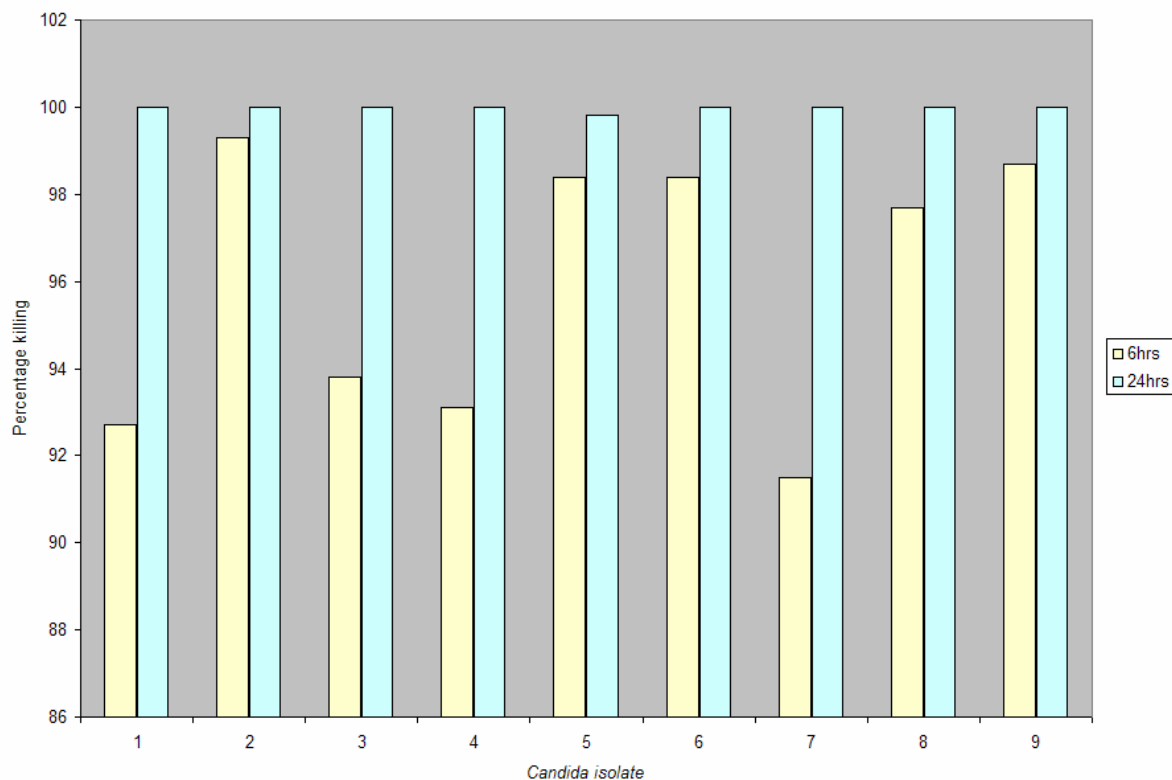


Figure 1: Time Kill (%) of *Candida* cells treated with 0.5µg/ml Amphotericin B at 37°C.

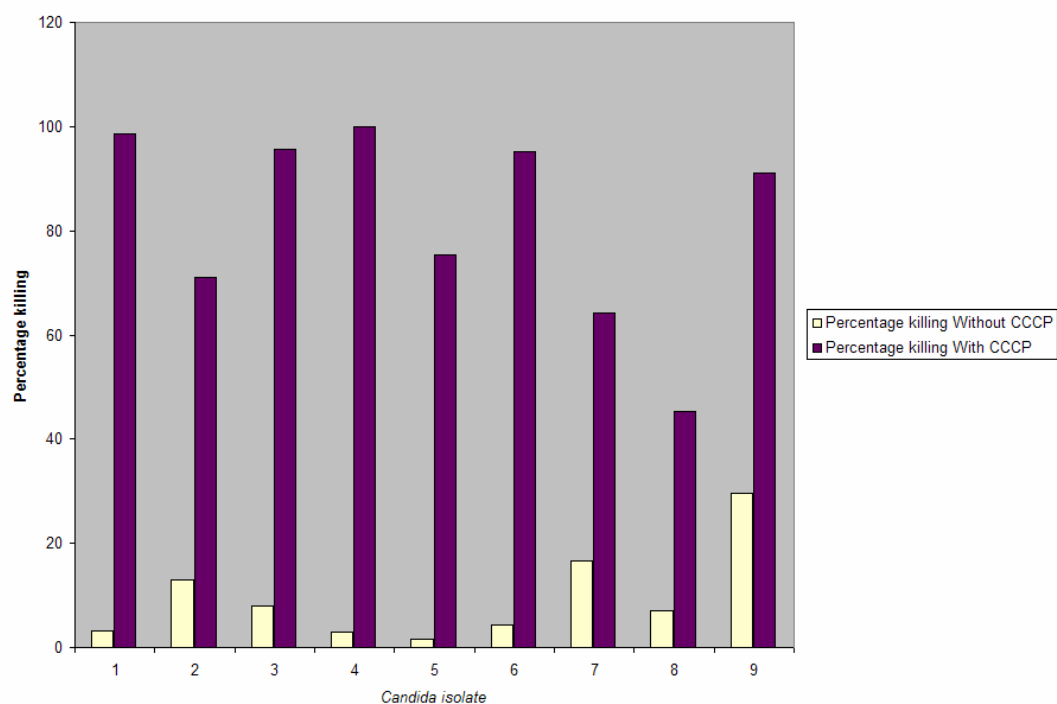


Figure 2: Effect of CCCP on percentage killing of *Candida* cells by 64µg/ml imidazole at 37°C .

Table 1: Susceptibility of *Candida* isolates to Amphotericin B and Imidazole.

Candida Isolate	MIC ( $\mu\text{g/ml}$ )	
	Amphotericin B	Imidazole
1.	1.0	> 64
2.	0.25	> 64
3.	1.0	> 64
4.	1.0	> 64
5.	1.0	> 64
6.	1.0	> 64
7.	1.0	> 64
8.	1.0	> 64
9.	1.0	> 64
<i>S. cerevisiae</i>	0.25	2.0

## Discussion

The MICs of AMB were low and in agreement with previous findings [11,12]. Amphotericin B is still one of the drugs of choice for many fungal diseases, but the adverse side effects associated with its use have limited the use of the drug in clinical practice [11, 13]. It also has a shorter time-course of activity than the azoles, to which IMI belongs [11]. The AMB MICs of the *C. albicans* strains (2, 3, 5, 8, 9, and 10) were similar except strain number 2 which had a relatively lower value (0.25 $\mu\text{g}$ ). The generally low MICs for all the isolates justify the popularity of AMB as an antifungal drug for treating many cases of mycoses. On the other hand, IMI is an old azole that has become less frequently used due to treatment failure and has been replaced with more potent so-called second-generation triazoles, such as fluconazole, voriconazole and posaconazole [14]. Several workers have reported cases of resistance of *Candida* species to azoles [12,15,16,17]. It is not surprising, therefore, that all the *Candida* isolates reported here were resistant to imidazole even at 64 $\mu\text{g/ml}$ . There is no information on the MIC interpretation for IMI but for fluconazole which is a newer azole than IMI, MIC greater than 64 $\mu\text{g/ml}$  is regarded as resistance [18].

Evidence exists that suggests active efflux as an important mechanism for the azole antifungals [19,20]. Student *t*-test at 95% probability showed significant differences between values before and after treatment with CCCP. An uncoupler of one of the efflux systems (ATP-binding cassette), CCCP is present in fungi. Those isolates in which there was 100% killing by IMI after the addition of CCCP (isolates 4 and 6) appear to have their resistance to IMI solely dependent on efflux pump(s), whereas in others, to varying degrees, the pump does not seem to be the only mechanism in agreement with previous report [20]. The isolates with the least killing when treated with IMI alone (1, 4, 5) were also the ones with the highest killing on addition of CCCP (with the exception of isolate number 9) indicating that active efflux is the main mechanism of resistance to the drug. Active efflux leads to reduced accumulation of drug in the cell, resulting in sub-lethal dose for the pathogen [8, 17, 20]. The significant variation in killing among the *C. albicans* strains (the highest number of same species) shows the diversity in these strains and suggests the presence of other resistance mechanisms. It also corroborates the assertion that many *Candida* strains have developed resistance to IMI.

The results from this study have shown that *Candida* species are diverse in antifungal susceptibility and as a result, susceptibility patterns for *Candida* should not be generalized but determined for individual strains. They also show that efflux pump is involved in the resistance of *Candida* species to IMI. The action of efflux pump in the resistance of *Candida* to other azoles should be investigated.

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