

Original Research Article

## In Vitro Susceptibility of *Malassezia Furfur* to Azoles

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### ABSTRACT

The lipophilic yeast *Malassezia furfur* and related species are members of the normal cutaneous microflora of humans and other warm blooded animals. It is also considered a facultative pathogen as associated with clinical disease including invasive infections under conditions that permit massive growth of the fungus. However, till now no standardized in vitro susceptibility testing method and minimum inhibitory concentration (MIC) breakpoints for *Malassezia* species is available. Here we studied the in vitro activity of fluconazole (FCZ), ketoconazole (KTZ), itraconazole (ITZ), voriconazole (VCZ) against one of the major pathogenic *Malassezia* species, *M. furfur*. We used Tween supplemented Christensen's urea broth to determine the minimum inhibitory concentration (MIC) of ketoconazole, fluconazole, itraconazole and voriconazole against 107 strains of *M. furfur* following CLSI M27-A3 protocol. *Malassezia furfur* reference strains, *Candida albicans* ATCC 90028 and *Candida krusei* ATCC 6578 were used as controls. Susceptibility to antifungal agents showed high endpoints (MIC<sub>90S</sub>, 1 to 16µg/ml) for *M. furfur*. Ketoconazole, itraconazole and voriconazole displayed lower MIC than fluconazole. Ketoconazole was found to be the most effective drug against *M. furfur* from pityriasis versicolor lesions. Modified Christensen's Urea Broth might be suitable for AST of *Malassezia*. However, this method still requires optimization of the testing conditions and standardization.

**Key words:** *Malassezia furfur*, Antifungal susceptibility, azoles

### INTRODUCTION

*Malassezia* is a basidiomycetous yeast and have been recognized for more than 150 years as members of the human cutaneous flora and etiologic agents of certain skin diseases. <sup>(1,2)</sup> The genus includes 14 lipophilic species, with *M. furfur*, *M. sympodialis*, *M. globosa* and *M. restricta* being the major pathogenic fungi causing many dermatological disorders like pityriasis versicolor, seborrhoeic dermatitis, dandruff, atopic eczema and folliculitis in immunocompetent patients.

<sup>(3-6)</sup> *Malassezia* yeasts are unique as they comprise almost exclusively the single eukaryotic member of the microbial flora of the skin. <sup>(1)</sup>

One unusual feature of this fungus is that, it is absolutely dependent on externally provided lipids which the fungus hydrolyses by lipolytic activity to release fatty acids necessary for both growth and pathogenicity. Moreover, *Malassezia* do not grow on a standard fungal culture medium, and they require specialized media supplemented with fatty

acids such as Leeming-Notman agar (LNA), Modified Dixon agar (MDA) or Littman agar (LA) for their growth. (7,8) The unstable morphological variations and strict lipid dependency lead to difficulty in growing this fungus, thereby limiting study on this fungus. (9-11) Because of its fastidious growth behaviour, the Clinical Laboratory Standard Institute (CLSI) recommended broth microdilution method (BMD) for yeast is not applicable to them and in vitro antifungal susceptibility test (AST) for *Malassezia* yeasts is still a problem.

Various workers have evaluated different methods of antifungal susceptibility tests for *Malassezia furfur* with different modifications of the CLSI broth microdilution (BMD) technique, using different culture media. Significant variations in MIC ranges have been observed in these studies, depending on the medium used, incubation temperature and duration of incubation, eventually resulting in wide variation in the susceptibility classification. (12) As *M. furfur* was found to be the most frequent isolate (72.8%) from the lesional areas of pityriasis versicolor patients in our study, in vitro antifungal susceptibility testing of the *M. furfur* was considered for selection of the appropriate antifungal agent for treatment of the cases.

In vitro antifungal susceptibility tests for *Malassezia* yeasts still require standardization. We conducted this study to evaluate the in vitro susceptibility of *Malassezia furfur* using the modified Christensen's Urea Broth (CUB) medium following CLSI BMD protocol (M27-A3) to the azoles ketoconazole (KTZ), fluconazole (FLZ), itraconazole (ITZ) and voriconazole (VCZ).

## **MATERIALS AND METHODS**

Before conducting the study, approval from the Institutional Ethics Committee was obtained. A total of 107 *M. furfur* strains isolated from pityriasis versicolor (PV) patients were included in

the study. An informed written consent was taken from every patient enrolled in the study. The isolates were identified by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of 26SrDNA region followed by sequencing. (13,14) Two reference *Malassezia furfur* strains, quality control strains *Candida albicans* ATCC 90028 and *Candida krusei* ATCC 6258 was tested as controls. All the *Malassezia* species were positive for urease activity when grown in modified CUB medium.

*Malassezia* yeasts were grown on modified Leeming Notman agar medium (LNA) (1% Bacteriological Peptone, 1% glucose, 2% yeast extract, 8% desiccated ox bile, 1.5% agar (all from HiMedia, Mumbai, India), 1% glycerol, 0.5% Tween 60, 2% olive oil [all from Sigma-Aldrich, USA), 0.05% glycerol monostearate (Central Drug House-CDH, New Delhi, India), and incubated for 72 hours ( $32^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ). Uniform suspensions of the yeasts were prepared in sterile distilled water with 0.04% Tween 80 (Sigma-Aldrich, USA). Inoculum suspensions ( $10^6$  CFU/ml) were adjusted by using spectrophotometer (Spectrascan UV2700, Thermo-Scientific, USA) to an absorbance of 0.425 to 0.435. (15) Stock suspensions of ketoconazole, fluconazole, itraconazole and voriconazole (all from Sigma-Aldrich, USA), were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and gradually diluted with distilled water. The final drug concentrations in each medium were 0.0313 to 16  $\mu\text{g/ml}$  for all antifungals; for FLC, drug concentrations ranged from 0.125 to 64  $\mu\text{g/ml}$ . The urea broth microdilution method of susceptibility testing was performed in 96-well microtiter plates (HiMedia, Mumbai, India). Modified Christensen's urea broth (100  $\mu\text{l}$ ), fungal suspension (50 $\mu\text{l}$ ), and the test drugs (50 $\mu\text{l}$ ) were added to these wells. The microtiter plates were arranged in twofold dilution from left to right and in triplicate and were incubated at  $32^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for 72 hours. The growth control wells

contain 100µl of sterile drug-free medium and are inoculated with 100µl of the corresponding diluted (2x) inoculum suspensions. The quality control (QC) organisms *Candida albicans* ATCC 90028 and *Candida krusei* ATCC 6258 were tested in the same manner and were included in each assay. Row 12 of the microdilution plate was used to perform the sterility control (drug-free medium only). The results were measured by an automatic microtitre plate reader. The final mean OD obtained for each antifungal concentration was expressed as a percentage of the control growth. For azoles, the MIC endpoints of the antifungal agents were defined as the lowest drug concentrations that showed an optical density of  $\leq 50\%$  (Multiskan EX, Thermo-Scientific, 450nm filter) of that of the (drug-free) growth control. Triplicate assays were conducted with all antifungals and results were reproducible (within one to two dilutions). Data were reported as MIC ranges, MICs where 50% and 90% of the isolates were inhibited (MIC<sub>50</sub> and MIC<sub>90</sub>), MIC mode and geometric mean. The results were compared with the triplicate MICs obtained with the reference strains. [15-18] *M. furfur* (Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, MTCC1374) and *M. furfur* CBS1878 (Centraalbureau Schimmelcultures-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands), were used as reference strains.

## RESULTS

Antifungal susceptibility test for *Malassezia furfur* was performed using modified Christensen's urea and CLSI

broth microdilution method. MICs of each drug against *M. furfur* could be recorded after 72 hours of incubation at 32°C. For the quality control strains, the MIC of the antifungals were within the expected range. (17,18) (Table 1) Comparison of MIC ranges of the azoles against *M. furfur* reference strains and the 107 clinical isolates are summarized in Table 1. Comparison of geometric mean, MIC mode, MIC<sub>50</sub> and MIC<sub>90</sub> values obtained for the azoles included in this study against the 107 *M. furfur* clinical isolates are presented in Figure 1. ITZ and VCZ MICs 1 µg/ml was observed for 90% of the *M. furfur* (MIC<sub>90</sub> 1 µg/ml) and a lower MIC 0.5 µg/ml was shown by 90% of *M. furfur* strains for KTZ (MIC<sub>90</sub> 0.5 µg/ml). FCZ MIC was higher than those observed for other azole drugs and ranged from  $\leq 0.125$  to  $>8$  µg/ml for *M. furfur*. *M. furfur* showed the highest geometric mean, MIC mode, MIC<sub>50</sub>, and MIC<sub>90</sub> values for FCZ. The triplicate MICs obtained with the 107 isolates evaluated with each antifungal agent showed that, MICs were reproducible (within 1 to 2 dilutions) (Table 1), including consistently high MICs for fluconazole.

In this study, according to the susceptibility to the antifungal agents, (which is based on the MIC<sub>90</sub>s, MIC at which 90% of the isolates tested are inhibited), the ketoconazole was found to be the most effective drug against *M. furfur*. In this study, a good correlation between the number of fungal cells and OD values was observed. Every result obtained using this modified Christensen's urea broth microdilution method demonstrated good reproducibility.

Table 1: Comparison of MIC ranges of different azoles against *M. furfur* reference strains and clinical isolates

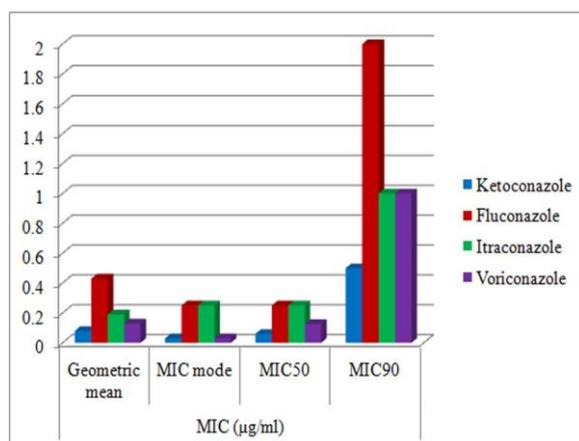
Azoles	MIC ranges (µg/ml)		
	<i>M. furfur</i> CBS1878	<i>M. furfur</i> MTCC1374	<i>M. furfur</i> Clinical isolates
Ketoconazole	0.125-0.5	0.125-0.25	$\leq 0.03-1$
Fluconazole	0.5-1	0.5-1	0.125-2
Itraconazole	0.125-0.25	0.5-1	$\leq 0.03-1$
Voriconazole	0.125-0.5	0.125-0.25	$\leq 0.03-1$

MIC: Minimum inhibitory concentration

**Table 2: Summary of reports on antifungal susceptibility of *Malassezia furfur* against azoles**

Reference no.	Author (Year)	Method	Culture medium	MIC range			
				KTZ	FLZ	ITZ	VCZ
20	Marcon <i>et al.</i> (1987)	Broth microdilution	Buffered YNB or Casitone -yeast extract-glucose media, both supplemented with 0.5% Tween 80	0.025-0.05µg/ml	-	-	-
19	Ahn <i>et al.</i> (1996)	Agar dilution	LNA	0.03-0.06µg/ml	-	0.03-0.12µg/ml	-
21	Schmidt <i>et al.</i> (1996)	Broth microdilution	Modified LNA with almar blue	<0.06-0.12µg/ml	-	-	-
22	Strippoli <i>et al.</i> (1997)	Broth microdilution	Yeast extract olive oil glucose broth	-	0.07-25mg/l	0.05-3.12mg/l	0.11-3.12mg/l
27	Gupta <i>et al.</i> (2000)	Agar dilution	Diagnostic sensitivity test medium	≤0.03-0.12µg/ml	-	≤0.03-0.12µg/ml	≤0.03-0.12µg/ml
28	Hammer <i>et al.</i> (2000)	Broth microdilution	Medium A broth	0.06-0.25µg/ml	-	-	-
16	Nakamura <i>et al.</i> (2000)	Broth microdilution	Modified CUB	-	-	3.2-25µg/ml	-
25	Garau <i>et al.</i> (2003)	Broth microdilution	LNA with almar blue	≤0.03µg/ml	2-4µg/ml	≤0.03-0.06µg/ml	≤0.03-0.12µg/ml
24	Velegraki <i>et al.</i> (2004)	Broth microdilution	Fatty acid RPMI1640	0.03-1µg/ml	0.05-32µg/ml	0.03-0.06µg/ml	0.03-16µg/ml
15	Rincon <i>et al.</i> (2006)	Broth microdilution	Modified CUB	0.03-1µg/ml	-	0.03-0.5µg/ml	0.03-1µg/ml
30	Faergmann <i>et al.</i> (2006)	Agar dilution	Diagnostic sensitivity test medium	0.05µg/ml	-	-	-
31	Tiwari <i>et al.</i> (2011)	Broth microdilution	RPMI1640	6.438µg/ml	10.538µg/ml	-	-
26	Rojas <i>et al.</i> (2013)	Broth microdilution	RPMI1640 with Tween and other supplement	≤0.03-0.25µg/ml	<0.12->64µg/ml	≤0.03-0.5µg/ml	≤0.03-0.12µg/ml
23	Latta <i>et al.</i> (2014)	Broth microdilution	CUB, SD broth, RPMI	-	2->128µg/ml*	0.03->0.25µg/ml*	0.03-1µg/ml*

MIC: Minimum inhibitory concentration; KTZ: ketoconazole; FCZ: Fluconazole; ITZ: Itraconazole; VCZ: Voriconazole  
 YNB: Yeast nitrogen base; LNA: Leeming-Notman agar; CUB: Christensen's urea broth; SD broth: Sabouraud's dextrose broth



**Figure 1: Comparison of antifungal activity of azoles against *M. furfur***

## DISCUSSION

Various studies have reported numerous antifungal susceptibility profiles for *M. furfur*.<sup>(19-23)</sup> In this study, low MIC (≤1µg/ml) was observed for ketoconazole,

itraconazole and voriconazole and comparatively a higher MIC range (0.125-2µg/ml) and higher endpoint (MIC<sub>90</sub> 2µg/ml) was observed for fluconazole (Table 1). These findings are similar to other reports, where a higher MIC ranges and high MIC endpoints have been reported in comparison to other azoles.<sup>(23-26)</sup>

Voriconazole MICs have been similar (≤0.03 to 1µg/ml) to other studies reported by Rincon *et al.* and Latta *et al.*<sup>(15,23)</sup> But there are lot of variations observed in other studies. These contradictory results may have resulted from use of non-standardized methodologies with either ≤10 isolates per species<sup>(16,24,27-29)</sup> or testing with a much lower inoculum<sup>(24)</sup> or by an agar dilution method.<sup>(29)</sup> The differences in the methodology, culture medium, inoculum

size, incubation time, and the criteria used to determine MIC endpoints adopted in these studies, limit comparison of the study results. However, a summary of the methodology, culture medium and MIC ranges reported in other studies on antifungal susceptibility profile of *M. furfur* are presented in Table 2.

The overall data shows that, various modifications of the CLSI M27-A3 protocol have been done for determination of MIC for *M. furfur* and other species as the this protocol is not applicable to most of the *Malassezia* species. In all the papers, the methodology has been altered from the original CLSI protocols in order to overcome four main problems involved in testing this yeast: a) finding a suitable growth medium specially for the lipophilic species of *Malassezia* b) increasing the inoculum size to counteract the slower growth rate of *Malassezia* compared to that of *Candida* species; c) increasing the incubation time to 72 hours, again to counteract the slower growth rate of *Malassezia* compared to that of *Candida* species; d) altering the definition of the MIC end point. Many authors refer to the MIC breakpoint as the level of 50% inhibition of growth, whilst others use a 90% or 100% inhibition level. When testing the azole group of antifungal agents, there is also the well known trailing end point problem with MIC determination. Trailing occurs when the turbidity continually decreases as the drug concentration increases but the suspension fails to become optically clear (partial inhibition of growth over an extended range of antifungal concentrations).<sup>(30)</sup>

In conclusion, based on the findings described here, modified Christensen's urea broth with 0.1% Tween 80 and 0.5% Tween 40 is a suitable culture medium in CLSI broth microdilution method to determine the antifungal susceptibility of *Malassezia furfur* to azole drugs. However, the effectiveness of CUB for antifungal susceptibility testing of

azoles requires further studies for determination of optimum testing conditions like standardization of inoculum suspension, incubation temperature and time for CLSI broth microdilution. Further collaborative studies are essential to investigate the inter-laboratory reproducibility of this method.

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