In vitro antifungal susceptibility of Malassezia isolates from pityriasis versicolor lesions

Sir,

Pityriasis versicolor is the only human disease for which *Malassezia* has been fully established as a pathogen. The genus *Malassezia* includes 15 lipophilic species with the recent addition of one new species *"Malassezia arunalokei"*. Traditionally, *Malassezia furfur*, *Malassezia sympodialis*, *Malassezia globosa* and *Malassezia restricta* have been considered the major pathogenic species implicated in dermatological disorders.[1,2] Since *Malassezia* species are a part of the normal flora of skin, it is impossible to eradicate them permanently by topical and systemic antifungals resulting in relapses in predisposed individuals. Antifungal susceptibility testing is warranted for *Malassezia* yeasts, as they are implicated in both cutaneous and invasive infections in humans.

Because of the lipophilic nature, antifungal susceptibility testing of *Malassezia* yeasts is still a problem and hence, little work has been published on the *in vitro* susceptibilities of *Malassezia* to various antifungal agents. Various workers have evaluated the antifungal susceptibility of *Malassezia* employing modified Clinical Laboratory Standard Institute (CLSI) broth microdilution technique, using different culture media. These studies have reported significant variations in minimum inhibitory concentrations resulting in erroneous susceptibility classification. Hence, the present study aimed at the evaluation of *in vitro* susceptibility of *Malassezia* species to amphotericin B, ketoconazole, fluconazole, itraconazole and voriconazole by Clinical Laboratory Standard Institute (CLSI) protocol M27-A3 using modified Christensen's urea broth.[3,4]

During the period 2012-2015, in the Department of Microbiology, Gauhati Medical college, Assam, *Malassezia* species were isolated from 290 patients with pityriasis versicolor and identified as *M furfur* (241), *M. globosa* (27), *M. restricta* (8), *M. obtusa* (7), *M. sympodialis* (5), *M. slooffiae* (1) and *M. japonica* (1) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of 26SrDNA region followed by sequencing.[5] Reference strains of *Malassezia* (*M. furfur* Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, MTCC1374, *M. globosa* Centraalbureau Schimmelcultures-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands CBS7886, *M. restricta* CBS7877, *M. japonica* CBS9432, *M. slooffiae* CBS7956 and *M. pachydermatis* MTCC1369) and quality control strains *C. albicans* ATCC (American Type Culture Collection) 90028 and *C. krusei* (American Type Culture Collection) 6258 were tested as controls.

The concentration of the yeast suspensions (10^6 cfu/ml) were adjusted by spectrophotometer.[4] Stock suspensions of amphotericin B, ketoconazole, fluconazole, itraconazole and voriconazole (Sigma-Aldrich, USA), were prepared in dimethyl sulfoxide. The different drug concentrations varied between 0.125-64 μg/ml for fluconazole and between 0.0313-16 μg/ml for all other antifungals. Antifungal susceptibility testing was performed in 96-well microtiter plates and cultures were incubated at 32°C ± 2°C for 96 hours for *M. globosa* and *M. restricta* and 72 hours for other species.[3,4] The final mean optical density obtained for each antifungal concentration was expressed as percentage of growth control. For azoles, the minimum inhibitory concentration endpoints of the antifungals were defined as the lowest drug concentrations that showed an optical density of ≤50% of that of the (drug-free) growth control. For amphotericin B, minimum inhibitory concentration endpoint was defined as the lowest concentration that completely inhibited growth.[4]

Table 1 summarizes the minimum inhibitory concentration (range, geometric mean & mode) and minimum inhibitory concentrations where 50% and 90% of the isolates were inhibited (MIC_{50} and MIC_{90}) obtained for the antifungal drugs. The minimum inhibitory concentration of ketoconazole, itraconazole and voriconazole was 1 μg/ml for 90% of the *M. furfur* and *M. globosa* isolates; however, the minimum inhibitory concentration of amphotericin B for *M. furfur* was higher than that for *M. globosa* (1 μg/ml versus 0.5 μg/ml). Fluconazole minimum inhibitory concentrations were higher than other azoles and ranged from ≤0.12 to >64 μg/ml for *M. furfur,* ≤0.12 to 8 μg/ml for *M. globosa*

Table 1: Minimum inhibitory concentration (MIC) ranges, geometric mean, mode, MIC50, and MIC_{90} obtained by broth microdilution method for 290 *Malassezia* isolates

Antifungal agents	MIC range (µg/ml)	Geometric mean	MIC Mode	MIC_{50}	MIC_{90}
Amphotericin B					
M. furfur[241]	≤0.03-16	0.19	0.06	0.12	1
M. globosa[27]	≤0.03-0.5	0.09	0.03	0.12	0.5
M. restricta[8]	≤0.03-4	0.24	NA[b]	0.12	4
M. obtusa[7]	≤0.03-0.12	0.07	0.12	0.12	0.12
M. sympodialis[5]	≤0.03-0.5	0.05	0.03	0.03	0.25
M. slooffiae[1]	0.06	NA[a]	NA[a]	NA[a]	NA[a]
M. japonica[1]	0.5	NA[a]	NA[a]	NA[a]	NA[a]
Ketoconazole					
M. furfur[241]	≤0.03-8	0.15	0.03	0.12	1
M. globosa[27]	≤0.03-1	0.14	0.03	0.03	1
M. restricta[8]	0.06-0.25	0.14	0.25	0.25	0.25
M. obtusa[7]	≤0.03-0.12	0.07	0.12	0.12	0.12
M. sympodialis[5]	≤0.03-0.25	0.05	0.12	0.12	0.25
M. slooffiae[1]	0.5	NA[a]	NA[a]	NA[a]	NA[a]
M. japonica[1]	0.5	NA[a]	NA[a]	NA[a]	NA[a]
Fluconazole					
M. furfur[241]	≤0.12->64	0.84	0.25	0.5	16
M. globosa[27]	≤0.12-8	0.85	2	1	4
M. restricta[8]	≤0.12-2	0.37	0.12	0.25	2
M. obtusa[7]	≤0.25-8	1.2	NA[b]	1	8
M. sympodialis[5]	≤0.12-1	0.05	0.12	0.12	0.25
M. slooffiae[1]	1	NA[a]	NA[a]	NA[a]	NA[a]
M. japonica[1]	1	NA[a]	NA[a]	NA[a]	NA[a]
Itraconazole					
M. furfur[241]	≤0.03-16	0.21	0.03	0.25	1
M. globosa[27]	≤0.03-8	0.36	1	0.5	1
M. restricta[8]	≤0.03-1	0.14	NA[b]	0.12	1
M. obtusa[7]	≤0.03-0.5	0.15	NA[b]	0.25	0.5
M. sympodialis[5]	≤0.03-1	0.05	NA[b]	0.06	1
M. slooffiae[1]	0.25	NA[a]	NA[a]	NA[a]	NA[a]
M. japonica[1]	≤0.03	NA[a]	NA[a]	NA[a]	NA[a]
Voriconazole					
M. furfur[241]	≤0.03-16	0.19	0.03	0.25	1
M. globosa[27]	≤0.03-8	0.22	0.25	0.25	1
M. restricta[8]	0.06-8	0.86	1	1	8
M. obtusa[7]	0.06-0.5	0.12	0.06	0.06	0.5
M. sympodialis[5]	≤0.03-0.25	0.05	0.03	0.03	0.25
M. slooffiae[1]	0.5	NA[a]	NA[a]	NA[a]	NA[a]
M. japonica[1]	0.06	NA[a]	NA[a]	NA[a]	NA[a]

n=No. of isolates MIC: Minimum inhibitory concentration, MIC_{50} and MIC_{90}: MIC values that indicate 50% and 90% of the isolates were inhibited, NA[a]: Not applicable, single isolate NA[b]: Not applicable, all strains had different MIC value

and *M. restricta. M. globosa* showed higher minimum inhibitory concentration ranges to all the azoles. The geometric mean and mode for all drugs tested were higher for *M. furfur, M. globosa* and *M. restricta* especially for fluconazole, itraconazole and voriconazole.

In this study, the *Malassezia* species could be divided into two groups. *M. sympodialis, M. obtusa, M. slooffiae* and *M. japonica* were more susceptible to antifungals while *M. furfur, M. globosa* and *M. restricta* comprised the less susceptible group. Every result obtained using this method demonstrated good reproducibility. The overall data shows that, the non-applicability of Clinical Laboratory Standard Institute M27-A3 protocol for *Malassezia* species has resulted in variations in methodologies for minimum inhibitory concentration determination with limited inter laboratory agreement. The original Clinical Laboratory Standard Institute protocol has been modified by using a more suitable growth medium, increasing the inoculum size to counteract the slower growth of *Malassezia,* increasing incubation time and altering the definition of the minimum inhibitory concentration end point.

In conclusion, modified Christensen's urea broth may be used for antifungal susceptibility testing of *Malassezia* with optimum testing conditions such as standardization of inocula, incubation temperature and time.

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Conflicts of interest

There are no conflicts of interest.

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