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ANTIFUNGAL SUSCEPTIBILITY OF *MALASSEZIA SPECIES* ISOLATED FROM PITYRIASIS VERSICOLOR CASES

Remya V. S.*[1], Arun B.[2], Sarala Devi[3], Deepak K. S.[4] and Philip George Theckel[5]

[1]Department of Microbiology, Pariyaram Medical College, Kannur, Kerala, India.

[2]School of Health Sciences, Kannur University, Kannur, Kerala, India.

[3]Research Guide, Kannur University, Kannur, Kerala, India.

[4]Department of Community Medicine, Govt. Medical College, Palakkad, Kerala, India.

[5]Electron Microscope Unit, Faculty of Medicine, Kuwait University, Kuwait.

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***Corresponding Author**
Remya V. S.
Department of Microbiology, Pariyaram Medical College, Kannur, Kerala, India.

ABSTRACT

Objective: To evaluate the *in vitro* antifungal susceptibility of *Malassezia* spp isolated from PV cases against three commonly used antifungal agents, ketoconazole, itraconazole and fluconazole. **Methods**: Antifungal susceptibility was assessed using CLSI (Clinical and Laboratory Standards Institute) broth micro dilution M27-A3 protocol (CLSI 2008) with fatty acid RPMI 1640**. Result**: All the tested *Malassezia* spp produced detectable growth after the incubation. The overall MICs range for fluconazole, itraconazole and ketoconazole were ≤ 0.125 – 16µg/mL, ≤ 0.03 – 1µg/mL and ≤ 0.03 – 8µg/mL respectively. **Conclusion**: High MICs and variability displayed in the antifungal activity of different antifungal drugs with inter-species variations of isolates from PV evinced the importance of performing the *in vitro* susceptibility of each species to capture reliable information for determining an effective treatment regimen.

KEYWORDS: Pityriasis versicolor (PV), *Malassezia*, Antifungal susceptibility testing.

INTRODUCTION

Pityriasis versicolor (PV) is a chronic superficial fungal disease of the stratum corneum etiologically associated with the *Malassezia* spp.[1] Although PV had been described in the beginning of nineteenth century,[1] obstacles in culturing of *Malassezia* spp *in vitro* due to its inherent lipophilic nature had delayed the determination of the taxonomic status.[2] Currently, 14 species are accommodated in the genus *Malassezia* namely *M. furfur*, *M. sympodialis*, *M.*

globosa, *M. obtusa*, *M. slooffiae*, *M. restricta*, *M. pachydermatis*, *M. dermatis*, *M. japonica*, *M. yamatoensis*, *M. nana*, *M. caprae*, *M. equina* and *M. cuniculi*. *M. nana*, *M. caprae*, *M. equina*, and *M. cuniculi* were non-human animal species that have never been isolated from humans.[3]

PV is relatively easy to cure but frequently recurs despite of adequate treatment, therefore posing considerable therapeutic challenges to practicing dermatologists. In predisposed individuals, the fungus is a part of normal flora that resides deep in hair follicles and is impossible to eradicate permanently. As a consequence, relapses may occur sooner or later.[4] The disease can be cured by using topical antifungal agents like ketoconazole and some patients who do not respond or experience multiple relapses may require systemic antifungal treatment with fluconazole or itraconazole. Hitherto, several methods are available for doing antifungal susceptibility of *Malassezia* spp *in vitro*[5-10] Only limited data was available on the susceptibility of *Malassezia* spp against topical imidazole and oral azole derivatives.[5]

MATERIALS AND METHODS

In vitro antifungal susceptibility testing was done against 247 *Malassezia* species isolated from clinically suspected PV patients attending the out patients clinics of dermatology department of a tertiary care hospital in north Kerala. All strains were identified by standard methods, which included, by morphological, physiological and biochemical tests as described by the Guillot *et al.*[11] Mayser *et al.*[12], comprising of *M. globosa* (n=82), *M. furfur* (n=76), *M. restricta* (n=63) *M. sympodialis*, (n=17), *M. obtusa* (n=5) *M. slooffiae* (n=3) and *pachydermatis* (n=1) as per CLSI (Clinical and Laboratory Standards Institute) broth micro dilution M27-A3 protocol (CLSI 2008) with fatty acid RPMI 1640 medium buffered to pH 7 with 0.165 M Morpholine propane sulphonic acid (MOPS) buffer with glucose (20 g/L), ox bile (4 g/L), glycerol monostearate (0.5 g/L), 2mL of oleic acid and Tween 20 (0.4mL/L).[6,13]

Initially ketoconazole and itraconazole stock solution was prepared in 100% dimethyl sulfoxide (DMSO) at a concentration of 5120µg/mL. Fluconazole stock solution was prepared in sterile distilled water at a concentration of 5120µg/mL. The tests were performed in sterile, round-bottomed, 96-well micro titre plates. From the stock solution of the drug, ten successive dilutions were prepared. The highest concentrations for ketoconazole and itraconazole were 16 µg/mL (16, - 0.03 µg/mL), but for fluconazole, it was 128 µg/mL (128-0.25 µg/mL). 100 µL of each antifungal were distributed in wells of the micro titer plate, from column three to 12. Column 3 corresponded to larger and the column 12 to the lower

concentration of each antifungal tested. Thus, all the wells, the rows A to H, were filled with antifungal, except in the first and second columns. These columns were reserved for testing positive and negative growth controls respectively.[13]

Six days old fresh cultures of *Malassezia* grown on mDA at 32°C was used for inoculum preparation. All inoculum suspensions were prepared with 5 mL of sterile saline and Tween-20 in 1: 1 ratio containing sterile glass beads of 1 mm diameter. Suspension was homogenized by vortexing for 20 seconds to disperse the lumps of *Malassezia* colonies. The inoculum was standardized spectrophotometrically at 530 nm and adjusted by adding modified RPMI medium to match the turbidity of the tube scale of 0.5 Mac Farland standards. From this suspension, dilutions were made in modified RPMI medium.[6,13]

Each well of micro titer plates containing serially diluted antifungal drug were inoculated with 100µL of freshly prepared inoculum suspensions in such a way that each row corresponded to one yeast sample. The first column (positive control) of all the micro titer plates received 100µL of the corresponding standard strain inoculum suspension plus 100µL of modified RPMI medium and the second column served as sterility control (negative control) which received only 200µL modified RPMI medium belonging to the same lot used to prepare the inoculum suspension and this column was free from antifungal drug and test inoculum suspension.[6,13]

Inoculated micro titer plates were sealed and incubated at 32°C for 72 hours in an incubator. Turbidity testing was performed after 72 hour. The inhibition of growth of the tested *Malassezia* spp on the wells containing antifungal agent was indicated by the decrease in turbidity. The growth in each well is compared with that of the growth control (drug free) well. *C. parapsilosis* ATCC 22019 was included as internal quality control. The test was repeated if the wells in the negative control had growth; the positive control wells had no growth and the standard strains in the positive control wells did not show the expected results. Comparison between antifungal drugs was carried out by calculating geometric mean MICs.[6,13]

RESUTS

All the tested *Malassezia* spp produced detectable growth after the incubation. The overall MICs range for fluconazole, itraconazole and ketoconazole were ≤ 0.125–16µg/mL, ≤ 0.03–1µg/mL and ≤ 0.03–8µg/mL respectively. When all the strains were considered together, this

study shows higher MIC values for fluconazole followed by ketoconazole and itraconazole. Antifungal susceptibility pattern showing MIC ranges and geometric means of MICs fluconazole, ketoconazole and itraconazole are shown in the table –1.

Table – 1: Antifungal Susceptibility pattern of *Malassezia* species

Species	No.	Antifungal agents	Pityriasis Versicolor Cases		
			MIC range (µg/mL)	Geometric Mean (µg/mL)	Std deviation
M. globosa	82	Fluconazole	0.125– 16	3.16	4.9
		Ketoconazole	0.125– 2	0.62	0.49
		Itraconazole	0.03– 1	0.32	0.3
M. furfur	76	Fluconazole	0.06– 16	3.53	5.14
		Ketoconazole	0.03– 8	1.10	2.38
		Itraconazole	0.03– 0.5	0.13	0.14
M. restricta	63	Fluconazole	0.03– 2	0.67	0.5
		Ketoconazole	0.03– 1	0.31	0.28
		Itraconazole	0.03– 0.25	0.12	0.071
M. sympodialis	17	Fluconazole	0.125– 0.5	0.25	0.15
		Ketoconazole	.03– 0.125	0.08	0.04
		Itraconazole	.03– 0.125	0.06	0.03
M. obtusa	5	Fluconazole	0.125– 0.5	0.25	0.18
		Ketoconazole	0.03– 0.06	0.05	0.03
		Itraconazole	0.03	0.03	0
M. slooffiae	3	Fluconazole	0.125– 0.5	0.25	0.16
		Ketoconazole	0.03– 0.06	0.05	0.015
		Itraconazole	0.03– 0.06	0.04	0.017
M. pachydermatis	1	Fluconazole	0.50	0.50	0
		Ketoconazole	0.03	0.03	0
		Itraconazole	0.03	0.03	0

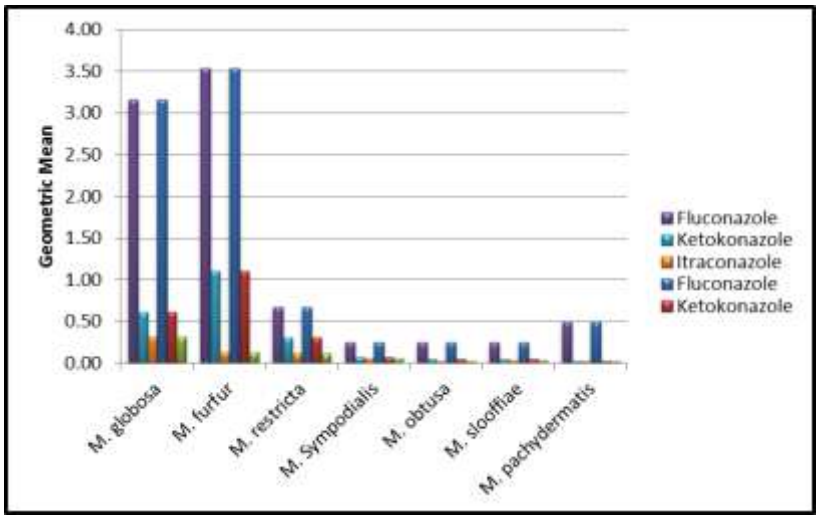

Figure 1: Antifungal Susceptibility of Itraconazole, Ketoconazole and Fluconazole Against Different *Malassezia* Spp Isolated from PV Cases.

DISCUSSION

With the global concern over high minimum inhibitory concentrations (MICs) to antifungal agents with emerging resistance and an increase in reports of recurrence of PV cases and *Malassezia* associated infections, there is every possibility that the organism may develop resistance to such drugs.[14,15] Hence, studying *Malassezia* susceptibility might provide better information about the susceptibility pattern of different species. The ketoconazole MICs range from ≤ 0.03 – 8 µg/mL with all the species tested, These values are similar to or higher than those documented in other studies.[14,15,8,17] Garau M *et al.*[8] registered MICs of ≤0.03µg/mL against 70 isolates of *Malassezia*. Gupta AK *et al.*[15] demonstrated that out of 55 of strains of *Malassezia* the MIC ranges as ≤ 0.03µg/mL – 0.125µg/mL with 95% of them having an MIC of ≤ 0.03µg/mL. This study is in contrast with the above mentioned studies as four *M. furfur* strains showed an increased MIC of 8µg/mL. Higher MIC values of ketoconazole observed with *M. furfur* indicate a possible manifestation of resistance.

In this study, itraconazole registered MICs ranging from ≤ 0.03 – 1µg/mL. These results are similar to or higher than those documented in various other studies.[15,18,19] MIC ranges against itraconazole shown in several studies are as follows: < 0.03–16µg/mL,[16] 0.007 – 0.05µg/mL,[19] 3µg/mL – 0.125µg/mL[6] and ≤ 0.03 – 0.06 µg/mL.[4] Gupta AK *et al.*[17] found the MIC data obtained for itraconazole were similar to MICs for itraconazole and ketoconazole with an MIC of 0.8µg/ml for both. An itraconazole MICs ≤ 0.03µg/mL (95% of the isolates) were observed by Rojas FD *et al.* without noticeable intra and inter species variability and with *M. furfur* ranging from ≤ 0.03µg/mL – 0.125µg/mL, *M. globosa* and *M. sympodialis* both had a MIC range of ≤ 0.03µg/mL – 0.06µg/mL.[15] The good *in vitro* activity of itraconazole shown in this study makes it a possible choice for the treatment of *Malassezia* related infections in this particular geographic area. In agreement with other reports, this study also found higher *in vitro* antifungal activity against all the tested clinical isolates of *Malassezia* spp, with itraconazole.[8,14,16]

In this study fluconazole MICs were significantly higher than those observed for other azole drugs and ranged from ≤ 0.125 µg/mL – 16µg/mL for all the isolates in PV cases. This is similar to or lower than those documented in various other studies.[15,16,18] The various MIC ranges against fluconazole shown in various studies are ≤ 0.125 – > 64 µg/mL,[16] 0.06 µg/mL – 1.0 µg/mL,[18] ≤ 0.5 µg/mL –32 µg/mL,[6] 0.25µg/mL – 4 µg/mL,[8] 0.25µg/mL – 256 µg/mlL[14] and ≤ 0.125 – > 64µg/mL.[15] Iatta R *et al.* opined that the high fluconazole

MIC values might be due to the hydrophilic nature of the drug which reduces its permeability in lipid rich cell membranes of *Malassezia* yeast cells.[20]

M. globosa isolated from four PV cases and six cases of *M. furfur* showed highest MIC values at 16µg/mL for fluconazole. All these isolates belonged to chronic recurrent and extensive forms of PV. In this study, *M. furfur* followed by *M. globosa* had geometric mean MIC of 3.53µg/mL, 3.16µg/mL respectively was the two species with higher fluconazole MICs indicating a lower susceptibility. This is similar to the reports of Rojas FD *et al.*[15] in which a geometric mean MICs of 3.29µg/mL was observed for *M. furfur*.

This was in contrast with the studies done by Velegraki A *et al*. in which they observed a lower fluconazole geometric mean MIC values for *M. furfur* and *M. globosa* at 1.89 µg/mL, 2.34µg/mL respectively.[6] Iatta *et al.* reported the highest number of resistant *M. furfur* strains for fluconazole and amphotericin B.[20] Carillo-Munoz *et al.* reported higher MIC values than this study for fluconazole particularly with *M. furfur* followed by *M. globosa* with a fluconazole geometric mean MIC of 7.4 µg/mL for 76 clinical isolates of *Malassezia* spp.[14]

Higher MIC values especially with fluconazole shown in this study was noticed only with the clinical strains particularly from chronic and recurrent cases. No published studies are available to compare these findings. This may be due to a change in susceptibility pattern of *Malassezia* yeast cells after their encounters with the antifungals during treatment. In this study, the highest geometric mean MIC values were shown by *M. furfur* with 3.53µg/mL for fluconazole and 1.10µg/mL for ketoconazole indicating this species as the least susceptible species and second being *M. globosa* was second with geometric mean MIC of 3.16 µg/mL for fluconazole and 0.62µg/mL for ketoconazole. *M. restricta*, *M. sympodialis*, *M. obtusa*, *M. slooffiae* and *M. pachydermatis* showed comparable lower MIC values with fluconazole, ketoconazole and itraconazole indicating a higher susceptibility than *M. furfur* and *M. globosa*. The lower MIC values registered for itraconazole indicated it as the most effective antifungal.

The intra and inter species variability of the *Malassezia* yeasts in susceptibility profiles of different antifungal agents has also been demonstrated in several other studies. Rojas FD *et al.* noted that *M. sympodialis* was the most susceptible and *M. furfur* the least susceptible species.[15] Carillo-Munoz *et al.* showed higher MIC values for *M. furfur* indicating a lower

susceptibility. *M. sympodialis* and *M. pachydermatis* with lower MIC values were found to be more susceptible than *M. furfur*, *M. globosa*, *M. slooffiae* and *M. restricta*.[14] Kim BJ *et al.* noticed a variation of MIC values and found itraconazole (0.007 – 0.05µg/mL), and ketoconazole (≤ 0.03 µg/mL – 0.15µg/mL) as the most effective agents. *M. sympodialis*, *M. globosa* and *M. obtusa* showed comparable MIC values; whereas MIC values for *M. furfur* were significantly higher, suggesting that *M. furfur* was the less susceptible species when compared to *M. obtusa*, *M. globosa* and *M. sympodialis.*[18] Rincon S *et al.* found *M. globosa* and *M. restricta* as the least susceptible species.[7] Rojas FD *et al.* recorded MICs of ≥ 8 µg/mL in 28.2% of *M. furfur* and 8.3% of *M. globosa* spp by fluconazole, indicating its poor therapeutic response. Ketoconazole showed excellent activity against *M. globosa, M. sympodialis*, and *M. furfur* whereas fluconazole showed high MICs with wide MIC ranges.[15]

CONCLUSION

If clinical resistance to antifungals is suspected, *in vitro* antifungal susceptibility testing can be utilized. In this study, the higher MIC values reported for fluconazole indicates a possibility of resistance. The lower MIC values registered for itraconazole indicate that it is the most effective antifungal drug. Ketoconazole exhibited lower MIC values except for *M. furfur* and *M. globosa*. *M. restricta, M. sympodialis, M. obtusa M. slooffiae* and *M. pachydermatis* showed comparable lower MIC values with fluconazole, ketoconazole and itraconazole indicating a higher susceptibility than *M. furfur* and *M. globosa.* High MICs and variability displayed in the antifungal activity of different antifungal drugs with inter-species variations of isolates from PV cases evinced the importance of performing the *in vitro* susceptibility of each species to capture reliable information for determining an effective treatment regimen.

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