Microbes and Infectious Diseases 2023; 4(3): 988-993

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Antifungal susceptibility testing of *Malassezia* spp isolated from patients with *Pityriasis versicolor* and healthy individuals

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ARTICLE INFO

Article history:
Received 27 February 2023
Received in revised form 19 March 2023
Accepted 20 March 2023

Keywords:
Antifungal susceptibility
Pityriasis versicolor
Drug resistance
Malassezia

ABSTRACT

Background: The genus *Malassezia* is a normal flora of our body. It needs lipid supplements for its growth and is seen in the area rich in sebaceous glands. It is a causative agent of *Pityriasis versicolor* (PV), *Malassezia* folliculitis, seborrheic dermatitis and also associated with some systemic infection like fungemia, catheter acquired sepsis in patients receiving lipid parenteral nutrition. Increased incidence of *Malassezi*a infection emphasizes the need of susceptibility to choose a specific and accurate treatment. The aim of this study was to evaluate the *in-vitro* activity of amphotericin B, fluconazole, ketoconazole and voriconazole against the *Malassezia* spp isolated from PV and healthy individuals. **Methodology:** Modification of the CLSI M27-A3 document method using Christensen's urea broth with the addition of 0.5% Tween 40 and 0.1% Tween 80 was performed to evaluate the optimal antifungal susceptibility patterns of the isolates. **Results:** The *Malassezia* spp from healthy and PV patients shows variability in susceptibility pattern. Among the different species the lowest Microbial Inhibition Concentration (MICs) were found in amphotericin B, ketoconazole and Fluconazole with MIC_{50} values of 0.62, 0.03 and 0.125 in healthy individuals respectively. Isolates from PV patients showed slight highest value of MIC_{50} 0.5, 0.25 and 0.5 respectively. **Conclusion:** The susceptibility pattern showed intra species variation and difference between healthy and PV patients as well. This emphasizes the need to identify the species and to evaluate the antifungal susceptibility of *Malassezia*. Further investigation is needed to correlate the *in-vitro* activity of antifungal agents with clinical outcomes.

Introduction

Malassezia spp are natural inhabitants of the healthy skin of human and animals. Since many years, *Malassezia* was considered as commensals however, under certain predisposing factors, they may cause or exacerbate several skin diseases such as *Pityriasis versicolor* (PV), malassezia (Pityrosporum) folliculitis, seborrheic dermatitis (dandruff), atopic dermatitis, psoriasis systemic and blood stream infection in immunocompromised patients thus finally leads to mortality [1,2]. Invasive *Malassezia* outbreaks has been reported in intensive neonatal units with neonates who

DOI: 10.21608/MID.2023.196612.1473
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receiving total parental nutrition. This alarming the importance of *Malassezia* in the field of medical mycology [3].

Treatment for infection related to *Malassezia* is great challenging because of its chronicity and recurrence. The topical and systemic antifungal drugs used to treat infection were not satisfactory as the susceptibility cannot be predicted based on the identity of the genus. However the antifungal treatment for *Malassezia* related infection is not clinically efficient [4,5]. This may be due to antifungal resistance of *Malassezia* spp. hence we intend to study in-vitro antifungal susceptibility of *Malassezia* species isolated from PV patients and from healthy individuals.

Materials and Methods

A cross sectional study was done in a tertiary care hospital Puducherry from July 2019 to September 2022. After obtaining institutional ethical clearance.

Samples

A total of 200 isolates 100 from PV patients and 100 from healthy individuals. These isolates were obtained as a part of a previous study.

Antimicrobial susceptibility testing

A modification of the Clinical and Laboratory Standard Institute (CLSI 2008) recommended (Document M27-A3) broth microdilution method for testing the antifungal susceptibility of yeasts using Christensen's urea broth with the addition of Tween 40 (0.5%) and Tween 80 (0.1%), was used for antifungal susceptibility testing of *Malassezia* isolates [6].

Antifungal agents

For each drug the following range of concentration was used

Fluconazole - 0.125 to 64 µg/ml

Ketoconazole - 0.125 to 64 µg/ml

Voriconazole - 0.125 to 64 µg/ml

Amphotericin B - 0.0313 to 16 µg/ml

Inoculum preparation

Malassezia isolates grown on Modified Dixon's Agar (MDA) for 72 h at 32°C ± 2°C was used. Prepared antifungal stock solutions were stored at −70°C.

Quality control

Each microtiter plate included growth control well with inoculum and supplemented urea broth without the antifungal agent. *Malassezia* MTCC was used as a quality control strain for AFST.

Reading of results

Minimal inhibitory concentration was determined by comparing the amount of growth in the growth control well with the amount of growth in the wells containing the antifungal agent and a numerical score was given as follows:

Numerical Score :

0- Optically clear or absence of growth

1-Approximately 25% of the growth control or slight growth

2-Approximately 50% of the growth control or prominent reduction in growth

3-Approximately as of the growth medium or slight reduction in the growth (75% of growth control)

4-No reduction in the growth

Interpretation

Complete absence of growth, corresponding to a numerical score of 0 was taken as the end point for amphotericin B. 80% growth inhibition corresponding to a numerical score of 3 was taken as the end point for azoles [7,8]. MIC50 and MIC90 were determined for the isolates. Minimal inhibitory concentration of MTCC control strain was confirmed to be within the expected range, for the validity of the interpretation for the test strains.

Results

Among the total of 200 *Malassezia* isolates, *Malassezia furfur (M furfur)* (n=62), *M globosa* (n=83), *M sympodialis* (n=19), *M restricta* (n=4), *M obtusa* (n= 7), *M pachydermatis* (n=25) which was shown in **table (1)**. Amphotericin B and voriconazole showed lowest MIC (0.125-1) for the *Malassezia* isolates from healthy individuals as well isolates from PV patients. Fluconazole and ketoconazole showed highest MIC (1-32).

Table 1. Species distribution of isolates from healthy individuals and *Pityriasis versicolor* patients.

Species	**Healthy individuals (N=100)**	***Pityriasis versicolor* (N=100)**
Malassezia furfur (n=62)	30	32
Malassezia globosa (n=83)	27	56
Malassezia sympodialis (n=19)	16	3
Malassezia restricta (n=4)	2	2
Malassezia obtusa (n=7)	2	5
Malassezia Pachydermatis (n=25)	20	5

Table 2. Minimum inhibitory concentration (MIC) of *Malassezia* isolates from heathy individuals.

***Malassezia spp* N=100**	**Amphotericin B 0.031-16 µg/ml**		**Ketoconazole 0.125 to 64 µg/ml**		**Fluconazole 0.125-64 µg/ml**		**Voriconazole 0.125-64 µg/ml**	
	MIC_{50} µg/ml	**MIC_{90} µg/ml**	**MIC_{50} µg/ml**	**MIC_{90} µg/ml**	**MIC_{50} µg/ml**	**MIC_{90} µg/ml**	**MIC_{50} µg/ml**	**MIC_{90} µg/ml**
***M furfur* (n=30)**	0.25	4	0.125	2	0.25	1	0.25	1
***M globosa* (n=27)**	0.5	2	0.25	4	0.5	8	0.25	2
***M sympodialis* (n=16)**	0.125	1	0.625	0.25	0.5	4	0.5	1
***M pachydermatis* (n=20)**	1	4	0.25	4	0.25	4	0.125	0.25
***M restricta* (n=2)**	0.031	1	0.031	1	0.125	1	0.25	1
***M obtusa* (n=2)**	0.625	0.5	0.125	1	0.25	0.5	0.5	2

Table 3. Minimum inhibitory concentration (MIC) of *Malassezia* isolates from *Pityriasis versicolor* patients.

***Malassezia* spp N=100**	**Amphotericin B 0.031-16 µg/ml**		**Ketoconazole 0.125 to 64 µg/ml**		**Fluconazole 0.125-16 µg/ml**		**Voriconazole 0.125-16 µg/ml**	
	MIC_{50} µg/ml	**MIC_{90} µg/ml**	**MIC_{50} µg/ml**	**MIC_{90} µg/ml**	**MIC_{50} µg/ml**	**MIC_{90} µg/ml**	**MIC_{50} µg/ml**	**MIC_{90} µg/ml**
***M furfur* (n=32)**	0.5	8	1	4	2	32	0.125	1
***M globosa* (n=56)**	0.125	2	2	8	2	16	1	8
***M sympodialis* (n=3)**	0.125	1	0.625	0.5	1	2	0.125	0.5
***M pachydermatis* (n=5)**	0.625	2	2	4	0.5	2	0.5	4
***M restricta* (n=2)**	0.5	16	0.5	1	0.25	0.5	1	2
***M obtusa* (n=5)**	0.25	1	4	16	0.5	2	2	8

Discussion

Malassezia spp cause PV, seborrheic dermatitis, atopic dermatitis in addition to this it was associated with deep seated infection such as pneumonia, catheter related fungemia with lipid parenteral administration and peritonitis in dialyzed patients [9]. As the *Malassezia* related infections are

chronic and recurrent the treatment was ineffective. Antifungal susceptibility testing for *Malassezia* can be done by agar or broth dilution method. To determine the appropriate antifungals, there is no standard guidelines for antifungal susceptibility testing of *Malassezia* spp. The CLSI and EUCAST recommend the microbroth dilution method for *Candida* and *Cryptococcus* spp, as the *Malassezia* is a lipophilic yeast slight modification and variation of incubation time is adopted [10]. In our study Christensen's urea microbroth method was applied in addition to this Tween 40 and Tween 80 was added to the medium. This method was adopted from **Rincon et al.** were he compared the CLSI broth dilution methods with Modified Christensen's urea microbroth dilution method, which showed the significant results [7]. Some studies show different time interval for reading MIC values.

Malassezia furfur was incubated for 48 h and other species for 72 h. *Malassezia globosa* was incubated for 96h as they were slow growers [4,7]. The growth time of *Malassezia* vary from species to species, to overcome this and to have a constant time to read the micro broth dilution results, the inoculum size has been increased. In this study, the inoculum size we used is (2.5 × 105 CFU/ml) which is larger than in CLSI M27-A3 in order to achieve the uniform reading time of 72h.

In our study we have not found the MIC breakpoints and the correlation between in vitro and in vivo of antifungal agent. In this study *Malassezia* from healthy individuals showed, lowest MIC50 of *M furfur* was ketoconazole (0.125 μg/ml) and amphotericin B, fluconazole and voriconazole was 0.5 μg/ml. In case of *M furfur* from PV patient shows higher MIC to the ketoconazole and Fluconazole. Which was contradictory to the study conducted by **Romald et al.** [4] M furfur showed higher MIC value for all the antifungals especially which is higher resistant to ketoconazole.

Amphotericin B is an effective antifungal for the treatment of systemic infection by *Malassezia* in neonates and adults [11]. In our study amphotericin B showed lowest MIC for the isolates from healthy individuals, whereas *M furfur* and *M restricta* isolate from PV patients shows highest MIC value. Amphotericin B is a drug of choice for blood stream infection caused by *M pachydermatis*, with limited susceptibility to other species of *Malassezia* [11]. In this study *M pachydermatis*, *M globosa*, *M sympodialis* and *M obtusa* were susceptible to amphotericin B except *M furfur* and *M restricta*. Similar other studies showed the same results [4,7,8,13].

The mechanism of action of azoles is to inhibit the synthesis of 14 alpha-demethylase, which result in inhibition of ergosterol formation. Ergosterol is present in the fungal cell membrane its malfunction will leads to death of fungi [12].

Fluconazole shows wide range of MICs, there is a variation of sensitivity of species isolates from healthy and PV patients. The CLSI M27-S4 establishes the species-specific clinical break point for *Candida* species. An isolates FCZ MIC 8 μg/ml is categorized as resistant [13]. In this study *M globosa* and *M furfur* for PV patients showed highest MIC of 32 μg/ml and 16 μg/ml respectively. These results are similar to those of **Velegraki et al.** who found the high MICs of Fluconazole for *Malassezia* this indicates that the FCZ is not a good option for treating pityriasis versicolor [18]. This has been coinciding with the study conducted by **Rojas et al**, *M globosa*, *M furfur* and *M sympodialis* were found to be resistant to fluconazole and amphotericin B

Ketoconazole and voriconazole were the most active drugs showing the lowest MIC. Ketoconazole has an excellent activity on all the six species of *Malassezia* from healthy individuals. In case of isolates from PV patients, except *M obtusa* and *M globosa* all others are sensitive [15,16]. Though most of the *Malassezia* spp is sensitive to ketoconazole, because of its toxicity it is no longer recommend as first line choice. In our study, the MICs of ketoconazole for *Malassezia* spp ranged from 0.125-16 μg/ml but 53% of the isolates showed a MIC of 0.125. These data are similar to those found by **Miranda et al.** who reported MIC values of 0.03 μg/ml for 33.6% of strains tested [13].

Comparing the antifungal susceptibility of *Malassezia* spp from PV and healthy individuals shows major differences. *Malassezia furfur* isolated from PV patients showed higher resistance to amphotericin B, ketoconazole, fluconazole than the isolate from healthy individuals. As within the same species it shows variation among PV and healthy individuals.

Conclusion

There is no standard recognized method for antifungal susceptibility of *Malassezia* spp. Therefore, it is difficult to determine the resistance of *Malassezia.* In this study the susceptibility pattern

showed intra species variation and difference between healthy and PV patients as well. This emphasizes the need to identify the species and to evaluate the antifungal susceptibility of *Malassezia*. Further investigation is needed to correlate the *in-vitro* activity of antifungal agents with clinical outcomes.

Conflicts of interest

Authors declare no conflict of interest.

Financial disclosure: None.

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Balaji VK, Ragunathan L, Kannaiyan K, Duraipandian J. Antifungal susceptibility testing *of Malassezia* spp isolated from patients with *Pityriasis versicolor* and healthy individuals. Microbes Infect Dis 2023; 4(3): 988-993.