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Antifungal susceptibility of *Malassezia furfur*, *Malassezia sympodialis*, and *Malassezia globosa* to azole drugs and amphotericin B evaluated using a broth microdilution method

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Abstract

We studied the *in vitro* activity of fluconazole (FCZ), ketoconazole (KTZ), miconazole (MCZ), voriconazole (VCZ), itraconazole (ITZ) and amphotericin B (AMB) against the three major pathogenic *Malassezia* species, *M. globosa*, *M. sympodialis*, and *M. furfur*. Antifungal susceptibilities were determined using the broth microdilution method in accordance with Clinical and Laboratory Standards Institute reference document M27-A3. To support lipid-dependent yeast development, glucose, peptone, ox bile, malt extract, glycerol, and Tween supplements were added to Roswell Park Memorial Institute RPMI 1640 medium. The supplemented medium allowed good growth of all three species studied. The minimal inhibitory concentrations (MICs) were recorded after 72 h of incubation at 32°C. The three species showed different susceptibility profiles for the drugs tested. *Malassezia sympodialis* was the most susceptible and *M. furfur* the least susceptible species. KTZ, ITZ, and VCZ were the most active drugs, showing low variability among isolates of the same species. FCZ, MCZ, and AMB showed high MICs and wide MIC ranges. Differences observed emphasize the need to accurately identify and evaluate antifungal susceptibility of *Malassezia* species. Further investigations and collaborative studies are essential for correlating *in vitro* results with clinical outcomes since the existing limited data do not allow definitive conclusions.

Key words: *Malassezia*, susceptibility, broth microdilution, azole drug, amphotericin B.

Introduction

Yeasts of the genus *Malassezia* have been recognized as members of the normal skin biota of humans and other warm-blooded animals. Since they are unable to synthesize fatty acids, all are lipophilic and almost all are lipid dependent, requiring an external source of lipids. For this reason, they prevail in body areas rich in sebaceous glands [1–9].

Malassezia species are considered to be the etiological agents of pityriasis versicolor and *Malassezia* folliculitis, associated agents in seborrheic dermatitis and contributory factors in other skin disorders such as atopic dermatitis, psoriasis, confluent and reticulate papillomatosis, and neonatal pustulosis [1,4,5,7,8,10–16]. Furthermore, *Malassezia* spp. have been associated with systemic infections such as catheter-acquired sepsis, fungemia, and pulmonary infection in neonates and immunocompromised patients who receive lipid parenteral nutrition. In addition, they are involved in other infections such as septic arthritis, otitis externa, abscess, and peritonitis in ambulatory peritoneal dialysis patients [1,8,11,17–20].

The skin diseases associated with *Malassezia* spp. are often chronic and recurrent. Usually, superficial skin disorders are treated with topical and systemic antifungal therapy, but not always with successful outcomes [1]. In addition, the increased incidence of systemic infections associated with this genus emphasizes the need to know the susceptibility profile of these yeasts in order to choose a specific and accurate treatment [21].

The Clinical and Laboratory Standards Institute (CLSI) approved document M27-A3, which describes a broth dilution method for testing the *in vitro* antifungal susceptibility of *Candida* species and *Cryptococcus neoformans* for determination of minimal inhibitory concentrations (MICs). However, this method is not applicable to *Malassezia* species due to their nutritional requirements.

The aim of this study was to evaluate the *in vitro* activity of fluconazole (FCZ), ketoconazole (KTZ), miconazole (MCZ), voriconazole (VCZ), itraconazole (ITZ), and amphotericin B (AMB) against the three major pathogenic *Malassezia* species, *M. furfur*, *M. sympodialis*, and *M. globosa*.

Material and methods

A total of 73 isolates, 39 *M. furfur*, 20 *M. sympodialis*, and 14 *M. globosa,* that had been deposited in the culture collection of the Departamento de Micología, Instituto de Medicina Regional, Universidad Nacional del Nordeste, Argentina, were tested. All of these yeasts were isolated from clinical samples obtained from human patients with different dermatological pathologies. Identifications were performed using polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLP) [22]. *Malassezia* isolates were grown on modified Dixon agar [3] for 72 h at 32°C ± 2°C in order to obtain pure cultures for the testing.

MICs were determined by broth microdilution method in accordance with CLSI M27-A3 [23]. To support development of these lipid-dependent yeasts, the base medium, RPMI 1640 (Gibco, Life Technologies, Argentina), was added with glucose (1.8%, Cicarelli, Reagents, San Lorenzo, Argentina), peptone (1%, Laboratorios Britania, Argentina), ox bile (0.5%, Laboratorios Britania, Argentina), malt extract (0.5%, Laboratorios Britania, Argentina), glycerol (1%, Biopack, Argentina), Tween 40 (0.5%, Sigma-Aldrich, Argentina), Tween 80 (0.05%, Anedra, Research AG, San Fernando, Argentina), and chloramphenicol (250 mg/l, Laboratorios ELEA, CABA, Argentina).

All stock inoculum suspensions were prepared in sterile saline solution and standardized spectrophotometrically at 530 nm (10^7 colony-forming units [CFU]/ml). This inoculum was diluted 1:100 in supplemented RPMI medium to achieve a final concentration of 0.5–2.5 × 10^5 CFU/ml, as verified by viable colony counts in modified Dixon agar.

Antifungal stock solutions of FCZ and VCZ (both from Pfizer, Inc., Groton, CT, USA) and KTZ, MCZ, ITZ, and AMB (all from Sigma-Aldrich, Argentina) were prepared in dimethyl sulfoxide (Sigma-Aldrich, Argentina) and stored at −70°C until used. The final concentration for all drugs was 0.03–16 μg/ml, except for FCZ with concentrations ranging from 0.125 to 64 μg/ml.

Test microtiter plates with 96 U wells (Greiner bio-One, GBO Argentina, Buenos Aires, Argentina) were incubated for 4 days at 32°C. The MIC endpoint for azole drugs was defined as the lowest concentration at which there was a ≥50% inhibition of growth as compared with the (drug-free) growth control. For AMB, the MIC endpoint was defined as the lowest concentration that completely inhibited growth. Growth and sterility control wells were also included in each test. Quality controls strains, *Candida parapsilosis*, American Type Culture Collection ATCC 22019, and *C. krusei*, ATCC 6258, were included in each assay. Duplicate 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_{50} and MIC_{90}), mode and geometric mean.

Results

Optimal growth of the three *Malassezia* species was obtained with the supplemented RPMI 1640 medium. MICs of each drug against the three species could be recorded after 72 h of incubation at 32°C. Due to the partial inhibition of growth over a range of antifungal concentration (trailing effect), a rise in azole MICs was observed with 16.4% of isolates. In these cases, final MIC endpoints were clearly observed after an additional 24 h of incubation (96 h).

MIC range, geometric mean, mode, MIC_{50} and MIC_{90} values obtained for the antifungal drugs included in this investigation against 73 isolates are summarized in Table 1. KTZ MICs ≤0.06 μg/ml and ITZ MICs ≤0.03 μg/ml were

Table 1. Minimal inhibitory concentration (MIC) ranges, geometric mean, mode, MIC_{50}, and MIC_{90} obtained by broth microdilution method for 73 *Malassezia* isolates.

	MIC range (μg/mL)	Geometric mean	Mode	MIC_{50}	MIC_{90}
Fluconazole					
M. furfur [39]	≤0.125–>64	3.29	4	4	16
M. sympodialis [20]	≤0.125–4	0.58	0.25	0.5	2
M. globosa [14]	≤0.125–8	0.56	0.5	0.5	2
Ketoconazole					
M. furfur [39]	≤0.03–0.25	0.04	0.03	0.03	0.06
M. sympodialis [20]	≤0.03–0.06	0.03	0.03	0.03	0.03
M. globosa [14]	≤0.03	0.03	0.03	0.03	0.03
Itraconazole					
M. furfur [39]	≤0.03–0.125	0.04	0.03	0.03	0.06
M. sympodialis [20]	≤0.03–0.06	0.04	0.03	0.03	0.06
M. globosa [14]	≤0.03–0.06	0.03	0.03	0.03	0.06
Miconazole					
M. furfur [39]	0.125–16	1.29	2	1	4
M. sympodialis [20]	≤0.03–8	0.32	0.03	0.25	4
M. globosa [14]	≤0.03–4	0.23	0.03	0.25	2
Voriconazole					
M. furfur [39]	≤0.03–0.5	0.08	0.06	0.06	0.25
M. sympodialis [20]	≤0.03–0.06	0.04	0.06	0.06	0.06
M. globosa [14]	≤0.03–0.25	0.06	0.03	0.03	0.125
Amphotericin B					
M. furfur [39]	0.25–4	1.02	1	1	2
M. sympodialis [20]	0.125–4	0.56	0.25	0.5	2
M. globosa [14]	0.06–4	0.5	1	0.5	1

n: number of strains MIC, minimal inhibitory concentration; MIC_{50} and MIC_{90}, MIC values that indicate 50% and 90% of the isolates were inhibited.

observed for 90% and 95% of the isolates, respectively, showing little intra- and interspecies variability. FCZ MICs were higher than those observed for other azole drugs and ranged from ≤0.125 to >64 μg/ml for all isolates. The highest geometric mean, mode, MIC_{50}, and MIC_{90} values for all drugs tested were those for *M. furfur*, especially for FCZ and MCZ. The three species tested showed similar MICs against AMB.

Discussion

In vitro susceptibility of *Malassezia* species has been studied using either agar [24–26] or broth-dilution methods [2,25,27–32]. However, differences in these studies regarding methodology, culture medium, inoculum, incubation time, and the criteria used to determine MIC endpoints limit comparison of the study results. No reference method has been developed for lipid-dependent yeasts, which would allow for the integration of different assay procedures [21]. On the other hand, some reports on the susceptibility of *Malassezia* spp. were published prior to the recognition of new species through molecular methods [26] or conducted with isolates identified by conventional methods that do not differentiate all species [2,24]. Therefore, the data obtained from these studies are questionable and should be carefully reviewed.

The nutritionally supplemented RPMI1640 medium used in this study allowed for suitable growth of the three *Malassezia* spp. tested and good MICs visual readings. The addition of malt extract, ox bile, and Tween is essential for development of these species [2,3,27,29], and the minimal quantities added do not increase turbidity of the medium.

Although the inoculum size used was larger than that recommended in CLSI M27-A3 ($0.5–2.5 \times 10^5$ CFU/ml), it provided suitable growth at 72 h of incubation. In contrast to other studies in which supplemented RPMI 1640 medium was used, MIC reading times were not species dependent in our study. Velegraki et al. incubated for 48 h *M. furfur* isolates and 72 h the other species [29]. In other investigations, readings were made between 96 h and 5 days after inoculation for slow-growing species such as *M. globosa* [2,27,29,31,32]. These different incubation times could be due to the different sizes of final inocula. We used higher inoculum concentrations in order to achieve unified reading times for the three species and, in turn, to become independent of factors such as the decreased potency of drugs.

KTZ and ITZ were the most active drugs tested. KTZ showed excellent activity against the three species, and

the results are similar to those found by others who used either agar or broth dilution methods [24,25,30,32,33]. Despite its well-known activity against *Malassezia* species, KTZ is no longer recommended as first-line treatment because of its toxicity. Comparable with results described in other reports [2,29,30,32,33], low MICs for ITZ were observed in 95% of *Malassezia* strains tested (MIC $\leq$ 0.03 μg/ml), which indicates that ITZ is an effective treatment option.

FCZ showed high MICs and wide MIC ranges compared with the other drugs. This variability was also observed in previous investigations [2,29,30,32]. The CLSI M27-S4 supplement establishes species-specific clinical breakpoints for species of *Candida* and FCZ. An isolate is categorized as being resistant when the FCZ MIC is $\geq$8 μg/ml [34]. In our study, these MIC results were noted in 28.2% of *M. furfur* and 8.3% of *M. globosa* isolates. Although working breakpoints and the correlation between *in vitro* and *in vivo* results for *Malassezia* spp. have not been established for any antifungal agent, the high MICs observed with FCZ could possibly indicate that it is not a good treatment option.

Using a similar methodology, Velegraki et al. obtained lower MICs for *M. furfur* against FCZ than those observed in this study (MIC_{90} = 8 μg/ml and geometric mean = 1.89 μg/ml). Furthermore, they reported higher MICs for *M. sympodialis* and *M. globosa* [29]. In contrast, Carrillo-Muñoz et al. reported higher MICs than those obtained in our studies using a similar methodology for the same species [32]. High MICs and variability in the antifungal activity of FCZ against *Malassezia* isolates emphasize the importance of performing *in vitro* susceptibility testing for these yeasts.

A wide range of MICs was found with MCZ. Information about the effectiveness of this drug against *Malassezia* spp. is limited. Carrillo-Muñoz et al. reported geometric mean values and MIC ranges that were in agreement with our results with the same species [32]. Van Gerven and Odds [26], using an agar culture medium, evaluated the effectiveness of MCZ against 23 isolates of *M. furfur* and obtained MICs greater than those noted in this study. However, since the test isolates in their study were not identified by molecular methods, the results should be interpreted with caution [26]. Since MCZ is one of the most widely used topical drugs, it is important to obtain more information about its activity.

Although VCZ was active against all three species, MICs were higher than those obtained for KTZ and ITZ, especially for *M. globosa* and *M furfur*. These data are consistent with those obtained by other authors [2,24,30].

The M27-S4 supplement establishes that VCZ MICs $\geq$0.25 μg/ml for *Candida* species are correlated with susceptible dose-dependent strains. Even though these breakpoints are not applicable for *Malassezia* spp., 6/39 of *M. furfur* and 2/14 *M. globosa* showed results $\geq$0.25 μg/ml for VCZ in our study, supporting the importance of additional studies to set epidemiological and clinical susceptibility breakpoints for this genus.

Similar AMB MIC ranges were found with all three species and there were some isolates of each with MICs >1 μg/ml. The M27-A3 document states that *Candida* species with MICs >1 μg/ml are likely resistant to AMB. In our study, 24.6% of the isolates showed MIC >1 μg/ml. Despite the fact that there are no breakpoints for categorizing these yeasts as resistant to AMB and the clinical significance is unknown, this finding is remarkable. These results are similar to those of Velegraki et al., who observed high MICs for this drug against *M. furfur* and *M. globosa* [29]. This is important because AMB is a primary treatment option for systemic infection, especially in neonatal patients [35–38].

Regarding *Malassezia* species, the highest MICs for all drugs tested were for *M. furfur*. Approximately 31% of *M. furfur* had MICs $\geq$2 μg/ml for AMB and 28% showed MICs $\geq$8 μg/ml for FCZ. Considering the standards established by the CLSI for *Candida*, these isolates would be categorized as resistant. In contrast, ITZ and KTZ were the most active drugs against this species, with low MICs and limited variation in susceptibility among different isolates. Similar results were obtained by others under comparable conditions [29,32] or using other methods [2,24,27,30,31,39]. KTZ was the most active drug against *M. sympodialis*, FCZ the most variable, and MCZ the least active. *Malassezia sympodialis* is considered one of the most susceptible species to antifungal drugs [32]. All isolates of *M. globosa* tested were susceptible *in vitro* to KTZ (MIC $\leq$ 0.03 μg/ml) and showed wide MIC ranges and more intraspecies variation to FCZ and AMB. Previous studies have shown that *M. globosa* is one of the least susceptible species to antifungal drugs [2,27,29,31,32]. In our study, one isolate showed high MICs for FCZ, MCZ, and AMB (8 μg/ml, 4 μg/ml, and 4 μg/ml, respectively). Miranda *et al.* described a similar case of one isolate showing low susceptibility to ITZ, VCZ, and FCZ [2].

Malassezia furfur, *M. sympodialis*, and *M. globosa* showed different susceptibility profiles to the drugs tested. *Malassezia sympodialis* was the most susceptible and *M. furfur* the least. KTZ, ITZ, and VCZ were the most active drugs against the three species and low variability among different isolates of the same species was observed. In contrast, high MICs were obtained with FCZ, MCZ, and AMB and wide MIC ranges for the three species tested.

Differences observed among *M. furfur*, *M. sympodialis*, and *M. globosa* emphasize the need to accurately identify and evaluate the antifungal susceptibility of the three major pathogenic *Malassezia* species. Further investigations and

collaborative studies are essential for correlating *in vitro* results with clinical outcomes since collected data so far do not allow definitive conclusions.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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