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Azole susceptibility of *Malassezia pachydermatis* and *Malassezia furfur* and tentative epidemiological cut-off values

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Abstract

This study aims to determine the minimal inhibitory concentration (MIC) distribution and the epidemiological cut-off values (ECVs) of *Malassezia pachydermatis* and *Malassezia furfur* isolates for fluconazole (FLZ), itraconazole (ITZ), posaconazole (POS), and voriconazole (VOR). A total of 62 *M. pachydermatis* strains from dogs with dermatitis and 78 *M. furfur* strains from humans with bloodstream infections (BSI) were tested by a modified broth microdilution Clinical and Laboratory Standards Institute (CLSI) method. ITZ and POS displayed lower MICs than VOR and FLZ, regardless of the *Malassezia* species. The MIC data for azoles of *M. pachydermatis* were four two-fold dilutions lower than those of *M. furfur*. Based on the ECVs, about 94% of *Malassezia* strains might be categorized within susceptible population for all azoles, except for FLZ, and azole cross-resistance was detected in association with FLZ in *M. pachydermatis* but not in *M. furfur*.
The study proposes, for the first time, tentative azole ECVs for *M. pachydermatis* and *M. furfur* for monitoring the emergence of isolates with decreased susceptibilities and shows that the azole MIC distribution varied according to the *Malassezia* species tested, thus suggesting the usefulness of determining the susceptibility profile for effective treatment of each species.

Key words: Azole susceptibility, *Malassezia pachydermatis*, *Malassezia furfur*, bloodstream infections, skin, epidemiological cut-off values.

Introduction

Malassezia pachydermatis and *Malassezia furfur* are lipophilic yeasts, part of the normal animal and human skin microbiota, which can cause various forms of dermatitis in both animals and humans, as well systemic infections in immunocompromised patients [1–6]. Although guidelines for the therapy of *Malassezia* spp. infections have not yet been assessed, topical therapy with azole drugs may be generally sufficient to resolve the clinical signs of *M. pachydermatis* dermatitis and/or otitis in dogs when underlying *M. pachydermatis* overgrowth is controlled. Importantly, relapsing infections are common, thus regular maintenance therapy is essential for successful management [5,7]. For *Malassezia*

fungemia in humans, infusion of amphotericin B is the preferred and the most useful treatment [1,2]. Moreover, the induction of *in vitro* fluconazole (FLZ) resistance in *M. pachydermatis* [8,9] and the clinical evidence of FLZ treatment failure in preventing *M. furfur* fungemia in humans and relapses in animals, [10,11] suggest a probable occurrence of resistance phenomena in these species. Thus far, *in vitro* susceptibility testing for *Malassezia* spp. has not yet been standardized neither by Clinical and Laboratory Standards Institute (CLSI) nor by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [1] because of slow growth, the lipid dependency and a tendency to form clusters of *Malassezia* yeasts, thus resulting in an absence of clinical breakpoints (CBPs). Recently, a consensus among experts asserts that in the absence of CBPs, the epidemiological cut-off values (ECVs) might be useful for separating susceptible (i.e., wild-type [WT]) and resistant (i.e., non-WT) isolates [12,13]. Thus, this study aims to determine the Minimal Inhibitory Concentration (MIC) distribution for FLZ, itraconazole (ITZ), posaconazole (POS), and voriconazole (VOR) of *M. pachydermatis* isolated from the skin of dogs with dermatitis and *M. furfur* of clinical specimens from BSI patients. Tentative azole ECVs were proposed in order to interpret the results of *in vitro* susceptibility tests and to monitor the isolates with low susceptibility.

Materials and methods

Malassezia strains

A total of 140 *Malassezia* strains (i.e., n = 62 *M. pachydermatis* and n = 78 *M. furfur*) were tested. *M. pachydermatis* strains were isolated from the skin of 62 dogs with localized or generalized dermatitis characterized by erythema and pruritus. The dogs came from different veterinary clinical centres and had no known history of antibiotic or antifungal treatment in the preceding 5 months. *M. furfur* was isolated from blood, urine, gastric aspirate and catheter tip (total of n = 60 strains), and from the skin of the arm (n = 15 strains) and/or chest (n = 3 strains) of 15 BSI patients. All the enrolled patients were admitted in intensive care units of a hospital in southern Italy. A blood sample from each patient was collected at the onset of clinical signs (i.e., apnea caused by respiratory distress, elevated or depressed leukocyte count, increased C-reactive protein level, abdominal distension, or thrombocytopenia). In addition, clinical specimens (i.e., urine, gastric aspirate), catheter tip, and skin swabs were collected from each BSI patient. All biological specimens and swabs were cultured on Sabouraud dextrose agar with 0.5% chloramphenicol and incubated at 37°C for 5 d and on Dixon agar and incubated at 32°C for 10 d. The isolates grew only on Dixon agar were sent to the Department of Veterinary Medicine, University of Bari, Italy, for species identification.

The isolates were identified by standard methods and sequencing of the internal transcribed spacer (ITS) of nuclear ribosomal DNA [2,14]. The strains were stored at −80°C. Prior to testing, each isolate was sub-cultured at least twice onto modified Dixon agar [15] to ensure purity and viability.

In vitro susceptibility testing

The antifungal susceptibility of *M. pachydermatis* and *M. furfur* strains was performed using the reference CLSI broth microdiluition (BMD) M27-A3 [16] protocol with some modifications. Specifically, Sabouraud dextrose broth (SDB, Liofilchem Diagnostici®, Roseto degli Abruzzi, Italy) containing 1% Tween 80 (Sigma Co, Milano, Italy) was used instead of RPMI 1640 medium as previously reported [8,10]. Stock inoculum suspensions were prepared from 4-day-old colonies on modified Dixon agar at 32°C. The final concentration of the stock inoculum in sterile distilled water was adjusted to an optical density of 2.4 McFarland using a turbidimeter (DEN-1 McFarland Densitometer, Biosan), which is equivalent to $1–5\times10^6$ colony forming units (CFU)/ml, as inferred by quantitative plate counts of CFU in Dixon agar.

The following antifungal drugs were supplied by the manufacturers as pure standard compounds: ITZ (Sigma-Aldrich, Milan, Italy), FLZ and VOR (Pfizer Pharmaceuticals, Groton, Connecticut, USA) and POS (Schering-Plough Corporation, Kenilworth, New Jersey, USA). The antifungal drug concentrations ranged from 0.008 to 16 μg/ml, except for FLZ (from 0.06 to 128 μg/ml). Visual reading of plates was performed, after 48 h of incubation at 32°C, and the growth of each strain at various drug concentrations was assessed in comparison to growth in a drug-free medium control. Following incubation, MIC endpoints were determined as the lowest concentration of the drug that produces a decrease in turbidity (i.e., ≥50% of inhibition) when compared to that of the drug-free control [8]. The MIC values were determined by three independent experiments and evaluated by three different operators.

Quality control

Quality control (QC) strains (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258; American Type Culture Collection, Manassas, Virginia, USA) were included in each run in order to assess the accuracy of the drug dilutions and reproducibility of the results [16]. Five

repeats were performed for each isolate. All QC values were within the ranges established by CLSI [16].

Definitions

A microorganism was defined as wild-type (WT) when it did not display acquired or mutational resistance mechanisms to the drug tested [12,13,17,18]. The typical MIC distribution for WT organisms covers three to five two-fold dilutions surrounding the modal MIC [12,13,17]. Testing a single isolate/clinical specimen, for each infectious episode, ensured the inclusion of WT strains in the present study.

The epidemiological cut-off value was obtained with consideration of the MIC distribution, the modal MIC and the inherent variability of the test, usually within one two-fold dilution. In general, the ECV should encompass at least 95% of the isolates in the WT distribution and should be calculated as two-fold dilution steps higher than the modal value [13,17]. Organisms with acquired or mutational resistance mechanisms may be included among those for which the MIC results are higher than the ECV [13,17,18]. Data were also reported as MIC ranges and mean values, MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the strains were inhibited.

Statistical analysis

Both on-scale and off-scale results were included in the analysis. The low and high off-scale MICs were converted to the lowest MIC or the highest MIC, respectively. MIC mean values of azoles in different groups were screened using paired Student t-tests. Data were statistically analysed using the R software (version 2.8.1, http://www.r-project.org/) and a *P*-value less than 0.05 was considered statistically significant.

Results

The azole MIC data for *M. pachydermatis* and *M. furfur* from different sources are shown in Table 1 and Figure 1. ITZ and POS displayed lower MIC values than VOR and FLZ regardless of the *Malassezia* species or the source of isolate (Table 1; Figure 1). The MIC data for all azoles of *M. pachydermatis* were at least four two-fold dilutions lower than those registered for *M. furfur* (Table 1; Figure 1).

The MIC_{50} and the modal values for VOR and FLZ of *M. furfur* from skin were higher than those registered for *M. furfur* from blood or other sterile sites (Table 1). Based on ECVs, about 94% of *Malassezia* strains, herein tested, were susceptible to all azoles, except FLZ (Table 1). A total of four *M. pachydermatis* strains showed MIC>ECV for FLZ, of which two strains also for ITZ and two for VOR. Four *M. furfur* strains showed MIC>ECV for ITZ with two also for POS and one for POS and VOR (Table 1). A total of 11 *M. furfur* strains (i.e., four from blood or other sterile sites and seven from skin) revealed MIC>128 μg/ml for FLZ only and were categorized as resistant.

Table 1. Fluconazole (FLZ), itraconazole (ITZ), posaconazole (POS), and voriconazole (VOR) minimum inhibitory concentration (MIC, μg/ml) data, standard deviation (SD), and Epidemiological Cut-off Values (ECV) of *Malassezia pachydermatis* and *Malassezia furfur* from different sources.

Isolates	Antifungal drug	Range	MICm (SD)	MIC_{50}	MIC_{90}	Modal MIC	ECV	No. of isolates (%) MIC>ECV
M. pachydermatis	FLZ	4–>64	13.8 (14.8)	8	32	8	32	4/62* (6.4)
Dog skin	ITZ	0.008–0.125	0.013 (0.024)	0.008	0.016	0.008	0.032	2/62 (3.2)
	POS	0.008–0.032	0.013 (0.007)	0.016	0.032	0.016	0.064	0 (0)
	VOR	0.016–0.50	0.074 (0.091)	0.064	0.064	0.064	0.25	2/62 (3.2)
M. furfur	FLZ	8–>128	85 (41.8)	64	128	128	512	4/60° (6.6)
Human blood and sterile site	ITZ	0.032–8	0.6 (1.5)	0.25	1	0.25	1	4/60[V] (6.6)
	POS	0.016–8	0.4 (1.1)	0.25	0.5	0.25	1	4/60 (6.6)
	VOR	0.064–8	1.3 (1.3)	1	2	1	4	1/60 (1.7)
M. furfur	FLZ	8–>128	77 (60.3)	128	>128	>128	>512	7/18° (38.9)
Human skin	ITZ	0.064–16	1.1 (3.7)	0.25	0.5	0.25	1	1/18^(5.5)
	POS	0.032–0.25	0.6 (1.8)	0.125	0.25	0.25	1	1/18 (0)
	VOR	0.064–8	1.9 (2.4)	2	2	2	8	0/18 (0)

Note: Percentage of isolates for which the MIC was greater than the ECV is also reported. MIC_{50} and MIC_{90}: MICs at which 50% and 90%, respectively, of isolates tested were inhibited.

*Two strains showed MIC>ECV for FLZ and ITZ and two for FLZ and VOR.

° The isolates showed MIC>ECV only for FLZ.

[V] Two strains showed MIC>ECV for ITZ and POS and one for ITZ, POS, and VOR.

^ The strain showed MIC>ECV for ITZ and POS.

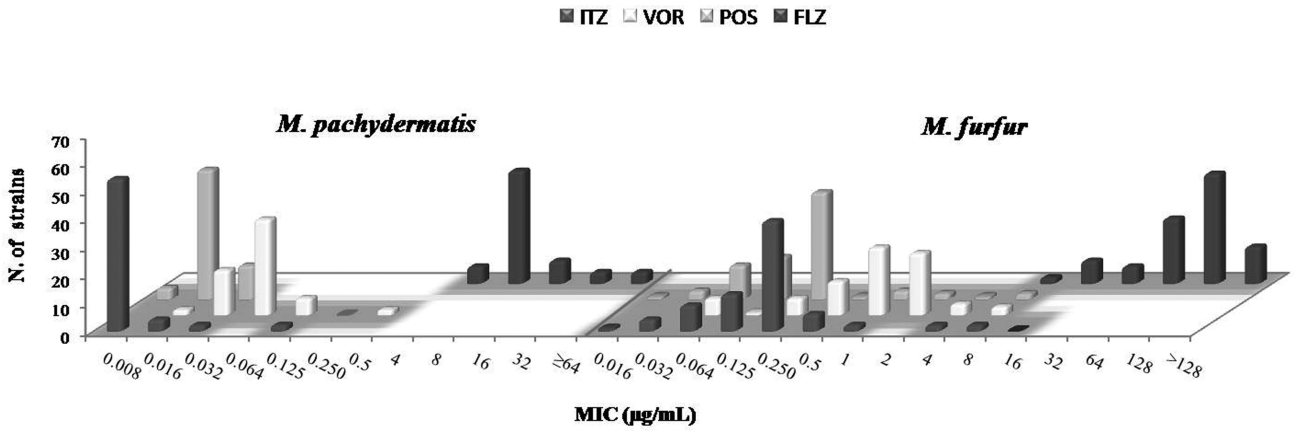

Figure 1. Distribution of minimum inhibitory concentrations (MIC) of itraconazole (ITZ), voriconazole (VOR), posaconazole (POS) and fluconazole (FLZ) of *Malassezia pachydermatis* and *Malassezia furfur.*

Discussion

Malassezia yeasts were highly susceptible to ITZ and POS and less to FLZ, regardless of the species and the source of sampling. Nevertheless, all the azole derivatives tested, except FLZ, showed a high *in vitro* antifungal activity against *M. furfur* and *M. pachydermatis*. In particular, ITZ and POS displayed the best *in vitro* activity and might be used as optimal antifungal agents in the management of *Malassezia* skin or systemic diseases both in humans and/or in veterinary clinical practice.

Since a standard method for determining the antifungal susceptibility of *Malassezia* spp. is not validated by a consensus procedure, the data need to be considered with caution. However, the results herein obtained were in general agreement with those previously reported, even if different media were employed through the use of the CLSI BMD protocol [8,10,20–23].

Accordingly, higher azole MIC values were recorded for *M. furfur* than *M. pachydermatis*, therefore suggesting the usefulness of determining the susceptibility profile for effective treatment of each species. In addition, the MIC values for FLZ, ITZ, and VOR of *M. furfur* were higher in BSI patients than those previously reported for human skin diseases [20,21,23,24]. Although the results above could be due to either the method employed for testing antifungal susceptibility or to the source of the isolates (i.e., BSI patients), the similarity in POS MIC values of *M. furfur* with those reported for *M. furfur* from skin using different protocols, suggests that the source of isolation is pivotal in strain susceptibility [20,23,24].

Accordingly, the higher VOR and FLZ MIC data of *M. furfur* from skin than from blood or other sterile sites might be due to the synergic effects of drugs usually administered to neonatal intensive care unit patients for preventing catheter-related infections [25–31] or to a different source of BSI from the complex ecosystem inhabiting the skin [32]. Moreover, since the number of *M. furfur* skin isolates were limited (i.e., n = 18), it is difficult to make a conclusion about the low susceptibility to VOR and FLZ of the *M. furfur* skin isolates and thus requires further validation.

The ECVs, herein proposed, demonstrated that 93.3% of the strains were within the WT population for all azoles, except for FLZ. Noteworthy, isolates with MIC values exceeding the ECV show reduced susceptibility compared with the WT population. The clinical relevance of these *in vitro* results require further investigation. However, the high ECVs of *M. furfur* for FLZ, ITZ and POS were similar to those previously reported for *Candida glabrata* and/or *Candida krusei* which usually show intrinsic azole resistance and require correct therapeutic management [13,17,19]. This evidence suggests that *Malassezia* BSI patients might be monitored using the same procedures employed for uncommon *Candida* yeasts infections [33]. Interestingly, the VOR ECVs, herein proposed, for *M. furfur* were higher than those previously reported for *Candida* spp. and/or *Aspergillus* spp., thus showing a lower efficacy of this drug for this yeast species [1,18,19,34]. However, even if high variation in the susceptibility profile to VOR was previously registered in *M. furfur*, the MIC values, herein recorded, were higher than those reported in literature [24,35], thus suggesting that the reduced efficacy of VOR might be related to the source of *Malassezia* isolation.

Interestingly, the proposed azole ECVs indicate that different resistance mechanisms may occur in *M. pachydermatis* and *M. furfur*. Indeed, *M. pachydermatis* showed cross-resistance to FLZ and the other azoles, whereas no such cross-resistance occurred for *M. furfur*. The mechanisms of azole resistance in *Malassezia* spp. have never been investigated, although they might be due to enhanced efflux mediated by ATP-binding cassette (ABC) transporters as

reported for *Candida* spp. [36]. FLZ resistance might be due to the induction of the efflux pumps encoded by multidrug resistance (i.e., MDR1) genes, whereas cross-resistance to the other azoles may be mediated by other genes (i.e., CDR), as previously reported for *Candida* spp. [36]. In addition, the similarity of FLZ ECV between *Malassezia* spp. herein tested and *C. krusei* [13,17,19] suggests a probable intrinsic mechanism of azole resistance also in *Malassezia* yeasts. Further investigations on resistance mechanisms in these yeast species are essential.

In conclusion, the results of this study suggest that *Malassezia* yeasts are susceptible to ITZ and POS and less so to FLZ and VOR regardless of the species. Nonetheless, the interspecies variability of the MIC distribution suggests the importance of defining the susceptibility profile of each species in order to obtain reliable information for implementing an effective treatment regimen. The proposed ECVs for ITZ, FLZ, and POS for both *M. pachydermatis* and *M. furfur* compare favourably to those proposed for uncommon *Candida* species and indicate the presence of azole cross-resistance.

Finally, this study proposes for the first time "tentative" azole ECVs for *M. pachydermatis* and *M. furfur* to interpret results of *in vitro* susceptibility tests and shows that they were useful for monitoring the emergence of isolates with decreased susceptibilities. However the data need to be confirmed, since a multicenter evaluation of MIC data was not herein performed being the occurrence of *Malassezia* fungemia lower than other mycoses. Future studies should be focused on the molecular mechanisms of drug resistance of the strains that fall outside the ECVs to validate these data and to promptly develop therapeutic guidelines for *Malassezia* infections.

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Declaration of interest

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

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