mycoses
Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Original article

In vitro antifungal activity of topical and systemic antifungal drugs against *Malassezia* species

Alfonso Javier Carrillo-Muñoz,[1] Florencia Rojas,[2] Cristina Tur-Tur,[3] María de los Ángeles Sosa,[2] Gustavo Ortiz Diez,[4,5] Carmen Martín Espada,[5] María Jesús Payá[5] and Gustavo Giusiano[2]

[1]*Dept. Microbiología-Micología, ACIAM, Barcelona, Spain,* [2]*Instituto de Medicina Regional, Universidad Nacional del Nordeste, Resistencia, Argentina,* [3]*SPDI, CAP Manso, Barcelona, Spain,* [4]*Hospital Clínico Veterinario, Universidad Alfonso X el Sabio, Madrid, Spain and* [5]*Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain*

Summary

The strict nutritional requirements of *Malassezia* species make it difficult to test the antifungal susceptibility. Treatments of the chronic and recurrent infections associated with *Malassezia* spp. are usually ineffective. The objective of this study was to obtain *in vitro* susceptibility profile of 76 clinical isolates of *Malassezia* species against 16 antifungal drugs used for topical or systemic treatment. Isolates were identified by restriction fragment length polymorphism. Minimal inhibitory concentrations (MIC) were obtained by a modified microdilution method based on the Clinical Laboratory Standards Institute reference document M27-A3. The modifications allowed a good growth of all tested species. High *in vitro* antifungal activity of most tested drugs was observed, especially triazole derivatives, except for fluconazole which presented the highest MICs and widest range of concentrations. Ketoconazole and itraconazole demonstrated a great activity. Higher MICs values were obtained with *Malassezia furfur* indicating a low susceptibility to most of the antifungal agents tested. *Malassezia sympodialis* and *Malassezia pachydermatis* were found to be more-susceptible species than *M. furfur*, *Malassezia globosa*, *Malassezia slooffiae* and *Malassezia restricta*. Topical substances were also active but provide higher MICs than the compounds for systemic use. The differences observed in the antifungals activity and interspecies variability demonstrated the importance to studying the susceptibility profile of each species to obtain reliable information for defining an effective treatment regimen.

Key words: *Malassezia*, susceptibility, topical antifungals, systemic antifungals.

Introduction

During the last decades, *Malassezia* taxonomy has undergone a great transformation to its expansion to the 14 species known today.[1–3] Species of the *Malassezia* genus are lipophilic yeast which are present in the normal microbiota of skin in humans and warm-blooded animals.[4–7] Under some predisposing factors, these yeast can act as opportunistic pathogens producing superficial and systemic infections in humans and other animals.[3,5–10] *Malassezia* yeast cause pytiriasis versicolor and can be related as an associated agent or a contributory factor in other dermatological entities such as seborrhoeic dermatitis, atopic dermatitis, seborrhoeic blepharitis, folliculitis, confluent and reticulated papillomatosis of Gougerot-Carteaud, etc.[3,4,8,11] In addition, *Malassezia* species have been associated with deep seated infections such as pneumonia, catheter-related fungemia with lipid parenteral administration and also peritonitis in dialysed patients.[3–5,7]

Correspondence: G. Giusiano, Instituto de Medicina Regional, Universidad Nacional del Nordeste, Av. Las Heras 727, Resistencia 3500, Argentina.
Tel.: +54 3624 422793. Fax: +54 3624 422793.
E-mail: gustavogiusiano@yahoo.com.ar

Submitted for publication 27 November 2012
Revised 22 February 2013
Accepted for publication 24 February 2013

doi:10.1111/myc.12076

Veterinary infections by *Malassezia* are also well documented in superficial dermatitis and otitis in cats and dogs.[9,10,12–14]

Treatments tend to be ineffective due to the chronic and recurrent nature of infections associated with *Malassezia*. Usually treatments are simultaneously oral and topical but not always with good outcome. In addition, *Malassezia*-related systemic infections are reported to be increasing in the last few years.[3,4,15] All these facts emphasise the importance of the knowledge of *in vitro* susceptibility profiles of the species of this genus. This information can be used in clinical practice to establish a treatment regimen and also for adequate monitoring of the patient's clinical evaluation. Currently, the treatments do not follow a particular schema and multiple antifungal drugs for oral and topical therapies are used.

The purpose of this study was to obtain the *in vitro* antifungal susceptibility profile of the clinical isolates of *Malassezia* species against 16 drugs used for topical or systemic treatment by a microdilution method.

Materials and methods

We studied the *in vitro* susceptibility of 76 clinical isolates of *Malassezia* spp., including *Malassezia globosa* (*M. globosa*) ($n = 29$), *M. furfur* ($n = 20$), *M. sympodialis* ($n = 18$), *M. slooffiae* ($n = 2$) and *M. restricta* ($n = 1$) isolated from pytiriasis versicolor lesions. In addition, *M. pachydermatis* ($n = 6$) obtained from otitis lesions in dogs.

The employed strains were molecularly identified and characterised in previous studies by restriction fragment length polymorphism analysis (RFLP).[16,17]

Malassezia yeast isolates were grown on modified Dixon's medium at 32 °C for 5 days.[18]

CBS 7019 *M. furfur*, CBS 7705 *M. globosa*, CBS 7991 *M. restricta*, CBS 7222 *M. sympodialis*, CBS 7956 *M. slooffiae*, CBS 10533 *M. pachydermatis*, CBS 9169 *M. dermatis*, CBS 9558 *M. nana*, CBS 9432 *M. japonica*, CBS 9725 *M. yamatoensis*, CBS 7876 *M. obtusa*, were included as reference strains.

Minimal inhibitory concentrations (MIC) were obtained by following a modified CLSI (Clinical Laboratory Standards Institute) M27-A3 microdilution method.[19]

Antifungal drugs as pure compounds were used: selenium sulphide (Se), zinc pyrithione (ZnP), amorolfine (AMR), miconazole (MCN), ciclopiroxolamine (CIC), tioconazole (TCZ), ketoconazole (KTC), bifonazole (BFN), clotrimazole (CLT), terbinafine (TRB), voriconazole (VRC), posaconazole (PSC), albaconazole (ABC), ravuconazole (RVC), itraconazole (ITC) and fluconazole (FLC). All the drugs were procured from Sigma Aldrich Química™, Madrid, Spain.

Antifungal drugs dilutions were prepared by the microdilution method as mentioned in document CLSI M27-A3.[19] Drug concentrations ranged between 0.016 and 16 μg ml^{-1} were used for all the substances, except for FLC (0.25–256 μg ml^{-1}).

Modifications to adapt the CLSI microdilution method M27-A3 to favour the development of lipid-dependent *Malassezia* spp. were made. The *Malassezia*-adapted versions included: (1) use of RPMI 1640 supplemented ox-bile (0.5%) (Sigma Aldrich) as culture medium, (2) microplates (96 flat-bottomed wells Corning™, Corning Incorporated, New York, USA) incubated at 32 °C for 72 h, (3) inocula suspensions in saline solution with Tween® 20 (Oxoid, Saint Louis, MO, USA) prepared by adjusting the turbidity to a 0.5 McFarland scale and further diluted to get a final inoculum density of 2×10^3–4×10^3 CFU ml^{-1}.[19–21] Inoculated microplates were incubated in humidity chambers to avoid desiccation. MICs were defined as the lowest concentration of antifungal drug which visually inhibited 50% of fungal growth for azole antifungals and 100% for non-azole drugs as compared with the control wells with no antifungal drug.

Clinical Laboratory Standards Institute quality control strains (QC) *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included in this assay.[19]

Data obtained were reported as MIC ranges, MIC at which 50% (MIC50) and 90% (MIC90) of the isolates were inhibited. Comparison between antifungal drugs was carried out by calculating geometric mean MICs and applying Student's *t*-test ($P \leq 0.05$).

Results and discussion

Despite the fact that broth microdilution method recommended by CLSI is accepted as a standard protocol to study *Candida* spp. and *Cryptococcus neoformans* susceptibility,[19] the *Malassezia*-adapted versions used in this study allowed a good growth of this lipophilic yeast and showed good reproducibility for all the species and drugs tested.

To determine the end point, uniform incubation time of 72 h was not sufficient for all the species tested. As *M. globosa* and *M. restricta* are slow growing, they required more incubation time before reading the results. The end point was read at a different time interval for these species including extended time to 5 days.

To study the *in vitro Malassezia* species susceptibility to certain antifungals, different diffusion and dilution methods with a variation in the culture media composition have been tried with the purpose to enable the growth of these lipo-dependant yeasts.[20–25] Different MICs values had been shown using the same clinical isolates with different experimental variables.[23,26,27]

Although the experimental modifications used for susceptibility testing in this study were in agreement with those described by other authors, comparison of data was difficult due to the recent expansion of the genus in new species by PCR methods.[21,22,26,28,29] Results from previous studies which did not employ molecular methods may correspond to false identification of the species. Gupta *et al.* [30] showed a misidentification rate of 13.8%.

Similar to other previous findings, our results also showed a higher *in vitro* antifungal activity for most tested drugs against all clinical isolates of *Malassezia* species (Table 1), especially with triazole derivatives (ITC, VRC, ABC, RVC and PSC) without showing any significant differences.[20,24,29] However, FLC showed significantly high MICs with a wide range of concentrations (Table 1). The lower antifungal activity of FLC observed was in agreement with previous studies that used different culture media composition.[20,26,29]

In this study, MICs values obtained for ABC, KTC, VRC and ITC were considerably lower in comparison with those obtained with different experimental conditions using other culture media such as Leeming-Notman medium agar and modified Christensen's urea.[20,26,29,31,32]

The geometric MIC of KTC ranged from 0.01 to 0.06 μg ml^{-1} for all species tested. This higher activity of KTC observed in this study has been reported by various authors in different studies.[20,21,26,29,32] KTC is probably the most extensively tested azole compound that has shown good *in vitro* activity and hence can be a good choice for the topical treatment of *Malassezia*-related conditions.

In contrast with other studies, our results have described a higher *in vitro* activity values for MCN, CLT, BFN and TCZ.[24,32] Relatively fewer studies have examined the antifungal activity of azole derivates that can be formulated for topical use. Compared with triazole derivatives and imidazoles, these drugs for topical use were less active (Table 1). MICs values obtained for AMR and TRB in this study were found to be lower than those reported in earlier studies.[23,24]

Schmidt *et al.* [25] have reported higher mean MICs values for Se and ZnP (1 μg ml^{-1} for Se and 8 μg ml^{-1} for ZnP, using a Leeming Notman medium)

Table 1 *In vitro* antifungal activity (geometric mean, MIC_{50}, MIC_{90} and MIC ranges in μg ml^{-1}) of 16 antifungal drugs against 76 clinical isolates of *Malassezia* spp.

Species	ABC	AMR	BFN	CIC	CLT	FLN	ITC	KTC	MNC	PSC	Se	ZnP	TCZ	TRB	RVC	VRC
Malassezia furfur (*n* = 20)	0.01	0.13	0.4	0.26	0.98	38.05	0.01	0.01	1.39	0.02	0.28	0.87	0.11	1	0.02	0.03
Malassezia globosa (*n* = 29)	0.01	0.09	0.07	0.24	0.24	8.94	0.02	0.02	0.21	0,01	0.08	0.21	0.05	0.1	0.02	0.01
Malassezia pachydermatis (*n* = 6)	0.01	0.01	0.05	0.01	0.36	1.2	0.02	0.02	0.01	0.01	0.21	0.08	0.02	0.01	0.01	0.01
Malassezia restricta (*n* = 1)	0.01	0.25	0.5	8	8	128	0.01	0.06	2	0.01	1	1	0.06	0.5	0.01	0.01
Malassezia slooffiae (*n* = 2)	0.01	0.01	0.76	0.14	6.06	5.86	0.01	0.01	0.1	0.01	0.14	1.32	0.03	3.03	0.01	0.02
Malassezia sympodialis (*n* = 18)	0.01	0.04	0.05	0.05	0.11	2.9	0.01	0.01	0.03	0.01	0.05	0.08	1	0.06	0.01	0.02
Geometric mean (*n* = 76)	0.01	0.06	0.1	0.13	0.33	7.4	0.01	0.02	0.12	0.01	0.1	0.21	0.03	0.13	0.01	0.02
Minimal MIC (*n* = 76)	0.01	0.01	0.01	0.01	0.01	0.25	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Maximal MIC (*n* = 76)	0.06	16	16	16	16	256	0.06	0.13	16	0.13	16	8	16	16	0.13	0.25
MIC_{50} (*n* = 76)	0.01	0.01	0.13	0.01	1	32	0.01	0.01	0.01	0.01	0.03	0.5	0.01	0.01	0.01	0.01
MIC90 (*n* = 76)	0.01	2	2	4	8	256	0.03	0.06	8	0.01	2	2	0.5	8	0.01	0.13

ABC, albaconazole; MR, amorolfine; BFN, bifonazole; CIC, ciclopiroxolamine; CLT, clotrimazole; FLC, fluconazole, ITC, itraconazole; KTC, ketoconazole; MNC, miconazole; PSC, posaconazole, Se, selenium sulphide; ZnP, zinc pyritione; TCZ, tioconazole; TRB, terbinafine; RVC, ravuconazole; VRC, voriconazole.

than those observed in this study (Table 1).Interestingly, our susceptibility pattern for Se and ZnP was similar to that described by Van Cutsem *et al.* [33], where they have demonstrated a good *in vitro* antifungal activity in Dixon and Sabouraud culture media.

Triazole derivatives ITC, VRC, ABC, RVC and PSC offered a similar and uniform *in vitro* activity pattern against the different *Malassezia* species tested. The only exception was FLC that was found to be less active against *M. furfur*, *M globosa, M slooffiae* and *M. restricta*. Lower MICs were also observed for *M. sympodialis* and *M. pachydermatis* (Table 1).

Malassezia furfur, *M. globosa* and *M. sympodialis* are the species that are most frequently described worldwide in dermal conditions related to *Malassezia*. In our study, *M. furfur* was the species with higher MICs values indicating a lower susceptibility to the most of antifungal agents tested (Table 1). These values are lower than those published by Van Gerven *et al.* [32], who reported geometric MIC values of 0.51 μg ml^{-1} for KTC; 8.1 μg ml^{-1} for BFN; 14 μg ml^{-1} for MCN and 15 μg ml^{-1} for CLT.

Lower MICs values were also observed for *M. pachydermatis* with all the antifungals tested. Our results were lower than those obtained with MCN and CLT by Peano *et al.* [34] under similar experimental conditions. In contrast, Cafarchia *et al.* [35] reported clinical isolates of *M. pachydermatis* from animal samples with a high susceptibility to ITC, KTC, PSC but resistant to TRB, FLC and MCN at the same time. Few previous studies have also reported azolic cross resistance in *M. pachydermatis* isolates.[27,35,36]

Although *Malassezia* species show differences in their *in vitro* antifungal susceptibility, the *in vivo* efficacy needs to be further evaluated to obtain the correlation between *in vitro* MICs and the clinical outcomes.

In conclusion

With the exception of FLC, all the triazole derivatives tested showed clearly a high *in vitro* antifungal activity against all *Malassezia* species studied. These drugs could be a good option for the systemic management of *Malassezia* infections, mainly in those extended, severe or recurrent forms.

Although FLC is one of the most commonly used antifungal agent worldwide, our results showed higher MICs for FLC than other molecules. In addition, FLC was the antifungal agent that showed more variability between the species.

Ketoconazole and ITC proved being the antifungals with best *in vitro* activity against all *Malassezia* species tested. ITC is currently used worldwide as an oral antifungal agent and KTC has been reported as optimal for the topical management of *Malassezia* infections in human and veterinary clinical practice.

Topical alternative substances, such as BFN, CLT, MCN, Se and ZnP used for the treatment and prophylactic purposes in dermatological problems, were also active *in vitro* but provides higher MICs values than the majority of azole derivates.

Diverse antifungal profiles and interspecies variability observed necessitates the development of *in vitro* tests to know the susceptibility profile of each species to obtain reliable information for the development a treatment that can be efficient.

Conflict of interest

The authors have no conflict of interest to declare.

References

1 Cabañes FJ, Vega S, Castellá G. *Malassezia cuniculi* sp. nov., a novel yeast species isolated from rabbit skin. *Med Mycol* 2011; **49**: 40–48.
2 Cafarchia C, Gasser RB, Figueredo LA, Latrofa MS, Otranto D. Advances in the identification of *Malassezia*. *Mol Cell Probes* 2011; **25**: 1–7.
3 Gaitanis G, Magiatis P, Hantschke M, Bassukas ID, Velegraki A. The *Malassezia* genus in skin and systemic diseases. *Clin Microbiol Rev* 2012; **25**: 106–41.
4 Giusiano GE. *Malassezia*. Current knowledge and study perspectives. *Rev Argent Microbiol* 2006; **38**: 41–48.
5 Batra R, Boekhout T, Guého E *et al. Malassezia* Baillon, emerging clinical yeast. *FEMS Yeast Res* 2005; **5**: 1101–13.
6 Guého E, Boekhout T, Ashbee HR *et al.* The role of *Malassezia* species in the ecology of human skin and as pathogens. *Med Mycol* 1998; **36**(Suppl. 1): 220–9.
7 Ashbee HR. Update on genus *Malassezia*. *Med Mycol* 2007; **45**: 287–303.
8 Gupta AK, Batra R, Bluhm R, Boekhout T, Dawson TL Jr. Skin diseases associated with *Malassezia species*. *J Am Acad Dermatol* 2004; **51**: 785–98.
9 Cafarchia C, Gallo S, Capelli G, Otranto D. Occurrence and population size of *Malassezia* spp. in the external ear canal of dogs and cats both healthy and with otitis. *Mycopathol* 2005; **160**: 143–9.
10 Cafarchia C, Gallo S, Romito D *et al.* Frequency, body distribution, and population size of *Malassezia* species in healthy dogs and in dogs with localized cutaneous lesions. *J Vet Diag Invest* 2005; **17**: 316–22.
11 Sugita T, Boekhout T, Velegraki A, Guillot J, Hadina S, Cabañes FJ. Epidemiology of *Malassezia*-related skin diseases. In: Boekhout T, Guého-Kellermann E, Mayser P, Velegraki A (eds), *Malassezia and the Skin*. Berlin, Heidelberg: Springer Verlag, 2010: 65–121.
12 Chen TA, Hill PB. The biology of *Malassezia* organisms and their ability to induce immune responses and skin disease. *Vet Dermatol* 2005; **16**: 4–26.
13 Faergemann J, Bergbrant IM, Dohsé M, Scott A, Westgate G. Seborrhoeic dermatitis and *Pityrosporum* (*Malassezia*) folliculitis: characterization of inflammatory cells and mediators in the skin by immunohistochemistry. *Br J Dermatol* 2001; **144**: 549–56.
14 Nardoni S, Corazza M, Mancianti F. Diagnostic and clinical features of animal malasseziosis. *Parasitol* 2008; **50**: 81–83.

15 Gupta AK, Kogan N, Batra R. Pityriasis versicolor: a review of pharmacological treatment options. *Expert Opin Pharmacother* 2005; **6**: 165–78.
16 Mirhendi H, Makimura K, Zomorodian K, Yamada T, Sugita T, Yamaguchi H. A simple PCR-RFLP method for identification and differentiation of 11 *Malassezia* species. *J Microbiol Methods* 2005; **61**: 281–4.
17 Giusiano G, Sosa MA, Rojas F, Toma Vanacore S, Mangiaterra M. Prevalence of *Malassezia* species in pityriasis versicolor lesions in northeast Argentina. *Rev Iberoam Micol* 2010; **27**: 71–74.
18 Guillot J, Gueho E, Lesourd M *et al.* Identification of *Malassezia* species: a practical approach. *J Med Mycol* 1996; **6**: 103–10.
19 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard*, 3rd edn. M27-A3. Wayne, PA: CLSI, 2008.
20 Garau M, Pereiro M Jr, del Palacio A. *In vitro* susceptibilities of *Malassezia* species to a new triazole, albaconazole (UR-9825), and other antifungal compounds. *Antimicrob Agents Chemother* 2003; **47**: 2342–4.
21 Velegraki A, Alexopoulos EC, Kritikou S, Gaitanis G. Use of fatty acid RPMI 1640 media for testing susceptibilities of eight *Malassezia* species to the new triazole posaconazole and to six established antifungal agents by a modified NCCLS M27–A2 microdilution method and Etest. *J Clin Microbiol* 2004; **42**: 3589–93.
22 Ahn KJ, Ashbee AR. Determination of minimun inhibitory concentration of several azole antifungal for *Malassezia furfur*. *Ann Dermatol* 1996; **8**: 187–94.
23 Gupta AK, Kohli Y, Li A, Faergemann J, Summerbell RC. In vitro susceptibility of the seven *Malassezia* species to ketoconazole, voriconazole, itraconazole and terbinafine. *Br J Dermatol* 2000; **142**: 758–65.
24 Nakamura Y, Kano R, Murai T, Watanabe S, Hasegawa A. Susceptibility testing of *Malassezia* species using the urea broth microdilution method. *Antimicrob Agents Chemother* 2000; **44**: 2185–6.
25 Schmidt A, Rühl-Hörster B. In vitro susceptibility of *Malassezia furfur*. *Arzneimittelforschung* 1996; **46**: 442–4.
26 Rincón S, Cepero de García MC, Espinel-Ingroff A. A modified Christensen's urea and CLSI broth microdilution method for testing susceptibilities of six *Malassezi*a species to voriconazole, itraconazole, and ketoconazole. *J Clin Microbiol* 2006; **44**: 3429–31.
27 Nijima M, Kano R, Nagata M, Hasegawa A, Kamata H. An azole-resistant isolates of *Malassezia pachydermatis*. *Vet Microbiol* 2011; **149**: 288–90.
28 Prado MR, Brito EHS, Brilhante SNR *et al.* Subculture on Potato dextrosa agar as a complement to the broth microdilution assay for *Malassezia pachydermatis*. *J Microbiol Methods* 2008; **75**: 341–3.
29 Miranda KC, de Araujo CR, Costa CR, Passos XS, de Fátima Lisboa Fernandes O, do Rosário Rodrigues Silva M. Antifungal activities of azole agents against the *Malassezia* species. *Int J Antimicrob Agents* 2007; **29**: 281–4.
30 Gupta A, Boekhout T, Theelen B, Summerbell R, Batra R. Identification and typing of *Malassezia* species by amplified fragment length polymorphism and sequence analyses of the internal transcribed spacer and large-subunit regions of ribosomal DNA. *J Clin Microbiol* 2004; **42**: 4253–60.
31 Murai T, Nakamura Y, Kano R, Watanabe S, Hasegawa A. Susceptibility testing of *Malassezia pachydermatis* using the urea broth microdilution method. *Mycoses* 2002; **45**: 84–87.
32 Van Gerven F, Odds FC. The anti-*Malassezia furfur* activity in vitro and in experimental dermatitis of six imidazole antifungal agents: bifonazole, clotrimazole, flutrimazole, ketoconazole, miconazole and sertaconazole. *Mycoses* 1995; **38**: 389–93.
33 Van Cutsem J, Van Gerven F, Fransen J, Schrooten P, Janssen PA. The in vitro antifungal activity of ketoconazole, zinc pyrithione, and selenium sulfide against *Pityrosporum* and their efficacy as a shampoo in the treatment of experimental pityrosporosis in guinea pigs. *J Am Acad Dermatol* 1990; **22**: 993–8.
34 Peano A, Beccati M, Chiavassa E, Pasquetti M. Evaluation of the antifungal susceptibility of *Malassezia pachydermatis* to clotrimazole, miconazole and thiabendazole using a modified CLSI M27–A3 microdilution method. *Vet Dermatol* 2012; **23**: 131–5.
35 Cafarchia C, Figueredo LA, Iatta R, Montagna MT, Otranto D. In vitro antifungal susceptibility of *Malassezia pachydermatis* from dogs with and without skin lesions. *Vet Microbiol* 2012; **155**: 395–8.
36 Jesus FP, Lautert C, Zanette RA *et al.* In vitro susceptibility of fluconazole-susceptible and resistant isolates of *Malassezia pachydermatis* against azoles. *Vet Microbiol* 2011; **152**: 161–4.