

Short Communication

Susceptibility variation of *Malassezia pachydermatis* to antifungal agents according to isolate source

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Abstract

Malassezia pachydermatis is associated with dermatomycoses and otomycosis in dogs and cats. This study compared the susceptibility of *M. pachydermatis* isolates from sick (G1) and healthy (G2) animals to azole and polyene antifungals using the M27-A3 protocol. Isolates from G1 animals were less sensitive to amphotericin B, nystatin, fluconazole, clotrimazole and miconazole.

Key words: *Malassezia pachydermatis*, susceptibility, antifungal agents.

Currently, the genus *Malassezia* includes fourteen species, thirteen of which are lipid-dependent and are most frequently recovered from humans, ruminants and horses (*Malassezia furfur*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, *M. dermatis*, *M. nana*, *M. japonica*, *M. yamatoensis*, *M. equina*, *M. caprae* and *M. cuniculi*); the non-lipid-dependent species, *M. pachydermatis*, is commonly recovered from dogs and cats (Cabañas *et al.*, 2011). The distribution of different species, the colonization prevalence and the population density of *Malassezia* spp. varies by carrier and affected sites of the body, which are influenced by the skin lipid composition and the presence of competitive microbiota (Sugita *et al.*, 2010).

M. pachydermatis is the species most adapted to animals and is often recovered as part of the microbiota of the ear canal and skin of dogs, cats and other species of domestic and wild animals. In dogs and cats, *M. pachydermatis* has been linked to both localized (otitis externa and dermatitis) and systemic disease (Guillot and Bond, 1999). Antifungal triazoles are commonly used for treatment, and treatment should be continued until the clinical signs re-

solve and yeast are no longer observed during direct examination (Daigle, 2007).

The aim of this study was to compare the susceptibility of *M. pachydermatis* isolates recovered from healthy animals and animals with otitis to ketoconazole, fluconazole, itraconazole, voriconazole, clotrimazole, miconazole, nystatin and amphotericin B.

We studied two groups of *M. pachydermatis* isolates taken from dogs and cats at the Cellular and Molecular Biology Laboratory of the Paulista University (UNIP, São Paulo, Brazil). Group 1 (G1) was comprised of 40 isolates recovered from the ear canals of animals with otitis externa; group 2 (G2) was comprised of 40 isolates recovered from the ear canals of healthy animals. Isolate identification was confirmed by randomly amplified polymorphic DNA using the Mpa-F (CTGCCATACGGATGCGCAAG) and 58S-R (TTCGCTGCGTTCTTCATCGA) primers (Sugita *et al.*, 2003).

Stock solutions of antifungal agents were obtained from the dilution of each antifungal drug in dimethyl sulfoxide or sterile distilled water for fluconazole. The drugs were serially diluted in RPMI 1640 broth (GIBCO™) to obtain the following final concentrations: ketoconazole

(16 µg/mL-0.007 µg/mL) (Janssen Beerse), itraconazole (16 µg/mL-0.007 µg/mL) (Janssen Beerse), clotrimazole (64 µg/mL-0.125 µg/mL) (Bayer), voriconazole (16 µg/mL-0.007 µg/mL) (Pfizer), miconazole (64 µg/mL-0.125 µg/mL) (Labware), nystatin (64 µg/mL-0.125 µg/mL) (Bristol-Myers), amphotericin B (16 µg/mL-0.007 µg/mL) (Bristol-Myers) and fluconazole (64 µg/mL-0.125 µg/mL) (Pfizer). Inocula were obtained from 48-h pure Dixon agar cultures and consisted of micro-organism suspensions in sterile saline (0.85%) plus Triton X-100 (0.05%) (Merck), whose turbidity was adjusted to 0.5 on the McFarland scale. Inocula were diluted 1:50 in sterile distilled water and then at 1:20 in RPMI 1640 broth. For each isolate, microdilution plates containing 10 µL of antifungals in RPMI 1640 diluted in different concentrations were inoculated with 10 µL of the standardized inocula. As positive control, the standardized inocula were cultured alone; the negative control was the antifungal alone diluted in RPMI 1640 broth. Culture plates were incubated at 37 °C for 48 h. Minimum inhibitory concentrations (MICs) were recorded following the M27-A3 protocol (CLSI, 2007). All tests were performed in duplicate. The Mann-Whitney test was used to compare the two groups of isolates to determine whether they had similar susceptibility patterns to the antifungal agents tested.

Because no standardized susceptibility testing procedures exist for *M. pachydermatis*, the present study was based on the M27-A3 protocol (CLSI, 2007); in the absence

of specific breakpoints for *Malassezia* spp., we utilized breakpoints described for *Candida* spp.

In this work, the lowest MICs were observed with ketoconazole, itraconazole and voriconazole (Table 1). Voriconazole showed the smallest variations of MICs with the MICs for G1 isolates ranging from 0.01-0.25 µg/mL, while the MICs for G2 isolates ranged from 0.01 to 0.125 µg/mL. These data are consistent with findings from Gupta *et al.* (2000) that reported MICs ranging from 0.03 to 0.25 µg/mL. Hammer *et al.* (2000) reported the lowest MICs with ketoconazole, with MIC₅₀ and MIC₉₀ values similar to values we recorded for G2 isolates (Table 2). Although the susceptibility testing for itraconazole, fluconazole and amphotericin B showed that 100% of the isolates were susceptible to these antifungals, the MIC range was higher in G1 isolates compared to G2 isolates (Table 2). Nakamura *et al.* (2000) tested seven *Malassezia* species and all were susceptible to itraconazole; however, *M. pachydermatis* exhibited the highest MIC range among the species evaluated.

Statistical analysis revealed significant differences in susceptibility between G1 and G2 isolates. In the susceptibility tests with amphotericin B, nystatin, fluconazole, miconazole and clotrimazole, G1 isolates had significantly higher MICs compared to G2 isolates ($p < 0.05$). The existence of a different susceptibility profile between the two groups revealed higher antifungal resistance in the isolates obtained from infected animals (G1). The study by

Table 1 - Distribution of MICs (µg/mL) for *M. pachydermatis* isolated from ear canals of animals with and without otitis.

Groups	No. (%) of isolates sensitive to concentrations of:								
	0.01	0.03	0.06	0.125	0.25	0.5	1.0	2.0	
KTZ ^a	G1	19 (47.5)	-	3 (7.5)	8 (20)	2 (5)	8 (20)	-	-
	G2	17 (42.5)	2 (5)	5 (12.5)	10 (25)	4 (10)	2 (5)	-	-
FLZ ^b	G1	2 (5)	2 (5)	3 (7.5)	9 (22.5)	5 (12.5)	15 (37.5)	4 (10)	-
	G2	3 (7.5)	2 (5)	2 (5)	11 (27.5)	5 (12.5)	7 (17.5)	-	-
ITZ ^c	G1	22 (55)	2 (5)	4 (10)	5 (12.5)	3 (7.5)	4 (10)	-	-
	G2	17 (42.5)	3 (7.5)	10 (25)	9 (22.5)	1 (2.5)	-	-	-
VRZ ^d	G1	10 (25)	10 (25)	9 (22.5)	7 (17.5)	4 (10)	-	-	-
	G2	16 (40)	9 (22.5)	6 (15)	9 (22.5)	-	-	-	-
CTZ ^e	G1	5 (12.5)	4 (10)	1 (2.5)	7 (17.5)	7 (17.5)	12 (30)	4 (10)	-
	G2	6 (15)	11 (27.5)	4 (10)	9 (22.5)	5 (12.5)	5 (12.5)	-	-
MCZ ^f	G1	6 (15)	6 (15)	2 (5)	4 (10)	8 (20)	14 (35)	-	-
	G2	9 (22.5)	10 (25)	5 (12.5)	5 (12.5)	4 (10)	7 (17.5)	-	-
NYS ^g	G1	3 (7.5)	6 (15)	3 (7.5)	5 (12.5)	7 (17.5)	11 (27.5)	1 (2.5)	4(10)
	G2	7 (17.5)	8 (20)	5 (12.5)	6 (15)	5 (12.5)	7 (17.5)	2 (5)	-
AMB ^h	G1	4 (10)	4 (10)	1 (2.5)	7 (17.5)	7 (17.5)	13 (32.5)	4 (10)	-
	G2	12 (30)	3 (7.5)	2 (5)	8 (20)	3 (7.5)	12 (30)	-	-

^aKetoconazole, ^bFluconazole, ^cItraconazole, ^dVoriconazole, ^eClotrimazole, ^fMiconazole, ^gNystatin, ^hAmphotericin B. G1: isolated from animals with otitis; G2: saprophytic isolates.

Table 2 - *In vitro* susceptibility of *M. pachydermatis* isolates to antifungal agents.

Antifungal	Groups	MIC range ^a (µg/mL)	MIC ₅₀ ^b (µg/mL)	MIC ₉₀ ^c (µg/mL)	GM ^d (µg/mL)
Ketoconazole	G1	0.01-0.5	0.06	0.5	0.048
	G2	0.01-0.5	0.06	0.25	0.041
Fluconazole	G1*	0.01-1.0	0.25	0.5	0.219
	G2**	0.01-0.5	0.125	0.5	0.068
Itraconazole	G1	0.01-0.5	0.01	0.25	0.032
	G2	0.01-0.25	0.03	0.125	0.032
Voriconazole	G1	0.01-0.25	0.03	0.125	0.042
	G2	0.01-0.125	0.03	0.125	0.029
Clotrimazole	G1*	0.01-1.0	0.25	0.5	0.163
	G2**	0.01-0.5	0.06	0.5	0.069
Miconazole	G1*	0.01-0.5	0.25	0.5	0.124
	G2**	0.01-0.5	0.06	0.5	0.061
Nystatin	G1*	0.01-2.0	0.25	1.0	0.181
	G2**	0.01-1.0	0.06	0.5	0.084
Amphotericin B	G1*	0.01-1.0	0.25	0.5	0.180
	G2**	0.01-0.5	0.125	0.5	0.081

G1: isolated from animals with otitis; G2: saprophytic isolates; ^aInterval between the lowest and highest MICs; ^bMinimum concentration of the antifungal agent able to inhibit the growth of 50% of the isolates; ^cMinimum concentration of the antifungal agent able to inhibit the growth of 90% of the isolates; ^dGeometric mean of MICs. (*)(**)Statistically different susceptibility profiles (p < 0.05).

Lyskova *et al.* (2007) using *M. pachydermatis* isolates from animals with and without otitis media found that all isolates exhibited high susceptibility to all of the antifungal agents tested in this study, except for fluconazole, to which 4.4% of the isolates were resistant. However, in this same study, significant differences in susceptibility between the two isolate groups were not observed. Fluconazole resistance has been described previously as Eichenberg *et al.* (2003) observed fluconazole resistance in 2.4% of the 82 isolates tested. Further, Jesus *et al.* (2011) induced resistance to fluconazole *in vitro*, demonstrating that *M. pachydermatis* isolates can become resistant during treatment with this antifungal. Bernardo *et al.* (1998) found a *M. pachydermatis* isolate that was resistant to several antifungals, including amphotericin B, nystatin and miconazole.

Malassezia species have a structure resembling a capsule around the cell wall that provides protection and is similar in form to the capsule of *Cryptococcus* spp. (Ashbee and Bond, 2010). In saprophytic strains, this capsule contains high levels of lipids, blocking the exposure of fungal antigenic proteins. Factors such as humidity, high temperature and high fat environment favor rapid *M. pachydermatis* multiplication, which is accompanied by decreased lipid content and exposure of antigenic particles. The reduced susceptibility observed in G1 isolates against azole antifungal agents might be linked to variations in lipid composition. The main mechanism of action of azoles is to inhibit ergosterol synthesis. However, these drugs also act on the cell membrane through direct interaction with its

lipid components (Hitchcock *et al.*, 1986). Thus, during infection, the body's immune cells release substances that promote a lipid imbalance in the constitution of the fungal membrane, affecting membrane permeability (De Kruyff *et al.*, 1973) and interfering with the action of azole antifungal agents. In the case of polyene antifungal agents, the reduced susceptibility of the G1 isolates may be related to the high activity of fungal intracellular enzymes such as catalase and/or superoxide dismutase. In microorganisms, catalase plays an important role in the detoxification of reactive oxygen species that are released by phagocytic cells during the host immune response (Hampton *et al.*, 1998). Because polyenes act directly on fungal ergosterol causing direct oxidative damage to the cell membrane, the high concentration of catalase in fungal cells could protect the fungal cells from the oxidative action of polyene antifungal agents, possibly explaining the higher MICs in isolates obtained from animals with malasseziosis.

This study demonstrated that *M. pachydermatis* isolated from animals with otitis are less sensitive to some antifungal agents than yeasts isolated from animals without otitis. This finding may explain because some malasseziosis treatment failure and emphasizes the importance to evaluate the susceptibility of this pathogenic fungi.

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