



Susceptibility of *Malassezia pachydermatis* to azole antifungal agents evaluated by a new broth microdilution method*

Avaliação da susceptibilidade da *Malassezia pachydermatis* aos antifúngicos azólicos através de um novo método de microdiluição em caldo

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ABSTRACT

Malassezia pachydermatis is considered an opportunistic pathogen of the outer ear duct in dogs and cats. This yeast can also be found in the skin, rectum, anal sacks and vagina. Eighty-two samples of this yeast isolated from dogs with the symptoms of external otitis from the Porto Alegre region were tested for their susceptibility to antifungal agents using the Broth Microdilution Method. The testing antifungal agents were Ketoconazole, Fluconazole and Itraconazole. Experimental essays determined that Sabouraud dextrose broth supplemented with 1% Tween 80 was the most appropriate medium for culture, for a ten-fold dilutions for the inocula, and 48 hours as the interval of readings. The ranges of the Minimal Inhibitory Concentrations (MICs) for the 82 samples were (a) Ketoconazole, from 0.015 to 0.25 mg/mL (mean of 0.08 mg/mL), (b) Fluconazole, from 1 to 32 mg/mL (mean of 9.22 mg/mL), and (c) Itraconazole, from 0.007 to 0.125 mg/mL (mean of 0.05 mg/mL). The isolates of *M. pachydermatis* showed an excellent level of susceptibility to antifungal azole agents, with all strains being susceptible to Itraconazole, and with only 2.4% and 3.7% being resistant to Fluconazole and Ketoconazole, respectively. The use of the broth microdilution method allows the assessment of the susceptibility of large numbers of samples from *M. pachydermatis* isolates to the most common antifungal agents. The proposed procedure is easy to perform and considerably inexpensive compared with other existing tests, which makes this a method of choice for laboratorial use. A standardization of procedures currently used in veterinary mycology laboratories is required. Consistent results among laboratories could greatly benefit the establishment of proper comparisons between studies on antifungal susceptibility and therapeutic trials.

Key words: Antifungal susceptibility, *Malassezia pachydermatis*, broth microdilution, otitis, ketoconazole, fluconazole, itraconazole.

RESUMO

Malassezia pachydermatis é uma levedura, habitante normal e patogênica oportunista do conduto auditivo externo de cães e gatos, mas também pode ser encontrada na pele, reto, sacos anais e vagina. Oitenta e duas amostras desta levedura, isoladas de cães com sintomatologia de otite externa oriundas da região de Porto Alegre foram testadas quanto à susceptibilidade antifúngica pelo teste de Microdiluição em Caldo. Os antifúngicos utilizados foram cetoconazol, fluconazol e itraconazol. Ensaios experimentais indicaram que o Sabouraud dextrose líquido acrescido de 1% Tween 80 era o meio de cultivo mais adequado, assim como a diluição de 1:10 e o tempo de incubação de 48 horas. A faixa de variação da Concentração Inibitória Mínima (CIM) foi a seguinte: cetoconazol de 0,015 a 0,25 mg/mL (média de 0,08 mg/mL); fluconazol de 1 a 32 mg/mL (média de 9,22 mg/mL) e itraconazol de 0,007 a 0,125 mg/mL (média de 0,05 mg/mL). Os isolados de *M. pachydermatis* apresentaram uma excelente sensibilidade aos agentes antifúngicos testados sendo todos sensíveis ao itraconazol, com apenas 2,4% resistentes ao fluconazol e 3,7% ao cetoconazol. O emprego desta proposta de metodologia de microdiluição em caldo, permite a avaliação da susceptibilidade *in vitro* de um grande número de isolados clínicos de *M. pachydermatis* aos agentes antifúngicos mais comuns. O fato do protocolo ser de fácil execução, aliado ao baixo custo em relação aos testes similares, torna esta técnica adequada para a rotina laboratorial. Para que seja possível o estabelecimento de comparações criteriosas entre os resultados dos antifungogramas e avaliações cooperativas de ensaios terapêuticos, existe uma necessidade imperiosa de uma padronização mínima dos protocolos utilizados em laboratórios de Micologia Veterinária.

Descritores: susceptibilidade antifúngica, *Malassezia pachydermatis*, microdiluição em caldo, otite externa, cetoconazol, fluconazol, itraconazol.

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INTRODUCTION

The yeast *Malassezia pachydermatis* is a common inhabitant of the ear canal of dogs, cats, and other animals, but it can also be found in the skin, rectum, anal sacks and vagina. This yeast can also become an opportunistic pathogen [3,6,14]. An infectious state caused by the *M. pachydermatis* usually parallels or follows a primary established illness in the animal [2-4,11,13,20,22,23,26,29]. Recently, an increase in the incidence of canine otitis by *M. pachydermatis* has been reported in the region covered by this study [12,18].

The treatment of yeast infections has been rather empirical and is most often unsuccessful. In aggravation, susceptibility tests are seldom performed [8]. The *in vitro* susceptibility tests for *Malassezia pachydermatis* was first described in 1976 by Maestrone *et al.* [19]. In Brazil, Coutinho & Paula [6,7] described the results of similar test in 1997, utilizing an expensive and non-standardized method, named ETEST. Several other studies have reported distinct procedures and non-standard methods [7,8,15,17,24,26,31,32]. Clearly, a standardization of procedures currently used in veterinary mycology laboratories is still lacking.

The aim of this study was to determine the susceptibility profile of 82 *Malassezia pachydermatis* cultures obtained from the ear canal of dogs and cats to the common antifungal agents Ketoconazole, Fluconazole and Itraconazole, using a Broth Microdilution Method modified from procedures described by the National Committee for Clinical Laboratory Standards (NCCLS, USA) [25].

MATERIALS AND METHODS

Isolates and culture conditions: Eighty-two *Malassezia pachydermatis* cultures obtained from the ear canal of dogs and cats were used in this study. Initially, the samples were isolated on Sabouraud dextrose agar supplemented with chloramphenicol¹ (500 mg/L), following incubation at 35°C during four to seven days. All cultures were maintained on the same medium described above, at room temperature, with subcultures being carried out on a monthly basis. Prior to the assays, isolates were subcultured twice on the same medium above, and incubated at 35°C for 48h to ensure purity and optimal growth.

Antifungal agents: Media containing each azole agent used in these experiments were prepared in stock solutions. Fluconazole² and Itraconazole³ were dissolved in sterile distilled water and in polyethylene glycol 400⁴, respectively, to obtain stock solutions containing 500µg/mL. The stock solution of Ketoconazole³ was diluted in a hydrochloric acid solution (0.2N) to a final concentration of 10.000µg/mL, to be subsequently aliquoted and stored frozen at -70°C until use.

Assay medium: Based on preliminary studies with RPMI 1640⁵ and Yeast Nitrogen Base⁶ media (data not shown), Sabouraud dextrose broth, supplemented with 1% Tween 80 [16] was chosen for the assays.

Broth microdilution method: Testing was performed in 96-well round-bottom microtiter plates. Cell suspensions were prepared in Sabouraud dextrose supplemented with 1% Tween 80, and inoculum concentrations were adjusted to approximately 0.5 x 10⁶ to 3.0 x 10⁶ cells/mL. Stock solutions were diluted with Sabouraud dextrose broth supplemented with 1% Tween 80. The final antifungal agent concentrations ranged from 0.125 to 64 µg/mL for Fluconazole, 0.078 to 4.0µg/mL for Itraconazole, and 0.015 to 8.0µg/mL for Ketoconazole. Volumes of 100µL of each antifungal concentration were dispensed in each well, following procedures described elsewhere (M27 NCCLS protocol). Plates were frozen until assayed, when 100µL of the adjusted inoculum were enclosed. Subsequently, plates were incubated at 35°C, to be read after 48h. The MICs (minimal inhibitory concentrations) were defined as the lowest azole concentration at which there was 50% inhibition of growth (MIC-50) compared with a drug-free growth control. A culture of *Malassezia pachydermatis* with a known clinical sensibility to Itraconazole treatment was employed as a control reference.

RESULTS

A total of 246 MICs for the azoles used in this study were determined from the isolates of *Malassezia pachydermatis* obtained from clinical cases of canine otitis (Table 1). The MICs varied from 0.015 to 0.25 mg/mL (mean of 0.08 mg/mL), 1.0 to 32.0 mg/mL (mean of 9.22 mg/mL), and 0.007 to 0.125 mg/mL (mean of 0.05 mg/mL) to Ketoconazole, Fluconazole, and Itraconazole, respectively. The variation observed

for the MIC to Ketoconazole was greater than to the other azoles used in these experiments.

The patterns of susceptibility for the 82 isolates of *M. pachydermatis*, considering the MIC values (in mg/mL) for Ketoconazole (MIC-50 ≤ 0.06 and MIC-90 ≤ 0.125), Fluconazole (MIC-50 ≤ 8 and MIC-90 ≤ 16), and Itraconazole (MIC-50 ≤ 0.06 and MIC-90 ≤ 0.125), are presented in Table 2. Overall, for the range of azole concentrations used in the susceptibility study, a very low frequency of resistance was observed in the isolates, with all samples being susceptible to Itraconazole.

DISCUSSION

A better understanding of the fungal susceptibility profiles to antifungal agents is essential for therapeutics, providing epidemiological elements to unravel the reality of mycotic infections.

After intensive investigations by research groups [5,9,10,21,27,28,30,31], *in vitro* susceptibility tests have been recently standardized (NCCLS M27-A document) [25] for *Candida* spp. and *Cryptococcus neoformans*, with some still being currently underway. However, the M27-A technique should not be readily applied to *Malassezia pachydermatis*, since this yeast has distinct biochemical demands than former species. Due to its excellent growth pattern demonstrated in preliminary studies [33], Sabouraud dextrose broth supplemented with 1% Tween 80, was chosen

as the assay medium to be used in these experiments.

The inoculum concentrations recommended by the M27-A document for *Candida* sp. and *Cryptococcus neoformans* did not confer an adequate pathogen growth in culture when applied for *Malassezia pachydermatis*. An optimal growth pattern was obtained only after a minimum of 100-fold increase in cell counts (0.5-3.0 x 10⁶ cfu/mL).

The MIC patterns observed in this study displayed an acceptable profile if confronted with assays carried out with those described for *Candida* sp. and *Cryptococcus neoformans*. Based on the results obtained, it appears that important variables such as medium, inoculum concentrations, pH, and temperature and time of incubation were adequately selected for the execution of these experiments.

The interpretation of the observed MIC values also requires caution because the classification as susceptible, susceptible-dose depending (SDD), or resistant, as recommended by the M27-A document, applies specifically to *Candida* sp. and *Cryptococcus neoformans*. After adapting this guideline to *Malassezia pachydermatis*, the results showed 28 (34.1%) SDD to Fluconazole, 3 (3.7%) resistant to Ketoconazole, with all isolates being susceptible to Itraconazole. The significance of these findings, however, can only be validated when standardized techniques become available.

Table 1. Minimal Inhibitory Concentrations of Ketoconazole, Fluconazole and Itraconazole to 82 *Malassezia pachydermatis* isolates from canine otitis, determined by broth microdilution after 48 h of incubation.

Azoles	Distribution of patterns									
	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8
Ketoconazole (mg/mL)	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8
isolates inhibited	1	15	40	23	3	-	-	-	-	-
Fluconazole (mg/mL)	0.125	0.25	0.5	1	2	4	8	16	32	64
isolates inhibited	-	-	-	2	7	25	20	26	2	-
Itraconazole (mg/mL)	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4
isolates inhibited	1	9	30	29	13	-	-	-	-	-

Table 2. Patterns of *in vitro* susceptibility of 82 *Malassezia pachydermatis* isolates from canine otitis to Ketoconazole, Fluconazole and Itraconazole.

Azoles	Number and percentage of isolates		
	Susceptible	Susceptible-dose depending(SDD)	Resistant
Ketoconazole ^a	56 (68.3%)	23 (28.1%)	3 (3.7%)
Fluconazole ^b	54 (65.9%)	26 (31.7%)	2 (2.4%)
Itraconazole ^c	69 (84.1%)	13 (15.9%)	-

^aKetoconazole: S ≤ 0.06 mg/mL; SDD ≤ 0.125 mg/mL; R > 0.125 mg/mL. ^bFluconazole: S ≤ 8.0 mg/mL; SDD ≤ 16.0 mg/mL; R > 16.0 mg/mL. ^cItraconazole: S ≤ 0.06 mg/mL; SDD ≤ 0.125 mg/mL; R > 0.125 mg/mL.

Further studies on *Malassezia pachydermatis*, as also valid for others species of importance in veterinary mycology, are still required for the development of standard methodologies that could be useful for comparison studies among MICs. Recently, molecular studies on clinical *M. pachydermatis* isolates from dogs and cats have demonstrated the existence of some genetic diversity, with strains being classified in subgroups named A, B, C and D [1]. The epidemiological implications of this classification regarding strain virulence, clinical presentation and susceptibility to antifungal agents are still unknown. Studies proposed to approach these questions must be encouraged.

Finally, it must be emphasized that the methodology employed in this study is easy to perform, has a very low cost, and could be readily adopted by veterinary laboratories currently investigating clinical and biological features of *Malassezia pachydermatis*.

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