Antifungal susceptibility of *Malassezia pachydermatis* biofilm

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Antifungal resistance has been associated with biofilm formation in many microorganisms, but not yet in *Malassezia pachydermatis*. This saprophytic yeast can cause otitis and dermatitis in dogs and has emerged as an important human pathogen, responsible for systemic infections in neonates in intensive care units. This study aims to evaluate the *in vitro* antifungal susceptibility of *M. pachydermatis* strains, in both their planktonic and sessile forms, to fluconazole, miconazole, ketoconazole, itraconazole, posaconazole, terbinafine and voriconazole using the XTT assay and Clinical and Laboratory Standards Institute (CLSI) microdilution method. The minimum inhibitory concentration (MIC) values recorded for each drug were significantly higher for sessile cells relative to planktonic cells to the extent that ≥ 90% of *M. pachydermatis* strains in their sessile form were classified as resistant to all antifungal agents tested. Data suggest that *M. pachydermatis* biofilm formation is associated with antifungal resistance, paving the way towards investigating drug resistance mechanisms in *Malassezia* spp.

**Keywords** *Malassezia pachydermatis*, antifungal resistance, sessile cells, planktonic cells, MIC values

**Introduction**

Biofilms are differentiated microorganism communities, formed by a single microbial agent or by a mixture of fungal and/or bacterial species, which adheres to a biotic or abiotic surface, and is difficult to remove [1–3]. This structure contributes to the innate physical and chemical resistance of the microorganisms and is responsible for cooperative degradation of complex nutrients and community-based regulation of gene expression [4,5]. Indeed, biofilm formation plays an important role in resistance to antimicrobial agents, with sessile cells up to ~2000 times more resistant than planktonic cells [3,6–9]. In addition, biofilm formation contributes to the chronic status of many diseases caused by microorganisms, resulting in recurring symptoms even after treatment [1,10]. The capability to form biofilms has been demonstrated in many yeast genera, such as *Candida*, *Trichosporon*, *Cryptococcus*, *Blastoschizomyces*, *Saccharomyces* and *Malassezia* [11–13]. For instance, *Candida albicans* biofilms are resistant to many antifungal agents, including fluconazole (FLZ), itraconazole (ITZ), ketoconazole (KTZ) and amphotericin B (AMB) [6,14,15].

*Malassezia pachydermatis* biofilm formation has been studied *in vitro*, and its structure consists of clusters of blastoconidia, organized in mono- or multilayers with variable production of extracellular matrices [13,16]. Although biofilm production is not associated with the strain of origin (e.g., from lesioned or healthy dog skin), it is proposed that it acts in synergism with phospholipase production, thus inducing or exacerbating skin lesions in dogs [13].

*Malassezia pachydermatis* is a saprophytic microorganism, but can become pathogenic under certain circumstances causing otitis and dermatitis, which is usually chronic and recurrent [17,18]. In addition, *M. pachydermatis* is an emerging pathogen in neonates in intensive care units, with the use of infused lipid hyperalimentation via deep vein catheters considered a risk factor for systemic infections [19–22]. The ability of fungi to colonize indwelling catheters (causing bloodstream infections) is usually associated with biofilm formation which, in turn, enhances antifungal resistance in many microorganisms,
e.g., Candida spp. [23]. Nonetheless, a potential relationship between antifungal resistance and biofilm formation in M. pachydermatis cells has never been explored. Thus, the aim of this study was to evaluate the antifungal resistance of sessile (i.e., biofilm) and planktonic M. pachydermatis cells to FLZ, KTZ, ITZ, miconazole (MICO), posaconazole (POS), terbinafine (TER) and voriconazole (VOR) antifungal drugs.

Material and methods

Malassezia isolates and identification

Malassezia pachydermatis isolates were collected from 60 dogs, with or without skin lesions, and cultured using modified Dixon agar [24]. Isolates were separated into two groups, i.e., Group A comprising 31 isolates collected from skin of healthy dog and Group B, consisting 29 isolates recovered from dogs presenting skin lesions. M. pachydermatis strains were identified phenotypically (via macroscopic and microscopic morphology) and by their ability to grow on medium without lipid supplementation [25]. Isolates were maintained on modified Dixon agar and deposited in the fungal collection of the Department of Veterinary Medicine (Unit of Mycology and Parasitology) at the University of Bari (Italy).

In vitro susceptibility testing of planktonic cells

The in vitro antifungal susceptibility of M. pachydermatis planktonic cells was determined using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method as previously described [26–30]. Specifically, inoculum suspensions of M. pachydermatis strains were prepared from 7-day-old colonies grown on modified Dixon agar at 32°C. Sabouraud Dextrose broth (Liofilchem Diagnostici, Roseto degli Abruzzi, Italy) with 1% of Tween 80 (Sigma Co, Milano, Italy) was used as the growth medium in the susceptibility studies. The final concentration of the inoculum suspensions was equivalent to 1–5 × 10⁶ CFU/ml (optical density of 2.4 using a DEN-1 McFarland Densitometer (Biosan) [28]).

The following antifungal drugs were supplied by the manufacturers as pure compounds: KTZ, MICO, ITZ and TER (Sigma-Aldrich, Milan, Italy), FLZ and VOR (Pfizer Pharmaceuticals, Groton, CT, USA) and POS (Schering-Plough Corporation, Kenilworth, NJ, USA). The final concentrations of each antifungal drug ranged from 0.008–16 mg/l, with the exception of FLZ (0.03–64 mg/l). After 48 h at 32°C, plates were read and the growth of each strain at various drug concentrations assessed [27–30]. Drug-free medium was used as the positive control and each plate was run in duplicate. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug which produces a noticeable decrease in turbidity (i.e., 90% growth inhibition) compared to the positive control [28,31]. The MIC results reported are the concentrations where growth of 50% of tested isolates was inhibited (MIC50). Quality control strains (Candida parapsilosis ATCC 22019 and Candida krusei ATCC 6258; American Type Culture Collection, Manassas, VA, USA) were included to check the accuracy of the drug dilutions and reproducibility of the results [32].

In vitro susceptibility testing of Malassezia pachydermatis sessile cells

In vitro susceptibility of M. pachydermatis sessile cells was tested using the 2,3-bis(2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) based assay as previously described [33]. Specifically, M. pachydermatis strains were cultured on Dixon agar under aerobic conditions at 32°C for 4 days. The cells were transferred into 20 ml of yeast peptone dextrose (YPD; 1% yeast extract, 2% peptone, 2% dextrose) and incubated at 32°C, 150 rpm for 2 days. Cultures were centrifuged, the supernatant removed, the cells washed with 20 ml PBS, and then resuspended in YPD at a concentration of ~ 1 × 10⁶ CFU/ml. A total of 200 µl of each inoculum was deposited in each well of the plate, with the exception of the negative control wells, and the plate incubated at 32°C, 150 rpm for 4 days to allow biofilm formation [13]. The supernatant of each well was aspirated and the wells washed three times with 200 µl PBS. The plates were dried and 200 µl of antifungal dilutions deposited in each well containing sessile cells. For each antifungal agent, the lowest dilution tested represented two dilutions under the MIC50 value determined for planktonic cells. The highest dilutions tested represented three dilutions above the MIC50 value for planktonic cells. Hence, the following concentrations were tested; KTZ from 0.008–0.125 mg/l, ITZ from 0.008–0.064 mg/l, TER from 0.003–1 mg/l, VOR from 0.016–0.5 mg/l, POS from 0.008–0.125 mg/l, and FLZ 2–64 mg/l. Plates were incubated at 32°C for 2 days.

After incubation, 100 µl XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] with menadione (final concentration of 1 µM) was added to each well. Plates were incubated in darkness at 32°C for 3 h, and 80 µl aliquot from each well was transferred to another plate and growth measured at 490 nm using a microtiter plate reader [33]. Medium without M. pachydermatis cells, as well as drug-free medium containing fungal cells, were included as negative and positive controls respectively.

The Sessile Minimum Inhibitory Concentration (SMIC) was defined as the lowest concentration of the drug which
produces a noticeable decrease in turbidity (i.e., 80% growth inhibition) compared to the positive control [33]. SMIC results are reported as the drug concentration where growth of 50% of isolates tested was inhibited (SIMC50).

**Breakpoint interpretation for Malassezia pachydermatis**

Breakpoints have not yet been established for *M. pachydermatis*; therefore, the following criteria were used to categorize isolates as susceptible (S; MIC sample ≤ MIC50), susceptible dose-dependent (SDD; MIC50 < MIC sample ≤ MIC90) or resistant (R; MIC sample > MIC90) [28]. The interpretative categories (i.e., S, SDD, R) obtained from sessile cells and planktonic cells were compared. Essential agreement (EA) was assigned when there was no more than a two-fold difference between the MICs of sessile and planktonic cells. Categorical agreement (CA) was assigned when planktonic and sessile cells were classified in the same susceptibility category [28].

**Statistical analysis**

Normality of data was assessed using the Lilliefors test. The Chi-square test was used to compare the occurrence of S, SDD, and R of planktonic and sessile cells. A value of *P* ≤ 0.05 was considered statistically significant. Statistical analyses were performed using the BioEstat 5.0 software [34].

**Table 1** Ketoconazole (KTZ), itraconazole (ITZ), posaconazole (POS), terbinafine (TER), voriconazole (VOR), and fluconazole (FLZ) MIC50 values of planktonic and sessile cells of *Malassezia pachydermatis* isolated from dogs without (Group A) and with skin lesions (Group B). The MIC50 values, MIC range and mean values (MICm) with standard deviation (SD) were also calculated. Essential agreement (EA) among MICs of planktonic and sessile cells is reported. Statistically significant results (*P* ≤ 0.05) are indicated with same letters in superscript.

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Cells</th>
<th>Group A</th>
<th>Group B</th>
<th>All isolates</th>
<th>Range</th>
<th>MICm (SD)</th>
<th>EA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KTZ</td>
<td>Planktonic</td>
<td>0.016</td>
<td>0.016</td>
<td>0.016^a</td>
<td>&gt;0.008–0.064</td>
<td>0.02 (0.013)</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>Sessile</td>
<td>&gt;0.125</td>
<td>&gt;0.125</td>
<td>&gt;0.125^a</td>
<td>&gt;0.008–0.125</td>
<td>0.122 (0.018)</td>
<td></td>
</tr>
<tr>
<td>ITZ</td>
<td>Sessile</td>
<td>&lt;0.008</td>
<td>&lt;0.008</td>
<td>&lt;0.008^b</td>
<td>&gt;0.008–0.016</td>
<td>0.008 (0.002)</td>
<td>3.3</td>
</tr>
<tr>
<td>POS</td>
<td>Planktonic</td>
<td>&gt;0.064</td>
<td>&gt;0.064</td>
<td>&gt;0.064^c</td>
<td>&gt;0.008–0.064</td>
<td>0.060 (0.014)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sessile</td>
<td>&gt;0.125</td>
<td>&gt;0.125</td>
<td>&gt;0.125^c</td>
<td>&gt;0.008–0.125</td>
<td>0.117 (0.028)</td>
<td></td>
</tr>
<tr>
<td>TER</td>
<td>Planktonic</td>
<td>0.064</td>
<td>0.125</td>
<td>0.064^d</td>
<td>&gt;0.008–0.125</td>
<td>0.106 (0.077)</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Sessile</td>
<td>&gt;1</td>
<td>&gt;1</td>
<td>&gt;1^e</td>
<td>&gt;0.5–1</td>
<td>0.964 (0.165)</td>
<td></td>
</tr>
<tr>
<td>VOR</td>
<td>Planktonic</td>
<td>0.064</td>
<td>0.064</td>
<td>0.064^f</td>
<td>&gt;0.03–0.125</td>
<td>0.061 (0.037)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sessile</td>
<td>&gt;0.5</td>
<td>&gt;0.5</td>
<td>&gt;0.5^e</td>
<td>&gt;0.064–0.5</td>
<td>0.440 (0.147)</td>
<td></td>
</tr>
<tr>
<td>FLZ</td>
<td>Planktonic</td>
<td>8</td>
<td>8</td>
<td>8^f</td>
<td>&gt;0.5–64</td>
<td>12.34 (11.24)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Sessile</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64^f</td>
<td>&gt;8–64</td>
<td>56.8 (17.79)</td>
<td></td>
</tr>
</tbody>
</table>

EA, Essential Agreement (discrepancies in MIC results of no more than two-fold dilutions between the sessile and planktonic cells).

**Results**

The MIC values of sessile cells were at least three dilutions higher than those of planktonic cells (*P* ≤ 0.05) for all antifungal drugs tested (Table 1). No statistically significant differences in SMIC values were observed between *M. pachydermatis* collected from animals with (Group B) or without (Group A) skin lesions. For sessile cells of 60 *M. pachydermatis* isolates tested, 98.3% were classified as resistant to KTZ, followed by 96.7% to TER, 95% to ITZ, 93.3% to POS and 90% FLZ and VOR. Conversely, planktonic cell resistance varied from 8.3% (KTZ) to 0% (ITZ and TER) (Table 2), resulting in statistically significant differences between sessile and planktonic cells (*P* < 0.0001). Overall, the results showed an Essential agreement (EA) ranging from 1.7–15% and a Categorical agreement (CA) ranging from 1.7–10%, depending upon the antifungal drugs tested. The highest concordance was observed for FLZ (CA 10% and EA 15%) and KTZ (CA 8.3% and EA 13.3%), and the lowest for ITZ (CA 1.7%, and EA 3.3%) and TER (CA 1.7% and EA 1.7%) (Tables 1 and 2).

**Discussion**

This study provides essential information on the antifungal susceptibility of *M. pachydermatis* cells in both planktonic and sessile forms, and demonstrates that biofilm formation is responsible for increased antifungal resistance, in agreement with previous reports on other yeasts species [5,6,9,14,15,35]. A small number of Malassezia strains displayed azole resistance in the planktonic form, while...
resistance to all drugs tested was observed for sessile forms. Indeed, MIC values of sessile cells were at least three dilutions higher than those of planktonic cells for each antifungal agent. This observation is probably due to a range of factors, including density of biofilms populations and presence of extracellular matrix.

The *M. pachydermatis* biofilm structure, represented by clusters of blastoconidia, organized in multi- or monolayers with extracellular matrix production [13], is likely to act as a barrier against drugs. However, this physiological barrier cannot be solely deemed responsible for biofilm drug resistance. For example, natural selection of biofilm cells by apoptosis of sub-lethally damaged cells [36] or overexpression of efflux pumps encoded by *CDR1, CDR2*, and *MDR1* genes [37] could also contribute towards the biofilm resistance, as has been demonstrated for *Candida* spp. Nonetheless, as for *Candida* spp. and other microorganisms, the high antifungal resistance of sessile cells, as observed in this study for *M. pachydermatis*, could be responsible for treatment failure in *Malassezia* infections [14,35,38–41].

It is known that canine *Malassezia* infections are usually chronic, with conventional therapy largely ineffective [42,43]. This may be due to the ability of *Malassezia* to form biofilms, consequently requiring higher drug concentrations than are currently used to cure infection [5,44]. Since the highest CA observed was for FLZ, KTZ and VOR, and 10% of *M. pachydermatis* sessile cells remained S or SSD for FLZ and VOR, these results advocate these drugs being evaluated for topical and/or systemic treatment of recurrent *Malassezia* otitis/dermatitis in dogs.

Results from this study confirm the hypothesis that *M. pachydermatis* biofilm formation is associated with antifungal resistance and paves the way towards investigating drug resistance mechanisms in *Malassezia* spp.

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