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In vitro antifungal susceptibility of *Malassezia pachydermatis* from dogs with and without skin lesions

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ABSTRACT

Canine *Malassezia* dermatitis is frequently treated with systemic ketoconazole (KTZ) and itraconazole (ITZ). However, no information is available on the antifungal susceptibility to azoles and allilamine of *Malassezia pachydermatis* isolates from dogs with or without skin lesions. The present study was designed to evaluate the *in vitro* antifungal susceptibility of *M. pachydermatis* strains from dogs with or without skin lesions to KTZ, ITZ, miconazole (MICO), fluconazole (FLZ), posaconazole (POS), voriconazole (VOR) and terbinafine (TER) using the Clinical and Laboratory Standards Institute reference Broth Microdilution Method (CLSI M27-A2). The association between the susceptibility to antifungal compounds and the origin of *M. pachydermatis*, from skin with or without lesions has been also assessed. A total of 62 *M. pachydermatis* strains from healthy dogs (i.e., Group A = 30) or with skin lesions (i.e., Group B = 32) were tested. ITZ, KTZ and POS showed the highest activity against *M. pachydermatis* strains, whereas MICO TER and FLZ the lowest. A higher number of *Malassezia* resistant strains were registered among isolates from Group B than those from Group A.

This study indicates that *M. pachydermatis* strains were susceptible to ITZ, KTZ, and POS. However, dogs with lesions may harbour strains with low susceptibility to antifungal agents and displaying cross-resistance phenomena to azole. The antifungal therapy in *Malassezia* infections requires careful appraisal of choice of drugs especially in cases of unresponsiveness to antifungal treatment or recurrent infections.

1. Introduction

Malassezia pachydermatis is one of most frequent aetiological agents responsible for skin disease in dogs and, in severe infections, the disease requires long treatments and/or high doses of antifungal agents (Bond, 2010). *Malassezia* dermatitis is frequently treated with systemic ketoconazole (KTZ) and itraconazole (ITZ) (Bond, 2010). Nowadays, the CLSI M27-A2 method established by the Clinical and Laboratory Standards Institute (NCCLS, 2002) represents the gold standard technique for evaluating the susceptibility of the yeasts to antifungal compounds (Velegraki et al., 2004; Cantón et al., 2009; Nijima et al., 2011). Despite the fact that, to date, this method has only been standardized for *Candida* species and *Cryptococcus neoformans*, this technique was instrumental for the identification of resistant isolates of *M. pachydermatis* to KTZ and ITZ (Nijima et al., 2011). It has been suggested that the chemical composition of the skin might have an incidental effect on drug susceptibility and it may differ, depending on skin site, health and integrity (Chen and Hill, 2005; Sugita et al., 2005). The susceptibility of *M.*

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pachydermatis isolates from dogs with skin lesions to the azoles and allilamine has been evaluated with different procedures (Gupta et al., 2000; Eichenberg et al., 2003; Garau et al., 2003; Nascente et al., 2003; Velegraki et al., 2004; Sugita et al., 2005; Rincón et al., 2006; Prado et al., 2008; Jesus et al., 2011; Nijima et al., 2011), but their susceptibility profile has never been compared with that of strains isolated from skin without lesions. Thus, the aims of the study were: (i) to evaluate the *in vitro* antifungal susceptibility of *M. pachydermatis* strains from dogs with and without lesions to KTZ, miconazole (MICO), fluconazole (FLU), ITZ, posaconazole (POS), voriconazole (VOR) and terbinafine (TER) using a modified CLSI M27-A2 microdilution method; (ii) to critically assess the correlation between the antifungal susceptibility and the origin of *M. pachydermatis*, from lesioned or healthy dog skin.

2. Materials and methods

2.1. Malassezia isolates and phenotypic identification

Malassezia pachydermatis isolates were collected from 62 dogs with or without skin lesions and/or otitis, and identified phenotypically (macroscopic and microscopic morphology) and physiologically as previously reported (Guillot et al., 1996). Upon collection, isolates were divided into two groups: Group A consisting of 30 isolates collected from healthy dog skin; Group B comprising 32 isolates from dogs with localized skin lesions (i.e., $n = 5$) or chronic generalized dermatitis (i.e., $n = 27$). Isolates were deposited in the fungal collection of the Faculty of Veterinary Medicine at the University of Bari (Italy).

2.2. In vitro susceptibility testing

The susceptibility of *M. pachydermatis* strains to antifungal compounds was verified using the reference CLSI M27-A2 with some modifications. In particular, Sabouraud Dextrose Broth medium (Liofilchem Diagnostici®, Roseto degli Abruzzi, Italy) with 1% of tween 80 (Sigma Co, Milano, Italy) was used instead of RPMI 1640 medium which has been shown to be unsuitable for the growth of *M. pachydermatis* since it lacks the required lipid supplements (Rincón et al., 2006). Stock inoculum suspensions were prepared from 7-days-old colonies developed on modified Dixon agar at 32 °C. The final concentration of the stock inoculum suspensions in sterile distilled water was adjusted to an optical density of 2.4 using a turbidimeter (DEN-1 McFarland Densitometer, Biosan) (equivalent to $1–5 \times 10^6$ CFU/ml as validated by quantitative plate counts of Colony Forming Unit (CFU) in Sabouraud Dextrose agar).

The following antifungal drugs were supplied by the manufacturers as pure standard 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 concentrations of the antifungal drugs ranged from 0.008 to 16 μg/ml, with the exception of FLZ (from 0.03 to 64 μg/ml). After 48 h of incubation at 32 °C, visual reading of plates was performed and the growth of each strain at various drug concentrations was assessed. The growth

Table 1
Ketoconazole (KTZ), miconazole (MICO), itraconazole (ITZ), terbinafina (TER), voriconazole (VOR), posaconazole (POS) and fluconazole (FLZ) MICs (μg/ml) for *Malssezia pachydermatis* strains cultured from dogs without (Group A) and with (Group B) skin lesions. MIC mean values (MICm) with standard deviation (SD) are also reported.

	Group A Dogs without skin lesion = 30				Group B Dogs with skin lesions =32				All animals Total = 62			
	Range	MIC_{50}	MIC_{90}	MICm (SD)	Range	MIC_{50}	MIC_{90}	MICm (SD)	Range	MIC_{50}	MIC_{90}	MICm (SD)
KTZ	<0.008–0.060	0.016	0.030	0.038 (0.011)[a]	<0.008–0.125	0.016	0.030	0.025 (0.023)[a]	<0.008–0.060	0.016	0.030	0.016 (0.02)
MICO	0.125–1	0.250	1.000	0.375 (0.264)	0.125–2	0.500	1.000	0.546 (0.476)	0.125–2	0.500	1.000	0.45 (0.39)
ITZ	<0.008–0.016	<0.008	<0.008	ND	<0.008–0.016	<0.008	<0.008	0.008 (0.0)	<0.008–0.016	<0.008	<0.008	ND
TER	0.030–0.250	0.060	0.250	0.99 (0.077)[b]	0.030–0.250	0.125	0.250	0.112 (0.076)[b]	0.030–0.250	0.125	0.250	0.11 (0.08)
VOR	0.060–0.125	0.060	0.125	0.60 (0.029)[c]	0.030–0.500	0.060	0.125	0.154 (0.088)[c]	0.030–0.500	0.060	0.125	0.07 (0.07)
POS	<0.008–0.030	0.016	0.016	0.016 (0.004)	<0.008–0.060	0.016	0.030	0.018 (0.006)	<0.008–0.060	0.016	0.030	0.016 (0.005)
FLZ	4–32	8	16	ND	8–>64	8	16	13.9 (14.7)	4–>64	8	16	12.8 (11.2)

[a,b,c]Student's *t*-test – statistically significant differences ($p < 0.05$) were marked with the same letters.