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BIOFILM FORMATION IN *MALASSEZIA PACHYDERMATIS* STRAINS ISOLATED FROM DOGS DECREASES SUSCEPTIBILITY TO KETOCONAZOLE AND ITRACONAZOLE

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Malassezia pachydermatis is a commonly isolated yeast in veterinary dermatology that can produce biofilms *in vitro* and *in vivo*, lowering its susceptibility to antimicrobial drugs. The aim of this study was to determine and compare the *in vitro* susceptibility of planktonic cells and biofilms of *M. pachydermatis* isolates to ketoconazole and itraconazole. The presence of biofilm formation was confirmed by crystal violet staining and absorbance measurement at 595 nm wavelength, and by a scanning electron microscopy method. Cell viability was determined by the Celltiter 96 Aqueous One solution assay containing a water-soluble tetrazolium compound (MTS) with absorbance measurement at 490 nm. Planktonic cell minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of ketoconazole and itraconazole were very low: MIC_{90} and MFC_{90} were 0.032 and 0.125 µg/ml for ketoconazole, while 0.063 and 0.25 µg/ml for itraconazole, respectively. Also, the half maximal effective concentrations (EC_{50}) of itraconazole were higher for planktonic cells and biofilms compared to ketoconazole. The EC_{50} values of ketoconazole were 18–169 times higher and those of itraconazole 13–124 times higher for biofilms than for planktonic cells. Biofilm EC_{50} levels exceeded MICs 103–2060 times for ketoconazole and 84–1400 times for itraconazole. No significant difference was found between these values of the two substances. In conclusion, biofilms of all examined *M. pachydermatis* strains were much less susceptible to ketoconazole and itraconazole than their planktonic forms.

Key words: Biofilm, itraconazole, ketoconazole, *Malassezia pachydermatis*, planktonic

Malassezia pachydermatis is a frequently isolated commensalistic yeast in veterinary dermatology (Bond, 2010; Outerbridge, 2006). It is a member of the normal skin flora, but can cause secondary dermatitis or external otitis in dogs and

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cats with atopic dermatitis, cutaneous food adverse reactions, ectoparasite infestations, immunosuppression, primary keratinisation defects or endocrinopathies (Bond, 2010; Outerbridge, 2006). Common treatments for *Malassezia* dermatitis and otitis include antifungal agents like polyenes, azoles, allylamines or chlorhexidine. Antifungals belonging to the azole group are one of the most effective and the most frequently used drugs in the management of the infection, ketoconazole and itraconazole being the most active against this microorganism with very low MICs (Brito et al., 2007; Bond, 2010; Jesus et al., 2011; Jerzsele et al., 2013). However, yeasts including malasseziae can produce biofilms *in vitro* (Cannizzo et al., 2007; Figueredo et al., 2012), decreasing susceptibility to the antimicrobial drugs used. It was reported that *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Pneumocystis* spp. and *Coccidioides immitis* might also be able to produce fungal biofilms in humans (Cannizzo et al., 2007; Martinez and Fries, 2010). Biofilms are extracellular matrices produced by microorganisms (primarily bacteria and fungi) which help the pathogens attach to viable and non-viable surfaces (Van Minnebruggen et al., 2010). Biofilm formation in *Candida albicans* resulted in a 30- to 2000-fold decrease in sensitivity to certain antifungals including ketoconazole (Martinez and Fries, 2010; Vandeputte et al., 2012). Figueredo et al. (2013) reported that *M. pachydermatis* biofilms exhibited high-level resistance to several antifungals including azoles, at least 3 dilutions higher as compared to planktonic cells.

The aim of this study was to determine and compare the *in vitro* susceptibility of planktonic cells and biofilms of *M. pachydermatis* strains of canine origin to ketoconazole and itraconazole.

Materials and methods

Malassezia pachydermatis *isolates*

Sixteen strains of *M. pachydermatis* were examined. All of the strains were isolated between 2009 and 2011 in Hungary from dogs suffering from clinical signs of external otitis or superficial dermatitis. Clinical specimens were obtained using a sterile dry cotton swab. Malasseziae were identified on the basis of colony morphology on Sabouraud agar, microscopic identification and growth on agar without lipid supplementation (Guillot et al., 1996). The strains were stored at –80 °C in Sabouraud dextrose broth (Biolab Co. Ltd., Budapest, Hungary) and 20% glycerol.

Biofilm production and planktonic cells of M. pachydermatis

Strains grown in Sabouraud dextrose liquid broth at 32 °C for 72 h were centrifuged at 3000 *g* for 10 min and resuspended in physiological saline. The

optical density of the suspension at 600 nm was set to 0.1 resulting in an approx. 10^6 colony forming units (CFU)/ml viable cell count confirmed by agar plate counting.

Biofilm production and subsequent evaluation was carried out according to the method of Hawser and Douglas (1994), with some modifications as recommended by Nett et al. (2011). Briefly, a sterile, disposable silicone elastomer Foley catheter was cut into standardised segments of 10 mm. The surface of these catheters was scraped with sterile, gamma-sterilised scalpel blades to enhance the attachment of yeast cells. Twenty-five µl of the yeast suspension (10^6 CFU/ml) was pipetted on catheter segment surfaces, put in a thermostat and incubated at 32 °C for 1 h (attachment phase) to facilitate adherence of the yeast cells. The segments were submerged in 1 ml of Sabouraud broth in a 24-well microplate (Corning, California, USA) and incubated at 32 °C for 8 h. After 8 h the medium was replaced by Sabouraud broth containing different concentrations of ketoconazole (Sigma, St. Louis, USA) or itraconazole (Sigma, St. Louis, USA). Stock solutions of the azoles were prepared in dimethyl sulphoxide (DMSO) and the final concentrations in the microplates were set in the range of 0.004 to 32 µg/ml for both drugs. Fungal suspensions containing the antifungals were placed in triplicates in a thermostat and incubated at 32 °C for 72 h. By omitting the placement of cells on catheter segments and the attachment phase, they grew in the planktonic form.

The presence of biofilm formation was confirmed by crystal violet staining and absorbance measurement at 595 nm wavelength (Jin et al., 2003) and a scanning electron microscopy (SEM) method described by Figueredo et al. (2012) utilising a ZEISS EVO MA10 scanning electron microscope. The samples were dehydrated with increasing concentrations of ethanol (20%, 40%, 50%, 70% and 90%), then coated with gold with a high-vacuum evaporator.

Determination of half maximal effective concentrations (EC_{50}) and MFC values of ketoconazole and itraconazole

Cell viability was determined by the Celltiter 96 Aqueous One solution assay (Promega, Madison, USA), containing a water-soluble tetrazolium compound (MTS). Formazan produced by viable yeasts was measured by absorbance at 490 nm and was directly proportional to the number of living cells. Dose-response curves were used to determine EC_{50} values (effective concentration 50%) of ketoconazole and itraconazole (Hawser and Douglas, 1994; Nett et al., 2011). All assays were performed in triplicates. For planktonic cells, also the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of the drugs were determined. The threshold of resistance was ≤ 0.125 µg/ml in the case of both drugs according to the CLSI (Clinical Laboratory Standards Institute) guidelines. MFC was determined by agar plate CFU counting and defined as the concentration of the drug that decreased viable cell

number by 99.9%. MIC_{90} and MFC_{90} were calculated as the concentrations of drugs that inhibit/kill 99.9% of fungi in 90% of the strains investigated.

Statistical analysis

The paired Student's *t*-test was used to evaluate the differences between MIC, MFC and EC_{50} mean values of the two antifungal agents. A value of $P < 0.05$ was considered to be statistically significant.

Results

All of the tested 16 *M. pachydermatis* isolates were able to produce biofilms on the catheter surface, as confirmed by crystal violet staining and SEM. A SEM picture of an 8 hours old *M. pachydermatis* biofilm is shown in Fig. 1.

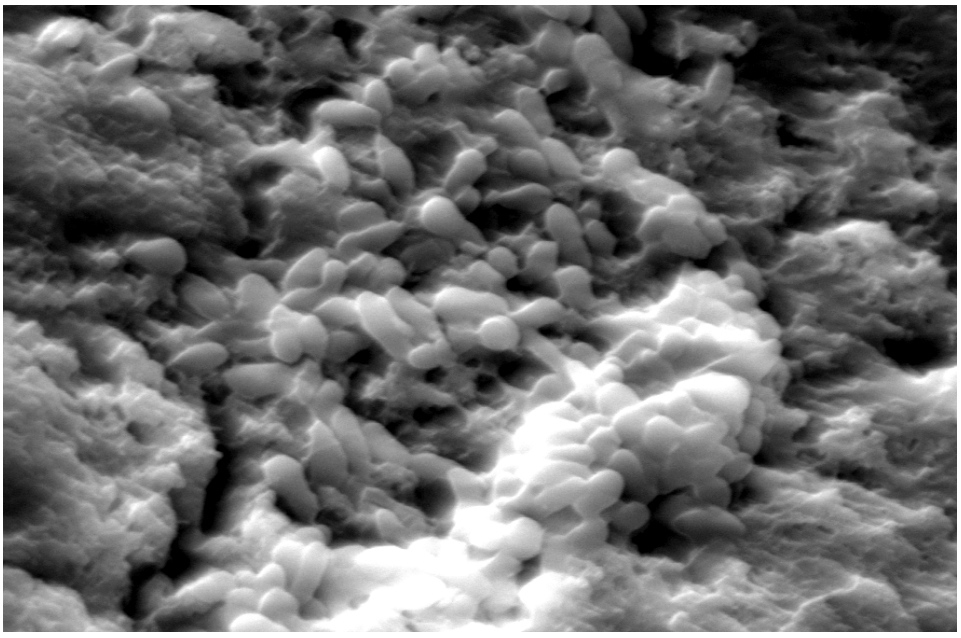

Fig. 1. Scanning electron micrograph of an 8 hours old *Malassezia pachydermatis* biofilm (Zeiss Evo MA10; magnification: × 2500)

Planktonic cells were much more sensitive than biofilms to both of the drugs tested. The susceptibility of the investigated *M. pachydermatis* strains to ketoconazole and itraconazole in biofilms and free-floating planktonic cells is shown in Table 1. MFC/MIC ratios were between 2 and 4, indicating excellent *in vitro* fungicidal effect of the azoles against *M. pachydermatis*. MIC_{90} and MFC_{90}

Table 1

Minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and EC_{50} (concentration able to reduce formazan formation by 50%) values of ketoconazole and itraconazole in planktonic cells and biofilms of *Malassezia pachydermatis* isolated from dogs

Strain No.	Ketoconazole					Itraconazole				
	Planktonic cells			Biofilm	Biofilm EC_{50}/ Planktonic EC_{50}	Planktonic cells			Biofilm	Biofilm EC_{50}/ Planktonic EC_{50}
	EC_{50}	MIC	MFC	EC_{50}		EC_{50}	MIC	MFC	EC_{50}	
1	0.09	0.016	0.063	2.06	23	0.09	0.032	0.125	2.7	30
2	0.06	0.008	0.032	2.68	41	0.11	0.016	0.063	2.64	24
3	0.11	0.016	0.063	1.85	18	0.17	0.016	0.063	2.23	13
4	0.05	0.004	0.016	2.47	51	0.20	0.032	0.125	3.1	16
5	0.19	0.008	0.032	14.42	74	0.23	0.016	0.063	9.8	43
6	0.26	0.032	0.125	13.39	52	0.40	0.032	0.125	17.9	45
7	0.07	0.008	0.032	3.19	44	0.09	0.008	0.032	4.8	53
8	0.10	0.008	0.032	16.48	169	0.09	0.008	0.032	11.2	124
9	0.21	0.032	0.125	12.36	59	0.17	0.016	0.063	9.95	59
10	0.11	0.016	0.063	4.64	41	0.18	0.032	0.125	11.3	63
11	0.34	0.032	0.125	8.76	26	0.39	0.032	0.125	9.67	25
12	0.26	0.063	0.25	6.49	25	0.33	0.063	0.25	8.9	27
13	0.07	0.016	0.063	2.78	38	0.10	0.016	0.063	4.72	47
14	0.09	0.008	0.032	4.02	45	0.15	0.032	0.063	6.87	46
15	0.25	0.032	0.125	10.82	43	0.35	0.063	0.25	14.3	41
16	0.22	0.016	0.063	9.99	46	0.47	0.125	0.25	13.76	29
Mean ± SD	0.16 ± 0.09			7.27 ± 5.01	49.5 ± 34.9	0.22 ± 0.13			8.37 ± 4.74	42.7 ± 26.3

for ketoconazole were 0.032 and 0.125 µg/ml, while for itraconazole 0.063 and 0.25 µg/ml, respectively. Although these values proved to be higher in the case of ketoconazole, differences in individual sensitivity were without statistical significance ($P > 0.05$). Also, the mean EC_{50} values for itraconazole were higher for planktonic cells and biofilms compared to ketoconazole but without significant differences ($P > 0.05$). EC_{50} values of ketoconazole were 18–169 times higher, while those of itraconazole 13–124 times higher for biofilms than for planktonic cells. Biofilm EC_{50} levels exceeded the MICs 103–060 times for ketoconazole and 84–1400 times for itraconazole. No significant difference was found between these values for the two substances.

Discussion

The MICs and MFCs of ketoconazole and itraconazole were very low as expected from previous studies (Jesus et al., 2011; Cafarchia et al., 2012), and no resistant strain was found to either of the azoles according to the CLSI breakpoints, similarly as reported in earlier studies (Brito et al., 2007). Figueredo et al. (2013) reported low MICs for ketoconazole and itraconazole, with higher MICs for ketoconazole. In our study, ketoconazole was more effective against both planktonic cells or biofilms. In our study, biofilm EC_{50} levels exceeded the MICs 103–2060 times for ketoconazole and 84–1400 times for itraconazole. In the study of Figueredo et al. (2013), although using a different methodology, *M. pachydermatis* biofilm MICs were approx. 8 times higher than those of planktonic cells. In our study, however, the EC_{50} values of ketoconazole and itraconazole were 18–169 and 13–124 times higher, respectively. These results show trends similar to those observed in earlier studies performed on *Candida albicans* strains in human medicine, where a 30- to 2000-fold higher EC_{50} was found as compared to the relevant MICs (Hawser and Douglas, 1995). The *M. pachydermatis* isolates examined in our study were more susceptible to ketoconazole and itraconazole than *Candida* yeasts with lower MICs and EC_{50}s, as has also been demonstrated by Brito et al. (2007).

The primary clinical significance of biofilm formation of yeasts in human medicine is their ability to colonise intravenous catheters and cause bloodstream infections (Leonidou and Gogos, 2010). The fungal species most commonly associated with these infections belong to the *Candida* genus, although *Malassezia* spp. are frequently isolated in neonatal intensive care units (Van Belkum et al., 1994). There are no reports on *M. pachydermatis* causing septicaemia in veterinary medicine. However, its occurrence in veterinary dermatology is common (Outerbridge, 2006). According to Cafarchia et al. (2012), dogs with skin lesions harboured strains of *M. pachydermatis* less susceptible to azoles *in vitro* than dogs without skin lesions. These observations, along with the results of our study, suggest that secondary *Malassezia* infections need a special approach for

evaluating *in vitro* susceptibility, and emphasise the importance of standardised methods for testing *M. pachydermatis* strains in veterinary medicine (Hector, 2005; Cafarchia et al., 2012).

In conclusion, all *M. pachydermatis* strains investigated in this study produced biofilms *in vitro*. Biofilms of all of the strains were much less susceptible to ketoconazole and itraconazole than their planktonic forms as demonstrated by the respective EC_{50} values.

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