

Antifungal agent susceptibilities and interpretation of *Malassezia pachydermatis* and *Candida parapsilosis* isolated from dogs with and without seborrheic dermatitis skin

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Malassezia pachydermatis and *Candida parapsilosis* are recognized as commensal yeasts on the skin of healthy dogs but also causative agents of seborrheic dermatitis, especially in atopic dogs. We determined and compared the susceptibility levels of yeasts isolated from dogs with and without seborrheic dermatitis (SD) using the disk diffusion method (DD) for itraconazole (ITZ), ketoconazole (KTZ), nystatin (NYS), terbinafine (TERB) and 5-fluorocytosine (5-FC) and the broth microdilution method (BMD) for ITZ and KTZ. The reliability between the methods was assessed using an agreement analysis and linear regression. Forty-five *M. pachydermatis* and 28 *C. parapsilosis* isolates were identified based on physiological characteristics and an approved molecular analysis. By DD, all tested *M. pachydermatis* isolates were susceptible to ITZ, KTZ, NYS and TERB but resistant to 5-FC. Only 46–60% of the tested *C. parapsilosis* isolates were susceptible to KTZ, TERB and 5-FC, but ITZ and NYS were effective against all. By BMD, over 95% of *M. pachydermatis* isolates were susceptible to KTZ and ITZ with an MIC₉₀ < 0.03 and 0.12 µg/ml, respectively. The frequency of KTZ- and ITZ-resistant *C. parapsilosis* was 29% and 7%, and the MIC₉₀ values were 1 µg/ml and 0.5–1 µg/ml, respectively. Regarding the agreement analysis, 2.2% of minor errors were observed in *M. pachydermatis* and 0.2–1% of very major errors occurred among *C. parapsilosis*. There were no significant differences in the yeast resistance rates between dogs with and without SD. KTZ and ITZ were still efficacious for *M. pachydermatis* but a high rate of KTZ resistant was reported in *C. parapsilosis*.

Keywords *Malassezia pachydermatis*, *Candida parapsilosis*, antifungal agent, seborrheic dermatitis, dog

Introduction

Yeast-associated seborrheic dermatitis (SD) and otitis externa are commonly found in atopic dogs with overgrowth of

Malassezia pachydermatis and *Candida parapsilosis*. In general, skin yeasts reversibly act as commensals and can be transient depending on the underlining factors that lead to SD [1]. *M. pachydermatis* was originally thought to be the sole yeast that caused canine skin diseases [2,3]. However, recently, over five yeast species were recognized as skin commensals [4], and *C. parapsilosis* was identified as a causative yeast concurrent with *M. pachydermatis* in SD dogs [5].

In animals with severe and widespread lesions, systemic ketoconazole (KTZ) and itraconazole (ITZ) are the

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most commonly used drugs with recommended doses of 5–10 mg/kg daily for up to 4 weeks [6–8]. Due to the recurring disease caused by SD, routine antifungal administration may possibly induce acquired resistance, leading to treatment failure. In addition, SD dogs can potentially be a route for zoonosis and nosocomial infection in circumstances where healthcare workers have transmitted the drug-resistant yeasts shared to human patients [9,10]. However, the absence of standardized methodology and interpretative values for animal yeasts has limited the available data on antifungal susceptibility [11]. The aims of this study were to: (i) evaluate the *in vitro* KTZ and ITZ susceptibility of *M. pachydermatis* and *C. parapsilosis* from dogs with and without SD using the CLSI M27-A3 broth microdilution method (BMD) and CLSI M44-A disk diffusion method (DD); (ii) compare the antifungal susceptibility levels between dogs with and without SD; and (iii) assess the correlation of reference between BMD and DD using an agreement analysis.

Materials and methods

Yeast strains

A total of 73 yeast strains were obtained from 45 dogs that had been examined by private small animal hospitals in Bangkok, Thailand from 2008–2011. Forty-five strains of *M. pachydermatis* isolated from dogs without SD ($n = 12$) and with SD ($n = 33$) and 28 strains of *C. parapsilosis* isolated from dogs without SD ($n = 13$) and with SD ($n = 15$) were used. Dogs without SD were defined from those who were less than six months of age, not atopic predisposing breeds, lack of from ectoparasites and clinical sign with normal skin and hair coat and untreated by steroids for at least 3 months prior to collection. SD lesions were defined based on clinical signs, including pruritus, moistness, erythema, mal-odor or hyperpigmentation, lichenification and self-induced alopecia [12]. Signalments and histories of all dogs are shown in Table 1.

M. pachydermatis isolates were confirmed based on morphology, lipid-independent properties, and the restriction patterns by PCR-RFLP [13,14]. *C. parapsilosis* isolates were characterized based on their morphological features, including germ tube production, chlamydoconidia and blastoconidia production on corn-meal agar and the sugar fermentation test [15]. The identifications were confirmed by partial sequencing of ribosomal DNA using a primer set for the internal transcribed spacer (ITS) located between ITS1 and ITS4 [16]. The DNA alignment and phylogenetic analysis were performed as previously described [5].

Screening for antifungal susceptibility

The disk diffusion method (DD) was performed according to the guidelines of CLSI M44-A [17]. Mueller Hinton

agar (Difco™, USA) supplemented with 2% dextrose and 0.5 mg/l methylene blue was used as the basic media. Commercial antifungal disks (Neo-Sensitabs, Rosco, Denmark) comprising itraconazole (ITZ), ketoconazole (KTZ), nystatin (NYS), terbinafine (TERB) and 5-fluorocytosine (5-FC) at concentrations of 8, 15, 50, 30 and 10 µg/disk, respectively, were used. The inocula included *C. parapsilosis* after one day of growth and *M. pachydermatis* after three days of growth on SDA and were adjusted to 0.5 McFarland standard absorption, which is equal to approximately $1-5 \times 10^6$ CFU/ml. The results were determined based on the inhibition zone diameter after incubating at 35°C for 24–48 h (*Candida* species) and 48–72 h (*M. pachydermatis*) [18]. The antifungal susceptibility levels were interpreted according to the manufacturer's breakpoint guidelines [19].

Minimum inhibition concentration (MIC) determination

The MIC values against itraconazole (Sigma-Aldrich, USA) and ketoconazole (Sigma-Aldrich, USA) were determined by the broth microdilution method (BMD) according to the CLSI M27-A3 guidelines [20]. The antifungals were dissolved in dimethyl sulfoxide (DMSO) (Merck Sharp & Dohme Ltd, Herts, UK) and diluted with standard RPMI-1640 medium with L-glutamine but without sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA) that was supplemented with 2% dextrose and buffered at pH 7.0 with 0.165 morpholinepropanesulfonic acid (MOPS) (Sigma Chemical Co., USA). The solutions were serially diluted two-fold from 0.06–32 µg/ml. The inocula included *C. parapsilosis* after one day of growth and *M. pachydermatis* after three days of growth on SDA. The inoculum sizes were adjusted to a final concentration of approximately $2-3 \times 10^3$ CFU/ml using a spectrophotometer at 530 nm and appeared 95% translucent when diluted with RPMI media [21]. BMD was performed in 96-well microtiter plates (Nunc, USA). Positive and negative controls of yeast growth, including no antifungal drug and sterile media, respectively, were included on each tested plate. After incubating at 35°C for 48 h (*Candida* species) and 48–72 h (*M. pachydermatis*), the MIC values were determined by the unaided eye. The MICs and MIC₉₀ were analyzed using WHONET version 5.5. The BMD breakpoints were interpreted according to the CLSI M44A. [22–24]. The minimum fungicidal concentrations (MFCs) were determined using a described standard [25].

Quality control

Both DD and BMD methods were validated at all steps using the quality control strains *C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028 and also

Table 1 Signalments and histories of all tested dogs.

Gr. of dogs	Breed	Age (years)				Lesions	Anatomical sites of lesions	History of drug usage		Concurrent bacterial infection
		<1	1–5	5–10	>10			KTZ	ITZ	
Healthy (13)	Mixed breed (10) Poodle (3) Shih tzu (4)	13	–	–	–	No	No	No	No	No
PSD (12)	Poodle (4) Basset hound (1) Beagle (1) Mixed breed (2) Shih tzu (5)	–	1	10	1	Recurrent moderate to severe moist, erythema, mal-odor, itch	Generalize; ears, muzzle, neck, interdigital areas, groin, ventral abdomen, perianal areas	12	10	<i>Staphylococcus pseudintermedius</i> (7)
SSD (21)	Poodle (5) Basset hound (2) Beagle (1) English cocker spaniel (2) Mixed breed (4) Pug (2)	–	5	13	3	Recurrent moderate to severe moist, hyperpigmentation, lichenification, alopecia, mal-odor, itch	Generalize; ears, muzzle, neck, interdigital areas, groin, ventral abdomen, perianal areas	21	18	<i>Staphylococcus pseudintermedius</i> (5)

PSD, primary seborrheic dermatitis; SSD, secondary seborrheic dermatitis; KTZ, ketoconazole; ITZ, itraconazole.

quantitatively determined by cultivating on SDA after unaided eye observation.

Statistical analysis and interpretation of results

Differences among the means of inhibition zone diameter, MIC and MFC for *M. pachydermatis* and *C. parapsilosis* between groups of dogs with and without SD were analyzed using the Student's *t*-test. The Chi-square test was used to compare the rates of susceptible (S), intermediate (I) or susceptible dose-dependent (SDD) and resistant (R) stains between these groups of dogs. $P < 0.05$ was considered statistically significant. To assess the correlation between the DD and BMD methods, the diameters of the inhibition zone were plotted against the respective BMD. MICs and statistical analysis were performed by linear regression. The results were also analyzed in terms of the agreement categorization as described by Vandebossche et al. (2002) [26]. The accuracy of determinations was assessed using very major errors (VME; the number of susceptible strains by DD that were considered resistant by BMD in which the number of resistant strains according to BMD was the denominator), major errors (ME; the number of resistant strains by DD that was judged as susceptible by BMD in which the number of susceptible strains according to BMD was the denominator) and minor errors (M; the number of susceptible strains according to DD that were evaluated as susceptible dose-dependent by BMD in which the total number of tested strains was the denominator) [26].

Results

Using Mueller Hinton agar supplemented with 2% dextrose and 0.5 mg/l methylene blue, an inhibition zone could be observed and verified based on the type of *Candida* strain. All tested *M. pachydermatis* isolates were susceptible to ITZ, KTZ, NYS and TERB but resistant to 5-FC (100%). By contrast, *C. parapsilosis* exhibited intermediate and resistant percentages for KTZ (32% I, 7% R), TERB (14% I, 7% R) and 5-FC (36% I, 50% R) but all tested *C. parapsilosis* isolates were sensitive to ITZ and NYS. The results of the DD test, including the susceptibility levels and mean of inhibition zone diameter, are shown in Tables 2 and 3.

By BMD, the MIC endpoint for *C. parapsilosis* could be observed within 48 h post incubation, while that of *M. pachydermatis* was observed at 72 h. All *M. pachydermatis* isolates from dogs without SD were susceptible to KTZ and ITZ (MICs < 0.03 µg/ml). SD-origin *M. pachydermatis* was susceptible with an MIC that ranged from < 0.03 – 0.12 µg/ml. In addition, one and two isolates derived from the SD group exhibited an intermediate and

susceptible dose-dependent level against KTZ and ITZ (MIC = 0.25 µg/ml), respectively.

C. parapsilosis from both groups showed a variation in the MIC values against KTZ and ITZ. In dogs without SD, eight KTZ-resistant strains (MIC ranging from 0.5 – 1 µg/ml) were detected and two of them showed cross-resistance for both KTZ and ITZ. The resistant rates or mean MIC values for KTZ and ITZ were not significantly different between dog groups with and without SD. Based on the BMD test, the susceptibility levels and mean MICs were determined and are summarized in Tables 2 and 3.

The MIC₉₀ of all tested *M. pachydermatis* isolates was similar to the MFC and thus could be used interchangeably. The *M. pachydermatis* isolates from dogs with SD had higher MFC ranges than those obtained from dogs without SD at < 0.03 – 0.5 µg/ml and < 0.03 µg/ml, respectively. There were three intermediate and one resistant *M. pachydermatis* isolate in dogs with SD and six susceptible dose-dependent strains for KTZ and ITZ, respectively. Nevertheless, the MIC₉₀ of *C. parapsilosis* was lower than the MFC but was still in the range of resistance for KTZ and ITZ. The MICs and MFCs for *M. pachydermatis* and *C. parapsilosis* are presented in Table 3.

The correlation between the DD and BMD results were analyzed based on the values of the correlation coefficient *r* and are shown in Fig. 1 and 2. By assessing the agreement between the methods, the minor errors for *M. pachydermatis* were only 2.2% and 4.4% for KTZ and ITZ, respectively, while there were no VME or ME. For *C. parapsilosis*, only 1% and 0.2% VME occurred for KTZ and ITZ, respectively. The susceptibility levels to azoles together with the error percentage between DD and BMD are shown in Table 4.

Discussion

The present study expanded our previous study on the frequency and distribution of canine skin yeasts at different stages of seborrheic dermatitis [5]. To treat mycoses, it is necessary to correctly identify the pathogen(s) and subsequently choose the proper antifungal drugs. Despite many available data on human yeast susceptibilities, the availability of susceptibility data on animal isolates is scant.

Since 1983, the CLSI, formerly the National Committee for Clinical Laboratory Standards (NCCLS), has published documentation for the standard reference microdilution methodologies for yeasts (CLSI, M27-A3) and molds (CLSI, M38-A). Agar-based diffusion methods such as DD (M44-A) and the E-test (ET) were also developed as a functional and simpler method and were consistent with the CLSI standard reference [27–29]. However, these document guidelines are only relevant for

Table 2 Summary of the ketoconazole (KTZ) and itraconazole (ITZ) susceptibility levels by the disk diffusion and broth microdilution methods for clinical yeast isolates from Thai dogs.

Disk diffusion method													
Organisms	Gr. of dogs	Antifungal agent	Mean inhibition zone diameter (mm.)			Minimum inhibitory concentration (µg/ml)					MIC ₉₀	Minimum fungicidal concentration (µg/ml)	
			S	I	R	S	I	R	R				
<i>Breakpoints</i>		KTZ	≥ 30	29–23	≤ 22	≤ 0.12	0.13–0.4	≥ 0.5					
<i>Candida albicans</i>	Quality controls	ITZ	≥ 16	15–11	≤ 10	≤ 0.12	0.13–0.4	≥ 1					
ATCC 90028		KTZ	40	–	–	0.06	–	–					
<i>Candida parapsilosis</i>		ITZ	22	–	–	0.06	–	–					
ATCC 22019		KTZ	44	–	–	0.06	–	–					
<i>Malassezia pachydermatis</i>	Healthy (12)	ITZ	24	–	–	0.12	–	–					
(45)		KTZ	72.4 (12)	0	0	< 0.03 (12)	0	0	< 0.03	< 0.03 (12)			
	SD (33)	ITZ	55.9 (12)	0	0	< 0.03 (12)	0	0	< 0.03	< 0.03 (12)			
		KTZ	48.3 (33)	0	0	< 0.03 (29)	0.25 (1)	0	< 0.03–0.5	< 0.03 (29), 0.25 (3), 0.5 (1)			
		ITZ	52.6 (33)	0	0	< 0.03 (26)	0.25 (2)	0	< 0.03–0.5	< 0.03 (26), 0.12 (1), 0.25 (4), 0.5 (2)			
						0.06 (1)							
						0.12 (4)							
<i>Candida parapsilosis</i> (28)	Healthy (13)	KTZ	39 (7)	25 (5)	24 (1)	< 0.03 (4)	0.03 (1)	0.5 (3)	1	< 0.03 (4), 0.12 (1), 0.25 (1), 0.5 (3), 1 (1), 2 (1), 16 (2),			
		ITZ	23 (13)	0	0	0.06 (1)		1 (2)	1	< 0.03 (3), 0.12 (3), 0.25 (5), 16 (2)			
						< 0.03 (4)	0.25 (1)						
						0.06 (1)							
	SD (15)	KTZ	36 (10)	25 (4)	21 (1)	< 0.03 (3)	0.03 (2)	0.5 (1)	1	< 0.03 (1), 0.03 (2), 0.06 (1), 0.12 (1), 0.25 (2), 0.5 (5), 1 (3)			
						0.12 (5)							
						< 0.03 (3)	0.12 (1)						
		ITZ	24 (14)	15 (1)	0	< 0.03 (3)	0.25 (3)	0	0.5	< 0.03 (2), 0.03 (1), 0.06 (1), 0.12 (3), 0.25 (6), 0.5 (1), 16 (1)			
						0.12 (9)							

S, susceptible; I, intermediate susceptible; R, resistant; MIC₉₀, MIC at which 90% of isolates were inhibited.

Table 3 Mean values for the inhibition zone diameter, MICs and MFCs for antifungal agents against clinical yeast isolates from healthy and seborrheic dermatitis dogs.

Organisms	Gr. of dogs	Mean values of inhibition zone diameter (mm) (SD)					Mean values of minimum inhibitory concentration (µg/ml)			Mean values of minimum fungicidal concentration (µg/ml)		
		Antifungal agent					Antifungal agent			Antifungal agent		
		5-FC	TERB	NYS	KTZ	ITZ	KTZ	ITZ	ITZ	KTZ	ITZ	ITZ
<i>Malassezia pachydermatis</i> (45)	Healthy (12)	0	130.4 ^a (11.1)	31.5 (3.7)	72.4 ^b (15.6)	56.3 (12.7)	0.03 ^c	0.03 ^d	0.03 ^e	0.03 ^f	0.03 ^f	0.03 ^f
	SD (33)	0	113.3 ^a (15.0)	32.5 (4.3)	48.3 ^b (14.1)	52.6 (15.5)	0.045 ^c (0.045)	0.055 ^d (0.058)	0.064 ^e (0.101)	0.088 ^f (0.130)	0.088 ^f (0.130)	0.088 ^f (0.130)
<i>Candida parapsilosis</i> (28)	Healthy (13)	12.8 ^g (13.0)	23.5 (10.0)	24.1 (2.6)	31.8 (7.8)	22.6 (4.2)	0.382 (0.401)	0.233 (0.346)	2.845 (5.86)	2.592 (5.95)	2.592 (5.95)	2.592 (5.95)
	SD (15)	24.8 ^g (7.6)	24.3 (9.8)	24.4 (1.4)	33.7 (9.0)	24.3 (9.2)	0.235 (0.251)	0.128 (0.072)	0.418 (0.36)	1.234 (4.09)	1.234 (4.09)	1.234 (4.09)

SD, seborrheic dermatitis; 5-FC, 5-fluorocytosine; TERB, terbinafine; NYS, nystatin; KTZ, ketoconazole and ITZ, itraconazole. ^{a, b, c, d, e, f} and ^g Statistically significant differences in the mean $\pm$ SD for the inhibition zone diameter, MICs and MFCs among the dog groups by Student's *t*-test at $P < 0.01$. The same letters indicate statistically significant differences.

Candida species and *Cryptococcus neoformans*, but also apply to *Malassezia* species and other emerging yeasts [21,22,30].

Mueller Hinton agar supplemented with 2% dextrose and 0.5 mg/l methylene blue was used for DD to allow growth of *C. parapsilosis* and *M. pachydermatis* and clearly presented an inhibition zone, except for KTZ and ITZ. As mentioned in the manufacturer's guidelines, the zones of tested yeast growth could be partially inhibited by these azoles disks. Our DD procedure was proposed to perform susceptibility screening for *M. pachydermatis* and *C. parapsilosis* in dogs.

In order to establish the susceptibility method for *Malassezia* yeasts, various media were tested to replace the standard RPMI 1640 recommended by the CLSI protocol, such as modified RPMI 1640 with lipid supplementation, modified Christensen's urea broth, Sabouraud dextrose broth with 1% Tween 80 and modified Dixon broth [22,31–33]. The BMD methodology used to test *M. pachydermatis* against various azoles followed that described by Jesus and colleagues together with the guidelines of the CLSI, M27-A3 and used the breakpoints for *Candida* species [34]. All lipid-free media allowed sufficient growth and susceptibility observation within 48–72 h, and the validity of the methods was assessed in parallel using reference controls. Since the age and concentration of the *M. pachydermatis* inocula were different in various studies, we confirmed that *M. pachydermatis* growth on day 3, equal to $2\text{--}3 \times 10^3$ CFU/ml, was the proper inoculum [22,25,30]. A higher density ($1\text{--}5 \times 10^6$ CFU/ml) should be recommended for lipid-dependent *Malassezia* species [31]. Therefore, an overly dense *M. pachydermatis* inoculum might yield an over incidence of antifungal resistance [33,35–37].

In this study, the scatter diagrams and the statistical analysis using linear regression could not explain the correlation between the CLSI MICs and inhibition zone diameters due to a lack of diversity among the susceptibility levels of *M. pachydermatis*. Therefore, a detection of errors or percentages of agreement were more appropriate, as recommended by the CLSI M23-A2 and a previous study [38]. Regarding the FDA criteria for acceptable categorical agreement, there was an excellent correlation between BMD and DD for *M. pachydermatis* and *C. parapsilosis* with $\leq 1.5\%$ VME and $\leq 3\%$ ME [39]. Thus, DD may be useful for clinical screening of antifungal susceptibility against *M. pachydermatis* and *C. parapsilosis* in animals. Regarding the DD results for MICs and MFCs, there was 99% agreement, which highlights the concordance and relationship between these methods. Herein, the CLSI document guidelines of *Candida* species could be employed and assessed for *M. pachydermatis* but an extended incubation time was essential (72 h). The

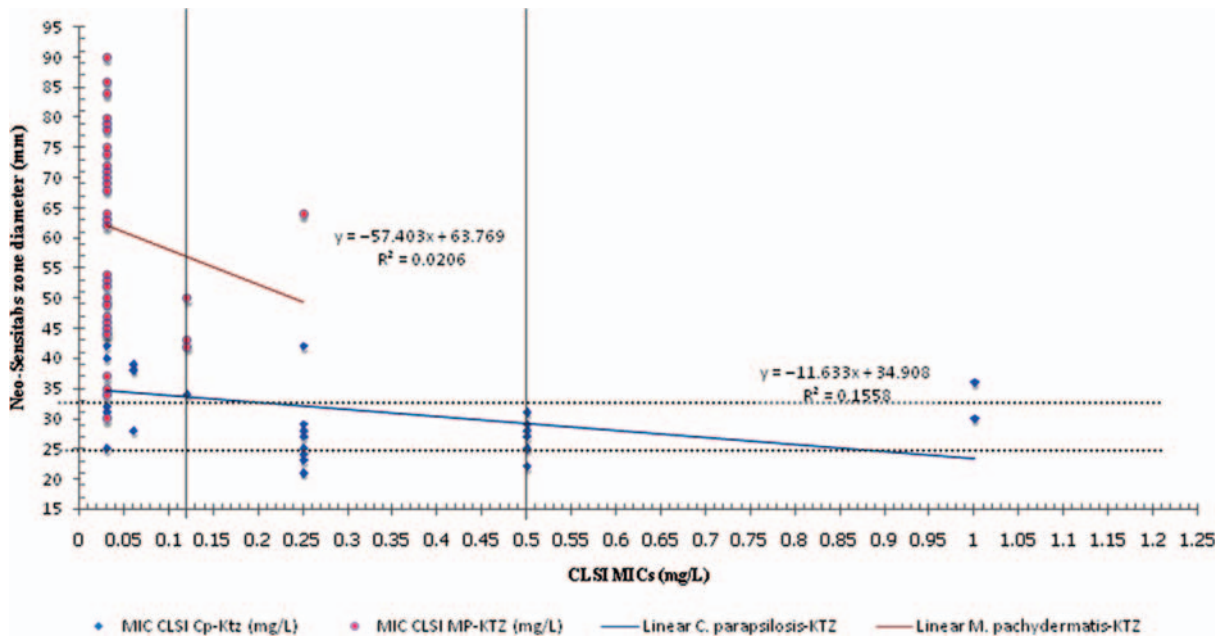


Fig. 1 Scatter diagram of CLSI MICs for ketoconazole, and the zone diameters obtained with this antifungal disk for *Candida parapsilosis* and *Malassezia pachydermatis*. The dots represent the susceptibility value for each tested strain and each line indicates the regression line with correlation coefficient r (R^2). Gray dotted and solid lines are the breakpoints for the disk diffusion (DD) and broth microdilution (BMD) MICs according to CLSI, respectively.

Candida species breakpoints were used to interpret the *M. pachydermatis* results with good agreement.

M. pachydermatis isolates were highly sensitive to all tested antifungal drugs, except 5-FC. These results are

consistent with previous studies where 5-FC resistance was shown to occur due to the absence of cytosine permease and deaminase and therefore is not recommended as a monotherapy to treat *Malassezia* infections [40,41]. Even

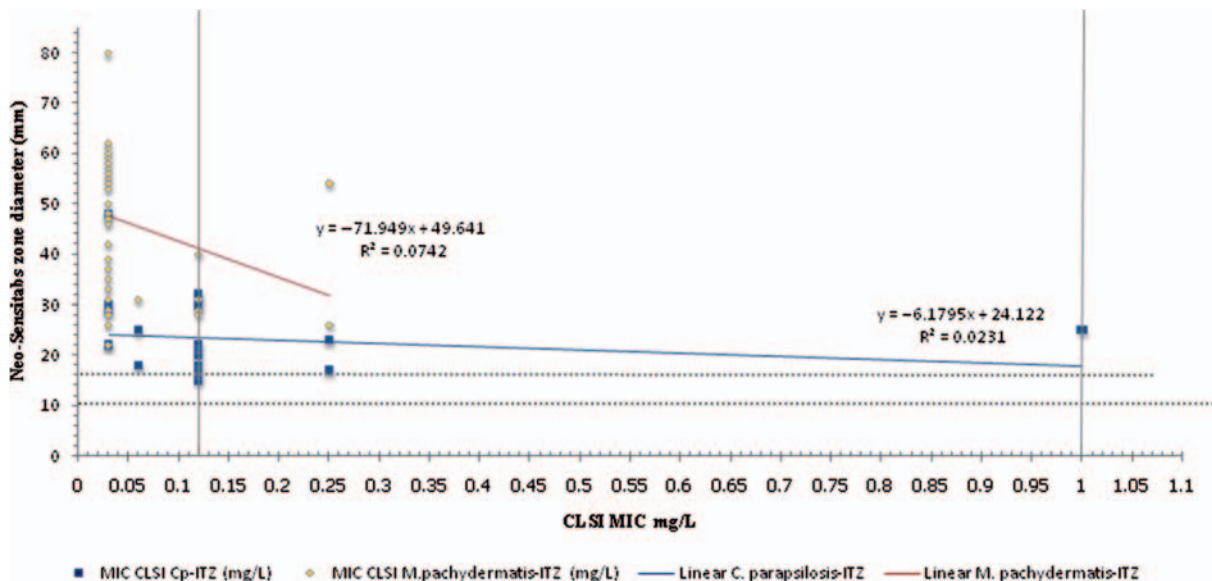


Fig. 2 Scatter diagram of CLSI MICs for itraconazole, and the zone diameters obtained with this antifungal disk for *Candida parapsilosis* and *Malassezia pachydermatis*. The dots represent the susceptibility value for each tested strain and each line indicates the regression line with correlation coefficient r (R^2). Gray dotted and solid lines are the breakpoints for the disk diffusion (DD) and broth microdilution (BMD) MICs according to CLSI, respectively.

Table 4 Summary of azole susceptibility levels and percentages of error detected between DD and BMD in clinical yeast isolates from Thai dogs.

Antifungal agent	Organism	Test method ^a	% of results in each category ^b (no. of strains)			% Error ^c		
			S	SDD	R	VME	ME	M
ITZ	<i>Malassezia pachydermatis</i>	DD	100 (45/45)	0	0	0	0	4.4 (2/45)
		MIC	95.6 (43/45)	4.4 (2/45)	0			
	<i>Candida parapsilosis</i>	DD	96.9 (31/32)	3.1 (1/32)	0	1 (1/1)	0	21.9 (7/32)
		MIC	78.1 (25/32)	18.8 (6/32)	1			
KTZ	<i>Malassezia pachydermatis</i>	DD	100 (45/45)	0	0	0	0	2.2 (1/45)
		MIC	97.8 (44/45)	2.2 (1/45)	0			
	<i>Candida parapsilosis</i>	DD	46.9 (15/32)	46.9 (15/32)	6.2 (2/32)	0.2 (2/10)	0	25 Aug-32
		MIC	40.6 (13/32)	28.1 (9/32)	31.3 (10/32)			

^aItraconazole and ketoconazole disk diffusion testing was performed according to CLSI method M44-A, and the broth microdilution method MIC testing was performed according to CLSI method M27-A2. ^bThe itraconazole susceptibility categories are as follows: S, susceptible; SDD, susceptible dose-dependent; R, resistant. ^cPercentage of error detection was calculated and described as follows: VME, very major errors; ME, major errors; M, minor errors.

though the resistant rates between groups of dogs did not exist, *M. pachydermatis* strains that originated from dogs with SD had a significantly reduced mean inhibition zone diameter against KTZ (48.3 mm) and TERB (113.3 mm) compared to those of dogs without SD (72.4 and 129.5 mm, respectively) ($P < 0.001$). The high resistant rates of canine *C. parapsilosis* for KTZ, TERB and 5-FC were detected by DD. Our results were consistent with those in human isolates, but there are no available data on animal-origin *Candida* species for comparison [42].

Generally, KTZ and ITZ are the routine antifungal drugs for canine seborrheic dermatitis, and their MIC values could be reliably used rather than values obtained by DD. Most *M. pachydermatis* isolates were highly susceptible to KTZ and ITZ at MICs $< 0.03 \mu\text{g/ml}$, which is consistent with previous reports [4,21,24,30,32–34]. The MIC values of *Candida* species and *M. pachydermatis* from dogs with SD were significantly higher than those in dogs without SD. To date, only one strain of *M. pachydermatis* that was resistant to KTZ and ITZ was isolated from a dog with SD (KTZ MIC = $1 \mu\text{g/ml}$, ITZ MIC = $2 \mu\text{g/ml}$) [32]. Since the breakpoints for *Malassezia* species have not been established, the MIC₅₀ and MIC₉₀ of *M. pachydermatis* represent a reliable level instead of the breakpoint criteria. In a previous study, KTZ- and ITZ-resistant canine *M. pachydermatis* was defined for isolates with values over the MIC₉₀ or MIC₅₀ and was detected in both groups of dogs with a higher number of resistant strains among dogs with lesions (12.5% for KTZ-resistant and 9.4% for ITZ-resistant). However, the incidence was possibly higher than expected when compared to CLSI interpretative breakpoints (KTZ; MIC₅₀ = $0.016 \mu\text{g/ml}$, MIC₉₀ = $0.03 \mu\text{g/ml}$, ITZ; MIC₅₀ $< 0.008 \mu\text{g/ml}$, MIC₉₀ $< 0.008 \mu\text{g/ml}$) [33].

Regarding zoonotic transmission, resistant strains of non-albicans *Candida* species, *Trichosporon* species, *Malassezia* species and *Rhodotorula* species are the

important causes of nosocomial infection [43]. Over 50% of strains with intermediate and KTZ/ITZ-resistance and an MIC ranging from 0.25– $1 \mu\text{g/ml}$ and MFC ranging from 0.25– $16 \mu\text{g/ml}$ were detected among the tested *C. parapsilosis* strains from dogs with and without SD. The results suggest a source of infection because 6–23% of the KTZ- and ITZ-resistant *C. parapsilosis* strains were recovered from both groups of dogs.

Because of insignificant *in vitro* resistant levels between dogs with and without SD, the evolution of resistance is difficult to access by *in vivo* and needed a monitoring of yeast population over time in patients undergoing antifungal therapy [44]. However, the history of antifungal use in each group was not recorded, and thus this hypothesis should be further examined in future follow-up studies. In addition, resistant progression was suggested the continuing exposure to sub-therapeutic concentration of antifungal drugs associated in resistant development in *M. pachydermatis* [45].

Our previous study clearly demonstrated that at the stage of primary lesions in seborrheic dermatitis, dogs were coexisting infected with over 1000 times of *C. parapsilosis* and 100 times of *M. pachydermatis* number [5]. Therefore, the diagnosis and treatment of yeast-associated canine dermatitis should be reconsidered. In cases of primary lesion SD, itraconazole may be a more appropriate antifungal choice because of the emergence of KTZ-resistant *C. parapsilosis*.

In conclusion, the clinical canine *M. pachydermatis* strains isolated from dogs with and without SD demonstrated *in vitro* susceptibility to all antifungal drugs tested except 5-FC whereas KTZ- and ITZ-resistant *C. parapsilosis* were found by BMD. However, the effectiveness of clinical therapeutic decisions based on these data would best be assessed by follow-up after treatment. There was very good agreement between CLSI DD and BMD,

indicating the reliability of these methods for antifungal susceptibility in dog-origin yeasts.

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