In vitro antifungal susceptibility of Malassezia spp. to azole drugs

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Original Article

In vitro antifungal susceptibility of *Malassezia* spp. to azole drugs

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Abstract

Objective To analyze *in vitro* the susceptibility of ketoconazole, fluconazole, and miconazole against *Malassezia* spp.

Methods Antifungal susceptibilities were determined from isolates of pityriasis versicolor lesions using disc diffusion method. The minimum inhibitory concentrations (MICs), based on Clinical and Laboratory Standard Institute (CLSI) guide were recorded after 24-48 hour of incubation at 35°C. *Malassezia* spp. showed different susceptibility profiles for the drug tested. The samples were tested for ketoconazole, fluconazole, and miconazole susceptibility. The susceptibility differences were analyzed using chi square test (x2).

Results According to statistic, the susceptibility of ketoconazole, fluconazole and miconazole showed a significant difference ($p<0.05$) in which 16 samples were sensitive to ketoconazole and fluconazole, while 10 samples were resistant and 6 samples were intermediate to miconazole.

Conclusion There was a significant difference in susceptibility of ketoconazole, fluconazole, and miconazole against *Malassezia* spp.

Key words
Pityriasis versicolor, susceptibility, disc diffusion, ketoconazole, fluconazole, miconazole, *Malassezia* spp.

Introduction

Pityriasis versicolor is a superficial fungal infection of the skin caused by *Malassezia* spp. yeast which is a normal flora of human skin.[1] Pityriasis versicolor shows typical alteration of skin pigmentation resulting in macula, as the outcome of yeast colonization in stratum corneum that can often be found in sebaceous gland rich area such as chest, back, and head.[2,3]

Clinically suspected pityriasis versicolor can be confirmed by microscopic examination with KOH smear. Positive result under microscope shows pseudohyphae and yeast cell or usually called by spaghetti and meatballs.[1] Pityriasis versicolor is often found in tropical area because of its high temperature and humidity or people who have a lot of physical activity and excrete plenty of sweat.[1]

Topical antifungals are still considered the first-

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line treatment of pityriasis versicolor, while systemic antifungals are the second-line and only used for severe cases of pityriasis versicolor, recurrent infection, and if the first line fail to treat.[4]

The Clinical Laboratory and Standards Institute (CLSI) has issued 4 standard methods for antifungal susceptibility testing,[5] this study used disc diffusion method. Based on Esteban study, both disc diffusion and dilution method can be used for antifungal susceptibility testing measurement with no significant difference.[6]

Antifungal resistance cases in pityriasis versicolor are increasing due to many factors from host, drug, and the yeast itself.[7-9] Moreover, the research about antifungal susceptibility is rarely done in Indonesia. High recurrent rate of pityriasis versicolor still remains a problem. This condition leads to free antifungal drug use without any proper doctor prescription and eventually make higher antifungal resistance rate. Helou *et al.*[7] reported one of the antifungal resistance case in a 52-year-old male who had resistance to fluconazole, ketoconazole, itraconazole, fenticonazole cream, because of the recurrent infection and continuously drug use without any doctor prescription.[7] Thus, we intended to assess the *in vitro* susceptibility of ketoconazole, fluconazole, and miconazole against *Malassezia* spp.

Methods

Malassezia spp. and susceptibility testing A total of 16 *Malassezia* spp. isolates were studied. All were isolated from clinical samples obtained from human patients with diagnosis of pityriasis versicolor. The yeasts were stored in the culture collection of Microbiology Department of Medical Faculty, Diponegoro University. Isolates were cultured using Sabouraud's dextrose agar (SDA) standardized onto a homogenous mixture using ($5x10^5$CFU/ mL/ 0,5) McFarland standard. Yeast colony inoculum was evenly cultured onto SDA media and after that ketoconazole, fluconazole, and miconazole disc placed on the media surface. After the incubation period in 35^0C within 24-48 hours, we measured the inhibitory zone diameter, which then being interpreted using CLSI provision table (**Table 1**). Written informed concern from the patients were obtained according to Ethical Committee of Faculty of Medicine/ Dr. Kariadi Hospital, Diponegoro University, Semarang.

Statistical analysis The susceptibility differences were analyzed using chi square (x2) test after performing interpretation of MICs. SPSS software was used for statistical analysis.

Results

Antifungal susceptibility test was performed using disc dilution method accordance to CLSI standard. MICs of each drug against *Malassezia* spp. could be recorded after 24-48 hours incubation at 35^0C. Diameter inhibitory zone result showed in **Table 2**.

Table 3 showed a significant *in vitro* susceptibility difference of ketoconazole, fluconazole, and miconazole against *Malassezia* sp. (p<0,05 ; x^2test).

Inhibitory zone measurement results are showed in **Figure 1.**

Table 1 Inhibitory zone provision by Clinical Laboratory and Standards Institute.

Antifungal disc	*Inhibitory zone (mm)*		
	Sensitive	*Intermediate*	*Resistant*
Ketoconazole	≥28	27-21	≤20
Miconazole	>20	19-12	<11
Fluconazole	>19	18-15	<11

Table 2 Inhibitory zone interpretation by Clinical Laboratory and Standards Institute.

No	*Ketoconazole*		*Miconazole*		*Fluconazole*	
	Inhibitory zone (mm)	*Interpretation*	*Inhibitory zone (mm)*	*Interpretation*	*Inhibitory zone (mm)*	*Interpretation*
1	57	Sensitive	10	Resistant	50	Sensitive
2	53	Sensitive	8	Resistant	56	Sensitive
3	45	Sensitive	7	Resistant	45	Sensitive
4	47	Sensitive	7	Resistant	45	Sensitive
5	47	Sensitive	10	Resistant	45	Sensitive
6	49	Sensitive	17	Intermediate	47	Sensitive
7	50	Sensitive	17	Intermediate	47	Sensitive
8	45	Sensitive	12	Intermediate	43	Sensitive
9	42	Sensitive	14	Intermediate	40	Sensitive
10	46	Sensitive	16	Intermediate	40	Sensitive
11	45	Sensitive	13	Intermediate	40	Sensitive
12	50	Sensitive	7	Resistant	40	Sensitive
13	47	Sensitive	7	Resistant	48	Sensitive
14	45	Sensitive	7	Resistant	50	Sensitive
15	35	Sensitive	7	Resistant	40	Sensitive
16	50	Sensitive	8	Resistant	50	Sensitive

16 samples were sensitive to ketoconazole, while miconazole had 10 samples resistant and 6 samples intermediate.

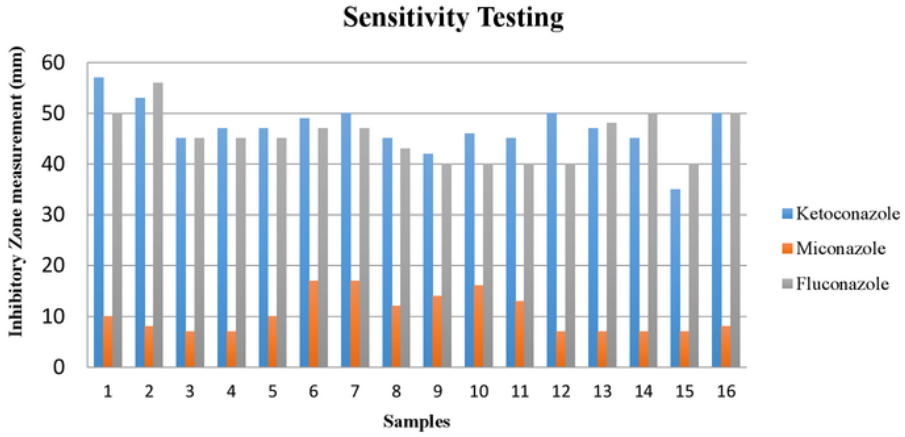

Figure 1 Inhibitory zones of different antifungals.

Table 3 Susceptibility difference of ketoconazole, fluconazole, and miconazole.

Antifungal	*Interpretation*			*p*
	Sensitive (%)	*Intermediate (%)*	*Resistant(%)*	
Ketoconazole	16 (100)	0 (0)	0 (0)	0,001
Miconazole	0 (0)	6 (37,5)	10 (62,5)	
Fluconazole	16 (100)	0 (0)	0 (0)	

Discussion

Based on statistical analysis, ketoconazole and miconazole showed a significant susceptibility difference. 16 samples were sensitive to ketoconazole while miconazole had 10 samples resistant and 6 samples intermediate. This study is in accordance with Alfonso *et al.*[10] who reported that ketoconazole had high sensitivity rate compared to other azoles including miconazole. Similar results were also reported by Rojas *et al.*[11] that ketoconazole had the best

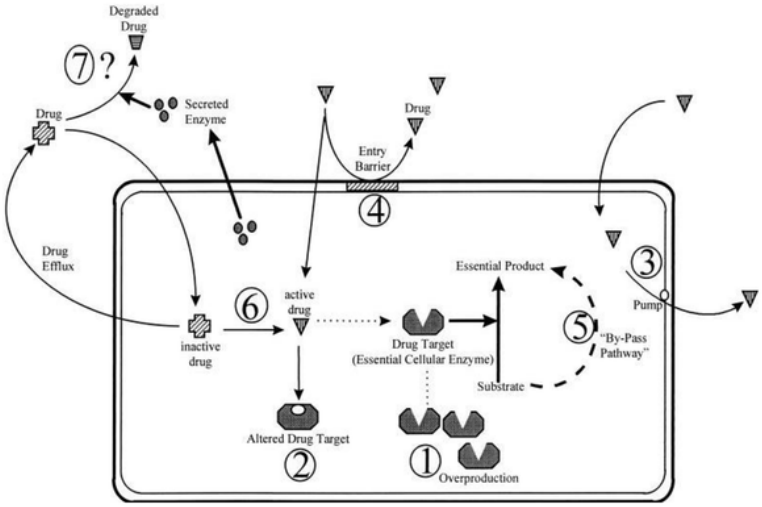

Figure 2 Resistance mechanism azole group antifungal drug in *Malassezia* spp.[16]

antifungal drug activities and minimal variability compared to fluconazole, miconazole, and amphotericin B. Miconazole itself showed resistant results.[11] The latest study in 2017 by Leong *et al*.[12] showed that ketoconazole still had the best sensitivity although it showed resistant result in few samples, nevertheless ketoconazole still being the recommendation antifungal drug for treating *Malassezia* spp. infection.[12]

Regarding fluconazole, in the previous study conducted in Dr. Kariadi hospital in 2012 from 36 total samples, all the samples were sensitive to fluconazole.[13] A research by Dheghan in 2010 also reported high sensitivity rate of fluconazole with 82% from 50 total samples.[4] Another study held by Rojas *et al*.[14] in 2016 with 50 total samples of *Malassezia*, reported that sensitivity rate for fluconazole was 72%.

The high sensitivity of fluconazole against *Malassezia* spp. might be caused by the rare use of this drug to treat pityriasis versivolor.[13] One of the reason was that fluconazole preparation is only available for systemic use, prescribed in patients with widespread lesions, recurrent infection or if the first-line fail to treat. Besides, the side effects of fluconazole are gastrointestinal inconvenience like nausea, vomit, and diarrhea. AIDS patients could had urticaria, Stevens-Johnson syndrome, hidden liver failure, and thrombocytopenia, which is another reason why the drug infrequently use.[15] However, as noted above there was 0% *Malassezia* spp. sensitive to miconazole. The acquired data was 62.5% resistant and 37.5% intermediate to miconazole. This result was similar to the previous study, in which 50 samples of pityriasis versicolor were 58% intermediate sensitive and 18% resistant.[11]

Whereas ketoconazole and fluconazole had no significant difference because both of them had 100% same sensitivity, this result was similar in the previous study, reported that *Malassezia* spp. showed 100% sensitive to ketoconazole and fluconazole.[13]

Resistance mechanism of miconazole could be caused by several process including (**Figure 2**): 1. Excessive production of target enzyme, so the drug could not inhibit the whole process; 2. Drug target alteration, so that drug could not bind to the target site; 3. The drug being pumped

out by efflux pump; 4. Entrance of the drug was blocked in cell membrane or cell wall level; 5. Cell has bypass route which can compensate functional loss by the antifungal drug activities; 6. Some of the yeast enzymes that transformed the inactive drug into active drug were inhibited; and 7. Yeast cell secreted some enzyme into extracellular medium, which then degraded the drug.[14]

In the previous study,[10-12] microdilution method was used, yet in this study used disc diffusion method. Esteban said in his study that both disc diffusion and dilution method was able to measured sensitivity rate of antifungal drug with no significant difference.[6] Cordoba *et al.*[14] in their study about comparison of disc diffusion and dilution method, said that disc diffusion method could not estimate MIC value of the drug, but it can determined the sensitivity of *Malassezia* spp.[14]

Conclusion

There was a significant in vitro susceptibility difference of ketoconazole, fluconazole and miconazole against *Malassezia* spp.

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