The Ability of Biofilms Formation and the Differences of Antifungal Susceptibility Pattern between Planktonic and Biofilm *Candida sp.*

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Abstract
Candida sp. as human body's flora causes infections and its ability to create biofilms antifungals and host immune responses cannot reach it caused resistance to broad-spectrum antifungal. In this study, the antifungal susceptibility patterns of ketoconazole, itraconazole, and fluconazole against biofilms of Candida sp. will be determined, as well as the differences in susceptibility patterns between biofilm and planktonic Candida sp. The antifungal susceptibility of clinical isolates of both planktonic and biofilm Candida sp. determine by liquid dilution methode and the intensity of biofilm formation determined by Crystal Violet methode. Of the twenty-four Candida sp., 70.8% exhibited strong biofilm intensity, while 29.2% had moderate intensity. Planktonic Candida sp. exhibited a 100%, 78.9%, and 57.9% susceptibility pattern to fluconazole, ketokonazole, and itraconazole, respectively. Meanwhile, the sensitivity percentage decreased for biofilm Candida sp., which had sensitivity values of 31.6%, 21.1%, and 31.6%, respectively. Planktonic Candida sp. had MIC values ranging from 2 to about 33 times lower than those of biofilm Candida sp. Compared to planktonic Candida sp., the susceptibility pattern of biofilms revealed a lower sensitivity percentage. Planktonic Candida sp. had an average value ratio of 1.5 to 3.1 times, whereas Biofilm Candida sp. had MIC values 2 to > 33 times higher.
Keywords*: antifungal susceptibility pattern, biofilm Candida sp and MIC values.*

I. INTRODUCTION

Candida sp. as normal flora of the human body causes fungal infections worldwide and is commonly found in environment (1). Invasive fungal infections by *Candida glabrata* show decreasing sensitivity to Fluconazole (2). The various *Candida sp.* infection manifestation is associated with biofilm's formation ability. Negative clinical implications of biofilm evade the host's immune response and antifungal effects (3). Differences between biofilm cells and planktonic form are that biofilm cells tend to be more resistant to antimicrobial agents and protection against hospes immune system (4). *Candida sp.* causes superficial fungal infections in the mucosa to systemic infection. One of the virulence factors is the ability of *Candida sp.* to form biofilms, where the eradication of biofilms becomes difficult and significantly less sensitive to antimicrobial agents. Recently 65% of all human infections involve biofilms (5)

Several studies of antifungal susceptibility patterns of *Candida albicans* showed 16.7% resistance against itraconazole and 5.6% resistance against flucytosine. Meanwhile, *candida glabrata* showed 50% resistance against itraconazole, *and candida tropicalis* showed 75% resistance against itraconazole and 25% resistance against fluconazole(6) . Research on Candidemia showed that the sensitivity test results in 101 (92.65) isolates susceptible to fluconazole. There were only three isolates dependent on dose (SDD), and five isolates were susceptible to fluconazole (7). Research on Candidiasis cutis lesions in Jakarta showed 100% sensitivity of susceptibility pattern of *C. albicans* against ketoconazole, 91.1% sensitivity, 6.7% intermediate, and 2.2% resistance against itraconazole. Meanwhile, 93.3% sensitivity and 6.7% intermediate were against fluconazole (8).

The antifungal susceptibility pattern of *Candida sp.* against Ketoconazole, Itraconazole, and Fluconazole in Yogyakarta has not been reported. This study aims to identify the pattern of antifungal

susceptibility of Biofilm *Candida sp.* against Ketokonazole, Itraconazole, and Fluconazole as well as the difference of antifungal Susceptibility pattern between Biofilm *Candida sp.* and Planktonic *Candida sp.*

II. METHOD

Biofilm formation ability was tested on a 96-wheel microplate, and the intensity was measured by Crystal Violet staining. Antifungal sensitivity test of Planktonic and Biofilms *Candida sp.* was conducted by utilizing the liquid dilution method on a 96-wheel microplate-based on Christopher G.P. methode (2010) (3). MIC (Minimal Inhibitory Concentration) values were determined based on the smallest antifungal concentrations that inhibited *Candida sp.* growth (µg / ml).

Results

Twenty four *Candida sp.* from Microbiology Laboratory of Faculty of Medicine UGM, demonstrating biofilm intensity resulted in a strong intensity as much as 70.8%, 29.2% of moderate intensity, and no weak intensity.

Table 1. The intensity of Biofilm formation ability of *Candida sp.* based on OD value at 595 nm wavelength

No	Intensity	OD Value	Quantity	Percentage
1	weak	< 0,2	0	0
2	Moderat	0,2 - 0,5	7	29,2
3	Strong	> 0,5	17	70,8

Antifungal susceptibility pattern of planktonic *Candida sp.* against antifungal Ketoconazole, Itraconazole, and Fluconazole was examined by the broth dilution method. Interpretation of Antifungal susceptibility pattern was Sensitive (S) or Resistant (R) based on MIC (Minimal Inhibitory Concentration) value.

Table2. Antifungal susceptibility pattern of planktonic *Candida sp.* against Ketoconazole, Itraconazol and Fluconazole

No	Antifungal	Sensitive		Resistant	
		quantity	Percentage	quantity	Percentage
1	Ketoconazol	15	78,9	4	21,1
2	Itraonazol	11	57,9	8	42,1
3	Fluconazol	19	100,0	0	0,0

Antifungal susceptibility of Planktonic *Candida sp.* showed 100% sensitivity against Fluconazole, which was higher than the sensitivity against Ketoconazole and Itraconazole with a percentage of 78.9% and 57.9% respectively (Table 2).\

Antifungal susceptibility of Biofilm *Candida sp.* showed less sensitive than planktonic *Candida sp.* Sensitivity percentage of Biofilm *Candida sp.* against Ketoconazole, Itraconazole, and Fluconazole was 31.6%, 21.1% and 31.6% respectively (Table 3)

Table 3. Antifungal susceptibility pattern of Biofilm *Candida sp.* against Ketoconazole, Itraconazol, and Fluconazole

No	Antifungal	Sensitivity		Resistance	
		quantity	Percentage	quantity	Percentage
1	Ketoconazol	6	31,6	13	68,4
2	Itraconazol	4	21,1	15	78,9
3	Fluconazol	6	31,6	13	68,4

The differences of antifungal susceptibility patterns between Planktonic *Candida sp.* and Biofilm *Candida sp.* against Ketoconazole, Itraconazole, and Fluconazole were shown in figure 1

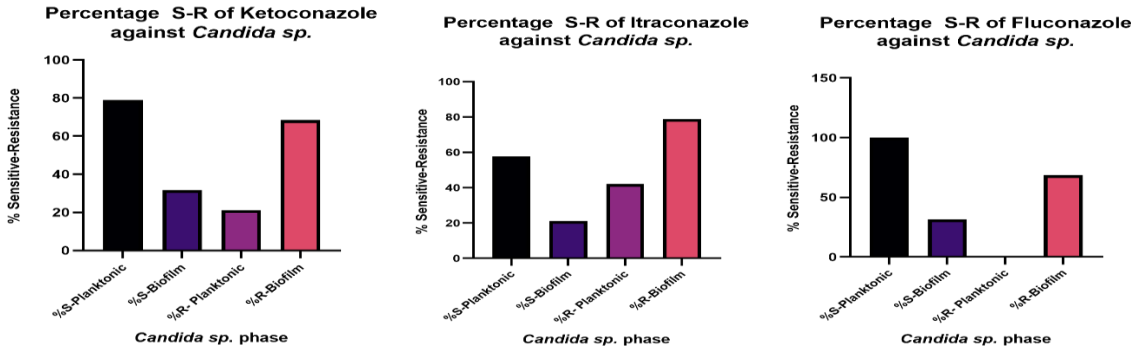

Fig. 1. Antifungal susceptibility pattern of Ketoconazole, Itraconazole, and Fluconazole against Planktonic and Biofilm *Candida sp.*

Figure 1 showed the differences in sensitivity percentage between planktonic *Candida sp.* and biofilm *Candida sp.* against Ketoconazole, Itraconazole, and Fluconazole. The sensitivity percentage of biofilm *Candida sp.* was lower than planktonic *Candida sp.* In detail, the sensitivity percentage of biofilm *Candida sp.* against Ketoconazole was 31.6%, while the sensitivity percentage of planktonic *Candida sp.* against Ketoconazole was 78.9%. Moreover, the sensitivity percentage of biofilm *Candida sp.* against Itraconazole and Fluconazole was lower than planktonic *Candida sp.* which was as much as 21.1% and 31.6% respectively. Meanwhile, the sensitivity percentage of planktonic *Candida sp.* against Itraconazole and Fluconazole was 57.9% and 100%, respectively . In contrast, the resistance percentage of Biofilm *Candida sp.* against three antifungal tested was higher than planktonic *Candida sp.*

Figure 2 showed an antifungal MIC value of Biofilm and Planktonic *Candida sp.* against Ketoconazole, Itraconazole, and Fluconazole and the ratio between the two MIC values. The antifungal MIC value of biofilm *Candida sp.* showed 2 - ≥ 33 times higher than an antifungal MIC value of planktonic *Candida sp.*

The antifungal MIC value of Ketoconazole against Biofilm *Candida sp.* was <0.015 to > 0.5 with an average value of 0.1, while against Planktonic *Candida sp.* was 0.015 to 0.25 with an average value of 0.1. The antifungal MIC value ratio of Ketoconazole between Biofilm *Candida sp.* and Planktonic *Candida sp.* is <1 to > 33,3 with the ratio mean value of 3.1 (Figure 3)

The antifungal MIC value of Itraconazole against Biofilm *Candida sp.* was <0.015 to > 0.5 with an average value of 0.1, while 0.015 to 0.5 with an average value of 0.2 was against Planktonic *Candida sp.* The ratio of antifungal MIC value of Itraconazole between Biofilm *Candida sp.* and Planktonic *Candida sp.* was <1 to > 33,3 with ratio mean value 1.5(Figure 3)

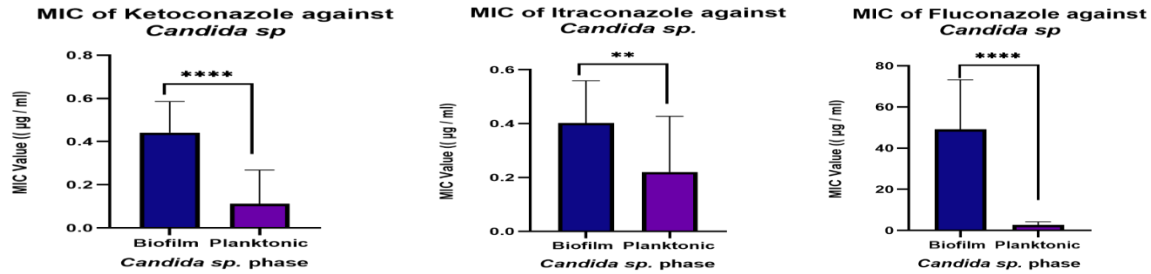

Fig. 2. Antifungal MIC Values Difference between Biofilm *Candida sp.* and Planktonic *Candida sp.* against Ketoconazole, Itraconazole, and Fluconazole

The antifungal MIC value of Fluconazole against Biofilm *Candida sp.* was < 2 to > 64 with an average value of 5.1, while <2 to 4 with an average value of 2,6 was against Planktonic *Candida sp.* The ratio of antifungal MIC value of Itraconazole between Biofilm *Candida sp.* and Planktonic *Candida sp.* was <1 to > 32 with the ratio mean value of 1.7 (Figure 3)

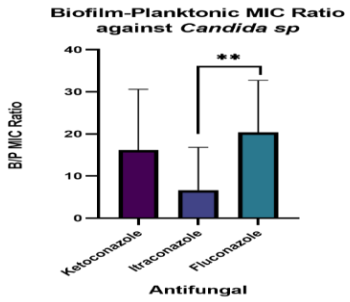

Fig. 3. Biofilm-Planktonic MIC Ratio of Antifungal against Candida sp

III. DISCUSSION

Candida sp. as normal flora of the human body caused infection worldwide whose manifestation is associated with the ability of biofilm formation and is unreachable by host immune responses and antifungal (3). Treatment has traditionally been primarily based on four kinds of systemically active antifungal medications: azoles, echinocandins, polyenes, and 5-flucytosine, an analogue of pyrimidine (9).(10)

Some *Candida sp.* in this study have the intensity in biofilm formation ability. The intensity is determined by Crystal Violet staining and measured by Optical Density (OD) value on a spectrophotometer at λ = 595 nm. Twenty-four *Candida sp.* have a strong intensity of biofilm formation ability (OD> 0.5) as much as 70.8%, medium intensity of biofilm formation ability (OD between 0.2 - 0.5) is as much as 29.2%, and weak intensity of biofilm formation ability is none (OD < 0.2) (11).

The result of the antifungal susceptibility test of planktonic *Candida sp.* showed various resistance percentages. The antifungal susceptibility test of planktonic *Candida sp.* against Ketoconazole showed 21.1% resistance, 42.1% resistance was against Itraconazole, 100% sensitivity was against Fluconazole and none of them were resistant.

The common source of invasive mycotic disorders is the *Candida species*. Some of these opportunistic pathogens, including *Candida glabrata, Candida albicans, Candida parapsilosis* and *Candida auris*, can infect immunocompromised people with life-threatening infections. Drug-resistant Candida species have reduced the efficacy of the conventional antifungal drugs, which includes polyenes, azoles, and echinocandins, which are used to treat invasive mycoses. (12)(13)

A study conducted by Srihartati (2010) on vulvo-vaginalis candidiasis in Surabaya showed 16.7% resistance of sensitivity percentage of *Candida albicans* against Itraconazole and 5.6% resistance against Flucitosin. Candida glabrata showed 50% resistance against Itraconazole while candida tropicalis showed 75% resistance against Itraconazole and 25% resistance against Fluconazole (6) Resistance Percentage of *Candida sp.* against Itraconazolee was similar to Candida glabrata. However, the percentage was lower compared to *C. tropicalis.* The resistance percentage of Fluconazole in this study is better than that of the research result conducted by Srihartati.

In another line, a study conducted by Agustina (2010) on Kandidosis Kutis lesion in Jakarta showed the susceptibility pattern of C. albicans isolates against Ketoconazole was 100% sensitivity, 91.1% sensitivity against Itraconazole, 6.7% at an intermediate level, and 2.2% resistance. Meanwhile, the susceptibility pattern of C. albicans isolates against Fluconazole was 93,3% sensitivity and 6.7% at intermediate level(8) . There are differences in an antifungal susceptibility pattern of *Candida sp.* against Ketoconazole, Itraconazole, and Fluconazole in this study showing 78.9%, 57.9%, and 100 % sensitivity, respectively. One of the most common fungus species in the human microbiome is *Candida albicans*, which asymptomatically colonizes the mouth, skin, genitourinary system, and gastrointestinal tract. (14)(15). However, *C. albicans* overgrowth can result from immunocompromised health states, dysbiosis of the microbiota, or environmental changes, causing infections ranging from superficial mucosal infections to severe hematogenously disseminated infections (15)

Furthermore, clinical manifestations of *Candida albicans* infection vary based on their biofilms formation ability on the biomaterial's surface. Biofilm cells show different phenotypic features compared to

planktonic cells. They show an increased resistance against antimicrobial agents and protection against host immune system(4). Biofilm-associated Candida infections are particularly difficult to treat because they are resistant to antifungal medication concentrations considerably higher than those generated by planktonic cell infections (15)

The antifungal susceptibility test results of biofilm *Candida sp.* showed various resistance percentages. The Antifungal susceptibility of Biofilm *Candida sp.* against Ketoconazole was 68,4% resistance, 78,9% resistance against Itraconazole, and 68,4% resistance against Fluconazole. There was an increasing resistance percentage of Biofilm *Candida sp.* compared to Antifungal susceptibility of Planktonic *Candida sp.* whose resistance percentage was 21.1% and 42.0% respectively against Ketoconazole, Itraconazole. Meanwhile, there was no resistance found against Fluconazole.

A study conducted by Mursinah et al (2016) on Candidemia showed that the susceptibility test result was 101 (92.65%) sensitivity against Fluconazole. Only three isolates depended on dose (SDD), and five isolates of resistance against fluconazole were found. It can be said that the data showed different results with this study(7)

The difference of resistance percentage between Planktonic *Candida sp.* and Biofilm *Candida sp.* showed the biofilm formation ability of *Candida sp.* was one of Candida's virulence factors. The biofilm represents the most prevalent and important way to grow microorganisms in nature and influence human infections. Sixty-five percents of human infections involve biofilms, including *Candida sp.* especially *Candida albicans* [12]. Biofilm protects microbes from the host cell's immune response and antimicrobial substances. The biofilm matrix inhibits the penetration of antibiotics. Antibiotics dose in killing biofilms bacteria will require 1000 times to achieve the same result in cell suspension(16).

The antifungal MIC value of Ketoconazole, Itraconazole, and Fluconazole against biofilm *Candida sp.* were larger than the one against planktonic *Candida sp.*

The antifungal MIC value of Ketoconazole against Biofilm *Candida sp.* was <0.015 to > 0.5 with an average value of 0.1, while 0.015 to 0.25 with an average value of 0.1 was against Planktonic *Candida sp.* The ratio of antifungal MIC value of Ketoconazole between Biofilm *Candida sp.* and Planktonic *Candida sp.* was <1 to > 33,3 with a ratio mean value of 3.1 (Figure 3) . The antifungal MIC value of Itraconazole against Biofilm *Candida sp.* was <0.015 to > 0.5 with an average value of 0.1, while 0.015 to 0.5 with an average value of 0.2 was against Planktonic *Candida sp.* The ratio of antifungal MIC value of Itraconazole between Biofilm *Candida sp.* and Planktonic *Candida sp.* was <1 to > 33,3 with a ratio mean value of 1.5 (Figure 3). The antifungal MIC value of Fluconazole against Biofilm *Candida sp.* was < 2 to > 64 with an average value of 5.1, while <2 to 4 with an average value of 2,6 was against Planktonic *Candida sp.* The ratio of antifungal MIC value of Fluconazole between Biofilm *Candida sp.* and Planktonic *Candida sp.* was <1 to > 32 with a ratio mean value of 1.7(Figure 3).

The antifungal MIC value against Biofilm *Candida sp.* increased from 2 to ≥ 33 times antifungal MIC value against planktonic *Candida sp.* with a ratio of an average value of 1.5 to 3.1 times. *Candida albicans* was highly resistant against eight antifungal compounds namely fluconazole, ketoconazole, amphotericin B, clotrimazole, miconazole, zaragoza acid B, terbinafine, and cerulenin in anaerobic growth conditions (16). Fungal biofilms exhibit a complex and multivariate resistance to traditional antifungal drugs. Because of their constitutive upregulation of drug-efflux pumps and their altered metabolic states (e.g., via metabolically inactive persister cells), biofilms offer physical protection against antifungal drugs. Cells within biofilms are also intrinsically resistant to antifungal drugs.(15)

Antimicrobial Resistance (AMR) reduces the potential efficacy of agents, including antifungal agents such as the azole and echinocandin classes, that are commonly used to treat or prevent serious fungal infections in target patient populations(17)(18)(19)(20) Comprising various aspects of patient populations and institutional practices, such as diagnostics and the proactive, empirical, preemptive, or targeted use of antifungals, the notable variations in the distribution of Candida species and the occurrence of resistance within those settings are intricate and multifaceted. (20)

Furthermore, biofilm Candida reduced antifungal agents' sensitivity compared to planktonic Candida(16). This study showed biofilm formation ability of *Candida sp.* influenced the antifungal sensitivity of ketoconazole, Itraconazole, and Fluconazole. Clinicians' recommendations of the difficulty in *Candida sp.* infection management showed some antifungal resistances that it is necessary to consider the detection of the role of biofilm as one of Candida virulence factors. Fungal illnesses have been disregarded for a long time despite their frightening effects on human health. Of fact, there are large disparities in the rates at which knowledge is generated. The pathogenic mechanisms that cause fungal infections are initiated by eukaryotic microorganisms, which makes it difficult to create medications that are toxic to the pathogen but do not damage the tissues of the host(21)

IV. CONCLUSION

Planktonic Candida sp. and Biofilm Candida sp. showed distinct patterns of antifungal susceptibility. Comparing Biofilms Candida sp. to planktonic Candida sp., the susceptibility pattern revealed a lower sensitivity percentage . In addition, the minimum inhibitory concentration (MIC) of Biofilm Candida sp. was 2 to about 33 times more than the MIC of planktonic Candida sp., which had an average ratio of 1.5 to 3.1 times.

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