

Susceptibility Profile of *Candida albicans* Biofilms on Polyvinyl Chloride Endotracheal Tube to Antifungal Azoles

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Abstract

One of the most prevalent causative organisms in human fungal infections is *Candida albicans*. The extensive utility of medical devices such as endotracheal tube has resulted in the increased cases of fungal infections. The present study visualized *Candida albicans* biofilm on polyvinyl chloride endotracheal tube and described the susceptibility profile of the biofilm to antifungal azoles. Biofilm was examined using light and fluorescence microscopy. The susceptibility profile of planktonic and sessile cells with posaconazole, miconazole, voriconazole, and fluconazole was assessed employing the standard disc diffusion method. Planktonic *C. albicans* was susceptible to the identified antifungal azoles, whereas the sessile cells had varying susceptibility profiles. The biofilms remained susceptible to voriconazole and fluconazole, susceptible-dose dependent to posaconazole, and resistant to miconazole. Findings of the present study can provide relevant information on the resistance mechanism of fungal biofilms with azoles and in the design of new pharmacologic interventions intended as treatment of mycoses.

Keywords: Fluconazole, fungal infections, miconazole, polymer-based material, posaconazole, standard disc diffusion method, voriconazole

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INTRODUCTION

Medical devices are utilized in clinical, therapeutic, and diagnostic procedures (Stoica *et al.* 2017). Endotracheal tube is one of the widely used devices in clinical practice particularly in ventilator-associated pneumonia, subglottic stenosis, and bronchopulmonary dysplasia (Gibbs & Holzman 2012). Polyvinyl chloride is one of the most common materials used in making endotracheal tube due to its capability to contain large volume and yet maintain a low pressure which controls the full inflation of the cuff preventing damage on tracheal wall (Mariyaselvam *et al.* 2017). The utility of medical devices was linked with device-associated infections with *Candida* as the most common fungal genus found to colonize these medical devices (Desai *et al.* 2014). There has been a lot of focus on the growth patterns of *Candida* in the recent years as these caused quite a number of infections in the body in their pathogenic phase (Tsui *et al.* 2016). They grow in a mucosal environment allowing them to thrive on medical devices such as the endotracheal tube (Tsui *et al.* 2016). The genus *Candida*, the fourth most common nosocomial bloodstream pathogen, was reported to cause almost 90% of invasive fungal infections with 45% mortality rate (Spampinato & Leonardi 2013).

Furthermore, Nobile and Johnson (2015) reported in their study that National Institutes of Health identified biofilm formation of *C. albicans* to be the cause of 80% of all microbial infections, ranging from superficial mucosa to more serious infections, with high mortality rates. In the treatment of these fungal infections, azoles are frequently used because of their availability for oral administration, economical price, and less toxicity (Whaley *et al.* 2016). Hence, the present study assessed the susceptibility of *C. albicans* biofilms on polyvinyl chloride endotracheal tube (PVC-ETT) to antifungal azoles using standard disc diffusion method. Information on model fungal biofilms with emphasis on susceptibility profile can provide possible explanations in the elucidation of resistance patterns of fungal biofilms with the currently available antifungal drugs designed primarily in the treatment of local and systemic mycoses. New features of these fungal biofilms can provide additional information in the subsequent synthesis of pharmacologic interventions intended

for biofilm-associated diseases. In addition, new insights will influence other disciplines such as biomedical engineering in the manufacture of medical devices to minimize occurrence of biofilm-related infections.

MATERIALS & METHODS

Organism and Inoculum

In this investigation, *C. albicans* was subcultured on Sabouraud dextrose agar (SDA) and incubated at 37°C for 48 h. The inoculum was prepared using an existing protocol (Andes *et al.* 2004) with minor adjustments. Specifically, three colonies were added in 10 mL sterile distilled water. Using serial dilution, viable fungal counts were adjusted to 7.46 (\pm 0.07) log₁₀ CFU/mL.

Biofilm Formation

To generate fungal biofilms, an existing protocol (Chandra *et al.* 2001) was followed with slight modifications. Disks with the dimensions of 0.2 cm x 0.8 cm were carefully cut from the PVC-ETT (Sacett, Portex) and subsequently sterilized. The dried disks were individually placed in 96-well culture plates and 100 μ L of the prepared inoculum with 200 μ L 50 mM glucose were introduced in each disk. The plates were then incubated at 37°C for a period of 72 h. For the microscopic studies of biofilms, nonadherent cells were removed by washing the disks with 300 μ L of 0.15 M phosphate buffer solution (PBS). The disks were then examined using a light microscope. For the fluorescence microscopy, the disks were stained with 2 mL dye (Sypro ruby) for 15 min prior to microscopic examination.

Antifungal Susceptibility Testing

Following biofilm formation, standard disc diffusion method was employed to determine zone of inhibition of fluconazole, miconazole, posaconazole, and voriconazole. Standard inoculum was adjusted to 10⁷ cells/mL. After biofilm formation, disks were washed with 0.15 M PBS (300 μ L). Varied number of strips were transferred to a test tube containing 2 mL of sterile distilled water until similar turbidity was obtained with the established standard test tube and confirmed by standard plating procedure. Once vortexed, a sterile cotton swab was dipped in the suspension and swabbed over Mueller-Hinton agar (MHA) plate. With a pair of sterile forceps, each antifungal drug was dispensed on the surface of

the agar. The plates were then incubated at 37°C for 24 h and subsequently the zone of inhibition was measured. All experiments were prepared in triplicate per monitoring time following biofilm formation.

Data and Statistical Analysis

Evaluation of the susceptibility of *C. albicans* biofilms was based on Clinical and Laboratory Standards Institute (CLSI, 2017) breakpoints for each drug and Susceptibility Testing for Yeasts (STY, 2011) guidelines. Zones of inhibition were reported as mean ± standard

deviation. Means were compared (analysis of variance and Bonferroni test) at 5% significance level using STATA.

RESULTS

Candida albicans adhered on PVC-ETT and subsequently formed biofilm (Figs. 1-2). The complexity of the aggregation increased as the biofilm forms with time. Representative images for the susceptibility testing of *C. albicans* biofilms on PVC-ETT to antifungal azoles are presented in Fig. 3. These *C. albicans* biofilms showed varying

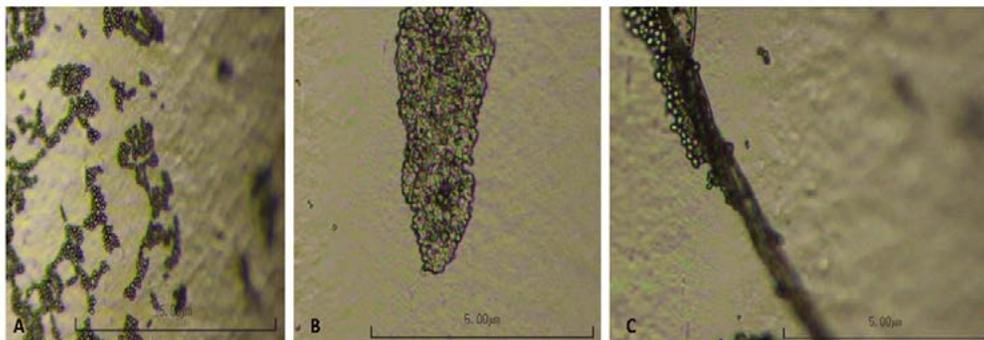


Fig. 1. *Candida albicans* biofilms on PVC-ETT at (A) 24 h, (B) 48 h, and (C) 72 h under light microscope at high power objective.

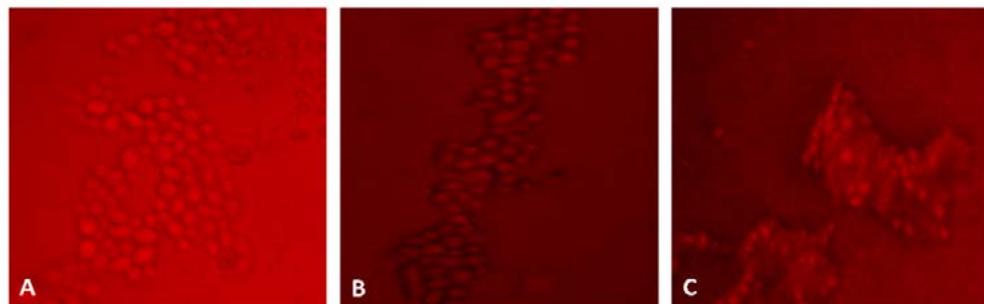


Fig. 2. Visualization of *Candida albicans* biofilms using fluorescence microscopy (X40 magnification). (A) 24-h, (B) 48-h, and (C) 72-h biofilms.

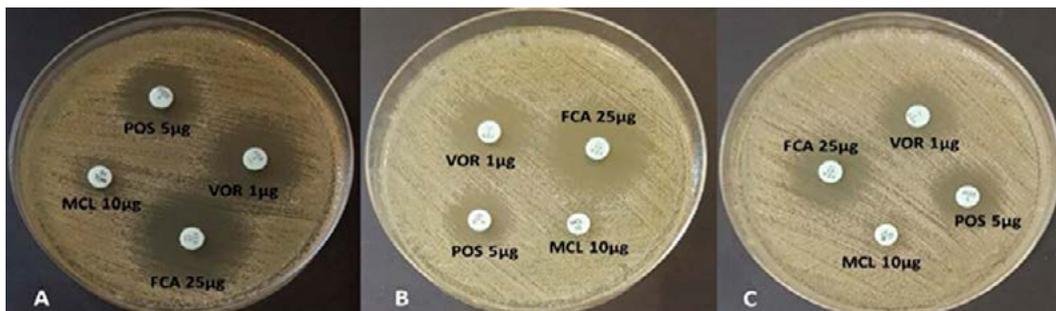


Fig. 3. Susceptibility pattern of *Candida albicans* biofilms on PVC-ETT against azoles: (A) 24-h, (B) 48-h, (C) 72-h; FCA: fluconazole, MCL: miconazole, POS: posaconazole, VO: voriconazole.

susceptibility patterns to the antifungal azoles (Table 1). The planktonic cells were susceptible to the antifungal azoles. In the serially grown biofilms, the sessile cells were resistant to miconazole. A change in the susceptibility profile of the sessile cells was observed with posaconazole, from susceptible 48-h biofilm to susceptible-dose dependent 72-h biofilm. However, *C. albicans* biofilms remained susceptible to fluconazole and voriconazole.

For fluconazole, the zones of inhibition of the planktonic cells and the biofilms at 24 to 48 h significantly differed from that of the 72-h biofilm. Significant differences between the zone of inhibition of planktonic cells and the *C. albicans* biofilms with miconazole and posaconazole were also observed. Finally, susceptibility pattern of 24-h and 48-h biofilms with voriconazole was found to be significantly different between the planktonic cells and the 72-h biofilm.

Table 1. Antifungal susceptibility of *Candida albicans* planktonic cells and biofilms on PVC-ETT.

Azoles	Zone of inhibition (mean \pm standard deviation, mm)			
	Planktonic cells	24-h biofilm	48-h biofilm	72-h biofilm
Voriconazole (1 μ g)	23.67 \pm 0.58 ^a	22.00 \pm 0.00 ^b	21.33 \pm 0.58 ^b	18.33 \pm 0.58 ^c
Miconazole (10 μ g)	21.33 \pm 0.58 ^a	10.67 \pm 1.15 ^b	10.00 \pm 1.00 ^b	9.33 \pm 0.58 ^b
Fluconazole (25 μ g)	28.33 \pm 0.58 ^a	26.67 \pm 0.58 ^a	26.33 \pm 0.58 ^a	21.00 \pm 1.00 ^b
Posaconazole (5 μ g)	24.00 \pm 1.00 ^a	18.00 \pm 1.00 ^b	18.00 \pm 1.00 ^b	16.67 \pm 1.53 ^b

In a given row, means with identical superscripts are not significant at 5%

DISCUSSION

Biofilms are surface-associated communities that are capable of colonizing the host's tissue or medical devices inside the body (Desai *et al.* 2014). An increase in the usage of medical devices among immunocompromised patients was found to be parallel with device-related biofilm infections caused by *C. albicans* (Shinde *et al.* 2012). Recurring infections and death resulted from these *Candida* biofilms (Pandolfi *et al.* 2019). Such clinical problem is further aggravated by its resistance to antifungal drugs. Azoles, the largest family of antifungal drugs, include imidazoles (e.g. miconazole) and triazoles (e.g. fluconazole, posaconazole, and voriconazole). In this study, planktonic cells and biofilms on PVC-ETT exhibited consistent susceptibility profile to fluconazole and voriconazole. Fluconazole, a broad spectrum antifungal drug, remains the first choice in the treatment of fungal infections because of its high efficacy, safety profile, and bioavailability (Liu *et al.* 2015). However, 14.1% fluconazole resistance to *C. albicans* was reported in 2014 due to repeated use of the drug (Liu *et al.* 2015). Fluconazole targets *C. albicans* by inhibiting lanosterol demethylase in the synthesis of ergosterol and such inhibition promotes toxic

accumulation of methylated sterols in fungal cell which eventually leads to cell death (Berkow & Lockhart 2017). Voriconazole and posaconazole are both broad-spectrum second-generation triazoles that are structurally similar to fluconazole and itraconazole, respectively, but are reserved for invasive infections (Ashbee *et al.* 2014). Voriconazole is also an active triazole derived from fluconazole with enhanced antifungal spectrum (Saravolatz *et al.* 2003). Furthermore, voriconazole and other second-generation azoles were also reported to be fungicidal for some filamentous organisms (Saravolatz *et al.* 2003).

Susceptibility pattern of *C. albicans* biofilms on PVC-ETT with posaconazole also changed from susceptible to resistant, although no significant difference was reported. Posaconazole is a broad-spectrum triazole used to treat infections caused by *Candida*, *Aspergillus*, and other opportunistic and dimorphic fungi encountered clinically (Gonzalez *et al.* 2007). However, increased resistance with posaconazole is presumably caused by a sixth mutation in ERG11, which functions to disrupt the binding segments of the drugs, elevating the level of posaconazole resistance (Li *et al.* 2015). Moreover, similar susceptibility pattern was also observed for miconazole, although only

the planktonic cells were susceptible and the rest of the serially grown biofilms were found to be resistant. Miconazole was the first azole identified to be effective against systemic infections (Loeffler & Stevens 2003). As was described for triazoles, miconazole also functions to affect membrane asymmetry and fluidity, and this altered plasma membrane composition may have contributed to the observed resistance to miconazole (Loeffler & Stevens 2003).

CONCLUSION

Biofilms on PVC-ETT exhibited varying susceptibility patterns with antifungal azoles. *Candida albicans* biofilms were susceptible to fluconazole, posaconazole, and voriconazole, but resistant to miconazole. The findings of the present study can provide additional information in the elucidation of resistance mechanism of the fungal biofilms with the existing antifungal drugs. Extensive study on the adherence of *C. albicans* on PVC-ETT would provide additional relevant information in the design and manufacture of biomedical devices potentially inhibiting biofilm formation and preventing development of possible fungal biofilm-associated diseases.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All data sets analyzed in the study are

included in the manuscript and presented as tables and figures.

ETHICS STATEMENT

The study protocol was approved by the University of the Philippines Manila Research Ethics Board (UPMREB 2018-486-01). The biosafety clearance was granted by the University of the Philippines Manila Institutional Biosafety and Biosecurity Committee (UPMIBBC 2018-018).

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