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Polymeric compositions of medical devices account for the variations in *Candida albicans* biofilm structural morphology

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Abstract

The increase in the number of fungal infections has been associated with the prevalent use of medical devices. This study assessed the morphological structure of *Candida albicans* biofilms on the surfaces of medical devices using scanning electron microscopy and characterized the polymeric compositions of these medical devices using infrared spectroscopic study. Biofilms on the surfaces of these medical devices exhibited variations in morphological topographies ranging from the presence of ellipsoid and spherical yeast cells joining end to end, to the growth of pseudohyphae and hyphae formation with chains of cylindrical cells, and the formation of several microcolonies entrenched in a polymeric matrix. The differences in the spectroscopic profiles of the medical devices accounted for the variations in the structural morphology of these biofilms. Spectral studies on polyvinyl chloride endotracheal tube revealed sp^3-CH stretching frequencies at 2959, 2926, and 2858 cm^{-1} with CCl stretching frequencies at 636 cm^{-1} and 693 cm^{-1}. Silicone polymer containing medical devices had SiOSi and SiC stretching frequencies identified at 1096 cm^{-1} and 804 cm^{-1} for the silicone urinary catheter, while the stretching frequencies were identified at 1005 cm^{-1} and 862 cm^{-1} for the silicone nasogastric tube, respectively. Given the information on the variations in the morphological appearance of the biofilms on medical device surfaces, these differences on the polymeric compositions of the medical devices can provide explanations on the adhesion potential, biofilm formation, structural morphology, and subsequent susceptibility pattern of the sessile organism to antifungal drugs.

Key words – Attenuated total reflectance-Fourier transform infrared spectroscopy – Biofilm – *Candida albicans* – Polyvinyl chloride polymer – Scanning electron microscopy – Silicone polymer

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Introduction

Over the years, the increase in the frequency of fungal infections, particularly candidiasis, has been linked with the utility of medical devices (Jarvis 1995, Richards et al. 1999, Shin et al. 2002, Douglas 2003, Taff et al. 2012, Mohamed & Al-Ahmadey 2013). When a microbial organism adheres on the surface of these devices, extracellular polymeric material is generated (Jin et al. 2003) which provides a scaffold for biofilm formation (Donlan 2001). These biofilms were implicated in the pathogenesis of infections (Potera 1999) and even in the persistence of diseases (Costerton 2001). Several studies have been done comparing variations in fungal biofilms as influenced by different environmental factors. The development of this polymeric biofilm on the surface of medical devices was dependent on the diversity and morphogenesis of *Candida albicans* (Hawser & Douglas 1994, Baillie & Douglas 1999, Kuhn et al. 2002, Shin et al. 2002, Li et al. 2003). The environmental conditions where the organism was introduced have implications on its growth and survival (Sevilla & Odds 1986, Hawser et al. 1998). Some of these environmental factors include pH (Stoodley et al. 1997, Marsh 2006), nutritional requirements (Jin et al. 2004, Fracchia et al. 2010), oxygen availability (Xu et al. 1998), temperature (Antley & Hazen 1988, Hazen 1989, Zeuthen & Howard 1989, Calderone & Braun 1991, Hazen et al. 1991, Karam et al. 2012, Weerasekera et al. 2016) and even the type of contact surface and substrate biomaterial (Hawser & Douglas 1994, Baillie & Douglas 1999, Douglas 2003, Li et al. 2003). In addition, polymeric compositions of medical devices may contribute to the variations in the formation of these fungal biofilms. Hence, this study examined the morphological structure of *C. albicans* biofilms on the surfaces of polyvinyl chloride endotracheal tube, silicone urinary catheter, and silicone nasogastric tube using scanning electron microscopy. The functional group compositions of the three medical devices were described using infrared spectroscopic analysis. Information on model biofilms with emphasis on structural changes of substrate materials influencing the structural architecture of these biofilms can provide possible explanations in the elucidation of resistance patterns of fungal biofilms to the currently available antifungal drugs designed primarily in the treatment of local and systemic mycoses. New features of 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 these medical devices to minimize occurrence of biofilm-related infections.

Materials & Methods

Biofilm Formation of *Candida albicans* on Medical Devices

A culture of *C. albicans* was provided by the Department of Medical Microbiology, College of Public Health, University of the Philippines Manila. In this investigation, the organism 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 (± 0.07) $\log_{10}$ CFU/mL.

To generate fungal biofilms, the protocols of Chandra et al. (2001) and Nett et al.(2014) were followed with slight modifications. Disks (0.2 cm x 0.8 cm) were carefully cut from the polyvinyl chloride endotracheal tube (Sacett, Portex), silicone urinary catheter material (Surgitech+, Fujian Bestway Medical Polymer Corp.), and silicone nasogastric tube (Medline®, NeoMed, Inc.), and subsequently sterilized. The dried disks were individually placed in 96-well culture plates and 100 μL of the prepared inoculum with 200 μL 50 mM glucose were introduced in each disk. The plates were then incubated at 37 °C for a period of 72 h.

Characterization of Biofilm Structural Morphology

Biofilms on the surfaces of the medical devices were described based on morphological structure using scanning electron microscope. Employing the procedures of Hawser & Douglas (1994) with slight modifications, the inoculated disks were carefully washed with 0.15 M phosphate

buffer solution (PBS, 5 mL). The dried biofilm specimens were then visualized by examining the porosity of the materials' surfaces and the biofilms surface morphologies.

Infrared Spectroscopic Study of the Medical Devices

For the analysis of the functional group compositions of the medical devices, spectral imaging using attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was employed (Barbes et al. 2014). The disks were individually examined by placing into the crystal suspended in the metal plate. Spectral analysis was performed using IR Affinity-1 spectrometer (Shimadzu) in the wavelength range of 3500-500 cm^{-1}.

Results

Initial examination of the surface topographies of the medical devices was done using scanning electron microscope. The polyvinyl chloride endotracheal tube and the silicone nasogastric tube revealed homogeneously smooth surfaces in contrast to the presence of several unevenly distributed grooves and depressions visualized on the surface of silicone urinary catheter (Fig. 1). The morphological topographies of *C. albicans* biofilms on the surfaces of these medical devices were also examined using a scanning electron microscope. For the 24-h biofilms on the surface of polyvinyl chloride endotracheal tube, *C. albicans* adhered to the surface of the material visualized as spherical yeast cells extending in a horizontal fashion (Fig. 2A-2B). The ellipsoid to spherical yeast cells joined end to end reflecting growth of pseudohyphae were observed in 48 h. Hyphae formation and chains of cylindrical cells were visualized at 72-h biofilm formation. These yeast cells continue to proliferate which appeared as clusters of microcolonies (Fig. 3A-3B) subsequently forming a basal layer of anchoring cells.

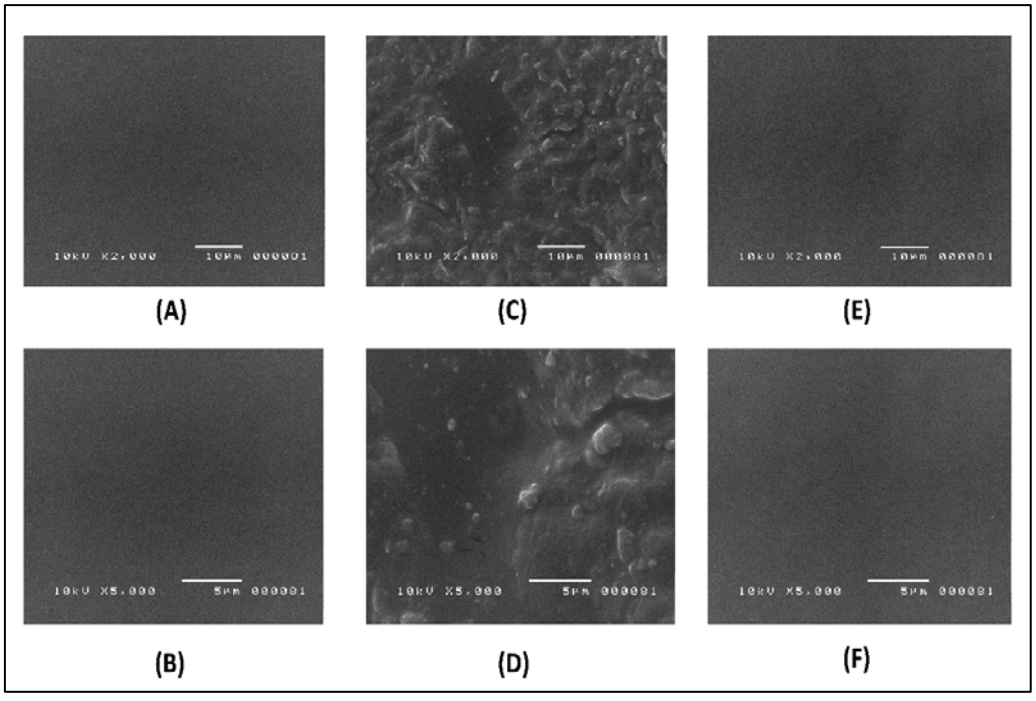

Fig. 1 – Scanning electron micrographs of medical devices: polyvinyl chloride endotracheal tube (A, B), silicone urinary catheter (C, D), and silicone nasogastric tube (E, F). Magnification: X2000 (A, C, E), X5000 (B, D, F).

For the silicone urinary catheter, the presence of grooves on the surface of the material (Fig. 1C-1D) favored yeast cells adhesion resulting in the basal layer formation and subsequent early biofilm formation (Fig. 2C-2D). Yeast cells were observed to propagate to filamentous hyphal forms as early as 24 h. By 72 h, sessile cells were eventually dispersed from the biofilm (Fig. 3C-3D). For *C.*

albicans biofilms on silicone nasogastric tube surface, adherence of fungal yeast cells to the surface eventually led to microcolony proliferation (Fig. 2E-2F). There is a subsequent basal layer formation of the anchoring cells. Moreover, extracellular polymeric production accompanied by pseudohyphae and hyphae formation was a consequence of the microcolonies high metabolic activity (Fig. 3E-3F). During the 72-h biofilm formation, the presence of the extracellular matrix can be visualized providing a niche for further fungal cell adhesion and eventually yeast-form cells dispersal from the biofilm.

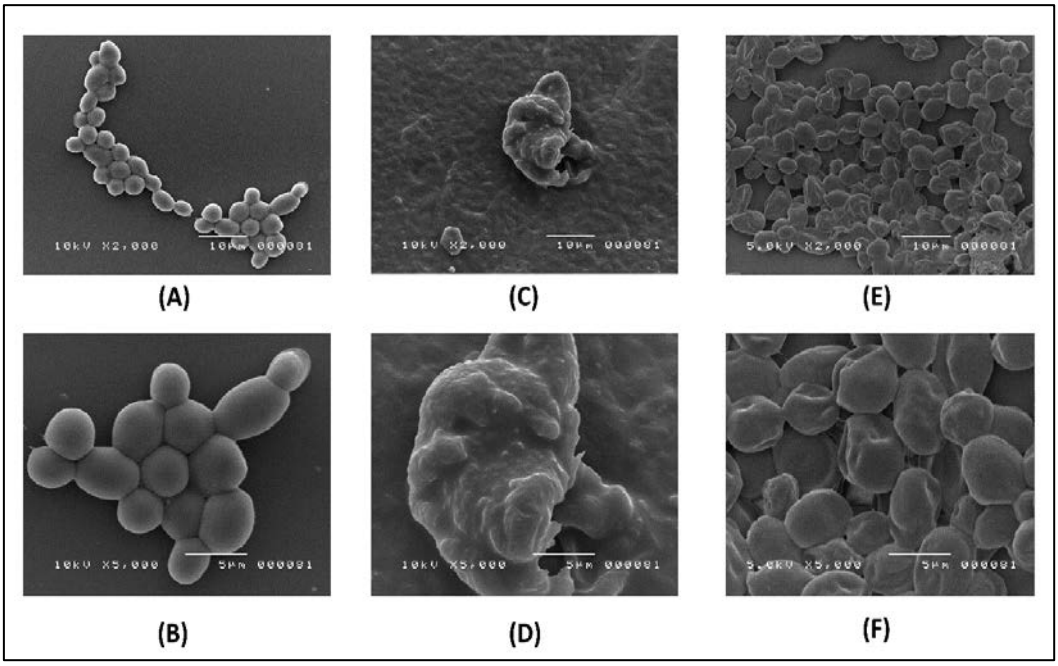

Fig. 2 – Scanning electron micrographs of *Candida albicans* biofilms on the surfaces of polyvinyl chloride endotracheal tube (A, B), silicone urinary catheter (C, D), and silicone nasogastric tube (E, F) at 24 h. Magnification: X2000 (A, C, E), X5000 (B, D, F).

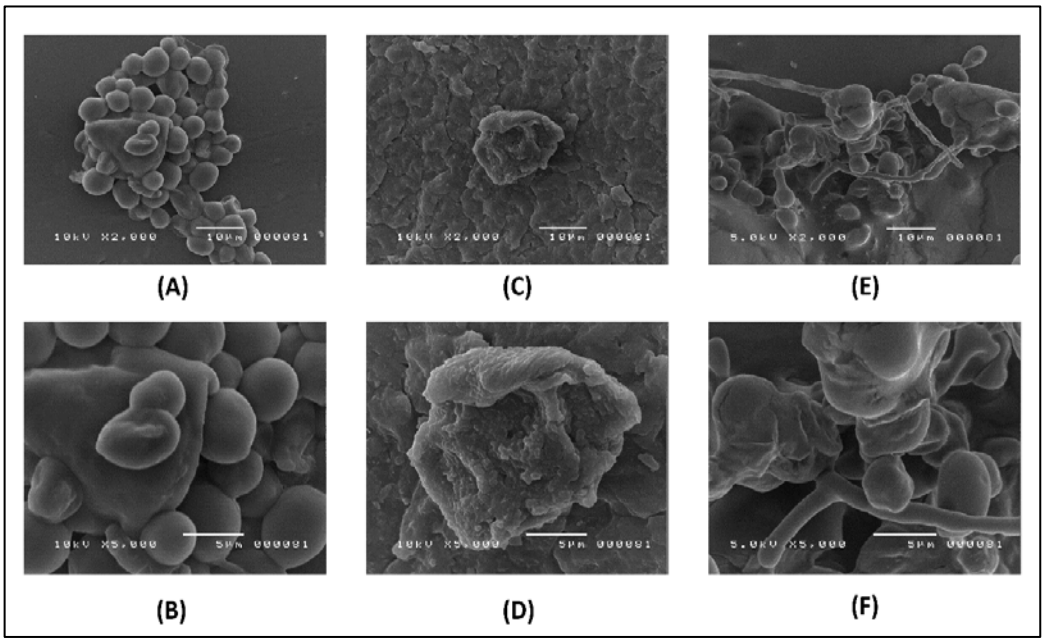

Fig. 3 – Scanning electron micrographs of *Candida albicans* biofilms on the surfaces of polyvinyl chloride endotracheal tube (A, B), silicone urinary catheter(C, D), and silicone nasogastric tube (E, F) at 72 h. Magnification: X2000 (A, C, E), X5000 (B, D, F).

Given the variations in the morphological topographies of the biofilms on the surface of the different medical devices, the organic functional group characteristics of these devices were examined using infrared spectroscopy. The spectral profiles were observed from 3500-500 cm^{-1} infrared regions. The ATR-FTIR spectral profile of polyvinyl chloride endotracheal tube revealed sp^3-CH (CH and CH_2) stretching frequencies at 2959, 2926, and 2858 cm^{-1} (Fig. 4A). Regarding the CCl stretching frequencies, the infrared spectral studies of polyvinyl chloride endotracheal tube identified these CCl stretching frequencies at 636 cm^{-1} and 693 cm^{-1} (Fig. 4A). For medical devices containing silicone polymers such as the silicone urinary catheter, the SiOSi and SiC stretching frequencies were identified at 1096 cm^{-1} and 804 cm^{-1}, respectively (Fig. 4B). For the spectral profiles of silicone nasogastric tube, the significant SiOSi and SiC stretching frequencies were observed at 1005 cm^{-1} and 862 cm^{-1}, respectively (Fig. 4C).

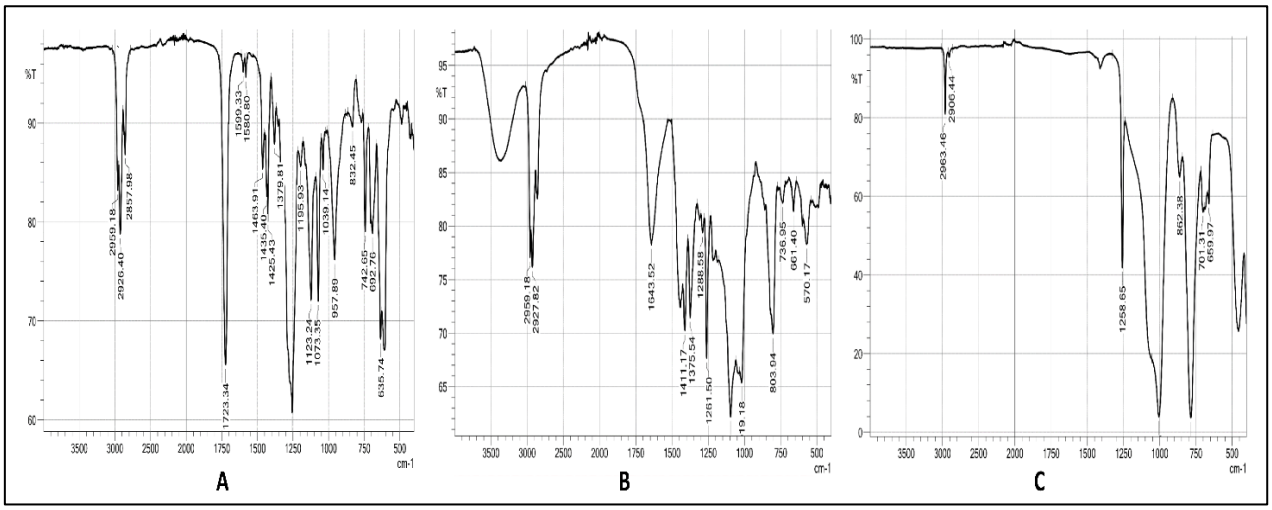

Fig. 4 – Infrared spectroscopic profile of (A) polyvinyl chloride endotracheal tube, (B) silicone urinary catheter, and (C) silicone nasogastric tube.

Discussion

Over the years, increasing cases of candidiasis have been reported and are linked with medical devices (Jarvis 1995, Richards et al. 1999, Shin et al. 2002, Douglas 2003, Taff et al. 2012, Mohamed & Al-Ahmadey 2013). When the microorganisms are in contact with the substrate surface, extracellular polymeric substances are generated (Jin et al. 2003) which support the development of a biofilm (Donlan 2001). Morphological examination showed that there was an initial attachment and adherence of the fungal yeast cells on the substrate surfaces. In the initial adherence stage, which occurred during the first 24 h, colonization of *C. albicans* was observed microscopically, with increasing aggregation complexity as the biofilm matures with time. The subsequent colonization of these adherent cells resulted to the formation of microcolonies with further cell proliferation and eventual biofilm maturation. On the 24 to 48 h, germ tube formation took place with subsequent hyphal development and formation. A mature biofilm on the 72-h formation was observed to be entrenched in a polymeric matrix, however, possible biofilm detachment and reduction in cell density was observed. This can be attributed to the possible dispersal of sessile cells and accumulation of the biochemical metabolites and waste products. With the dimorphic switching of the organism from yeast cells to hyphal form, it resulted to matrix formation and complex biofilm architectural structure. As a dimorphic organism, morphogenetic conversions of *C. albicans* are important for the several aspects of its biology, virulence, and pathogenicity (Odds 1988, Yamada-Okabe et al. 1999, Calderone & Clancy 2012). In response of the microorganism to various environmental stimuli, there is a subsequent hyphal formation which is controlled by its complex regulatory networks. These *C. albicans* hyphal elements were implicated in fungal infections (Yaar et al. 1997, Gale et al. 1998, Yamada-Okabe et al. 1999). In the development of highly organized architectural structure of *C. albicans* biofilms, hyphal filaments appeared to have an important role. These hyphae provide

structural integrity in fully matured biofilms which exhibited a heterogeneous three-dimensional morphology with a broad diversity comprised of a highly complex network of yeast cells and hyphal elements. Moreover, microscopic images in the present study revealed variations in the distribution of the fungal cells in the microcolonies resulting in the heterogeneity of the architecture of the mature biofilms. Variations on the extent of involvement and area coverage of mature biofilms on three medical device surfaces were observed and the appearance of individual yeast cells can be discerned in the less dense biofilm matrix.

Differences in the morphological appearance of the fungal biofilms which can be attributed to the medical device structural compositions was revealed through spectroscopic examination. The spectral profiles of the medical devices were observed from 3500-500 cm^{-1} infrared regions. For polyvinyl chloride endotracheal tube, the monomer units in polyvinyl chloride, with a chemical structure $(-CH_2CHCl-)_n$, are for the most part joined head-to-tail and the infrared spectral studies of the polymer revealed CH and CH_2 stretching frequencies corresponding to high frequency bands at 2967, 2920, 2849, and 2820 cm^{-1} (Krimm & Liang 1956). The stretching frequency observed at 2967 cm^{-1} is due to the CH out of phase stretching bonds on the adjoining CHCl groups. In the present study, the ATR-FTIR spectral profile of polyvinyl chloride endotracheal tube revealed sp^3-CH (CH and CH_2) stretching frequencies at 2959, 2926, and 2858 cm^{-1}. In the polyvinyl chloride, the CCl stretching was observed between 600 and 700 cm^{-1}, whereas CCl bending was detected at around 300 to 400 cm^{-1} (Krimm & Liang 1956). Regarding the CCl stretching frequencies, the ATR-FTIR spectral studies of polyvinyl chloride endotracheal tube identified these CCl stretching frequencies at 636 cm^{-1} and 693 cm^{-1}. For polymer materials containing silicone, spectral studies showed two major peaks observed at 1100 cm^{-1} and 800 cm^{-1} which correspond to SiOSi and SiC, respectively (Liu et al. 1997). For medical devices containing silicone polymers such as the silicone urinary catheter, the SiOSi and SiC stretching frequencies were identified at 1096 cm^{-1} and 804 cm^{-1}, respectively. For the spectral profiles of silicone nasogastric tube, the significant SiOSi and SiC stretching frequencies were observed at 1005 cm^{-1} and 862 cm^{-1}, respectively. Apparently, these medical devices have variable polymer compositions based on the spectral study findings which could possibly account in the variations on the *C. albicans* biofilms structural morphology.

Several studies have been done elucidating variations in fungal biofilms. The formation of these fungal biofilms was not only dependent on the type of contact surface and substrate biomaterial (Hawser & Douglas 1994, Baillie & Douglas 1999, Douglas 2003, Li et al. 2003), but also by the structural diversity and morphogenetic state of *C. albicans* (Hawser & Douglas 1994, Baillie & Douglas 1999, Kuhn et al. 2002, Shin et al. 2002, Li et al. 2003). Furthermore, the environmental conditions where the organism was introduced have also implications on its growth and survival (Sevilla & Odds 1986, Hawser et al. 1998) such as nutritional requirements (Jin et al. 2004, Fracchia et al. 2010), pH (Stoodley et al. 1997, Marsh 2006), temperature values (Antley & Hazen 1988, Hazen 1989, Zeuthen & Howard 1989, Calderone & Braun 1991, Hazen et al. 1991, Karam et al. 2012, Weerasekera et al. 2016), and oxygen availability (Xu et al. 1998). The present study demonstrated that glucose promotes formation of fungal biofilm. In the study conducted by Hawser & Douglas (1994) on *C. parapsilosis*, amendment of the medium with high-glucose concentration promoted biofilm formation. Furthermore, pH also influenced the morphological development (Ramon et al. 1999) and the regulation of morphogenesis and pathogenesis (Cottier & Mühlschlegel 2009) of *C. albicans*. When *C. albicans* invades at the infection site resulting in the influx of immune cells, tissue necrosis occurs which results in low oxygen concentrations, however, the organism has an adaptive mechanism of surviving even with this limited oxygen level (Grahl et al. 2012). Moreover, pH regulates proteolytic activity of *C. albicans* (Dostal et al. 2003), and this can alter the hydrophobicity of its cell surface, thus promoting host-cell surface adherence (Cutler 1991). When planktonic *C. albicans* cells were incubated in PBS (37 °C, pH 5), maximum attachment of the organism in the vaginal epithelium was observed (Karam et al. 2012). In contrast, *C. albicans* grown at room temperature exhibited substantial germ tube production and escaped phagocytosis (Antley & Hazen 1988). This suggests that the dimorphic nature of *C. albicans* makes it more virulent (Odds 1988, Calderone & Clancy 2012). Moreover, hydrophobicity of the yeast cell surface is also

influenced by temperature conditions (Calderone & Braun 1991, Hazen et al. 1991). At 25 °C, *C. albicans* appeared to be hydrophobic (Hazen 1989), whereas at 37 °C, it is hydrophilic (Hazen et al. 1991). However, at high temperature (45 °C), *C. albicans* synthesized heat-shock proteins (Zeuthen & Howard 1989).

Despite of the differences in the medical device polymeric compositions, *C. albicans* biofilms were still observed, suggesting that the organism can easily adapt and evolve in these microenvironments for its survival and can cause opportunistic infections (Cottier & Mühlschlegel 2009) resulting in antifungal resistance (Sumalapao et al. 2018). It was also observed that sessile cells were resistant to pharmacologic therapy when compared with planktonic cells (Donlan 2001, Ramage et al. 2001, Sumalapao et al. 2018). In conclusion, biofilms on the surfaces of polyvinyl chloride endotracheal tube, silicone urinary catheter, and silicone nasogastric tube exhibited variations in morphological topographies ranging from the presence of ellipsoid and spherical yeast cells joining end to end, to the growth of pseudohyphae and hyphae formation with chains of cylindrical cells, and the formation of several microcolonies entrenched in a polymeric matrix. *Candida albicans* exhibited remarkably variable structural topographies when monitored using these different medical devices. Spectral profiles of the medical devices afforded descriptions on the variations of the polymer compositions of the material which explained differences in the adhesion potential, biofilm formation, and structural morphology of the fungal biofilms. Information on model biofilms with emphasis on environmental conditions influencing the structural architecture of these biofilms can provide possible explanations in the elucidation of resistance patterns of these fungal biofilms with the currently available antifungal drugs primarily intended in the treatment of mycoses. New features of fungal biofilms can provide additional information in the subsequent synthesis of pharmacologic interventions designed for biofilm-associated diseases. In addition, these new insights can likewise influence other disciplines such as biomedical engineering in the manufacture of these medical devices to minimize occurrence of biofilm-related infections.

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