Susceptibility kinetic profile of Candida albicans biofilm on latex silicone surfaces with antifungal azoles

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Abstract

The present study examined the susceptibility profile of Candida albicans biofilm on latex silicone surfaces to antifungal azoles. Biofilms formed by C. albicans on latex silicone surfaces behaved in accordance with a second-order rate kinetic model. Nonadherent nonbiofilm C. albicans cells were initially susceptible to posaconazole, fluconazole, miconazole, and voriconazole. Biofilms formed by C. albicans on latex silicone surfaces remained susceptible to fluconazole, miconazole, and voriconazole, although a significant reduction in the zone of inhibition diameter was noted at 48-hour to 72-hour biofilm formation. The susceptibility kinetic profile of the fungal biofilm with posaconazole and voriconazole can be described using the zero-order rate kinetic model. Information obtained on these model biofilms with emphasis on susceptibility kinetic profile can possibly explain resistance of antifungal drugs and subsequent development of novel therapies for biofilm-based diseases.

Key words – Candida albicans – Fluconazole – Fungal biofilm – Kinetic equations – Miconazole – Posaconazole – Susceptibility profile – Voriconazole

Introduction

Candida albicans remains as the most common fungal pathogen in human infections with an unacceptably high mortality (Pfaller & Diekema 2007). The increase in these fungal infections has almost paralleled the prevalent use of varying medical implant devices (Richards et al. 1999, Shin et al. 2002). These implanted and indwelling devices, serving as substrates for biofilm growth (Shin et al. 2002, Mohamed & Al-Ahmadey 2013), with a detectable biofilm are mostly linked with these nosocomial infections (Douglas 2003, El-Azizi et al. 2015). In particular, the increasing rate of catheter-associated urinary infection, one of the most common nosocomial infections, resulted in over one million diagnosed patients (Nett et al. 2014), and more than a quarter of these catheter-
associated urinary infections were caused by Candida species. (Fisher et al. 2011).

In the treatment of systemic fungal infections and local mycoses, azoles remain as the currently used antifungal agents (Zonios & Bennett 2008). Hence, the present study assessed the susceptibility profile of the biofilm formed by C. albicans on latex silicone surfaces to antifungal azoles. In particular, the susceptibility profile of nonadherent nonbiofilm cells and biofilm formed at 24, 48, and 72 hours with posaconazole, miconazole, voriconazole, and fluconazole was recorded using different kinetic rate equations. Information obtained regarding model biofilms and parameter estimates describing susceptibility kinetic profile can provide plausible relationship between C. albicans biofilm formation and its possible resistance with some antifungal drugs. This baseline information can possibly lead to resistance mechanism elucidation and subsequent synthesis and development of novel therapies for fungal biofilm-based diseases.

Materials & Methods

Organism & Inoculum

A Candida albicans isolate obtained from a clinical specimen of a hospitalized patient was kindly provided by the Department of Medical Microbiology, College of Public Health, University of the Philippines Manila, Manila, Philippines. The identity of the organism was established using api20C-AUX system, germ tube formation, and urea and nitrate assimilation tests. The organism was maintained, grown, subcultured, and quantified on Sabouraud dextrose agar (SDA, Merck). Candida albicans was subcultured on SDA at 37°C for 24 hours prior to infection. The inoculum was prepared by placing three colonies in 10 mL sterile distilled water warmed to 37°C (Andes et al. 2004). The final inoculum was adjusted to 7.35 (± 0.05) log_{10} colony forming unit (CFU) per mL using serial dilution with viable fungal counts (7.34 – 7.36 log_{10} CFU/mL) of the inoculum confirmed by plating on SDA.

Biofilm formation

The fungal biofilms were grown using modified methods (Hawser & Douglas 1994, Chandra et al. 2001, Sumalapao et al. 2018). Catheter strips, with 0.16 cm² (0.2 cm by 0.8 cm) surface area, from latex silicone urinary Foley catheter material (Fr18, Surgitech+, Fujian Bestway Medical Polymer Corp.) were prepared and individually placed in a 96-well tissue culture plates. Standardized cell suspensions (100 μL) of the final inoculum were applied to the surface of each disc, supplemented with 100 μL 50 mM glucose (D(+)-glucose monohydrate, Unilab), and subsequently incubated for up to 72 hours at 37°C for the biofilm formation. In control experiments, discs without cells were incubated in medium containing 100 μL 50 mM glucose. All biofilm and control setups were prepared in triplicates.

Antifungal Susceptibility

Antifungal susceptibility of Candida albicans, both the nonadherent nonbiofilm cells and the biofilm formed on the catheter surfaces, was monitored serially at 24, 48, and 72 hours using the Clinical and Laboratory Standards Institute (CLSI 2009) standard disc diffusion method. To remove the nonadherent cells, catheter discs were individually submerged in a new 96-well plate containing 300 μL 0.15 M phosphate buffer solution (PBS), subsequently transferred to test tubes containing 10 mL sterile distilled water and were mixed vigorously using a vortex. For viability profile, samples were plated on SDA for viable fungal colony counts after incubation for 24 hours at 37°C with each test done in triplicate and results were expressed as the mean CFU/mL. For antifungal susceptibility, a sterile cotton swab was dipped in the suspension and streaked in many directions over the entire surface of the Mueller-Hinton agar (MHA) plate supplemented with 2% glucose and 0.5 μg/mL methylene blue. The four antifungal disks (Liofilchem Diagnostic), fluconazole (25 μg), posaconazole (5 μg), voriconazole (1 μg), and miconazole (10 μg), were placed in the MHA plates, incubated for 24 hours at 37°C, and then the zone of inhibition diameter was measured. In addition to the Clinical and Laboratory Standards Institute (CLSI 2009)
Table 1 Interpretive guidelines on antifungal susceptibility testing.

<table>
<thead>
<tr>
<th>Antifungal Azole</th>
<th>Zone of inhibition diameter interpretation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Susceptible-Dose Dependent</td>
</tr>
<tr>
<td>Posaconazole (5 μg)</td>
<td>≤ 13 mm</td>
<td>14 – 16 mm</td>
</tr>
<tr>
<td>Fluconazole (25 μg)</td>
<td>≤ 14 mm</td>
<td>15 – 18 mm</td>
</tr>
<tr>
<td>Miconazole (10 μg)</td>
<td>≤ 11 mm</td>
<td>12 – 19 mm</td>
</tr>
<tr>
<td>Voriconazole (1 μg)</td>
<td>≤ 13 mm</td>
<td>14 – 16 mm</td>
</tr>
</tbody>
</table>

STY: Susceptibility Testing of Yeasts (2011); CLSI: Clinical and Laboratory Standards Institute (2009)

Numerical Calculations & Statistical Analyses

All experiment setups were performed in triplicate in describing the susceptibility kinetic profile of *Candida albicans* biofilm with the antifungal azoles. Susceptibility of the biofilm formed with the different antifungal azoles was compared using the analysis of variance and Bonferroni test. Susceptibility kinetic profile of the biofilm with the antifungal azoles was described using different kinetic rate equations (Sumalapao 2017, 2018, Sumalapao et al. 2017, 2018). Employing the linearized forms of the kinetic equations (Table 2), the model parameters were estimated using linear regression analysis. The numerical calculations and statistical analyses were performed using Microsoft Excel® and STATA® version 12.0 at 5% level of significance.

Table 2 Nonlinear and linear forms of the susceptibility kinetic models.

<table>
<thead>
<tr>
<th>Kinetic Model</th>
<th>Nonlinear Model</th>
<th>Linear Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-order</td>
<td>( \frac{dz_t}{dt} = k_o )</td>
<td>( z_t = k_ot + z_o )</td>
</tr>
<tr>
<td>First-order</td>
<td>( \frac{dz_t}{dt} = k_1z_t )</td>
<td>( \log z_t = \log z_o + \frac{k_1}{2.303}t )</td>
</tr>
<tr>
<td>Second-order</td>
<td>( \frac{dz_t}{dt} = k_2z_t^2 )</td>
<td>( \frac{1}{z_r} = \frac{k_2}{z_n} + \left( \frac{1}{z_n} \right) )</td>
</tr>
</tbody>
</table>

\( z_t \) (mm): zone of inhibition at time \( t \) (hour); \( z_o \) (mm): zone of inhibition at \( t=0 \); \( k_0 \) (mm/hour): zero-order rate constant; \( k_1 \) (mm/hour): first-order rate constant; \( k_2 \) (mm\(^2\)·hour\(^{-1}\)): second-order rate constant

Results

The present study examined the susceptibility of *Candida albicans* nonadherent cells and the biofilm formed on latex silicone surfaces with the different antifungal azoles. *In vitro* susceptibility testing showed that *C. albicans* had varying susceptibility patterns across different antifungal azoles (Table 3). Some of these representative test plates are presented in Fig. 1. The nonadherent nonbiofilm *C. albicans* cells were initially susceptible to posaconazole, fluconazole, miconazole, and voriconazole. However, the susceptibility pattern of fungal biofilm with posaconazole significantly changed from susceptible-dose dependent to resistant in the 24-hour to 72-hour biofilm formation, respectively (Table 3). This change in susceptibility pattern of *C. albicans* biofilm with posaconazole has a kinetic profile described by a zero-order rate equation, with the zone of inhibition diameter diminishing at an average rate of 0.06 mm/hour (Table 4). The biofilms...
formed at 24, 48, and 72 hours remained susceptible to fluconazole, miconazole, and voriconazole. Susceptibility of nonadherent cells and biofilm formed at different monitoring points with fluconazole did not significantly differ (Table 3). However, susceptibility of 24-hour and 48-hour biofilms with miconazole significantly varied with the nonadherent nonbiofilm cells. *Candida albicans* biofilms when compared to nonadherent cells exhibited a significantly different susceptibility pattern with voriconazole. These variations on the susceptibility of the fungal biofilm with voriconazole can be described by a zero-order rate equation (Table 4).

**Table 3** Antifungal susceptibility of *Candida albicans* nonadherent cells and biofilm formed in different monitoring intervals.

<table>
<thead>
<tr>
<th>Azoles</th>
<th>Zone of inhibition diameter (mean ± standard deviation, mm)</th>
<th>Nonadherent cells</th>
<th>24-hr biofilm</th>
<th>48-hr biofilm</th>
<th>72-hr biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posaconazole (5 μg)</td>
<td>17.00 ± 0.00&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>15.67 ± 0.58&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>15.33 ± 0.58&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>12.67 ± 1.15&lt;sup&gt;a2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Fluconazole (25 μg)</td>
<td>23.50 ± 0.71&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>23.00 ± 2.00&lt;sup&gt;bc1&lt;/sup&gt;</td>
<td>21.67 ± 0.58&lt;sup&gt;bc1&lt;/sup&gt;</td>
<td>21.33 ± 0.58&lt;sup&gt;bc1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Miconazole (10 μg)</td>
<td>22.50 ± 0.71&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>20.33 ± 0.58&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>20.33 ± 0.58&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>20.67 ± 0.58&lt;sup&gt;b12&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Voriconazole (1 μg)</td>
<td>27.00 ± 0.00&lt;sup&gt;c1&lt;/sup&gt;</td>
<td>26.33 ± 1.53&lt;sup&gt;c12&lt;/sup&gt;</td>
<td>23.33 ± 1.15&lt;sup&gt;c2&lt;/sup&gt;</td>
<td>23.33 ± 0.58&lt;sup&gt;c2&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values with identical superscript letters in a given column and with identical superscript numbers in a given row do not differ at 5% level of significance using Bonferroni test.

**Fig. 1** – Susceptibility profile of a (A) 24-hour, (B) 48-hour, and (C) 72-hour *Candida albicans* biofilm to the different antifungal azoles: posaconazole (POS), fluconazole (FLU), miconazole (MCL), and voriconazole (VO).

**Table 4** Parameter estimates of the different kinetic rate equations describing the susceptibility of *Candida albicans* biofilm formed on latex silicone surfaces to antifungal azoles.

<table>
<thead>
<tr>
<th>Kinetic Model</th>
<th>Parameter</th>
<th>Posaconazole</th>
<th>Fluconazole</th>
<th>Miconazole</th>
<th>Voriconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-order</td>
<td>$k_0$</td>
<td>-0.0565</td>
<td>-0.0329</td>
<td>-0.0190</td>
<td>-0.0589</td>
</tr>
<tr>
<td></td>
<td>$z_o$</td>
<td>17.2174</td>
<td>23.5652</td>
<td>21.5652</td>
<td>27.1304</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.7679</td>
<td>0.4305</td>
<td>0.2713</td>
<td>0.6656</td>
</tr>
<tr>
<td></td>
<td>$p$</td>
<td>0.0004</td>
<td>0.0284</td>
<td>0.1004</td>
<td>0.0022</td>
</tr>
<tr>
<td>First-order</td>
<td>$k_1$</td>
<td>-0.0039</td>
<td>-0.0015</td>
<td>-0.0009</td>
<td>-0.0024</td>
</tr>
<tr>
<td></td>
<td>$z_o$</td>
<td>17.3622</td>
<td>23.5469</td>
<td>21.5289</td>
<td>27.1451</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.7399</td>
<td>0.4355</td>
<td>0.2625</td>
<td>0.6623</td>
</tr>
<tr>
<td></td>
<td>$p$</td>
<td>0.0007</td>
<td>0.0271</td>
<td>0.1071</td>
<td>0.0023</td>
</tr>
</tbody>
</table>
Table 4 Continued.

<table>
<thead>
<tr>
<th>Kinetic Model</th>
<th>Parameter</th>
<th>Antifungal Azole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_2$</td>
<td>Posaconazole</td>
</tr>
<tr>
<td>Second-order</td>
<td>0.0003</td>
<td>6.5e-5</td>
</tr>
<tr>
<td>$z_0$</td>
<td>17.5594</td>
<td>23.5283</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.7096</td>
<td>0.4395</td>
</tr>
<tr>
<td>$p$</td>
<td>0.0011</td>
<td>0.0262</td>
</tr>
</tbody>
</table>

$z_0$ (mm): zone of inhibition at $t=0$; $k_0$ (mm/hour): zero-order rate constant; $k_1$ (mm/hour): first-order rate constant; $k_2$ (mm$^1$.hour$^{-1}$): second-order rate constant; $R^2$: coefficient of determination; $p$: p-value

Discussion

Urinary catheters are widely used in hospital patients and there is an increasing rate of catheter-associated urinary infection (Nett et al. 2014), with 26.5% of these catheter-associated urinary infections caused by *Candida* species (Fisher et al. 2011). Medical devices including catheters promote colonization and biofilm formation by *Candida* species (Vaidyanathan et al. 2013, El-Azizi et al. 2015). *Candida albicans* formed biofilms in the surfaces of latex silicone urinary catheters and the viability and growth kinetic profile of *C. albicans* on this material behaved in accordance with the second-order rate equation (Sumalapao et al. 2018). Nobile & Johnson (2015) described the developmental stages of *C. albicans* biofilm formation. Initially, fungal yeast cells adhere to a surface and proliferate to microcolonies subsequently forming basal layer of anchoring cells. Given the high metabolic activity of the microcolonies, extracellular matrix production is accompanied by pseudohyphae and hyphae formation, and eventually yeast-form cells dispersal from the biofilm.

The present study identified that the nonadherent nonbiofilm *C. albicans* was susceptible to antifungal azoles, namely posaconazole, fluconazole, miconazole, and voriconazole. Azoles, which include triazoles and imidazoles, remain to be the largest family of antifungal drugs. Triazoles, which include posaconazole, fluconazole, and voriconazole, are considered in the treatment of superficial and systemic fungal infections (Sheehan et al. 1999). Triazoles inhibit lanosterol-14α-demethylase, an important enzyme in ergosterol biosynthesis pathway, resulting in aberrant sterol intermediates accumulation and subsequent fungal growth restriction (Fera et al. 2009). Similarly, imidazoles such as miconazole respond to induce membrane damage by inhibiting ergosterol synthesis leading to the accumulation of lanosterol. Imidazoles were observed to have fungistatic activity, but a leak in membranes from sterol perturbations has eventually resulted in fungal death (Sud & Feingold 1981).

Fungal biofilms provide organisms with a protective environment from host defenses and antifungal therapy (Nicolle 2014), and these organisms are relatively refractory to medical therapy (Vaidyanathan et al. 2013). In the present study, biofilms formed by *C. albicans* exhibited a decreasing susceptibility pattern to the tested antifungal azoles. In particular, the susceptibility pattern of *C. albicans* biofilm with posaconazole significantly changed from susceptible-dose dependent to resistant in the 24-hour to 72-hour biofilm formation. This change in susceptibility pattern of *C. albicans* biofilm with posaconazole has a kinetic profile described by a zero-order rate equation. Posaconazole is a new triazole antifungal drug which exhibits a similar mechanism of action as otherazole derivatives. Moreover, *C. albicans* biofilms on latex silicone surfaces remained susceptible to fluconazole, miconazole, and voriconazole, although a significant reduction in the zone of inhibition diameter was noted at 48-hour to 72-hour biofilm formation. Nett et al. (2014) established the tolerance to fluconazole of *in vitro* and *in vivo* *C. albicans* biofilms on silicone urinary catheter. Fluconazole was selected primarily for its clinical utility in the treatment of urinary candidiasis since the presence of a halogenated phenyl ring increases its antifungal activity (Dash & Elmquist 2001). Fluconazole basically alters the cellular membranes which leads to increased membrane permeability, impaired uptake of precursor, and leakage of essential elements from the cell (Charlier et al. 2006). However, Nasrollahi et al. (2015) reported resistance of *C. albicans* to fluconazole since Pir1p, a protein responsible for cell wall maintenance,
was found overexpressed in the fungal cell wall due to prolonged fluconazole treatment. Moreover, another triazole examined in the present study was voriconazole, a second-generation triazole and a synthetic derivative of fluconazole. *Candida albicans* biofilm when compared to nonadherent nonbiofilm cells exhibited a significantly different susceptibility pattern, but the fungal biofilm remained susceptible to voriconazole. This might be attributed to the stronger affinity of voriconazole with lanosterol-14α-demethylase which results in complete disruption of ergosterol synthesis and eventual fungal cell death (Saralovatz et al. 2003). Lastly, the susceptibility of 24-hour and 48-hour *C. albicans* biofilm with miconazole significantly differed with the nonadherent nonbiofilm cells, although the biofilm remained susceptible to miconazole. Miconazole, as an imidazole, has its cumulative effect on *C. albicans* consistent with its direct interaction with the fungal plasma membrane causing growth inhibition, and its contact with the intracellular membranes resulting in cellular disorganization, and eventual fungal death (Cope 1980).

In this study, the nonadherent nonbiofilm *Candida albicans* is different from the *C. albicans* in formed biofilms in the urinary catheter. The fungal biofilm exhibited tolerance to some antifungal drugs and biofilms are becoming progressively resistant to the first-line and second-line antifungal drugs (Martin 1999). Some of the major mechanisms which lead to azole resistance include reduced drug intracellular accumulation resulting in decreased effective drug concentration, decreased target affinity for the drug due to alterations in drug targets (Moron & Cabrera 2018), and drug effect counteraction (Spampinato & Leonardi 2013, Sanglard 2016).

Due to these emerging cases of antifungal drug resistance, there is an increase in the synthesis and manufacture of new drugs. However, some newer generation azoles have no significant activity against *C. albicans* biofilms (Katragkou et al. 2008). In conclusion, information obtained regarding these model biofilms and the parameter estimates describing the susceptibility kinetic profile of *C. albicans* biofilm formation can further elucidate resistance mechanism with these current antifungal drugs, and eventually lead to the synthesis and development of even more effective novel therapeutic and pharmacologic interventions designed specifically for fungal biofilm-related infections.

**References**


