Patterns of Candida biofilm on intrauterine devices

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Biofilms are colonies of microbial cells encased in a self-produced organic polymeric matrix and represent a common mode of microbial growth. Microbes growing as biofilm are highly resistant to commonly used antimicrobial drugs. We aimed to screen and characterize biofilm formation by different isolates of Candida on removed intrauterine devices (IUDs), to perform experimental biofilm formation with isolated strains, and to examine biofilm by the crystal violet and XTT reduction assays and scanning electron microscopy (SEM). A total of 56 IUDs were examined for biofilm formation using Sabouraud’s dextrose chloramphenicol agar. Suspected colonies were identified by different methods. Antifungal susceptibility testing with fluconazole (FLU) and amphotericin B for the isolated strains and in vitro experimental biofilm formation was carried out. The biofilm was quantified by crystal violet, XTT reduction assay and SEM. Among the 56 IUDs investigated, 26 were Candida positive (46.4%). Candida albicans was recovered from 15 isolates. The biofilm MIC of FLU was increased 64 to 1000 times compared to the MIC for planktonic cells. The XTT method results were dependent on the Candida species; biofilm formation was highest in Candida krusei and Candida glabrata strains, followed by C. albicans and Candida tropicalis. SEM of Candida biofilm revealed a heterogeneous thick biofilm with a mixture of micro-organisms. The main conclusion from this study was non-albicans Candida represents more than a half of the Candida biofilm. Better understanding of Candida biofilms may lead to the development of novel therapeutic approaches for the treatment of fungal infections, especially resistant ones among IUD users.

INTRODUCTION

Biofilms are colonies of microbial cells encased in a self-produced organic polymeric matrix and represent a common mode of microbial growth. Microbes growing as biofilm are highly resistant to commonly used antimicrobial drugs. Recently, microbial biofilm has gained prominence because of the increase in infections related to indwelling medical devices. A variety of manifestations of infections caused by Candida spp., particularly Candida albicans, are associated with the formation of biofilms on implantable medical devices (Douglas, 2003). These devices provide the necessary surfaces for biofilm formation and are currently responsible for a significant percentage of clinical candidiasis. Once a Candida biofilm forms in vivo, removal of the substrate that is supporting the biofilm growth is almost always required to eliminate the infection (Egbe et al., 2011).

Candida species are frequent inhabitants of the female genital tract and the use of intrauterine devices (IUDs) has been linked to pelvic inflammatory diseases. IUDs removed from women have been shown to be severely contaminated with C. albicans (Ramage et al., 2001; Inabo, 2006). IUDs removed from patients with recurrent vulvovaginal candidiasis revealed the presence of Candida biofilm with a dense multi-layered network of cells of different microorganisms embedded within a cellular matrix on the IUDs (Auler et al., 2010). Several groups have demonstrated that the Candida biofilm lifestyle leads to dramatically increased levels of resistance to the most commonly used antifungal agents, fluconazole (FLU) and amphotericin B (AMB) (Chandra et al., 2001; Pruthi et al., 2003).

The tetrazolium salt XTT has been used to monitor by colorimetric determination the biofilm formation of C. albicans isolates recovered from infected IUDs. The salt is reduced by living C. albicans mitochondrial dehydrogenase to a brown coloured water-soluble tetrazolium formazan product, which can be measured spectrophotometrically.

Abbreviations: AMB, amphotericin B; FLU, fluconazole; IUD, intrauterine device; SEM, scanning electron microscopy; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.
Biofilm formation among *C. albicans* varied significantly both by the XTT and crystal violet methods (Pál et al., 2005).

As metabolic activity, dry biomass and c.f.u. numbers are surrogate markers for biofilm formation, it is necessary to confirm formation of biofilms using microscopy methods that allow their visualization. Commonly used microscopy methods for biofilm characterization include fluorescence microscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy, which can provide information about the gross morphology, surface topography and matrix production, and three-dimensional architecture and thickness, respectively (Chandra et al., 2001).

All parts of an IUD allow the *in vitro* adherence of yeast cells. IUDs are foreign surfaces that are implanted for long periods into the uterine mucosa and the tail acts as a reservoir for yeast cells. Evidence for biofilms on IUDs has been proven by SEM and transmission electron microscopy (Pruthi et al., 2003).

The aims of this study were to screen and characterize Candida species attached to IUDs removed from women, to examine biofilm formation by different isolates of Candida, and to perform susceptibility testing of Candida biofilm to antifungal agents. Additional aims were to perform experimental biofilm formation on microtitre plates with different Candida species isolated from infected IUDs, and to perform experimental biofilm formation on copper IUDs with *C. albicans* and *Candida glabrata* and examine the biofilm by using SEM.

**METHODS**

**Study subjects.** This cross-sectional study with experimental trial was carried out on 56 women who attended the outpatient family planning clinic at the Women’s Health Hospital of Assiut University, Assiut, Egypt. All participants completed a clinical sheet asking for age, occupation, any gynaecological problem, previous pregnancies, abortions, pelvic inflammatory diseases, type of IUD and its duration of use, and previous antifungal therapy. All participants also underwent clinical examination looking for signs of genital tract infections. Infected IUDs were collected under completely aseptic conditions, placed in 20 ml Sabouraud’s dextrose broth and brought to the Medical Microbiology Department for processing. The study protocol was approved by the ethical committee of the Assiut University Faculty of Medicine.

**Culture of removed IUDs.** Removed IUDs were incubated aerobically at 37 °C for 24–48 h and then subcultured on Sabouraud’s dextrose chloramphenicol agar (SDA) plates and incubated aerobically at 37 °C for 24–48 h. Identification of suspected colonies was performed by Gram stain, germ tube test, sugar assimilation test and subculture on Czapek–Dox agar with Tween 80. Separate colonies from all Candida isolated on SDA were subcultured onto HiCrome Candida differential agar (Hasan et al., 2009). The medium was prepared according to the manufacturer’s instructions. All media were provided by HiMedia.

**In vitro Candida biofilm formation.** Candida strains isolated from infected IUDs were cultured on SDA plates for 24 h at 35 °C. The cultures were grown on yeast extract peptone dextrose broth (YEPD) medium (HiMedia) containing 20 g peptone 1⁻¹, 10 g yeast extract 1⁻¹ and 20 g glucose 1⁻¹, and were incubated for 48 h at 35 °C with agitation. After incubation, the whole medium was centrifugated for 10 min at 3000 g. The supernatant was discarded, the pelleted yeasts were resuspended in sterile RPMI/MOPS and the optical density of the suspension was adjusted to 0.38 at 520 nm. A total of 100 μl of the standardized cell count (107 cells ml⁻¹) was transferred into each well of a commercially sterilized polysterene flat bottomed 96-well microtitre plate (Corning) with a pipette and the plate was incubated for 48 h at 37 °C. Microtitre plates were washed thoroughly twice with 0.15 M PBS to remove free-floating organisms. The biofilm was quantified by the crystal violet assay and the XTT reduction assay (Shin et al., 2002; Pruthi et al., 2003).

**XTT reduction assay.** XTT (MP) solution (1 mg ml⁻¹ in PBS) was prepared, filter-sterilized through a 0.22 micron pore size filter and stored at −70 °C. Menadione (MP Diagnostic, USA) solution (0.4 mM) was prepared and was filter-sterilized immediately before...
each assay. Prior to each assay, XTT solution was thawed and mixed with the menadione at a ratio of 5:1 (v/v). The biofilms were first washed five times with 200 μl PBS and then 200 μl PBS and 12 μl XTT-menadione solution were added to each of the washed wells and the control wells. The microtitre plate was then incubated in the dark for 2 h at 37 °C. Following incubation, 100 μl solution was transferred to new wells and the colour change in the solution was measured with a microtitre plate reader (Stat Fax) as the absorbance at 490 nm (Pruthi et al., 2003).

Crystal violet staining. Biofilm formation was quantified using the crystal violet assay as described by Jin et al. (2003). Briefly, the biofilm-coated wells of microtitre plates were washed twice with 200 μl PBS and air dried for 45 min. Each well was washed four times with 350 μl sterile distilled water and immediately destained with 200 μl 95% ethanol. After 45 min of destaining, 100 μl was transferred to a new well and the amount of the crystal violet stain in the destaining solution was measured with a microtitre plate reader (Stat Fax) as the absorbance at 595 nm (Pruthi et al., 2003).

Antifungal susceptibility tests. MICs were determined for the planktonic Candida isolates (free-living Candida) against FLU by the broth microdilution method: the isolate was considered susceptible if the MIC was \( \leq 8 \mu g \text{ ml}^{-1} \), intermediate if the MIC was 16–32 \( \mu g \text{ ml}^{-1} \) and resistant if the MIC was \( \geq 64 \mu g \text{ ml}^{-1} \) (NCCLS, 1997). For AMB, MICs were determined by the agar dilution method: the isolate was considered susceptible if the MIC was \(<1 \mu g \text{ ml}^{-1} \), intermediate if the MIC was 2 \( \mu g \text{ ml}^{-1} \) and resistant if the MIC was \( >4 \mu g \text{ ml}^{-1} \) (Yoshida et al., 1997).

Determination of antifungal susceptibilities of Candida species in biofilm. Intact Candida biofilms were challenged with antifungal concentrations for FLU of 1 to 1024 \( \mu g \text{ ml}^{-1} \) and for AMB of 0.125 to 32 \( \mu g \text{ ml}^{-1} \). A colorimetric metabolic assay, the XTT reduction assay, was used to assess fungal cell viability after treatment with antifungal agents. The colorimetric reading at 490 nm was used to compare the reduction of growth relative to the control as a result of co-incubation with antifungal drugs. The lowest concentration associated with a 50% reduction in absorption compared with the control well at 48 h was reported as the sessile (Candida formed into biofilm) MIC (SMIC\text{50}) (Pruthi et al., 2003).

SEM of C. albicans and C. glabrata biofilms on IUDs. Two sterile IUDs (model Cu 375) were incubated in C. albicans and C. glabrata yeast suspension formed in Sabouraud’s dextrose broth for 1 and 2 weeks. Microbial biofilms formed on infected IUDs (both from patients...
and experimentally infected) were fixed with 2.5 % glutaraldehyde in Dulbecco 0.15 M PBS for 1 h, rinsed with PBS and then dehydrated through an ascending series of ethanol solutions (Chassot et al., 2008). Samples were dried and gold palladium coated (using a JEOL JFC1100E ion sputtering device). Documentation was carried out in the JEOL-JSM 5400 LV scanning electron microscope (Joel) at the Centre of Electron Microscopy, Assiut University, Egypt.

Statistical analysis. Statistical analysis was performed using SPSS software version 16 (SPSS). Values are expressed as means ± SD (continuous variables) or as percentages of the group from which they were derived (categorical variables). Two-tailed tests of significance were used to determine statistical significance, which was assumed when P<0.05. Assessment of linearity of the results of the XTT reduction and crystal violet assays was performed with SPSS software.

RESULTS

Cases

In 56 women investigated, 26 cases were Candida species positive (46.4 %). The reason for removal of the IUD was inflammation of varying degrees in 36 cases (64.3 %), metrorrhagia in 10 cases (17.9 %), or the need to conceive in 10 cases (17.9 %). The duration of the IUD being in situ was less than 1 year in 14 women (25 %) with positive Candida culture in 4 cases, between 1 and 5 years in 30 women (53.6 %) with positive Candida culture in 15 cases, and between 5 and 10 years in 12 women (21.4 %) with positive Candida culture in 7 cases.

Culture results

A total of 27 Candida isolates were isolated from 26 patients (46.4 %). Growth on HiCrome Candida differential agar showed that 15 isolates (55.6 %) produced light green coloured colonies of C. albicans. Two (7.4 %) isolates produced light blue to purple coloured raised colonies of Candida tropicalis. Seven isolates (25.9 %) were C. glabrata, which appeared as cream to white smooth colonies, and three isolates (11.1 %) were Candida krusei, which appeared as purple fuzzy colonies. Only one IUD was infected by mixed infection of C. albicans and C. tropicalis.

XTT reduction by planktonic Candida cultures of various isolates and species

Results represent the mean of three independent experiments carried out in triplicate. The XTT metabolic activity was dependent on the Candida species. Biofilm formation was highest in C. krusei, followed by C. glabrata strains, then C. albicans, and the least biofilm producer was C. tropicalis. Fig. 1 shows biofilm formation by all Candida isolates. Data represent the mean of nine independent determinations ± SE. Biofilm was highly variable among Candida isolates. Fig. 2 shows biofilm formation by XTT reduction assay. It was highest in C. krusei, then C. glabrata strains, followed by C. albicans, and the least biofilm producer was C. tropicalis. Fig. 3 shows biofilm formation by crystal violet assay. It was highest in C. albicans, then C. glabrata strains, followed by C. krusei, and the least biofilm producer was C. tropicalis. There was insignificant positive correlation between crystal violet assay and XTT reduction assay (P=0.072).

In vitro susceptibility of Candida isolates to antifungal agents

Comparing the results of susceptibility to FLU and AMB, 25 cases (92.6 %) were susceptible to AMB versus 14 cases only (51.9 %) were susceptible to FLU, while 2 cases (7.4 %) were resistant to AMB versus 5 cases (18.5 %) were resistant to FLU, and only 8 cases (29.6 %) were intermediate to FLU. Tables 1, 2 and 3 summarize susceptibility testing of the 27 Candida isolates to FLU and AMB. It was

Table 1. Susceptibility pattern of Candida isolates to FLU

<table>
<thead>
<tr>
<th>FLU susceptibility</th>
<th>C. albicans (n=15)</th>
<th>C. glabrata (n=7)</th>
<th>C. tropicalis (n=2)</th>
<th>C. krusei (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Susceptible dose dependent</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Resistant</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
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</table>

Table 2. MIC of FLU for the Candida isolates

The lowest concentration associated with a 50 % reduction in absorption compared with the control well at 48 h was reported as the sessile MIC (SMIC).

<table>
<thead>
<tr>
<th>Candida species</th>
<th>Planktonic MIC&lt;sub&gt;50&lt;/sub&gt; range (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Sessile MIC&lt;sub&gt;50&lt;/sub&gt; range (µg ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans (n=15)</td>
<td>0.125–32</td>
<td>256–&gt;1024</td>
</tr>
<tr>
<td>C. glabrata (n=7)</td>
<td>4–64</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>C. krusei (n=3)</td>
<td>16, 16, 1</td>
<td>256, 512, &gt;1024</td>
</tr>
<tr>
<td>C. tropicalis (n=2)</td>
<td>0.5, 16</td>
<td>256, &gt;1024</td>
</tr>
</tbody>
</table>
noticed that C. albicans and C. glabrata were more susceptible to FLU than C. tropicalis and C. krusei. The breakpoints for FLU were: ≤8 mg l⁻¹, susceptible; 16–32 mg l⁻¹, susceptible dose dependent; and ≥64 mg l⁻¹, resistant. While the MICs for AMB were within the range of 0.06–4 μg ml⁻¹.

It was noticed that the FLU MIC for biofilm was increased 64 to 1000 times compared to planktonic cells. There was a dramatic increase in the MIC of FLU and AMB against C. albicans biofilm.

**SEM of Candida biofilm**

SEM provided useful information on the different cellular morphologies present in the biofilm. Fig. 4 is a scanning electron micrograph showing the copper coils of the IUD. It demonstrates the dense structural organization of C. albicans biofilm formed on the surface of the copper coil. Fig. 5 is a scanning electron micrograph showing the compact interlacing hyphae on a patient’s IUD tail (thread). Fig. 6 shows an *in vitro* experimental infection by C. glabrata. C. glabrata biofilms on an IUD copper coil after 2 weeks are sparse and consist of clumps of small yeast cells.

**DISCUSSION**

The introduction of artificial material into several body locations has been accompanied by the ability of microorganisms, including *Candida* species, to colonize them and form biofilms that protect the organisms from antimicrobial agents and host defences, leading to persistent infections. *Candida* species are frequent inhabitants of the female genital tract and the use of IUDs has been linked to pelvic inflammatory diseases. IUDs removed from women have been shown to be severely contaminated with *C. albicans* (Inabo, 2006).

The most common species isolated from infected IUDs in this study were *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei*. In our study, we found 46.4 % of cases to be *Candida* species positive, with 55.6 % of isolated *Candida* strains being *C. albicans*; this is concomitant with the work of Egbe *et al.* (2011), who studied the prevalence of female reproductive tract infections among contraceptive users in Nigeria and reported that *C. albicans* represented 55.17 % of isolates among IUD users.

In our study, we found that the longer the duration of IUDs being *in situ*, the greater the incidence of biofilm formation on such IUDs. This is concomitant with the study of Pál *et al.* (2005), in which they performed a survey of biofilms formed on IUDs and confirmed the presence of multiple bacterial pathogens, as well as *C. albicans*. They reported that the longer the IUD was in place, the greater the total microbial burden and heterogeneity of species isolated from the device (Pál *et al.*, 2005). Therefore, monomicrobial or polymicrobial biofilm formation on these devices might contribute to the incidence and recurrence of genital infection (Harriott & Noverr, 2011).

Comparing the results obtained in terms of biofilm biomass (by crystal violet assay) and biofilm activity (by XTT assay), it can be concluded that there was insignificant

<table>
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<tr>
<th><strong>Candida species</strong></th>
<th><strong>Planktonic MIC₅₀ range (μg ml⁻¹)</strong></th>
<th><strong>Sessile MIC₅₀ range (μg ml⁻¹)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> (n=15)</td>
<td>0.06–0.5</td>
<td>0.25–8</td>
</tr>
<tr>
<td><em>C. glabrata</em> (n=7)</td>
<td>0.125–0.5</td>
<td>0.25–16</td>
</tr>
<tr>
<td><em>C. krusei</em> (n=3)</td>
<td>0.5, 2, 4</td>
<td>2, 4, &gt;16</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (n=2)</td>
<td>0.125, 0.125</td>
<td>4, &gt;16</td>
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Fig. 4. Scanning electron micrograph showing the copper coils of an IUD (×2000 magnification).

Fig. 5. Scanning electron micrograph of an IUD tail (thread) (×500 magnification).
positive correlation between them, as the highest cell activity does not correspond to the highest biofilm biomass and vice versa.

It has been concluded that there is not necessarily a linear relationship between the number of cells and colorimetric signal, and that care must be taken because cells in the biofilm have different metabolic activities (Kuhn et al., 2003). However, elsewhere it has been reported that cell activity measured by XTT is linearly associated with the number of cells, confirming the reliability of XTT staining in biofilm activity quantification (Harriott et al., 2010). Nevertheless, as biofilm cells are enclosed in an exopolymERIC matrix, this may possibly impose an access limitation to nutrients and oxygen, resulting in alterations in cellular activity.

Sessile cells within a Candida biofilm are less susceptible to antimicrobial agents than are planktonic cells. In this study, the biofilms were not affected by high concentrations of FLU (>1024 µg ml⁻¹). It has been suggested that biofilm resistance may be related to contributions from the extra-cellular matrix that prevent active drug diffusion, the physiologic state of the cell, efflux pumps on the cell membrane that pump antifungal drugs out of the cell or differential gene expression patterns by sessile cells (Chandra et al., 2001; Tobudic et al., 2012).

SEM was used to examine IUDs removed from patients with recurrent vulvovaginal candidiasis and revealed the presence of a dense multi-layered network of cells of different microorganisms embedded within a cellular matrix. They suggested that the presence of the biofilm on the surface of the IUD was an important risk factor for recurrent vulvovaginal candidiasis (Auler et al., 2010).

Our results may explain this fact by demonstrating yeast cells have adhered strongly to the IUD, and those parts that were covered with copper seem to contribute to the retention of yeast cells. The high concentration of yeast cells on the IUD tail may indicate the importance of this segment in the maintenance of the yeast cell colonization, as the tail makes a bridge between the external environment, the vagina that is colonized by yeast cells and the upper genital tract where there is no colonization.

A better understanding of Candida biofilms may lead to the development of novel therapeutic approaches for the treatment of fungal infections due to implants, especially in gynaecological infections among women with implanted IUDs. New antifungal agents and preventive strategies such as antimicrobial coatings may offer new hope in the management of these recalcitrant infections.

**REFERENCES**


