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Synergistic effect of amphotericin B and tyrosol on biofilm formed by *Candida krusei* and *Candida tropicalis* from intrauterine device users

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Abstract

The presence of intrauterine contraceptive devices (IUDs) provides a solid surface for attachment of microorganisms and an ideal niche for the biofilm to form and flourish. Vaginal candidiasis is often associated with the use of IUDs. Treatment of vaginal candidiasis that develops in connection with IUD use requires their immediate removal. Here, we present *in vitro* evidence to support the use of combination therapy to inhibit *Candida* biofilm. Twenty-three clinical *Candida* isolates (10 *C. krusei* and 13 *C. tropicalis*) recovered from endocervical swabs obtained from IUD and non-IUD users were assessed for biofilm-formation ability. The rate of isolation of *Candida* did not differ significantly among IUD and non-IUD users ($P = 0.183$), but the biofilm-formation ability of isolates differed significantly ($P = 0.02$). An *in vitro* biofilm model with the obtained isolates was subjected to treatment with amphotericin B, tyrosol, and a combination of amphotericin B and tyrosol. Inhibition of biofilm by amphotericin B or tyrosol was found to be concentration dependent, with 50% reduction ($P < 0.05$) at 4 mg/l and 80 μM, respectively. Hence, a combination effect of tyrosol and amphotericin B was studied. Interestingly, approximately 90% reduction in biofilm was observed with use of 80 μM tyrosol combined with 4 mg/l amphotericin B ($P < 0.001$). This represents a first step in establishing an appropriate antibiofilm therapy when yeasts are present.

Key words: *Candida*, IUDs, biofilm, amphotericin B, tyrosol.

Introduction

Vulvovaginal candidiasis (VVC) is common among women of different age groups, irrespective of their sexual activity. It occurs in 75% of all women at least once in their lifetimes [1], most often during childbearing years [2]. VVC is treatable and symptoms are usually mild; however, when

left untreated, these lesions cause significant morbidity in women with human immunodeficiency virus infections [3]. The occurrence of VVC appears to have increased in recent decades, and many predisposing factors have been proposed to explain this trend. Yet, few studies have linked use of intrauterine contraceptive devices (IUDs) with *Candida* colonization or infection among women with VVC [4,5]. Studies have demonstrated the capacity of *Candida* spp. to adhere to both cellular and inanimate surfaces. As a result, biofilm formation by *Candida* spp. on inert surfaces of implanted devices such as catheters and prosthetic cardiac valves has been studied in detail [6–9]. Additionally, *C. albicans* was shown to form biofilms on oral mucosa in an immunosuppressed murine model of oropharyngeal candidiasis [10]. Further, Harriott et al. [11] proposed the vaginal mucosa to be a newly characterized biotic surface for *Candida* biofilm formation in an immunocompetent host. However, very little is known about the formation of candidal biofilm on genital devices such as the IUD [12–14], which can be of importance in the development of VVC.

Candida biofilm formation is of particular interest because *Candida* involved in biofilm exhibits a poor response to antifungal agents. The evolution of antifungal drug resistance poses a growing public health problem because of the sharp increase in the incidence of opportunistic fungal infections in recent years [15]. Resistance is documented for all antifungal drugs that have been widely deployed therapeutically. The increase in fungal biofilm resistance to conventional treatments has driven the search for novel antifungal compounds. Combination drug treatment is a possible solution to curb drug resistance in biofilms. Thus, we focused on the isolation of *Candida* species from IUD and non-IUD users to determine the biofilm-formation ability of the isolates. The isolates from IUD users showed high biofilm-formation ability, and the formed biofilm showed resistance to amphotericin B (considered to be the gold standard antibiotic used to treat candidal infection) and tyrosol (a known quorum-sensing molecule). Hence, an attempt was made to study the synergistic effect of tyrosol and amphotericin B on *Candida* biofilm in order to derive a new treatment strategy for drug-resistant biofilms.

Material and methods

Case and control patients

Women aged 20–35 years were recruited from December 2011 through July 2012, of which 50 eligible (group A) married women were selected from outpatients referred to the gynecological and obstetrics unit of Pankajam Seetharaman Hospital, Tiruchirapalli, Tamilnadu, India, for removal of an IUD. Reasons for removal included pelvic inflammatory disease, hemorrhage, pelvic pain, vaginal discharge, desire to conceive, or an IUD *in utero* for ≥2 years. Among the 50 women, 35 used copper T 380A and 18 used Multiload 250 (SMB Corporation, Mumbai, Maharashtra, India).

Fifty women attending the same hospital at the same time for advice on contraception were recruited as controls (group B). The criteria for this group were as follows: not currently pregnant, had an intact uterus, no current referral for hysterectomy or cervical conization, reported no use of vaginal medication, reported no treatment of gynecological disease in the previous 6 months, and reported no diagnosis for anogenital cancer or tobacco-related disease. All recruited women received detailed information regarding the objective of the study and gave written consent to participate. The Institutional Ethics Committee of Bharathidasan University approved the study (DM/2011/101/20).

Sample collection and processing

The cervix of each patient was exposed by sterile bivalve speculum, and vaginal swabs were obtained with sterile cotton swabs. The swabs were then placed in sterile thioglycolate broth and transported on ice to the Medical Microbiology Laboratory, Department of Microbiology, Bharathidasan University, Tiruchirappalli, for further processing on the same day. The swabs were directly streaked on Sabouraud glucose agar (SGA) for isolation of *Candida*. The inoculated plates were incubated at 37°C until visible colonies appeared. The creamy *Candida* isolates obtained were subcultured to fresh SGA plates.

Characterization of the candidal isolates

The *Candida* isolates were identified on HiCrome *Candida* differential agar (HiMedia, Mumbai, India), as it quickly facilitates selective and differential identification of *Candida* species without compromising the sensitivity of identification [16]. The differential agar was prepared following the manufacturer's instructions. *Candida* species were identified on HiChrome agar based on the characteristic color of the colony by subculturing from SGA plates; the inoculated plates were incubated at 37°C for 24–48 h.

Further, the *Candida* isolates were subjected for phenotypic identification by germ tube test, grown in SGA plus 6.5% sodium chloride, and grown at 42°C and 45°C. Briefly, for the germ tube test, the isolates were incubated in human serum at 37°C for 3 h to determine germ tube formation. To examine growth at 42°C and 45°C, all isolates were subcultured in SGA and incubated for 48 h at 42°C and 45°C in order to determine growth capacity at these temperatures. For sodium chloride resistance, the isolates

were inoculated in SGA medium containing 6.5% sodium chloride and incubated for 48 h at 37°C.

Biofilm formation and crystal violet assay

To observe the initial stages of biofilm formation, the static biofilm model, as previously described, was adopted [17]. Briefly, Thermanox (TMX) polyolefin polymer plastic cell-culture-treated coverslips (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 13 mm diameter × 0.2 mm thick (Nalge Nunc International, Rochester, NY) were placed into each well of sterile 24-well polystyrene plates (Falcon). Standardized cell suspensions of 1×10^6 cells/ml were seeded into the wells of a 24-well plate. Wells containing broth alone were used as negative controls. The plates were covered and incubated at 37°C for 48 h. The formation of biofilm was then assayed using crystal violet assay as previously described [17]. The extraction of crystal violet by ethanol and absorbance of the dye in solution at 595 nm corresponds to the strength of biofilm formed [17].

Examination of biofilm formation on copper surfaces

As the biofilm-formation ability of the isolates had been initially studied on coverslips, we decided to determine if the isolates from IUD users resist copper toxicity and form biofilm on copper surfaces. To test this hypothesis, two representative isolates, one each from IUD (*C. tropicalis* CA4) and non-IUD (*C. tropicalis* CA45) users, were analyzed for biofilm-formation ability on copper coupons as described previously [17], using scanning electron microscopy (SEM). Candidal cells from an overnight culture were harvested and resuspended in Sabouraud glucose broth (SGB). The suspensions were dispensed into each well that contained coupons. After 48 h of exposure, the coupons were subjected to SEM. The copper materials from static biofilms were processed as previously described [17]. The formed biofilms were visualized using a Hitachi S-3400N SEM.

Examination of biofilm formation on IUDs

In order to compare the ability of the isolates (from IUD and non-IUD users) to form biofilm on IUD surfaces, two candidal isolates, *C. tropicalis* strain CA4 (from IUD users) and *C. tropicalis* strain CA45 (from non-IUD users), were allowed to form biofilms on the surface of commercial IUDs (copper T 380A) for 72 h. The formed biofilms were then analyzed using atomic force microscopy (AFM) with a Quesant Q Scope 350 (Quesant Instruments, Agoura Hills, CA, USA) in contact mode in order to visualize the surface topography of the formed *Candida* biofilm.

Anticandidal susceptibility testing

Susceptibility testing of *Candida* isolates was conducted with amphotericin B and tyrosol. The minimal inhibitory concentration (MIC_{50}) was determined using the microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI) [18]. Standardized cell suspensions (1×10^6 cells/ml in SGB) were seeded into selected wells of a microtiter plate, and amphotericin B or tyrosol was added at a final concentration of 0.25–4 mg/l or 20–640 μM. The plates were incubated at 37°C for 24 h, and the optical density was then read at 600 nm. Phosphate-buffered saline (PBS; pH 7.2) and media alone served as negative and blank controls, respectively. MICs were determined as the lowest concentration that produced a 50% reduction in growth relative to that of the drug-free control.

Biofilm susceptibility assay

The biofilm susceptibility test was performed for amphotericin B and tyrosol at the MIC_{50} concentrations determined with the methods described in the previous section. Standardized cell suspensions of all isolates were seeded into respective wells of microtiter plates and incubated for 48 h at 37°C. Following the initial incubation period, the medium was aspirated and nonadherent cells removed by thoroughly washing the formed biofilm three times with PBS. Amphotericin B (1–4 mg/l) or tyrosol (40 and 80 μM) were added at different concentrations to the adherent cells. The plates were then incubated for 24 h at 37°C. The effect of amphotericin B or tyrosol on the preformed biofilm was then estimated using the crystal violet assay. Percentage reduction was calculated using the following equation: [1 − (A595 of the test/A595 of nontreated control)] × 100, where A595 is the absorbance at 595 nm.

Synergistic effect of tyrosol and amphotericin on *Candida* biofilms

The combined effect of both amphotericin B and tyrosol on preformed biofilms was assayed using different combinations of amphotericin B and tyrosol, including 40 μM tyrosol with 1–4 mg/l amphotericin B and 80 μM tyrosol with 1–4 mg/l amphotericin B.

Statistical analysis

Data from multiple experiments were quantified and expressed as mean ± standard error. The relationship between the ability of *Candida* isolates from IUD and non-IUD users to form biofilm was analyzed using the χ^2 test, and P values were determined. Data were plotted using Sigma Plot 11.0 software.

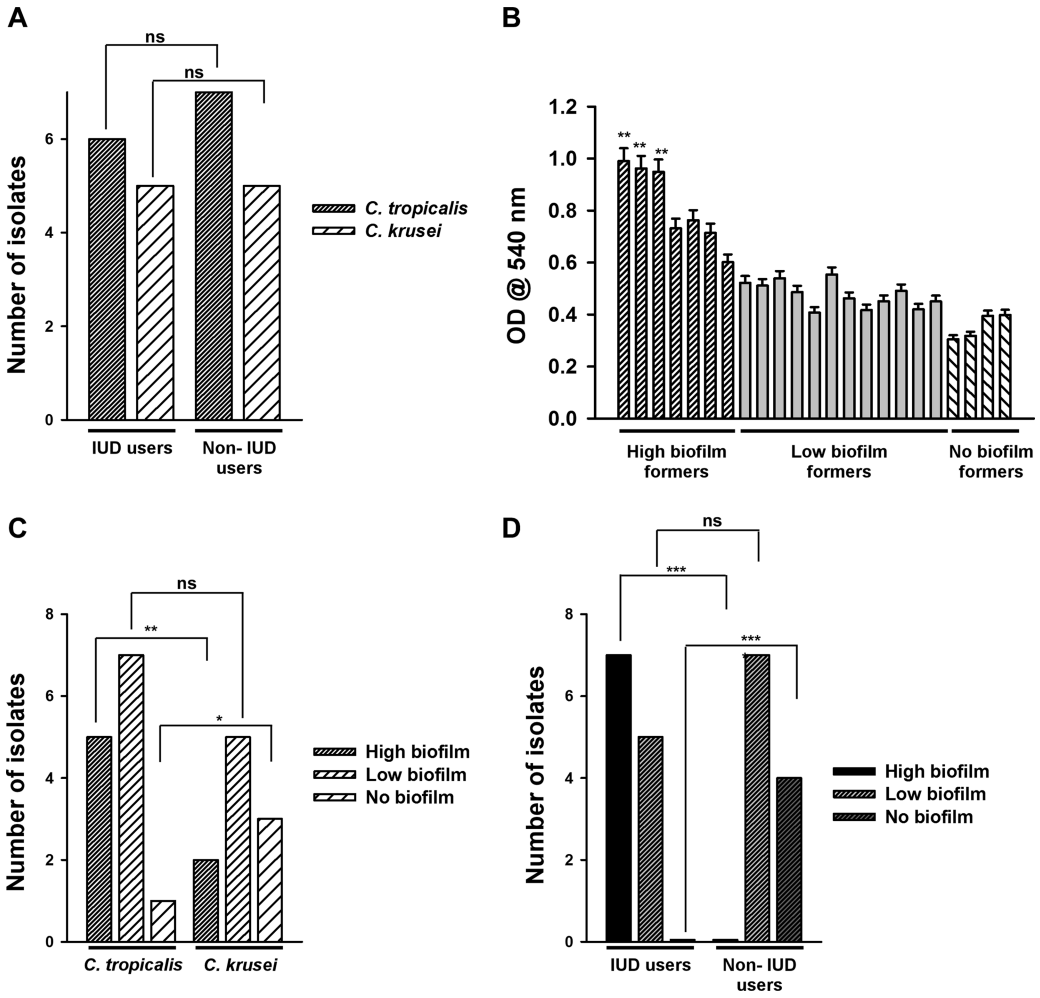

Figure 1. Biofilm-formation ability of the 23 *Candida* isolates. (A) The frequency of isolation of *Candida tropicalis* and *C. krusei* did not vary significantly between intrauterine contraceptive device (IUD) users and non-IUD users ($P = 0.183$). (B) Isolates were characterized as high biofilm formers ($n = 7$), low biofilm formers ($n = 12$), or no biofilm formers ($n = 4$). (C) Biofilm formation by *C. tropicalis* isolates differed significantly when compared with *C. krusei* isolates ($^{*}P < 0.05$; $^{**}P < 0.01$). (D) Biofilm formation was significantly higher in isolates from IUD users compared with non-IUD users (*** P < 0.001). ns, not significant; OD, optical density.

Results

Clinical presentation

Candida infection was found to be significant in IUD users aged 26–30 years (35%) and in non-IUD users aged 21–25 years (31.8%; $P < 0.05$). The culture-positive cases were more likely to have vaginal discharge (8.73%), weight loss (4.85%), itching and irritation while passing urine (6.79%), and abdominal pain (8.73%). Half (50%) of the women with IUDs were found to have vaginal candidiasis.

Isolation and identification of *Candida* isolates

A total of 23 *Candida* isolates were obtained from the study participants, with an isolation frequency of one isolate from one woman. Of these, 11 (47.8%) were from IUD users and 12 (52.2%) from non-IUD users. The frequency of isolation of *Candida* from IUD and non-IUD users did not differ significantly ($P = 0.183$). The isolated *Candida* species were identified on HiChrome agar based on the characteristic colony color and phenotypic growth characteristics. Of the 23 isolates, 10 were identified as *C. krusei* and 13 as *C. tropicalis*. The *C. tropicalis* and *C. krusei* were obtained in increased frequency; interestingly, no *C. albicans* were obtained. The frequency of isolation of *C. tropicalis* and *C. krusei* among IUD and non-IUD users also did not differ significantly ($P = 0.168$; Fig. 1A).

Biofilm formation

Under study conditions, all 23 isolates showed different levels of biofilm-formation ability (Fig. 1B). Seven, 12, and 4 of the isolates showed high, low, and no biofilm-formation abilities, respectively. The results showed *C. tropicalis* strains CA4, CA41, CA63, CA58, and CA69 and *C. krusei* strains CA54 and CA36 to be high biofilm

formers. These isolates showed several layers of *Candida* and contained several microcolonies. *Candida tropicalis* strains CA50, CA52, CA40, CA65, CA42, CA53, and CA32 and *C. krusei* strains CA59, CA56, CA43, CA3, and CA21 were characterized to have low biofilm-formation ability. The surfaces of the coverslips showed no biofilm development for *C. krusei* strains CA26, CA15, and CA18 and *C. tropicalis* strain CA45. Overall results indicate that *C. tropicalis* is more likely to be a good biofilm former compared with *C. krusei* (Fig. 1C). Further, it was observed that the biofilm-formation ability of the isolates from IUD and non-IUD users differed significantly ($P = 0.02$; Fig. 1D).

Examination of biofilm formation on copper surfaces

In order to determine the ability of the isolates to resist copper toxicity and form biofilm on the surface of copper sheets, the biofilms formed on copper sheets were analyzed using SEM. Adhesion on copper was observed in good detail with this technique (Fig. 2A, B). The isolates from IUD users were able to resist copper toxicity and formed biofilms on copper sheets when compared with isolates from non-IUD users.

Examination of biofilm formation on IUDs by atomic force microscopy

As the isolates were resistant to copper, their ability to form biofilm on IUDs was analyzed using AFM. The surface topography of the formed biofilm on IUDs was clearly visible using this technique. The AFM was operated in contact mode in which the cantilever measures the repulsion forces on the surface. From these observations, it was concluded that the dark zones belong to valleys, while light zones represent the crests on the top of the undulated surface. The morphology of the biofilm indicates protuberances hosting *Candida* colonies surrounded by the cumulative extrapolysaccharide products. This was corroborated by observation of the three-dimensional image shown in Figure 3.

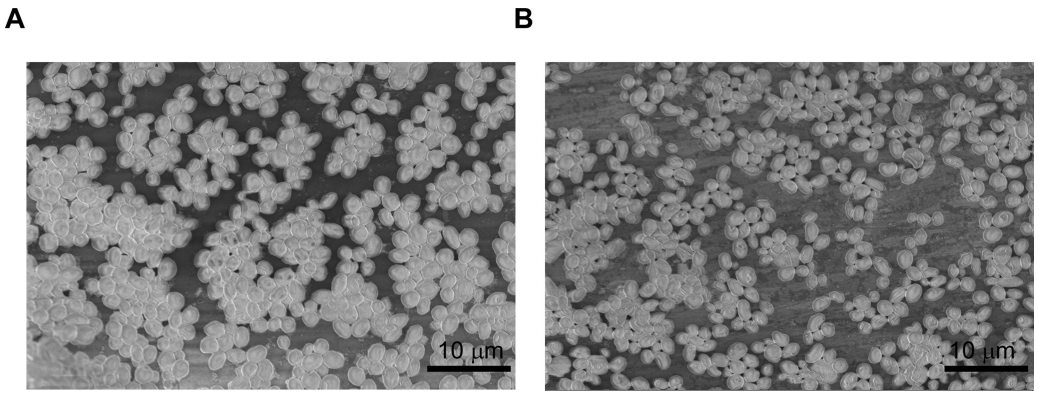

Figure 2. Biofilms formed by isolates on copper sheets. (A) *Candida tropicalis* strain CA4 isolated from intrauterine contraceptive device (IUD) users formed biofilm on copper sheets, visualized by scanning electron microscopy (SEM; 10 μm). (B) *Candida tropicalis* strain CA45 isolated from non-IUD users formed biofilm on copper sheets, visualized by SEM (10 μm).

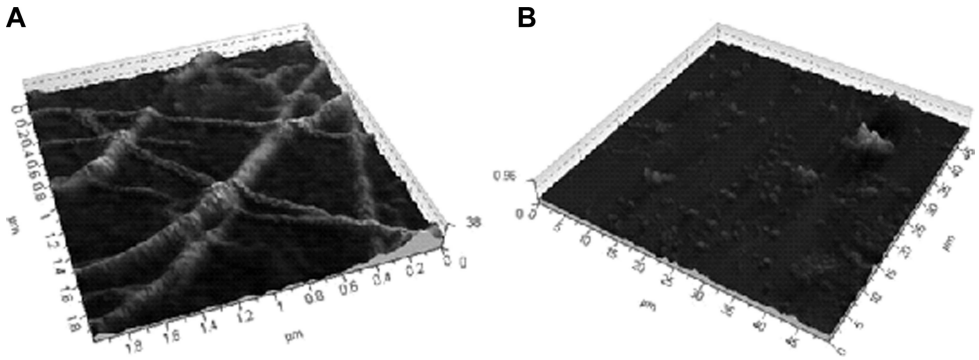

Figure 3. Biofilms formed by isolates on intrauterine contraceptive devices (IUDs) using atomic force microscopy (AFM) analysis. (A) *Candida tropicalis* strain CA4 isolated from IUD users formed biofilm on IUDs, visualized by AFM. (B) *Candida tropicalis* strain CA45 isolated from non-IUD users formed biofilm on IUDs, visualized by AFM.

Figure 4

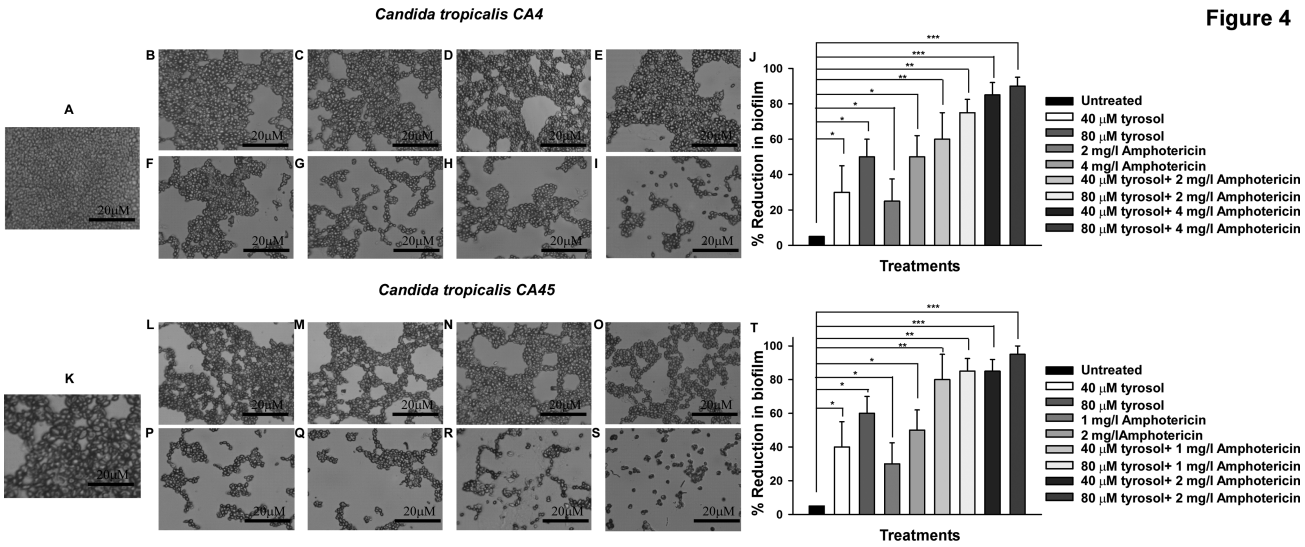

Figure 4. Inhibition of biofilm by amphotericin B and tyrosol. (Part I) Control biofilm without treatment. Treatment of preformed biofilm of the isolate from intrauterine contraceptive device (IUD) users (*Candida tropicalis* CA4) with 40 μM tyrosol (A), 80 μM tyrosol (B), 2 mg/l amphotericin B (C), 4 mg/l amphotericin B (D), 40 μM tyrosol combined with 2 mg/l amphotericin B (E), 40 μM tyrosol combined with 4 mg/l amphotericin B (F), 80 μM tyrosol combined with 2 mg/l amphotericin B (G), and 80 μM tyrosol combined with 4 mg/l amphotericin B (H). (Part II) Bar represents mean reduction of biofilm of various isolates from IUD users; error bar represents standard error (SE). (Part III) Control biofilm without treatment. Treatment of preformed biofilm of the isolate from non-IUD users (*C. tropicalis* CA45) with 40 μM tyrosol (I), 80 μM tyrosol (J), 1 mg/l amphotericin B (K), 2 mg/l amphotericin B (L), 40 μM tyrosol combined with 1 mg/l amphotericin B (M), 40 μM tyrosol combined with 2 mg/l amphotericin B (N), 80 μM tyrosol combined with 1 mg/l amphotericin B (O), and 80 μM tyrosol combined with 2 mg/l amphotericin B (P). (Part IV) Bar represents mean reduction of biofilm of various isolates from non-IUD users; error bar represents SE.

Anticandidal susceptibility testing

Amphotericin B is considered to be the gold standard antibiotic for *Candida* infections [19]. With this viewpoint, the effect of amphotericin on the isolates (grown planktonically) was determined. The MIC_{50} was determined using the microdilution method. The MIC_{50} for *C. tropicalis* strains CA4, CA41, CA63, CA58, and CA69 and *C. krusei* strains CA54 and CA36 (high biofilm formers) was 4 mg/l; for *C. tropicalis* strains CA50, CA52, CA40, CA65, CA42, CA53, and CA32 and *C. krusei* strains CA59, CA56, CA43, CA3, and CA21 (low biofilm formers) the MIC_{50} was 1–2 mg/l; and for *C. krusei* strains CA26, CA15, and CA18 and *C. tropicalis* strain CA45 (no biofilm formers) the MIC_{50} was 0.5 mg/l. The effect of tyrosol on the isolates was also determined. The MIC_{50} was 80 μM for high biofilm formers and 40–80 μM for low biofilm formers.

Biofilm susceptibility assay

Amphotericin B and tyrosol were used to treat adherent cells in order to determine whether different concentrations could adversely affect *Candida* biofilm formation. The microscopic and colorimetric analyses carried out to study the effect of amphotericin showed a 30% reduction in biofilm formation among high biofilm formers ($P < 0.05$) and a 50% reduction ($P < 0.05$) at concentrations of 2 mg/l and 4 mg/l, respectively (Fig. 4B, C, J). Similarly, tyrosol treatment caused a 25% reduction ($P < 0.05$) and a 50% reduction ($P < 0.05$) at a concentrations of 40 μM and 80 μM, respectively (Fig. 4D, E, J). Similarly, among low biofilm formers, amphotericin at concentrations of 1 mg/l and 2 mg/l reduced biofilm by 40% ($P < 0.05$) and 60% ($P < 0.05$), respectively (Fig. 4L, M, T); tyrosol at concentrations of 40 μM and 80 μM reduced biofilm by 30% ($P < 0.05$) and 50% ($P < 0.05$), respectively (Fig. 4N, O, T).

Synergistic effect of both amphotericin B and tyrosol on preformed biofilm

The effect amphotericin B combined with tyrosol on *Candida* biofilms showed significant inhibitory effects. A concentration-dependent synergistic effect was observed. There was reduction of approximately 90% in the formation of biofilm among high biofilm formers at the combined concentration of 80 μM tyrosol and 4 mg/l amphotericin B ($P < 0.001$; Fig. 4 F–I, J). Correspondingly, reduction of approximately 95% was observed among low biofilm formers with a synergistic treatment of

80 μM tyrosol and 2 mg/l amphotericin B ($P < 0.001$; Fig. 4P-S, T).

Discussion

Candidiasis is often associated with indwelling medical devices (eg, dental implants, catheters, heart valves, vascular bypass grafts, ocular lenses, artificial joints, and central nervous system shunts), which can act as substrates for biofilm growth [20,21]. In a multicenter study of 427 consecutive patients with candidemia, the mortality rate for patients with catheter-related candidemia was 41% [22]. Forty percent of patients with microbial colonization of intravenous catheters developed occult fungemia, with consequences ranging from focal disease to severe sepsis and death [22,23]. The tenacity with which *Candida* establishes on indwelling biomedical devices necessitates their removal to affect a cure [24]. In this scenario, IUDs are not exempt from the formation of *Candida* biofilms IUDs are cost effective and are the most popular method of contraception worldwide. It has been hypothesized that the formation of biofilm on IUDs is due to upward migration of the microbes from the vagina to the uterus. The vagina and surrounding regions of the reproductive tract are known to support a large number of bacteria and fungi. Their migration to the upper part of the female urogenital tract often leads to discomfort and infection [25]. Although normal uterine secretions actively manage such migrations, an IUD provides a solid surface for attachment and an ideal niche for the biofilm to form and flourish.

Parewijck et al. [26] showed that IUD users had significantly more yeast cells in the vagina, nearly 20% more than those who were non-IUD users. SEM analysis suggested that these microorganisms colonized the surface of the upper part of the coil and constituted a source of infection for patients with vaginal candidiasis. However, in the present study, there was no significant difference in the frequency of isolation of *Candida* from IUD and non-IUD users. The biofilm-formation ability of the isolates did differ significantly and, moreover, approximately 50% of IUD users reported vaginal candidiasis, supporting the hypothesis of IUD colonization as a source of vaginal candidiasis.

This suggests that IUDs allow adherence of the yeast cells and predispose the patient to develop vaginal candidiasis, as confirmed in another study [27]. Further, Auler et al. [28] recently studied IUDs removed from users and observed biofilms with yeasts using both SEM analysis and culture, suggesting that IUDs act as a solid support for yeast biofilm to form and flourish. High concentrations of yeast cells were observed principally on the IUD tail, possibly indicating the importance of this segment in maintaining the yeast cells colonization; the tail forms a bridge among the external environment, the vagina that is colonized by yeast cells, and the upper genital tract where there is no colonization. This may suggest that the sampling site in the present study represents the real site of infection of yeast cells among IUD users.

IUDs removed from women were shown to harbor *Streptococcus epidermidis, Streptococci* sp., *Corynebacterium* sp., *Micrococcus* sp., and anaerobic *Lactobacilli*. Medical implants are significant risk factors for *C. albicans* infection, and biofilms have been observed on device surfaces after removal from patients [9]. Thus, the present study is novel as non–*C. albicans* species have been isolated and studied for their biofilm formation potential, providing evidence for IUD colonization by non–*C. albicans* species.

SEM analysis showed adherence and viability of yeasts in the presence of copper. This was surprising because the role of copper ions is definitely important for this kind of contraception device, not only for their effects on estrogen receptors and reduction of pregnancy rates [29,30] but also for the protective role of copper ions against microorganisms [31]. The possible reason for colonization of *Candida* spp. may be that the copper released from the device decreases during the second year [32,33], which may, in part, explain why these infections occur predominantly in patients who use IUDs for long periods [34]. Thus, the SEM analysis carried out in the present study showed the ability of yeast isolates to resist copper toxicity and evidenced their increased ability to form biofilm on copper surfaces. Further, the AFM analysis of the formed biofilm on the surface of IUDs substantiated the biofilm-formation ability of the isolates on IUDs.

The increased resistance of *Candida* to amphotericin ($MIC_{50} \leq 1.0$ mg/l as susceptible and those with an $MIC_{50} > 2$ mg/l considered resistant) observed in the study may be due to the emergence of drug-resistant isolates. The misuse of antibiotic therapy worldwide has resulted in the emergence of higher levels of tolerance of target organisms against the available broad-spectrum antibiotics [35]. In the comparison of planktonic counterparts, biofilms were found to be highly resistant to amphotericin B. The mechanisms that protect microorganisms in biofilms from antimicrobial agents are poorly understood. Drug-tolerant or persister cells, usually ≤1% of the overall population, that neither grow nor die in the presence of microbiocidal agents are being considered as a possible reason for drug resistance [36,37]. Persisters can withstand drug concentrations substantially greater than the MIC and represent specialized survivor cells that are phenotypic variants of the wild type rather than mutants. The existence of persisters in *C. albicans* biofilms has been reported recently [38,39]. Hence, these persisters may play a role in the resistance of the candidal biofilm to amphotericin B.

In a recent study, tyrosol was identified as a quorum-sensing molecule that can play an important role in growth and morphogenesis of *C. albicans* [40]. The supernatant recovered from high-density cultures contained tyrosol. Further, in the present study, we found that the biofilm density was drastically altered by high concentrations of tyrosol at early stages of biofilm formation. As the concentration of tyrosol increased, the density of biofilm decreased in a dose-dependent manner. Further, the initial adherence time, that is, prior to amphotericin B–tyrosol addition, was found to be important in terms of the ability of amphotericin B–tyrosol to inhibit biofilm formation. Thus, amphotericin B–tyrosol may possess antibiofilm properties, although such properties are better suited for preventive strategies rather than for treatment.

The possibility of combined drug therapy as an alternate approach to the treatment of systemic mycoses started from the pioneering work of Medoff et al. [41]. Combinations of amphotericin B with 5-fluorocytosine, rifampin, and tetracycline acted synergistically against certain yeast-like fungi including *Candida* spp., *Aspergillus* spp., and *Coccidioides immitis in vitro* [42–45]. In clinical practice, simultaneous application of two antifungals is not yet recommended for the treatment of invasive candidiasis, except for the combination of amphotericin B with flucytosine in endocarditis, based on expert opinion [46]. Yet there may be circumstances where antifungal combination therapy could be of value. Persistent candidemia that originates from a biofilm-associated infection such as endocarditis might justify the use of an antifungal combination therapy to control the infection.

Conclusion

In conclusion, synergism and antagonism are *in vitro* concepts that are difficult to translate into clinical practice. Although there are experimental data on combination therapy, clinical studies, which could support the advantage of combination therapy over antifungal monotherapy in biofilm-associated infections, are needed but difficult to perform. Nevertheless, evidence of synergism of antifungal combination therapy *in vitro* might be the first step in establishing appropriate antifungal therapy. In addition, strategies involving antiseptic bonded biomaterials are now in common use, and future work on such biomaterials is likely to yield improved IUD devices. The results of the present study suggest that a synergistic coating of amphotericin B and tyrosol on an IUD could have broad-spectrum anticandidal activity and be a better prophylactic strategy in cases where IUD is the predisposing factor for VVC.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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