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Biofilm formation on intrauterine devices in patients with recurrent vulvovaginal candidiasis

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A biofilm is a complex community of surface-associated cells enclosed in a polymer matrix. They attach to solid surfaces and their formation can be affected by growth conditions and co-infection with other pathogens. The presence of biofilm may protect the microorganisms from host defenses, as well as significantly reduce their susceptibility to antifungal agents. Pathogenic microbes can form biofilms on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices (IUDs). The present study was carried out to analyze the presence of biofilm on the surface of intrauterine devices in patients with recurrent vulvovaginal candidiasis, and to determine the susceptibility profile of the isolated yeasts to amphotericin B and fluconazole. *Candida albicans* was recovered from the IUDs and it was found to be susceptible to the antifungal agents when tested under planktonic growing conditions. These findings indicate the presence of the biofilm on the surface of the IUD as an important risk factor for recurrent vulvovaginal candidiasis.

Keywords *Candida albicans*, biofilm, intrauterine device

Introduction

Biofilm is a complex structure that can be produced by various microorganisms, including members of the genus *Candida*. Its production varies according to the environment and the available substrate [1,2]. Biofilms attach to solid surfaces and their formation can be affected by growth conditions and co-infection with other microorganisms [3]. The presence of biofilms can serve as a reservoir of microorganisms, and can also lead to resistance to antimicrobial agents [4,5]. Although bacterial biofilms have been studied in detail [6], there have been few studies of medically-related fungal biofilms [7]. The intrauterine devices (IUDs) are the most commonly employed method of preventing fertilization [2,8,9,10]. The present paper describes two cases of patients with signs and symptoms of recurrent vulvovaginal candidiasis (RVVC) who used IUDs as a contraceptive method. After their removal, the IDU was analyzed for the presence of biofilm on their surfaces, and the susceptibility pattern of the yeasts recovered from the devices was investigated.

Materials and methods

Patients

Two patients with signs and symptoms suggestive of RVVC, defined by at least four symptomatic episodes in 1 year [11], were enrolled in the present study after obtaining Institutional Review Board approval. Microbiological studies were conducted at the Hospital of the University of São Paulo, São Paulo, Brazil.

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Case 1

The patient was 29 years old, married,with two children and presented clinical symptoms suggestive of RVVC (presence of vaginal discharge, vulval pruritus, itching and erythema). She was found to be negative for HIV infection and diabetes mellitus. The patient had used the IUD for three years and one month as a contraceptive method and reported that the symptoms had begun soon after the implantation of the device. These symptoms had intensified during the last year, primarily in terms of the pruritus and discharge. The patient mentioned that in several previous gynecological visits she had been treated with fluconazole. During her present gynecologic examination, a sample of the secretion from the patient's vagina was collected with sterile swabs from the lateral walls of the vagina and the fundus of the vaginal sack, with the aid of an unlubricated speculum. The clinical material was freshly harvested for immediate examination in 3 ml sterile saline solution (NaCl 0.85%). The vaginal secretions were prepared on the surfaces of two sterile slides for Gram-stain. Vaginal swabs were directly inoculated onto Petri dishes containing Sabouraud dextrose agar medium (Difco, Detroit, USA) as well as on Petri dishes containing CHROMagar Candida (Paris, France) in order to facilitate the isolation of *Candida* spp. All the cultures were incubated for 10 days at 37°C. The IUD was removed from the patient after gynecologic and laboratory examinations. Removal of the IUD was carried out under antiseptic conditions and was performed without the IUD touching the vaginal wall or the opener instrument to prevent contamination by the vaginal flora. Immediately after the removal of the IUD the patient was treated orally with a single 150 mg dose of fluconazole. Three months after removal of the IUD the patient returned for a second gynecologic examination where she did not present signs or symptoms suggestive of RVVC and laboratory examinations of vaginal secretions were negative for yeasts.

Case 2

The patient was 38 years old, married, and presented clinical symptoms suggestive of RVVC (presence of vaginal discharge with a clumpy, cottage-cheese appearance, vulval pruritus and itching). She too was found to be negative for HIV and diabetes mellitus. The patient reported that the discharge and itching had become worse over the last two years. She had been using an IUD for about five years as a contraceptive method. The patient mentioned that in previous gynecological visits she had been treated with fluconazole. During the gynecologic exam, vaginal secretions were collected with sterile swabs with the aid of an unlubricated speculum. The clinical material was freshly harvested for immediate examination in 3 ml sterile saline solution (NaCl 0.85%). The vaginal secretions were prepared on the surfaces of two sterile slides for Gram-stain. Vaginal swabs were directly inoculated onto Petri dishes containing Sabouraud dextrose agar medium (Difco, Detroit, USA), as well as on Petri dishes containing CHROMagar Candida (Paris, France) in order to facilitate the isolation of *Candida* spp. All the cultures were incubated for 10 days at 37°C. After the clinical and laboratory examinations the IUD was removed under antiseptic conditions and examined by scanning electron microscopy. The removal was performed without touching the IUD to the vaginal wall or the opener instrument to prevent contamination by the vaginal flora. Immediately after the removal of the IUD the patient was treated orally with a single 150 mg dose of fluconazole. Three months after the removal of the IUD, the patient returned for a second gynecologic exam, where she did not present signs or symptoms suggestive of RVVC and laboratory exams of vaginal secretion were negative for yeasts.

Isolation and identification of the yeasts

The yeasts isolated from the secretion samples of the patients were identified on the basis of germ-tube production in fetal calf serum at 37°C for 2 h, production of chamydospores on corn-meal agar (Oxoid) containing Tween 80 (Sigma) according to the Dalmau method, assimilation of carbon sources, as recommended by Kurtzman and Fell, 1998 [12] and the use of the commercial test API 20C AUX kit (bioMérieux). For comparative purposes, *Candida dubliniensis*, isolates were streaked onto the surface of SDA agar (Difco, Detroit, MI, USA) plate and incubated at 42°C as previously described [13]. All isolates that were recovered were suggestive of *Candida albicans* and to confirm this identification, PCR studies were performed as described previously with the oligonucleotide primer specie-specific forward CAL5 (5′ TGT TGC TCT CTG GGG GGC GGC CG 3′) and NL4CAL (5′ AAG ATC ATT ATG CCA ACA TCC TAG GTA AA 3′) reverse primer for confirmed *Candida albicans*. The American Type Culture Collection (ATCC) *Candida albicans* strain 90028 was used as the control. The PCR method used were performed in accord with that described by Mannarelli and Kurtzman [14].

Scanning electron microscopy

The IDUs were washed with sterile water to remove non-adhered yeast cells. The biofilms formed on the IDUs were

fixed with 2.5% (v/v) glutaraldehyde in 0.15 M PBS for 1 h at room temperature. They were then treated with 1% (w/v) osmium tetroxide for 1 h, washed thrice with distilled water, treated with 1% (w/v) uranyl acetate for 1 h and washed again with distilled water. The samples were then dehydrated in ethanol. All samples were dried to the critical point, submitted to gold-coat metallization, and observations were made with the JEOL-JSM 6100 scanning electron microscope (Jeol Ltda, Tokyo, Japan) at the Institute of Biomedical Sciences of the University of São Paulo, Brazil.

Susceptibility testing

The antifungal agents used in this study were amphotericin B (Sigma, St. Louis MO, USA) and fluconazole (Pfizer Central Research, New York, USA). Susceptibility testing was performed as described in document M27-A3 [15]. The stock solutions were prepared in water (for fluconazole) and dimethyl sulphoxide (for amphotericin B). Further dilutions of each antifungal agent were prepared with RPMI 1640 medium 0.2% glucose (Sigma) which had been buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (Sigma), with L-glutamine and phenol red as outlined in document M27-A3 (2008). The drug dilutions (containing double the final concentration) were dispensed into 96-well microdilution plates that were sealed and stored frozen at –70°C until the day of the test. The yeasts were subsequently subcultured on Sabouraud dextrose agar (Difco), for 24 h at 35°C prior to antifungal susceptibility testing. The inoculum was prepared for spectrophotometric analysis, conducted at 530 nm, and the yeast final inoculum concentration was 0.5×10^3 to 2.5×10^3 CFU/ml. The plates were incubated at 35°C for 48 h in a non-CO_2 incubator. The interpretive criteria for the minimum inhibitory concentration (MIC) in the susceptibility test were those published in document M27-A3 (2008).

Results

The first laboratory examination of the vaginal secretion from patient 1 did not reveal the presence of yeast cells, mycelia or motile trichomonads in the wet mount. Microscopic examination of the vaginal Gram-stained smears indicated the presence of yeasts with blastoconidia and pseudomycelia (Fig. 8A). *Candida albicans* was identified by phenotypic techniques and confirmed by PCR methods. The first laboratory exam of the vaginal secretion from patient 2 did not reveal the presence of yeast cells and motile trichomonads. Examination of Gram-stained smears demonstrated the presence of yeasts with blastoconidia

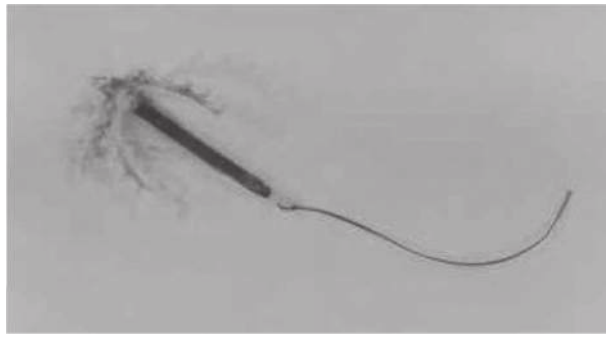

Fig. 1 Intrauterine device removed from patient 1.

(Fig. 8B), which were subsequently identified as *Candida albicans*, confirmed by PCR method. After removal of the IUDs, yeasts could not be found on the second laboratory exams of vaginal secretions of both patients. The *in vitro* susceptibility test results under planktonic conditions of

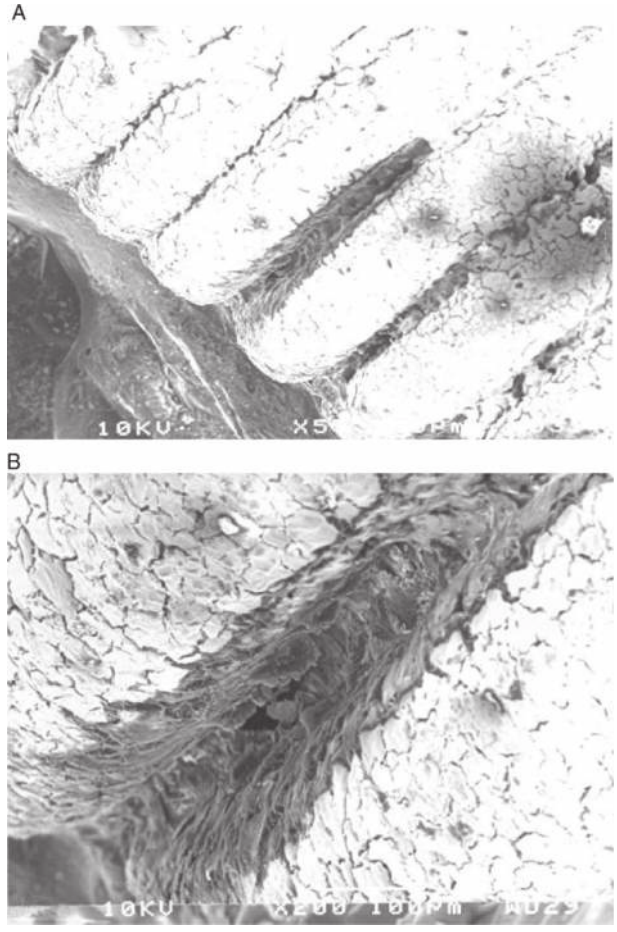

Fig. 2 (A) Scanning electron microscopy of the copper coil from patient 1. (B) Note the biofilm on the surface of the upper coil from patient 1. The depth of the crack revealed the biofilm matrix enclosed with microorganisms.

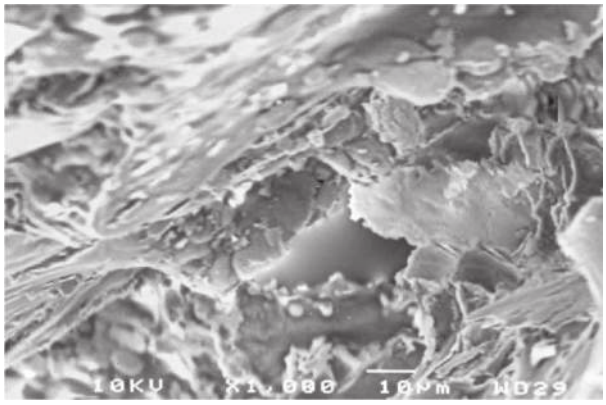

Fig. 3 Extracellular matrix with embedded microorganisms from patient 1.

the two *Candida albicans* strains recovered as part of the first laboratory exams revealed that they were susceptible to the antifungal agents tested. The MICs of the strain isolated from patient 1 were 0.25 µg/ml for amphotericin B and 0.5 µg/ml for fluconazole. The MICs of the isolate from patient 2 were 0.5 µg/ml for amphotericin B and 2.0 µg/ml for fluconazole. Fig. 1 shows the intrauterine device from patient 1 before scanning electron microscopy. The examination revealed a heterogeneous biofilm adherent (Figs. 2A, 2B, 3 and 4) on the surface of the IUD's copper coil. The analysis of the biofilm revealed the presence of a wide variety of morphologic types of bacteria that were embedded in a fibrous matrix (Fig. 3), with various types of cells – polymorphonuclear leukocytes and fibrin. Adherent yeasts were also observed on the surface of the biofilm (Fig. 4). Figs. [5–7] show the scanning electron microscopic results with the IUD from patient 2. Again, a wide variety of morphologic types of cells and microorganisms, embedded in a fibrous matrix present on the surface of the upper coil were noted. Fig. 6 shows a great aggregate of cells and mixed microorganisms on the IUD, while Fig. 7 also shows the yeast present on the surface of the upper coil from patient 2.

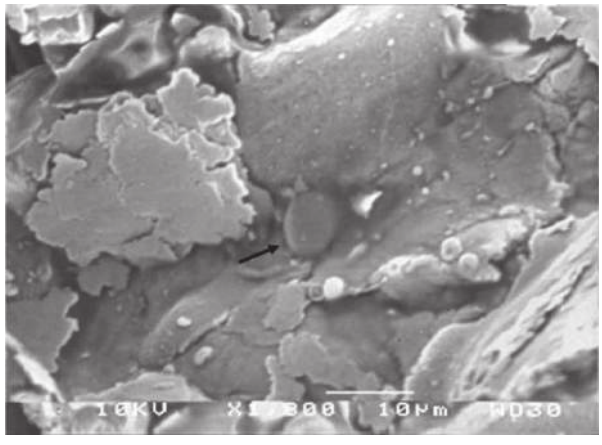

Fig. 4 Scanning electron microscopy showing evidence of the yeaston the surface of the biofilm from patient 1.

Fig. 5 Scanning electron microscopy of the biofilm formed on the intrauterine device from patient 2. Note the thick biofilm with a mixture of microorganisms.

Discussion

Biofilms are aggregates of unicellular microorganisms forming multicellular structures that adhere to surfaces [16]. Cells in biofilms are embedded within a complex matrix that may protect the microorganisms from host defenses, as well as decrease their susceptibility to antifungal agents [1]. Pathogenic bacteria and fungi can form biofilms on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and IUDs, and the presence of biofilm may constitute a source of infection for patients [2,4,17]. The use of the IUD is a highly effective,

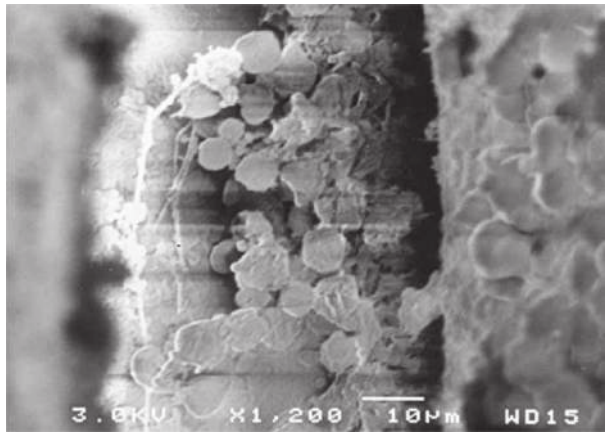

Fig. 6 The heavy biofilm on the surface of the upper coil from patient 2.

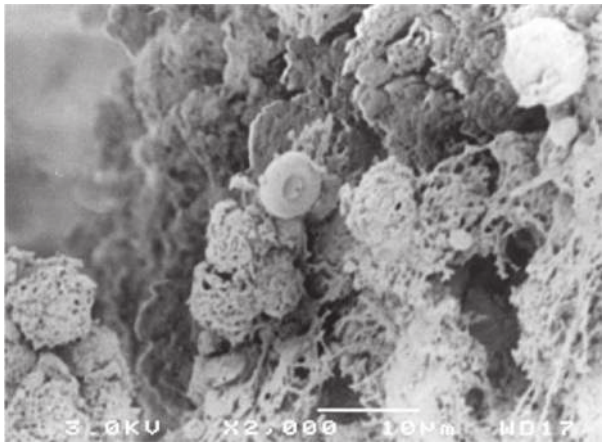

Fig. 7 Scanning electron microscopy showing a large amount of complex biofilm with the yeast present on the surface of the upper coil from patient 2.

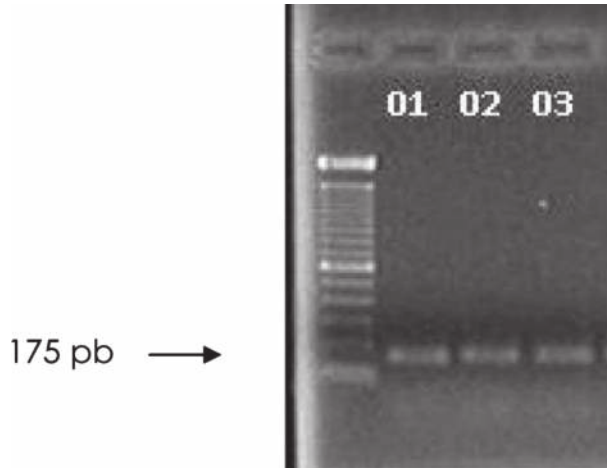

Fig. 9 Lane 1, molecular size marker 100 pb; lane 2, Patient 01, lane 3, Patient 02; lane 4, control 03, *Candida albicans* ATCC 90028.

cost efficient method of preventing pregnancy. It is one of the most popular methods of contraception in the world today [18]. However, if microbial biofilms form on the surface of IUDs, they may promote infection in a susceptible host [10]. Vulvovaginal candidiasis is a fungal infection that is extremely common and remains a significant problem worldwide. Several risk factors have been indicated in regard to its etiology, but many questions remain relative to recurrent infections concerning its pathogenesis due to relapses after ceasing of therapy [19,20]. The present study analyzed the presence of biofilm on the surface of IUDs as a possible risk factor for RVVC, and determined the susceptibility profiles of the yeasts isolated from these patients. The study involved two patients with clinical signs and symptoms of RVVC who used IUDs as a contraceptive method. The laboratory exam of the vaginal secretion of both patients indicated the presence of *Candida albicans* (Fig. 9). The scanning electron microscope revealed the presence of biofilm with a dense multilayered network of cells of different microorganisms embedded within a cellular matrix on both intrauterine devices. These data are in agreement with those published by Pruthi *et al*. (2003), who found on the IUD surface a consortium of microbes organized into biofilms [2]. Other authors who carried out *in vitro* tests have observed that yeast cells can adhere strongly to the parts of the IUD and form biofilms [5]. The data in the present study suggest that the presence of biofilm on the patients' IUDs served as a reservoir of yeasts and contributed to recurrent infection by *Candida albicans*. This fact can be verified by the observation of yeasts in the biofilm through electron scanning microscopic analysis, suggesting the possibility that the majority of these microorganisms had been present on these surfaces for possibly a long time. It should be noted that analysis of the images made by electron scanning microscopy (Figs. 5 and 6) of patient 2 revealed a heavier biofilm on the surface of the upper coil as compared to that of patient 1. Most likely, the longer use of the IUD by patient

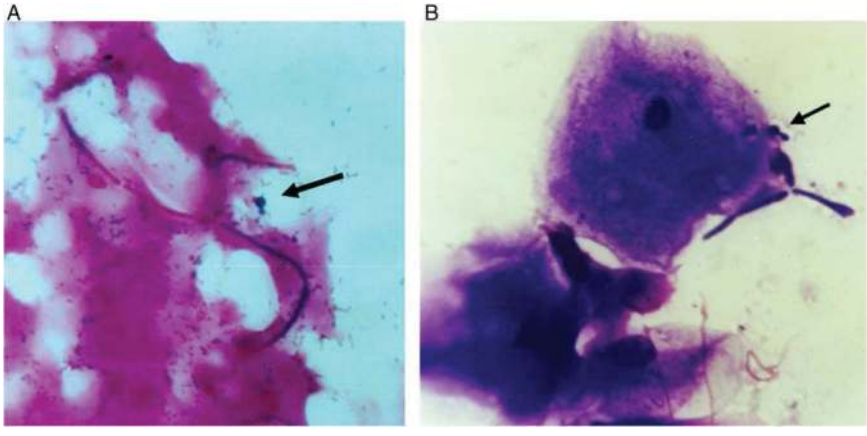

Fig. 8 (A) Vaginal Gram-stained smears showing yeasts with blastoconidia and pseudomycelium from patient 1. (B) Yeasts with the presence of blastoconidia from patient 2.

2 contributed to the formation of a larger mass of biofilm. This effect was observed by Pal *et al*. [10], who analyzed the biofilm formation on IUDs in relation to duration of use. They found that the longer use of IUDs was associated with a greater risk of chronic infection by microorganisms [10]. The adherent yeasts observed in Figs. 4 and 7 suggest that these microorganisms present on the surface of the upper coil may constitute a source of infection for patients with vulvovaginal candidiasis.

In the present study, three months after the removal of the IUD the patients returned for a new gynecological and laboratory exam, which did not reveal clinical signs or symptoms suggestive of RVVC. In addition, the laboratory data at this time were negative for *Candida albicans*, suggesting that the absence of the biofilm on the surface of the IUD contributed to the absence of infection. It is important to underscore that although the treatment with fluconazole aided in the cure, the absence of the source of the microorganisms contained in the biofilm was essential to avoid the relapses of vulvovaginal candidiasis in these patients.

In the present study, analysis of the data revealed that all of the recovered isolates were susceptible to the two antifungals studied when tested under planktonic growing conditions. The presence of the biofilm on the surface of the IUD contributed to protecting the yeasts from the action of the antifungal agent, while also contributing to the microorganism's persistence, leading to recurrent infections by vulvovaginal candidiasis.

In conclusion, biofilms are a complex matrix that can contain microorganisms forming multicellular structures that adhere to solid surfaces and may contribute to the source of infection for patients.

The present study suggests that the presence of the biofilm on the surface of the IUD may constitute a risk factor for persistent vulvovaginal candidiasis.

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