In Vitro Method To Study Antifungal Perfusion in *Candida* Biofilms

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Antimycotic perfusion through *Candida* biofilms was demonstrated by a modification of a simple in vitro diffusion cell bioassay system. Using this model, the perfusion of three commonly used antifungal agents, amphotericin B, fluconazole, and flucytosine, was investigated in biofilms of three different *Candida* species (i.e., *Candida albicans*, *Candida parapsilosis*, and *Candida krusei*) that were developed on microporous filters. Scanning electron microscopy revealed that *C. albicans* formed a contiguous biofilm with tightly packed blastospores and occasional hyphae compared with *C. parapsilosis* and *C. krusei*, which developed confluent biofilms displaying structural heterogeneity and a lesser cell density, after 48 h of incubation on nutrient agar. Minor structural changes were also perceptible on the superficial layers of the biofilm after antifungal perfusion. The transport of antifungals to the distal biofilm-substratum interface was most impeded by *C. albicans* biofilms in comparison to *C. parapsilosis* and *C. krusei*. Fluconazole and flucytosine demonstrated similar levels of perfusion, while amphotericin B was the least penetrant through all three biofilms, although the latter appeared to cause the most structural damage to the superficial cells of the biofilm compared with the other antifungals. These results suggest that the antifungal perfusion through biofilm mode of growth in *Candida* is dependent both on the antimycotic and the *Candida* species in question, and in clinical terms, these phenomena could contribute to the failure of *Candida* biofilm-associated infections. Finally, the in vitro model we have described should serve as a useful system to investigate the complex interactions that appear to operate in vivo within the biofilm-antifungal interphase.

Candida species are opportunistic pathogens which cause superficial and systemic disease in compromised individuals (26). *Candida albicans* is the main causative agent of oropharyngeal candidiasis, while the clinical significance of other non-*C. albicans* species such as *Candida parapsilosis* and *Candida krusei* are being increasingly recognized (16, 24, 28). The first step in the pathogenesis of candidal infection entails the adhesion of the yeast to the host surface or an associated prostheses, such as a denture or intravascular catheter material (18, 27, 34). It is thought that the presence of an indwelling denture or a catheter modifies the normal planktonic mode of growth of the yeasts in a yet unknown manner to a sessile biofilm mode of growth, leading to a structured community of cells encased in an extracellular polymer matrix. Once the biofilm mode of growth is established on prostheses such as heart valves and surgical implants (17, 19), the sessile yeasts begin to display unique characteristics, including resistance to antifungal agents (6). This in turn leads to failure of antifungal therapy and chronic infection, remediated by surgery and/or removal of these devices (33). Factors thought to contribute to antimycotic resistance in *Candida* biofilms include the growth rate of the yeast (2) and the low diffusion gradient of the antimicrobial through the extracellular polymer matrix of the biofilm (2, 3).

Although there are many reports on the phenomenon of incomplete penetration of antimicrobial agents through bacterial biofilms (1, 31, 32, 35), none so far have investigated this

particular feature in *Candida* biofilms. Therefore, the main objective of the present study was to evaluate qualitatively the antifungal perfusion in *Candida* biofilms with a modified, novel assay system. For this purpose, we used a representative isolate of each of *C. albicans*, *C. parapsilosis*, and *C. krusei* and three common antifungals: amphotericin B, fluconazole, and flucytosine. In addition, ultrastructural features were evaluated by scanning electron microscopy (SEM) to visualize and compare the cellular damage to the superficial layers of the *Candida* biofilm before and after antifungal perfusion.

MATERIALS AND METHODS

***Candida* strains.** Three *Candida* strains used in this study were *C. albicans* HK1Sa, from a human immunodeficiency virus-infected patient in Hong Kong, and *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, from the American Type Culture Collection. The identity of all three strains were reconfirmed by using the API20C AUX system, germ tube formation, and the CHROM agar technique prior to the experiments. All three *Candida* strains were maintained on Sabouraud's dextrose agar slopes at −4°C.

To prepare the yeast inoculum for the development of biofilms, a loopful of the candidal strain under investigation was transferred into 10 ml of liquid yeast nitrogen base (YNB; Difco) supplemented with 50 mM galactose and incubated at 37°C overnight on a rotary shaker at 75 rpm. Cells were harvested and washed with phosphate-buffered saline (PBS; pH 7.2), and a cell suspension of approximately 10^7 CFU/ml was prepared by using a spectrophotometer (at 520 nm). Two types of agar media were used. First, Sabouraud's dextrose agar supplemented with 500 mM galactose was used for the development of biofilms, and second, Sabouraud's dextrose agar supplemented with both 500 mM galactose and the specific antimycotic agent was used for the antifungal penetration studies (see below).

Antifungals. Three antifungals commonly used to treat oropharyngeal and systemic candidiasis were selected for the study, viz., amphotericin B (Sigma), fluconazole (Pfizer), and flucytosine (Sigma). Three concentrations of the drugs, i.e., 150, 300, and 600 µg/ml were prepared for each antifungal. Stock solutions of the drugs in dimethyl sulfoxide (amphotericin B), dimethyl formamide (fluconazole), and sterile distilled water (flucytosine) were prepared prior to use and
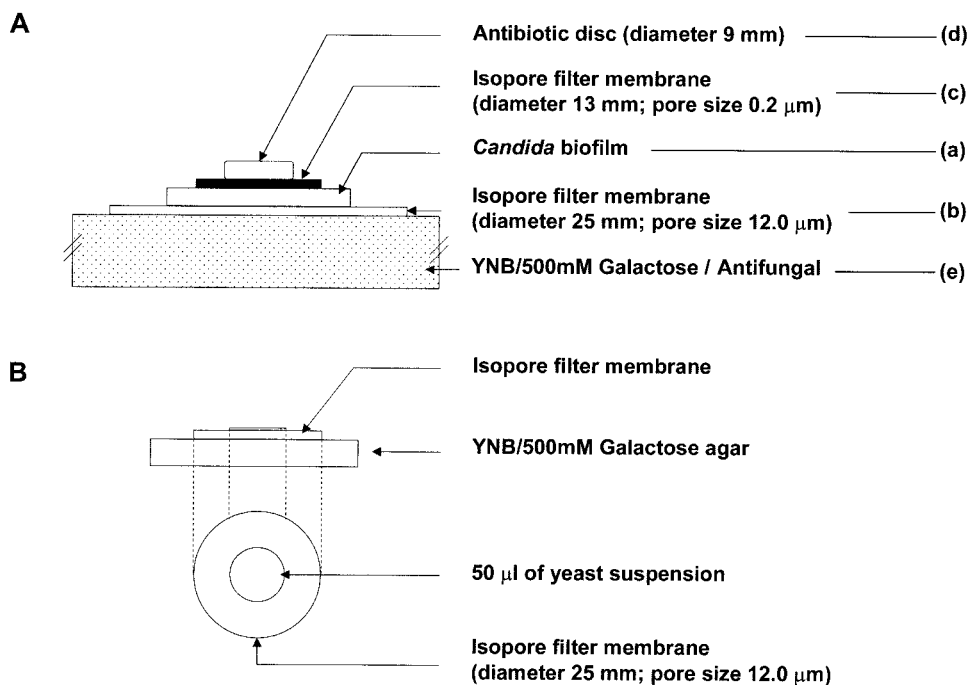
FIG. 1. (A) Schematic representation of the experimental system used to monitor antifungal penetration through *Candida* biofilms. The biofilm (a) was developed on a 25-mm-diameter microporous polycarbonate membrane (b) resting on the agar medium. A 13-mm-diameter microporous polycarbonate membrane (c) was placed on top of the biofilm, and then a moistened antibiotic disk (d) was placed on top of the polycarbonate membrane. The entire unit (components a through d) was then transferred to antifungal-laced agar (e). (B) Transverse view of the Isopore filter membrane placed on YNB–500 mM galactose agar and a surface view of the Isopore membrane inoculated with the yeast suspension (method adapted from Anderl et al. [1]).

added to a YNB–500 mM galactose agar solution at a temperature of 40°C for the preparation of the nutrient agar plates, which were then stored at 4°C and used within 1 day.

Development of *Candida* biofilms for antifungal penetration studies. *Candida* biofilms were developed on Isopore membrane filters (diameter, 25 mm; pore size, 12 μm; Millipore) by using a modification of a method described by Anderl et al. (1). A schematic presentation of this method is shown in Fig. 1. At first the filter membranes were sterilized by exposure to UV light (15 min per side), and each membrane was aseptically placed on a Sabouraud's dextrose agar (500 mM galactose) plate. Afterwards, 50 μl of an overnight *Candida* inoculum (10⁷ CFU/ml) was carefully deposited on the membrane with a pipette. The nutrient agar plates with the inoculum were incubated at 37°C for 1 h (to dry the deposit), and then the plate was inverted and reincubated at 37°C for a period of 48 h. During this incubation period, each filter with the growing biofilm was manually lifted and repositioned on a fresh location on the plate every 10 to 12 h.

After 48 h, the biofilms on the membrane filters were placed on an antibiotic-incorporated agar medium prepared as described above with the biofilm facing outwards. Black microporous membrane filters (diameter, 13 mm; pore size, 0.2 μm; Millipore) were then placed on top of 48-h-old *Candida* biofilms. A blank antibiotic (AB) disk (diameter, 9 mm; Difco) moistened with 10 μl of PBS (pH 7.2) was then placed on top of the 13-mm-diameter membrane (wetting the disk obviates passive capillary perfusion of the antifungal through the biofilm). The whole assembly was then incubated at 37°C for 4 h (Fig. 1).

A semiquantitative evaluation of the antifungals that diffused into an AB disk through the biofilm was performed as follows, with *C. parapsilosis* as an indicator organism. The disk was removed after the specified incubation time and placed on a fresh RPMI agar plate spread plated with 100 μl of (planktonic) *C. parapsilosis*. For this assay, *C. parapsilosis* was grown on Sabouraud's dextrose agar at 37°C, and the growth was diluted with PBS to an optical density of 0.05 (at 520 nm) prior to plating. The control used for the experiment was an AB disk placed on the two-membrane system devoid of a biofilm. Then the RPMI plates were incubated at 37°C for 24 h, and the diameters of the growth inhibition zones (with and without biofilms) were measured by a computerized image analysis system (Quantimet 500 Qwin; Leica, Cambridge, United Kingdom). The assay was conducted in duplicate on three separate occasions for each *Candida* strain tested.

Standard curves for antifungal concentration versus zone of inhibition. Standard curves of the drug concentration versus the radius of growth inhibition of the indicator organism for amphotericin B, fluconazole, and flucytosine were constructed by using the methodology described above.
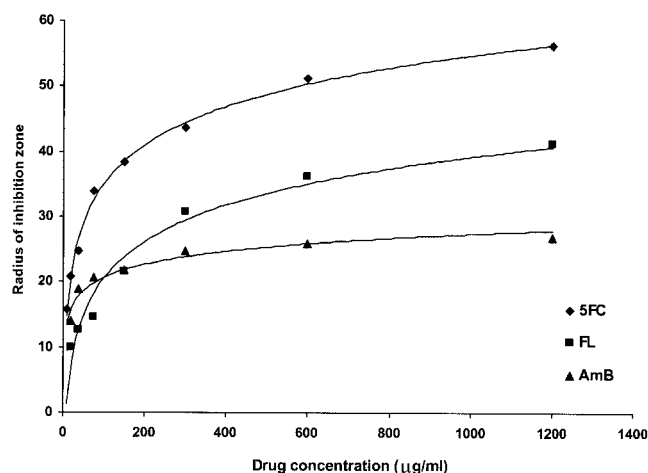

FIG. 2. Standard curves for the three antifungal agents, showing the relationship between drug concentration and the radius of growth inhibition of a lawn of *C. parapsilosis* on RPMI agar. 5FC, flucytosine; FL, fluconazole; AmB, amphotericin B.

TABLE 1. Percent drug penetration of the three antifungals through the three different *Candida* biofilms compared with biofilm-free controls[a]

Candida species	Drug	% Penetration (SD) at concn (µg/ml):			P value[b]
		150	300	600	
C. albicans	Amphotericin B	0.00 (0.00)	0.35 (0.05)	2.02 (0.72)	0.027
	Fluconazole	55.98 (4.57)	60.80 (6.12)	65.36 (4.42)	0.275
	Flucytosine	51.62 (6.94)	59.14 (7.91)	67.86 (6.66)	0.045
C. parapsilosis	Amphotericin B	0.00 (0.00)	1.28 (0.27)	1.68 (0.45)	0.027
	Fluconazole	63.81 (7.40)	74.64 (2.45)	86.87 (3.86)	0.037
	Fluocytosine	67.53 (12.50)	79.27 (12.04)	87.99 (5.59)	0.037
C. krusei	Amphotericin B	0.00 (0.00)	1.14 (0.32)	2.02 (0.72)	0.027
	Fluconazole	95.99 (12.27)	92.31 (3.08)	91.92 (3.33)	0.346
	Flucytosine	87.43 (12.89)	89.92 (12.63)	89.30 (7.59)	0.346

[a] Calculations were made, using the radii of growth inhibition zones, of lawns of *C. parapsilosis* grown of RPMI agar.
[b] Kruskel-Wallis H test.
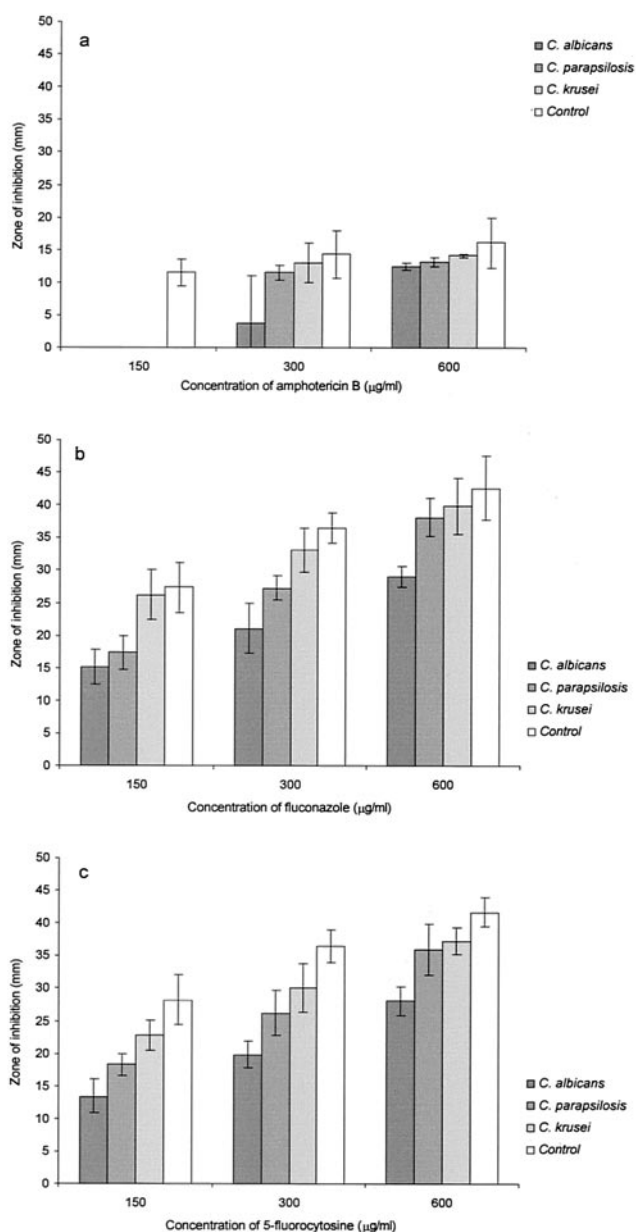
SEM. For SEM, biofilms were developed on microporous filters for 4, 9, 19, and 24 h, as described earlier. The biofilms were then rinsed in (4% [vol/vol] formaldehyde, 1% [vol/vol] phosphate buffer) and placed in 1% osmium tetroxide for 1 h. Samples were subsequently washed in distilled water, dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, and 100% for 20 min), and air dried in a desiccator prior to sputter coating with gold. Afterwards, the surface topographies of biofilms were visualized with a scanning electron microscope (Philips XL30CP) in high-vacuum mode at 10 kV, and the images were processed for display with Photoshop software (Adobe Systems, Inc., Mountain View, Calif.).

Statistics. The Kruskal-Wallis test was used to determine (i) significant differences in diffusion of the three concentrations of each of the drugs with each of the three *Candida* species and (ii) significant differences between a single *Candida* species and a similar concentration of the three respective drugs. The Mann-Whitney U test was used to compare significant differences between the control and each single drug concentration for each of the *Candida* species.

RESULTS

Methodology. All three *Candida* species evaluated developed satisfactory biofilms on membrane filters, and the whole assembly could be easily detached from the substrate surface for the diffusion studies. The use of the AB disk with the antifungal and the antifungal sensitivity assay with *C. parapsilosis* as the indicator organism yielded reproducible results on repeat experiments conducted on separate occasions, indicating satisfactory sensitivity of the assay system.

Standard curves for antifungal concentration versus zone of inhibition. Standard curves of the drug concentration versus the radius of growth inhibition of the indicator organism for the three antifungals were constructed as shown in Fig. 2. However, these standard curves were curvilinear and hyperbolic, indicating that the radius of growth inhibition could be used to derive the perfusion of only the very low drug concentrations. For instance, amphotericin B and flucytosine showed a concentration-dependent difference in the range of 10 to 100 µg/ml, while fluconazole exhibited a slightly higher range of 10 to 220 µg/ml. Therefore, the percent inhibition of drug penetration for each experiment was expressed in a semiquantita-

FIG. 3. Penetration of various concentrations (i.e., 150, 300, and 600 µg/ml) of the three antifungals, amphotericin B (a), fluconazole (b), and flucytosine (c), through 48-h-old *C. albicans*, *C. parapsilosis*, and *C. krusei* biofilms shown in terms of the radii of growth inhibition zones on a lawn of *C. parapsilosis* on RPMI agar.
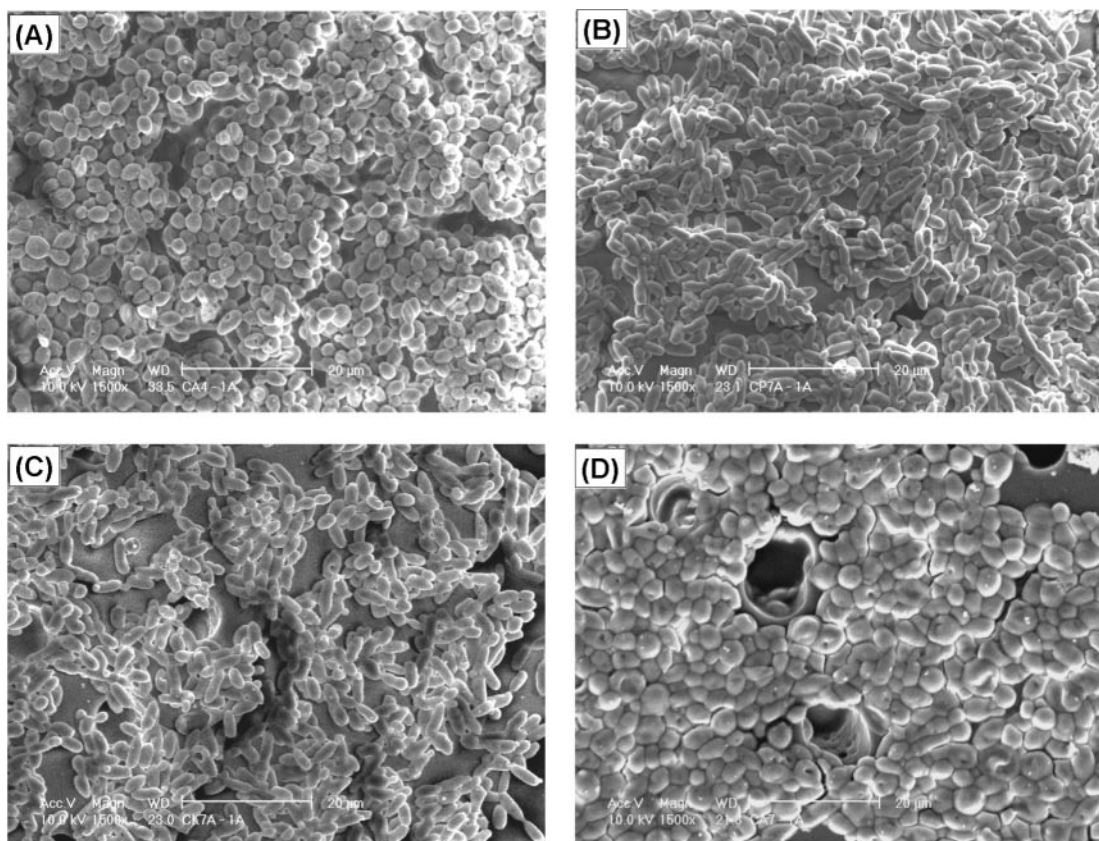
FIG. 4. SEM images of the *Candida* biofilms on polycarbonate microporous filters (diameter, 25 mm; pore size, 12 μm; Millipore). (A) *C. albicans* at 4 h; (B) *C. parapsilosis* at 7 h; (C) *C. krusei* at 7 h; (D) *C. albicans* at 7 h.

tive manner by using data obtained with a corresponding control AB disk without a biofilm (Table 1).

Antifungal perfusion. The variations in antifungal perfusion in the three different *Candida* biofilms for the three antimycotics studied were quantified in terms of the zone of inhibition of an indicator organism (*C. parapsilosis*) on RPMI agar. Statistical analysis revealed significant differences in drug penetration through the three different *Candida* biofilms for each of the drug concentrations tested ($P < 0.05$) (Fig. 3).

Amphotericin B. When the permeation of amphotericin B through the three different *Candida* biofilms was compared, a statistically significant reduction in drug diffusion was noted for all three *Candida* species for the 150-μg/ml concentration of the drug ($P < 0.037$). However, 300-μg/ml amphotericin B was inhibited significantly only by *C. albicans* ($P < 0.046$), while at a higher concentration of this drug, its penetration was significantly inhibited by *C. parapsilosis* ($P < 0.05$) and *C. albicans* ($P < 0.046$) but not by *C. krusei* ($P = 0.346$) (Fig. 3a).

Fluconazole. The profile of fluconazole penetration through the biofilms was noted to be different than that of either amphotericin B or flucytosine. Thus, fluconazole permeation through *C. albicans* biofilms was significantly lower for all of the tested drug concentrations ($P < 0.05$), while *C. parapsilosis* exhibited a significant difference only for 150 and 300 μg/ml ($P < 0.05$) and *C. krusei* exhibited a significant difference only for 150 μg/ml ($P > 0.05$). This implied that the *C. albicans* biofilm was the most resistant to fluconazole perfusion. However, with

increasing concentrations of fluconazole, a corresponding significant increase in drug perfusion ($P < 0.05$) was seen with all three *Candida* biofilms (Fig. 3b).

Flucytosine. For *C. albicans*, flucytosine did not demonstrate a significant difference in drug perfusion with either a 150-, 300-, or 600-μg/ml concentration compared with the control biofilm ($P = 0.05$). A similar trend in drug perfusion was observed for both *C. parapsilosis* and *C. krusei* biofilms. However, as observed for fluconazole, all three *Candida* biofilms demonstrated a significant increase in drug perfusion ($P < 0.05$) with increasing flucytosine concentrations (Fig. 3c).

Ultrastructural features. The ultrastructure of biofilm matrix observed through SEM varied depending on the *Candida* species investigated. Thus, *C. albicans* produced a more profuse biofilm relative to the two less commonly pathogenic *C. parapsilosis* and *C. krusei*. The latter two species first developed a basal blastospore cell layer sparsely dispersed on the filter surface devoid of either pseudohyphae or hyphae, whereas *C. albicans* essentially developed into a more contiguous biofilm intimately packed with blastospores and occasional pseudohyphae after 4 h of growth (Fig. 4A). Also, during the early phase of biofilm growth, *C. parapsilosis* cells adhered in relatively large numbers compared to *C. krusei*. These cells then gradually grew and developed a denser structure (at 7 h) mainly composed of noncontiguous small blastospore aggregates and channels in between (Fig. 4B and C). Both *C. parapsilosis* and *C. krusei* biofilms at this stage were less densely packed than *C.*
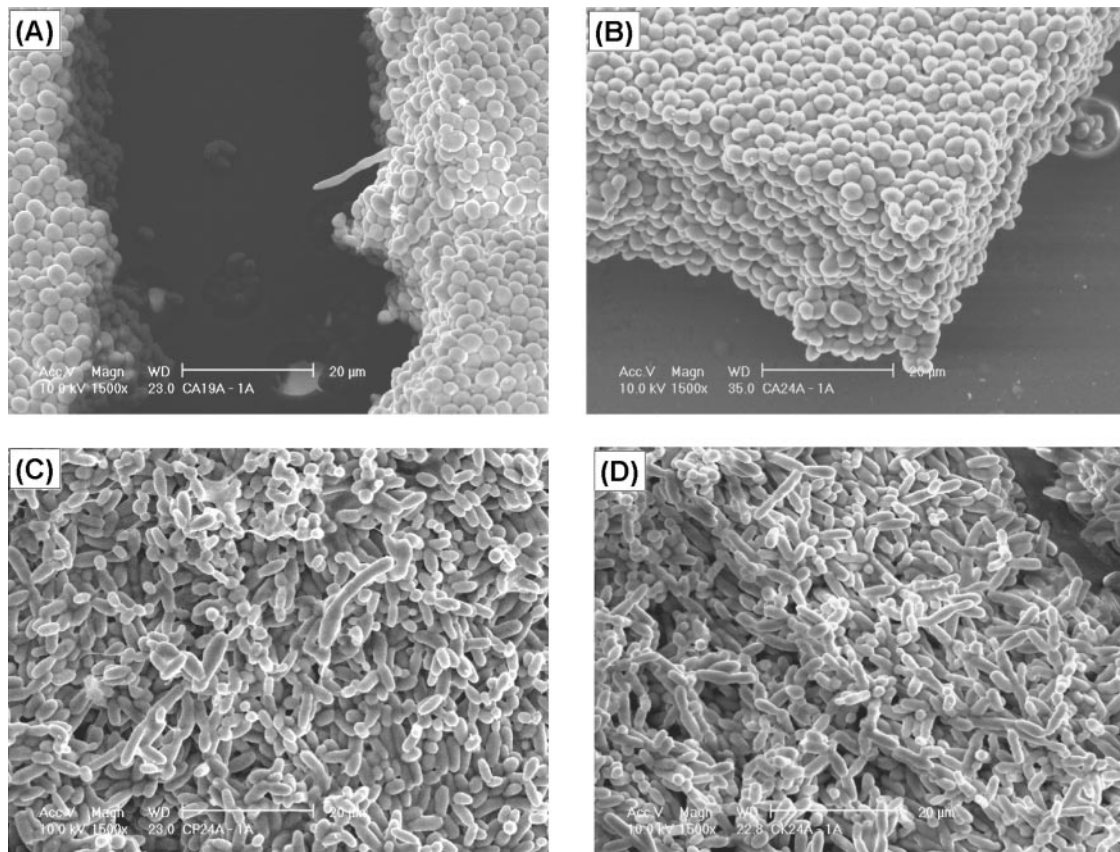
FIG. 5. SEM images showing the biofilm architecture of different *Candida* biofilms on polycarbonate microporous filters (diameter, 25 mm; pore size, 12 μm; Millipore). (A) *C. albicans* at 19 h; (B) *C. albicans* at 24 h; (C) *C. parapsilosis* at 24 h; (D) *C. krusei* at 24 h. Note the lesser cell density of the latter two species than that of *C. albicans*.

albicans (Fig. 4D) and were devoid of extracellular polymeric material.

After 19 h of growth, *C. albicans* exhibited a multilayer biofilm structure with a few hyphae penetrating the matrix (Fig. 5A). However, even after 24 h, extracellular polymeric material could not be visualized in *C. albicans* biofilms, which at this stage was 12 to 15 cell layers thick (Fig. 5B). *C. parapsilosis* and *C. krusei* also developed confluent biofilms after approximately 24 h. Characteristically, the latter two species displayed structural heterogeneity with a lesser cell density (Fig. 5C and D) and channels traversing the cell mass than *C. albicans*.

Visualization of the ultrastructure in general revealed that the most damage to the biofilm constituents was caused by the highest concentrations of the three antifungals. However, amphotericin B caused the most extensive damage, especially to the most superficial cell layers of all three *Candida* biofilms, compared to fluconazole and flucytosine (Fig. 6, 7, and 8). Amphotericin B-treated sessile *C. albicans* cells demonstrated a range of changes, such as shrinkage of cells, ballooning blastospores, ruptured cell walls, and shrunken, wrinkled, or fused *Candida* blastospores. This suggests that despite the relative minimal diffusion, amphotericin B, compared with other drugs, may be exerting a fairly severe metabolic interference in *Candida* biofilms. Amphotericin B-perfused *C. parapsilosis* cells also demonstrated a high degree of destruction with ruptured

and shrunken cells, while *C. krusei* blastospores exhibited the least damage.

The effect of fluconazole perfusion was more subtle. Effects of this drug were barely noticeable with *C. albicans* biofilms, whereas a small number of distorted cells were seen in the *C. parapsilosis* and *C. krusei* biofilms, especially on the superficial layers.

DISCUSSION

The development and characterization of *Candida* biofilms on bioprosthetic surfaces that are components of indwelling devices are well documented (5, 13, 14, 23). These studies have shown that *Candida* biofilm development is closely associated with the generation of an extracellular matrix and that mature biofilms show a highly heterogeneous structure and grow variably depending on the topography of the substrate. It has also been shown that *C. albicans* forms larger and more complex biofilms on silicone elastomer catheter material than *C. parapsilosis*, *Candida pseudotropicalis* and *Candida glabrata* and that differences occur between invasive and noninvasive *Candida* isolates from infective foci (12, 14).

It is now recognized that antibiotic resistance is a general trait associated with biofilm organisms, including bacteria (21, 22) and fungi (15, 23). Several theories have been proposed to explain this phenomenon, including the growth rate differen-
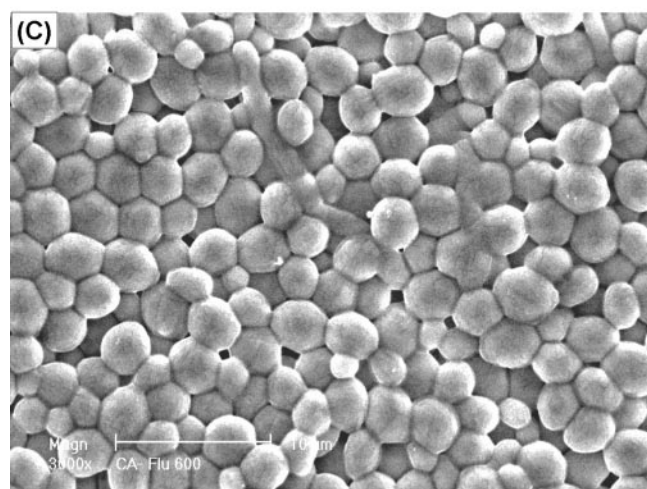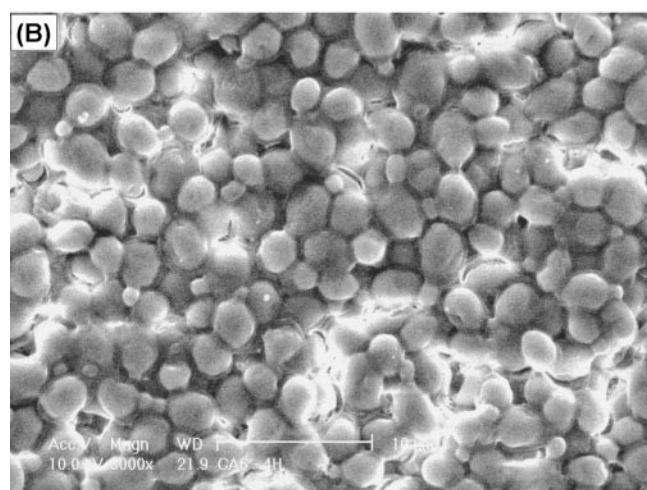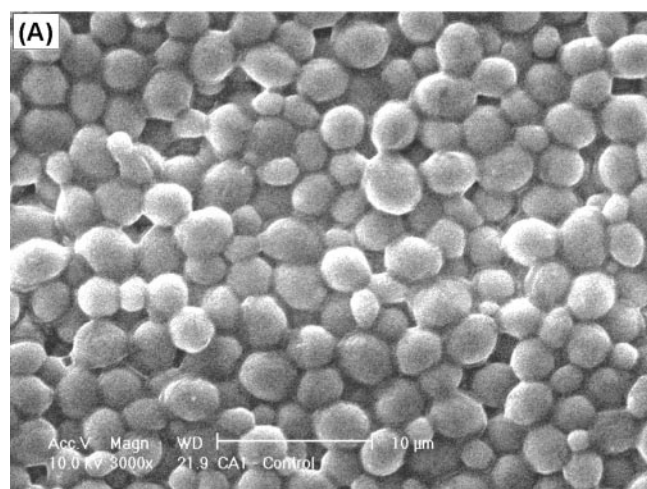
FIG. 6. SEM images of 48-h *C. albicans* HK1Sa. (A) Control *C. albicans* HK1Sa; (B) *C. albicans* HK1Sa biofilm exposed to 600-µg/ml amphotericin B for 4 h; (C) *C. albicans* HK1Sa biofilm exposed to 600-µg/ml fluconazole for 4 h. Note the wrinkled, shrunk, ruptured, and ballooning effect of the drug on yeast cells.
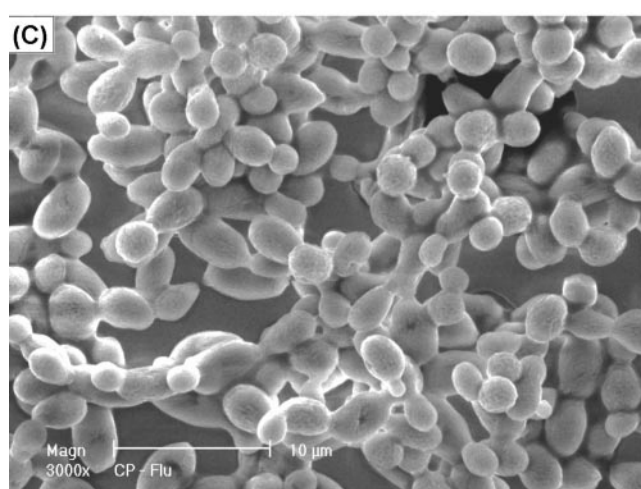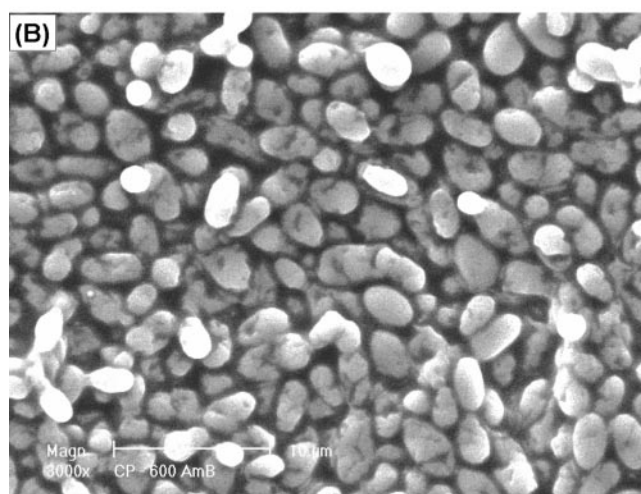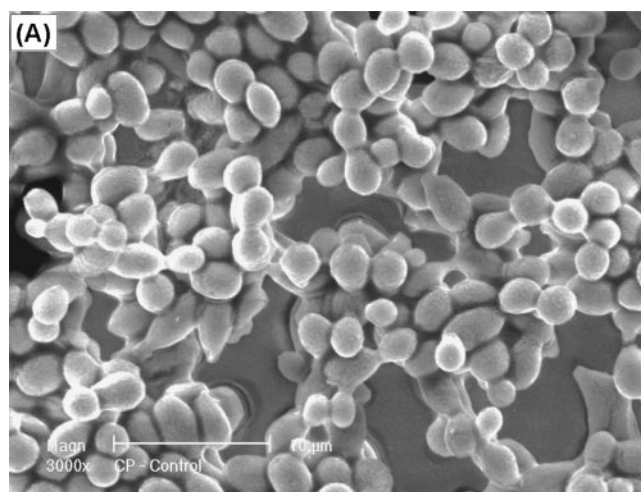
FIG. 7. SEM images of 48-h *C. parapsilosis* ATCC 22019. (A) Control; (B) *C. parapsilosis* ATCC 22019 biofilm exposed to 600-µg/ml amphotericin B for 4 h; (C) *C. parapsilosis* ATCC 22019 biofilm exposed to 600-µg/ml fluconazole for 4 h. Note the ruptured and shrunken effect of the drug on yeast cells.
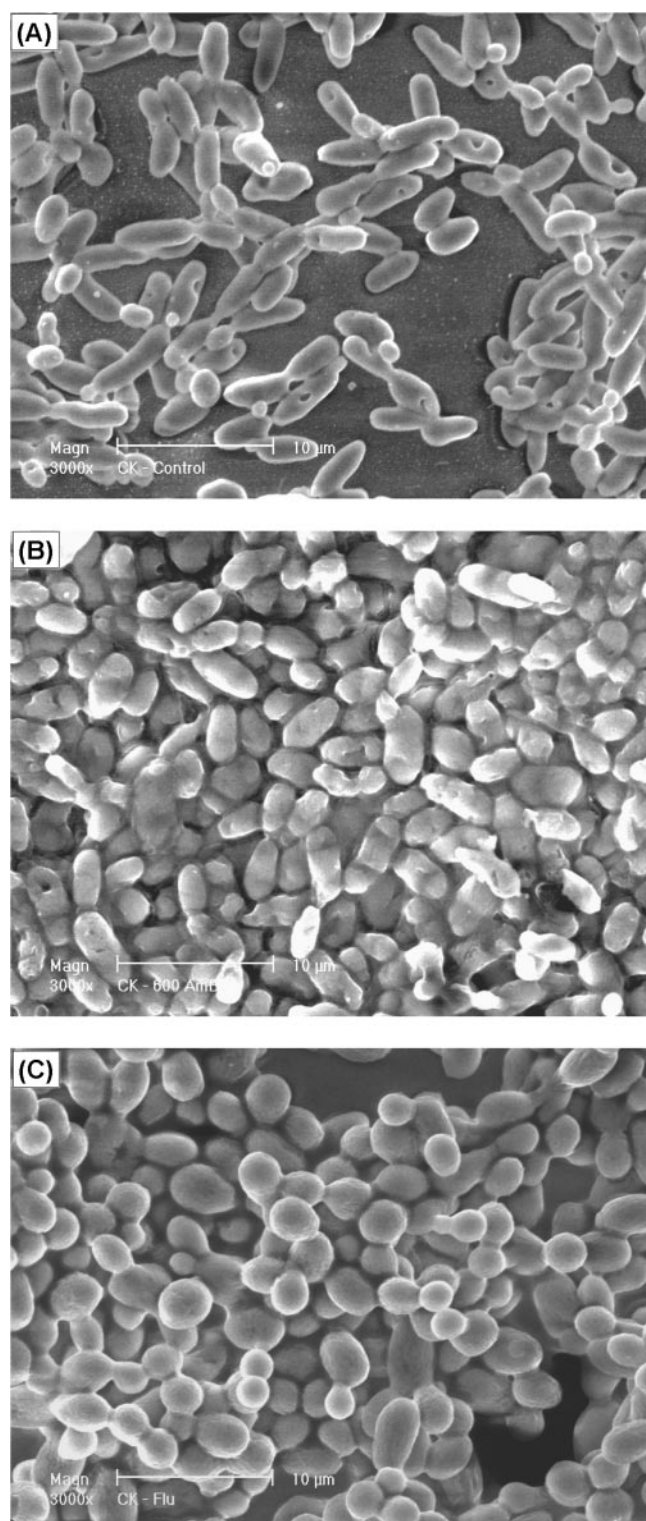
FIG. 8. SEM images of 48-h *C. krusei* ATCC 6258. (A) Control *C. krusei* ATCC 6258; (B) *C. krusei* ATCC 6258 biofilm exposed to 600-µg/ml amphotericin B for 4 h; (C) *C. krusei* ATCC 6258 biofilm exposed to 600-µg/ml fluconazole for 4 h.

tials (9, 10), a recalcitrant phenotypic state (4), the production of antibiotic-degrading enzymes (11), and the extracellular polymeric material that may act as an adsorbent or a reactant with the antimicrobial (30). Most of the foregoing studies, up to now, have been conducted with bacterial biofilms, and sparse information is available on the diffusion parameters of antimycotics in *Candida* biofilms. For instance, Baillie and Douglas observed that 20 times the MIC of commonly used antifungals such as amphotericin B, fluconazole, or flucytosine is required to cause a significant reduction in cell numbers (2). Further Chandra et al. (6) reported that *C. albicans* required low MICs of polyenes and fluconazole during the early biofilm development phase. However, during biofilm maturation, they became highly resistant to these drugs. These studies are hampered by the lack of an appropriately standardized model system to evaluate drug diffusion in candidal biofilms, and the present model was an attempt to address this issue. The advantages of such an in vitro membrane-supported biofilm system are (i) flexibility to investigate antifungal resistance by using simultaneous and parallel biofilm samples, (ii) the accessibility to both sides of the biofilm, after its removal from the membrane surface, and (iii) the possibility of using these biofilms as primitive models of pseudomembranous candidal infections. In our hands, we found this system to be relatively simple, inexpensive, and reliable for measurement of the qualitative differences in antifungal perfusion through *Candida* biofilms.

Our SEM studies revealed that the biofilms on micropore filters were distinct from those on polymethyl methacrylate strips or silicone elastomer disks, as reported by previous workers (5, 14). For instance, we observed large numbers of *C. albicans* microcolonies on the filter surface after only 4 h of incubation, unlike the smaller numbers that appeared during a similar incubation period with acrylic strips (5). There were only extremely sparse hyphal forms of *C. albicans* in mature filter surface biofilms, in comparison to profuse filamentation seen on polymethyl methacrylate strips (5), silicone elastomers (14), or polyvinyl chloride surfaces (13). This anomaly may be due to strain variation in germ tube and hyphal development (8, 25), and further studies with multiple *Candida* strains with the present system are required to confirm our findings.

Upon prolonged incubation, *C. albicans* biofilms developed into a dense mass stacked in a palisadic manner devoid of intracellular spaces. It is tempting to speculate that this exuberant growth mode may easily retard antifungal penetration into the basal layers of the biofilms, thus protecting them from the drug action. We also noted that *C. albicans* biofilms developed barely discernible matrix material under static incubation on nutrient agar plates, an observation similar to that of Hawser et al. (13).

In addition to *C. albicans*, we also used *C. parapsilosis* and *C. krusei* strains for the present pilot studies. The latter, in particular, exhibits intrinsic resistance to azoles, and to our knowledge, no one has evaluated its biofilms. Interestingly, the *C. parapsilosis* biofilms that were developed resembled their counterparts on polymethyl methacrylate disks cultured in YNB medium, where the yeast form was predominant (5). Compared with *C. albicans*, sparse numbers of *C. parapsilosis* and *C. krusei* microcolonies were seen on the filter surface after 4 h, and these slowly developed into less-dense biofilms, mostly of blastospores, during the ensuing 48-h period. When compared with *C. albicans*, these biofilms were of reduced thickness, less profuse, and devoid of hyphal elements.

When we evaluated antifungal perfusion in 48-h biofilms, it was noted that amphotericin B was likely to be the least penetrant through the biofilms of all three *Candida* species compared with fluconazole and flucytosine, both of which demonstrated almost a similar degree of drug penetration. However, further kinetic analyses have to be performed to confirm this data. One reason for the foregoing observation could be the large size of the amphotericin B molecule compared with flucytosine and fluconazole (20), which may hinder its diffusion. Another could be the hydrophobicity of amphotericin B, which lowers its solubility and, hence, its biofilm perfusion (7). Although, of the tested antifungals, amphotericin B was the least penetrant through the three *Candida* biofilms, SEM observations revealed that it caused the most damage to the biofilm surface layer in comparison to fluconazole and flucytosine.

In general, biofilms are encased within an exopolymer matrix which may restrict the diffusion of solutes and also bind antimicrobials. For example, extracellular polysaccharide matrices of bacterial biofilms are known to differentially regulate the diffusion of antibiotics (29). Stewart et al. (30) observed that chlorosulfamate penetrated bacterial biofilms more rapidly than hypochlorite due to a slower reaction rate with biofilm constituents. On the other hand, the ability of an antibiotic to penetrate the biofilm can be severely retarded if the antimicrobial agent is neutralized by its reactivity with the biofilm (30). These observations suggest that the extent of antibiotic penetration through bacterial biofilms is agent and organism specific, and it is likely that similar conditions may operate in candidal biofilms.

In conclusion, the results of our study indicate that the ultrastructure of different *Candida* biofilms is species specific, varies considerably, and may be affected by the substrate contents. The major differences in the spatial configurations of the three different *Candida* biofilms seen in our SEM images tend to suggest that genes encoding biofilm formation may be differentially regulated during this mode of growth. Finally, the present method appears to be simple and versatile for the study of differential perfusion of antifungals or other solutes through the biofilms of various *Candida* species. In clinical terms, it is difficult to determine if this type of in vitro biofilm morphology we observed resembles in vivo candidiasis on mucosal surfaces, inert catheters, or denture surfaces. Further studies with a large number of clinical *Candida* strains with the proposed method are required to demystify the complexities associated with the biofilm-solute interphase.

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