Rev Iberoam Micol 2002; 19: 139-143

Medical importance of biofilms in *Candida* infections

L. Julia Douglas

Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Summary

Many *Candida* infections involve biofilm formation on implanted devices such as an indwelling catheter, a prosthetic heart valve or a denture. *Candida* biofilms can be formed *in vitro* using several model systems. In the simplest of these, organisms are grown on the surfaces of small discs of catheter material or denture acrylic. Biofilms of *C. albicans* prepared in this way consist of matrix-enclosed microcolonies containing yeasts, hyphae and pseudohyphae, arranged in a bilayer structure. *Candida* biofilms are resistant to a range of antifungal agents in current clinical use, including amphotericin B and fluconazole. Current research suggests that multiple mechanisms are involved in biofilm drug resistance.

Key words Biofilms, *Candida,* Infection, Resistance

Importancia médica de las biopelículas en las infecciones por *Candida*

Resumen

En muchas candidiasis se produce la formación de biopelículas sobre material biomédico, como catéteres, válvulas cardíacas protésicas o prótesis dentales. Las biopelículas de *Candida* pueden reproducirse *in vitro* empleando diferentes modelos. Los modelos más sencillos consisten en que los microorganismos crezcan sobre la superficie de discos pequeños del material empleado para la fabricación de catéteres o de prótesis dentales acrílicas. Estas biopelículas de *Candida* están compuestas de microcolonias dentro de una matriz que contienen levaduras, hifas y pseudohifas, ordenadas en una estructura de doble capa. Las biopelículas de *Candida* son resistentes a un amplio rango de antifúngicos que incluye a anfotericina B y fluconazol. Los estudios realizados sugieren que están implicados varios mecanismos en esta resistencia antifúngica.

Palabras clave Biopelículas, *Candida,* Infección, Resistencia

In the majority of natural habitats, most microorganisms grow as structured biofilm communities on surfaces rather than individually in suspension. Cells in these biofilms are embedded within a matrix of extracellular polymeric material and display an altered phenotype; in particular, they are significantly less susceptible to antimicrobial agents [1-3]. Recently, it has been estimated that some 65% of all human microbial infections involve biofilms [4]. Many of these are implant-related infections in which adherent microbial populations can be demonstrated on the surfaces of devices such as catheters, prosthetic heart valves and joint replacements [5]. Biofilm microorganisms can also be detected in tissues taken from non-device-related chronic infections such as native valve endocarditis [6]. Biofilm infections may be caused by a single microbial species or by a mixture of bacterial or fungal species [7,8]. Bacterial biofilms and their role in disease have been investigated in detail over a number of years and there is now a considerable amount of information available on their structure and properties [9,10]. Much less is known about fungal biofilms. This review describes biofilm formation by pathogenic fungi in the genus *Candida*, the fungal system that has received most attention to date.

Candida infections and biofilms

Candida albicans and other closely related *Candida* spp. are now recognised as major agents of hospital acquired infection worldwide. Recent data from the US National Nosocomial Infections Surveillance system rank these organisms as the fourth most common cause of bloodstream infection, behind coagulase-negative staphylococci, *Staphylococcus aureus* and enterococci. Mortality rates are high and treatment costly [11]. *Candida* spp. are also frequently identified as agents of nosocomial pneumonias and urinary tract infections. Almost invariably, an implanted device such as an intravascular or urinary catheter, or endotracheal tube, is associated with these infections and a biofilm can be detected on the surface of the

Dirección para correspondencia:
Dr. L. Julia Douglas
Division of Infection and Immunity
Institute of Biomedical and Life Sciences
Joseph Black Building, University of Glasgow
Glasgow, G12 8QQ, UK.
Tel: +44 141 330 5842
Fax: +44 141 330 4600
E-mail: J.Douglas@bio.gla.ac.uk

Apdo. 699, E-48080 Bilbao (Spain)
1130-1406/01/10.00 Euros

device [12-14]. Other devices totally implanted into the body, such as prosthetic heart valves, cardiac pacemakers and joint replacements (hip, knee etc.) are also liable to infection by *Candida* spp., usually at the time of surgical placement.

The most commonly infected, surgically implanted device is the central venous catheter, which is used to administer fluids and nutrients as well as cytotoxic drugs. Infections may arise at any time during the use of the catheter, which is often prolonged. Sometimes the infusion fluid itself, or the catheter hub, is contaminated but more frequently, organisms are introduced from the patient's skin microflora or from the hands of nursing staff. The distal tip of the catheter may be contaminated at the time of insertion, or organisms may later migrate down the catheter wound [15]. Alternatively, if *Candida* spp. colonizing the gastrointestinal tract are able to invade the bloodstream, they may 'seed' the catheter tip endogenously. This is thought to be a common portal of entry with cancer patients undergoing therapy with cytotoxic drugs.

Superficial *Candida* infections associated with implanted devices are much less serious but can be troublesome and are encountered very frequently. The commonest is probably denture stomatitis which is a *Candida* infection of the oral mucosa that is promoted by a close-fitting upper denture. A mixed species biofilm is formed on the surface of the acrylic denture; it contains large numbers of bacteria, particularly streptococci, in addition to yeasts [16]. Silicone rubber voice prostheses which are fitted in laryngectomized patients are also subject to contamination by polymicrobial biofilms containing *Candida* spp. The prostheses often fail within months of placement because the biofilm causes malfunction of the valve mechanism [17].

Candida biofilm formation *in vitro*

A number of model systems (Table 1) have been used to characterize the overall properties and susceptibility to antifungal agents of *Candida* biofilms [18]. The simplest of these, and the first to be described, involves growing adherent populations on the surfaces of small discs cut from catheters [18-20]. Growth is monitored quantitatively by a colorimetric assay which depends on the reduction of a tetrazolium salt, or by [^{3}H]leucine incorporation; both methods give excellent correlation with biofilm dry weight [19]. A similar model system can be used to study biofilm formation on discs or strips of denture acrylic [21,22]. For rapid processing of large numbers of samples, biofilms may be grown in wells of 96-well microtitre plates [23].

Table 1. Model systems used for studying *Candida* biofilms.

Model system	Type of system (static / flow)	Reference
Catheter disc	Static	18, 19, 20
Acrylic disc	Static	21, 22
Microtitre plate	Static	23
Cylindrical cellulose filter	Flow	18, 24, 25
Perfused biofilm fermenter	Flow	18, 26
Modified Robbins device	Flow	27, 28

Various factors which affect fungal adhesion and biofilm formation are listed in Table 2. Comparison of biofilm formation by 15 different isolates of *C. albicans* on catheter discs failed to reveal any correlation with pathogenicity within this group [19], but there was some correlation with pathogenicity when different *Candida* species were tested. Isolates of *C. parapsilosis*, *C. pseudotropicalis* and *C. glabrata* all gave significantly less biofilm growth than the more pathogenic *C. albicans* [19]. On the other hand, it has been reported that non-*C. albicans* species, particularly *C. tropicalis* and *C. parapsilosis*, can produce significant amounts of biofilm when grown in medium containing 8% glucose [29]. This ability may be important in enabling these species to cause candidaemia in patients receiving total parenteral nutrition, where the glucose concentration of the solution being administered is usually high.

Table 2. Factors affecting *Candida* biofilm formation *in vitro.*

Factor	Reference
Candida species and strain	19, 29
Nature of colonized surface	19
Presence of conditioning film	21, 22, 30
Liquid flow	31
Bacteria	8, 32

Evaluation of various catheter materials, using the catheter disc model system, has shown that biofilm formation by *C. albicans* is slightly increased on latex or silicone elastomer, compared with polyvinylchloride (PVC), but substantially decreased on polyurethane or 100% silicone [19]. *In vivo*, catheter materials rapidly adsorb host proteins which form a conditioning film on the catheter surface. Preincubation of PVC catheter discs *in vitro* with fibrinogen or collagen enhanced biofilm formation by *C. albicans* [30]. Similarly, conditioning films of serum or saliva promoted biofilm formation on denture acrylic [21,22].

One defining characteristic of a biofilm is the presence of a matrix of extracellular polymeric material in which the microorganisms are embedded [6]. The matrix can be difficult to preserve when biofilms are examined by scanning electron microscopy and special drying procedures are required [18]. However, the amount of matrix visible depends not only on preparative techniques but also on incubation conditions during biofilm development. Substantially increased amounts of matrix are formed when biofilms of *C. albicans* are incubated with gentle shaking, instead of statically, to produce a flow of liquid over the surface of the cells (Figure 1). Under these conditions the microorganisms can be almost completely obscured by the enveloping matrix [31]. Matrix production is similarly increased when conventional flow systems such as the modified Robbins device or perfused biofilm fermenter are used (Table 1).

Bacteria are often found with *Candida* species in polymicrobial biofilms *in vivo*, and it is likely that extensive interspecies interactions take place in these adherent populations. *In vitro*, the catheter disc model system has been used to investigate mixed species biofilms consisting of *C. albicans* and *Staphylococcus epidermidis*, the commonest agent of bacterial catheter-related infection. Scanning electron microscopy revealed numerous physical interactions between the staphylococci and both yeasts and hyphae [32]. Moreover, drug susceptibility studies showed that fungal cells appear to modulate the action of antibiotics whereas bacteria can affect the activity of antifungal agents in these biofilms. Similar observations have been made with biofilms consisting of *C. albicans* and oral streptococci (*S. gordonii* and *S. salivarius*) on denture acrylic [8].

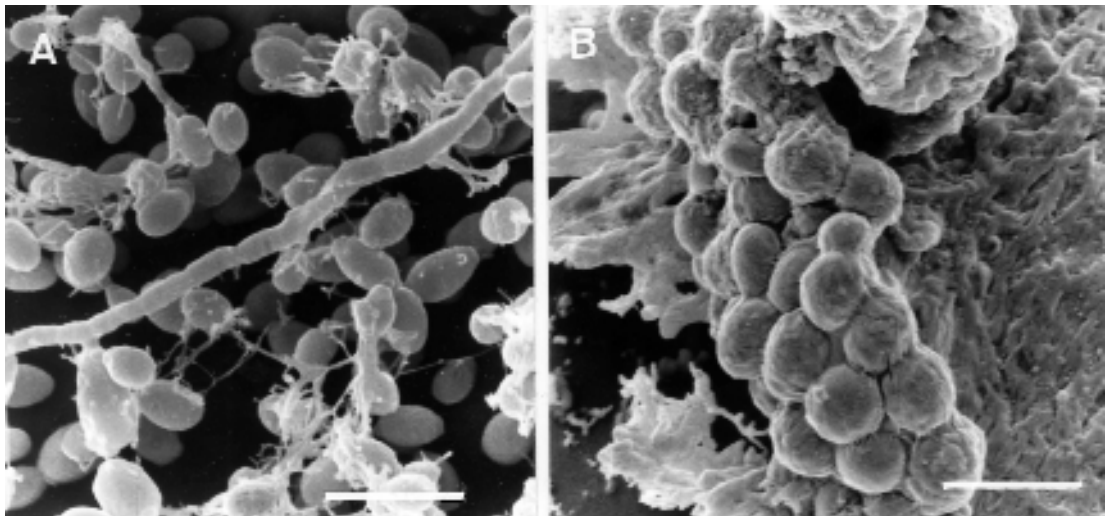

Figure 1. Scanning electron micrographs of biofilms formed by *C. albicans* on catheter discs. Bioflms were incubated statically (A), or with gentle shaking (B). Bar, 10 µm.

Biofilm ultrastructure

The detailed structure of *C. albicans* biofilms was first examined by scanning electron microscopy [19]. Initial attachment of yeast cells to a catheter disc was followed, after 3 to 6 h, by germ tube formation. Fully mature biofilms, produced after incubation for 24 to 48 h, consisted of a dense network of yeasts, hyphae and pseudohyphae [19]. This mixed morphology was not seen when the organism was grown in liquid culture or on an agar surface of the same medium (yeast nitrogen base with glucose), suggesting that morphogenesis was triggered by contact with the plastic surface. In this connection, it is interesting that a mutant of *C. albicans*, defective in filamentous growth and lacking the transcription factors Efg1p and Cph1p involved in morphogenetic signalling pathways, failed to colonize polyurethane catheters [33].

To assess the importance of dimorphism in biofilm development, biofilms produced by wild-type strains of *C. albicans* were compared with those formed by two morphological mutants, incapable of yeast and hyphal growth, respectively [25]. Scanning electron microscopy and thin sections of biofilms examined by light microscopy revealed that biofilms of wild-type strains formed on catheter discs consisted of two distinct layers: a basal region of densely packed yeasts and an overlying thicker, but more open, hyphal layer. The hypha$^-$ mutant produced only the basal layer, whereas the yeast$^-$ mutant formed a thicker, hyphal biofilm equivalent to the outer zone of the wild-type structures. Biofilms of the yeast$^-$ mutant were more easily detached from the catheter surface than the others, suggesting that the basal yeast layer has an important role in anchoring the biofilm to the surface.

Despite its excellent resolution properties, scanning electron microscopy has the disadvantage that all samples examined must be fully dehydrated. Confocal laser scanning microscopy (CLSM), on the other hand, allows the examination of fully hydrated, living biofilms. Using this technique, bacterial biofilms have been shown to consist of matrix-enclosed microcolonies whose appearance has been described as 'towers' or 'mushroom-shaped stacks'. The microcolonies are separated by water channels which provide a mechanism for nutrient circulation within the biofilm [6]. Recent CLSM studies suggest that biofilms of both *C. albicans* and *C. dubliniensis* have similar three-dimensional structures consisting of microcolonies surrounded by water channels [34-36]. Such studies have also confirmed the bilayer structure of *C. albicans* microcolonies when biofilms are grown on plastic surfaces [37].

Matrix polymers of bacterial biofilms are primarily exopolysaccharides and many of them are negatively charged. Smaller amounts of proteins, nucleic acids and various other components may also be present. However, much of the biofilm matrix - up to 97% - is water [38]. The matrix of *C. albicans* biofilms has been isolated and its composition compared with that of extracellular polymeric material obtained from culture supernatants of planktonically grown (suspended) organisms [39]. Both preparations consisted of carbohydrate, protein, phosphorus and hexosamine but the matrix contained significantly less carbohydrate (41%) and protein (5%). It also had a higher proportion of glucose (16%) than mannose, and contained galactose, suggesting that it might possess components unique to biofilms [39].

Drug resistance of biofilms

Probably the most significant feature of microbial biofilms is their notorious resistance to a variety of antimicrobial agents, including antibiotics, antiseptics and industrial biocides. For example, when bacteria exist in the biofilm form they are 10-1000 times more resistant to antibiotics than are planktonic cells [6]. Corresponding resistance of *Candida* biofilms to antifungal agents was first demonstrated in 1995 [20]. Clinically important antifungal agents - amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole - were tested using a catheter disc assay. All of these agents showed much less activity against *C. albicans* biofilms than against planktonic cells. Biofilms of non-*C. albicans* species, such as *C. tropicalis* and *C. parapsilosis*, were also drug resistant [20].

Subsequent studies have demonstrated drug resistance when *Candida* biofilms are grown on other types of surface including cellulose [24,25], polystyrene [23,34], and denture acrylic [22]. Recently, however, it has been claimed that some of the newer antifungal agents are active against *Candida* biofilms. Although biofilms of *C. albicans* and *C. parapsilosis* were clearly resistant to two new

triazoles (voriconazole and ravuconazole), there appeared to be some anti-biofilm activity with lipid formulations of amphotericin B and two echinocandins (caspofungin and micafungin) [40]. These are interesting findings which, if confirmed, could lead to important developments in the treatment of fungal implant infections.

Possible mechanisms of drug resistance

The mechanisms of biofilm resistance to antimicrobial agents are poorly understood. Possible mechanisms include: (i) restricted penetration of drugs through the biofilm matrix, (ii) phenotypic changes resulting from a decreased growth rate or nutrient limitation, and (iii) surface-induced expression of resistance genes [3,6]. Another recent suggestion is that a small number of 'persister' cells are responsible for resistance [41]. With bacteria, it already appears that multiple mechanisms operate, and that these vary with the bacteria present in the biofilm and the nature of the antimicrobial agent being administered [3].

To investigate whether the matrix plays a role in the resistance of *C. albicans* biofilms to antifungal agents, susceptibility profiles of biofilms incubated statically (which have relatively little matrix) were compared with those for biofilms incubated with gentle shaking (which produce much more matrix material). Biofilms grown with or without shaking failed to exhibit significant differences in susceptibility to any of the drugs tested, indicating that drug resistance is unrelated to the extent of matrix formation [39]. However, bacterial matrix material may act as a barrier to fluconazole penetration in mixed species biofilms of *C. albicans* and *S. epidermidis* [32].

Biofilm cells are thought to grow slowly because of the limited availability of nutrients, particularly at the base of the biofilm. To investigate a possible role for growth rate in drug resistance, a perfused biofilm fermenter (Table 1) was used to generate *C. albicans* biofilms at different growth rates. The susceptibility of the biofilm cells to amphotericin B was then compared with that of planktonic organisms grown at the same rates in a chemostat. The results showed that biofilms were resistant to the drug at all growth rates tested whereas planktonic cells were resistant only at low growth rates [26]. Biofilm resistance is therefore not simply due to a low growth rate but depends on some other feature of the biofilm mode of growth. A separate study [24] using the cylindrical cellulose filter model system (Table 1) demonstrated that glucose-limited and iron-limited biofilms grown at the same low rate were equally resistant to amphotericin B. However, daughter cells from iron-limited biofilms, which probably resemble more closely biofilms *in vivo*, were significantly more susceptible to the drug [24]. An acute disseminated infection produced by the release of such cells from an implant biofilm might therefore respond rapidly to amphotericin B, but the biofilm itself would be unaffected.

When microorganisms attach to a surface and form a biofilm they express an altered phenotype. Work is currently in progress to identify genes that are activated or repressed in biofilms compared with planktonic cells, and there is particular interest in genes that might contribute to drug resistance. For example, upregulation of genes coding for multidrug efflux pumps would result in a multidrug-resistant phenotype. *C. albicans* possesses two different types of efflux pump: ATP-binding cassette (ABC) transporters and major facilitators, which are encoded by CDR and MDR genes, respectively. A recent study has demonstrated that genes for both types of efflux pump are upregulated during biofilm formation and development. However, strains carrying single or double deletion mutations in some of these genes were highly susceptible to fluconazole when growing planktonically but still retained the resistant phenotype during biofilm growth [42]. These results strongly suggest that drug resistance in *C. albicans* biofilms, like that in bacterial biofilms, is complex and cannot be explained by a single molecular mechanism.

References

1. Costerton JW, Lewandowski Z, Caldwell DE *et al*. Microbial biofilms. Annu Rev Microbiol 1995; 49: 711-745.
2. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol 2000; 54: 49-79.
3. Mah T-FC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 2001; 9: 34-39.
4. Potera C. Forging a link between biofilms and disease. Science 1999; 283: 1837-1838.
5. Donlan RM. Biofilms and device-associated infections. Emerg Infect Dis 2001; 7: 277-281.
6. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002; 15: 167-193.
7. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284: 1318-1322.
8. Jenkinson HF, Douglas LJ. Interactions between *Candida* species and bacteria in mixed infections. In: Brogden KA, Guthmiller JM (Eds.) Polymicrobial Diseases. Washington, DC, ASM Press, 2002: 357-373.
9. Lappin-Scott HM, Costerton JW (Eds.). Microbial Biofilms. Cambridge, Cambridge University Press, 1995.
10. Allison DG, Gilbert P, Lappin-Scott HM, Wilson M (Eds.). Community Structure and Co-operation in Biofilms. Society for General Microbiology Symposium 59. Cambridge, Cambridge University Press, 2000.
11. Calderone RA. Introduction and historical perspectives. In: Calderone RA (Ed.) *Candida* and Candidiasis. Washington, DC, ASM Press, 2002: 3-13.
12. Crump JA, Collignon PJ. Intravascular catheter-associated infections. Eur J Clin Microbiol Infect Dis 2000; 19: 1-8.
13. Maki DG, Tambyah PA. Engineering out the risk of infection with urinary catheters. Emerg Infect Dis 2001;7: 1-6.
14. Adair CG, Gorman SP, Feron BM *et al*. Implications of endotracheal tube biofilm ventilator-associated pneumonia. Intensive Care Med 1999; 25: 1072-1076.
15. Goldmann DA, Pier, GB. Pathogenesis of infections related to intravascular catheterization. Clin Microbiol Rev 1993; 6: 176-192.
16. Budtz-Jorgensen E. *Candida* - associated denture stomatitis and angular cheilitis. In: Samaranayake LP, MacFarlane TW (Eds.) Oral Candidosis. London, Wright, 1990: 156-183.
17. Van der Mei HC, Free RH, Elving GJ *et al*. Effect of probiotic bacteria on prevalence of yeasts in oropharyngeal biofilms on silicone rubber voice prostheses in vitro. J Med Microbiol 2000; 49: 713-718.
18. Baillie GS, Douglas LJ. *Candida* biofilms and their susceptibility to antifungal agents. Meth Enzymol 1999; 310: 644-656.
19. Hawser SP, Douglas LJ. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect Immun 1994; 62: 915-921.
20. Hawser SP, Douglas LJ. Resistance of *Candida albicans* biofilms to antifungal agents in vitro. Antimicrob Agents Chemother 1995; 39: 2128-2131.
21. Nikawa H, Nishimura H, Yamamoto T *et al*. The role of saliva and serum in *Candida albicans* biofilm formation on denture acrylic surfaces. Microb Ecol Health Dis 1996; 9: 35-48.
22. Chandra J, Mukherjee PK, Leidich SD *et al*. Antifungal resistance of candidal biofilms formed on denture acrylic in vitro. J Dent Res 2001; 80: 903-908.

23. Ramage G, VandeWalle K, Wickes BL *et al*. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob Agents Chemother 2001; 45: 2475-2479.
24. Baillie GS, Douglas LJ. Iron-limited biofilms of *Candida albicans* and their susceptibility to amphotericin B. Antimicrob Agents Chemother 1998; 42: 2146-2149.
25. Baillie GS, Douglas L J. Role of dimorphism in the development of *Candida albicans* biofilms. J Med Microbiol 1999; 48: 671-679.
26. Baillie GS, Douglas LJ. Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. Antimicrob Agents Chemother 1998; 42: 1900-1905.
27. Kharazmi A, Giwercman B, Hoiby N. Robbins device in biofilm research. Meth Enzymol 1999; 310: 207-215.
28. Clark CM, Douglas LJ, unpublished observations.
29. Shin JH, Kee SJ, Shin MG *et al*. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. J Clin Microbiol 2002; 40: 1244-1248.
30. Hawser SP, Douglas LJ, unpublished observations.
31. Hawser SP, Baillie GS, Douglas LJ. Production of extracellular matrix by *Candida albicans* biofilms. J Med Microbiol 1998; 47: 253-256.
32. Adam B, Baillie GS, Douglas LJ. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. J Med Microbiol 2002; 51: 344-349.
33. Lewis RE, Lo H-J, Raad II *et al*. Lack of catheter infection by the efg1/efg1 cph1/cph1 double-null mutant, a *Candida albicans* strain that is defective in filamentous growth. Antimicrob Agents Chemother 2002; 46: 1153-1155.
34. Ramage G, VandeWalle K, Wickes BL *et al*. Biofilm formation by *Candida dubliniensis*. J Clin Microbiol 2001; 39: 3234-3240.
35. Ramage G, VandeWalle R, Wickes BL *et al.* Characteristics of biofilm formation by *Candida albicans*. Rev Iberoam Micol 2001; 18: 163-170.
36. Chandra J, Kuhn DM, Mukherjee PK *et al*. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J Bacteriol 2001; 183: 5385-5394.
37. Kuhn DM, Chandra J, Mukherjee PK *et al*. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. Infect Immun 2002; 70: 878-888.
38. Sutherland IW. The biofilm matrix - an immobilised but dynamic microbial environment. Trends Microbiol 2001; 9: 222-227.
39. Baillie GS, Douglas LJ. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. J Antimicrob Chemother 2000; 46: 397-403.
40. Kuhn DM, George T, Chandra J *et al*. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother 2002; 46: 1773-1780.
41. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother 2001; 45: 999-1007.
42. Ramage G, Bachmann S, Patterson TF *et al*. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. J Antimicrob Chemother 2002; 49: 973-980.