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In vivo Models for *Candida Albicans* Biofilms Study

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ABSTRACT

Biofilm is a common mode of fungal growth in clinical infection. In the mode of biofilm, *Candida albicans* tends to display high resistance to body immunity and antimicrobial agents, which has a significant impact on mortality. Biofilm models are essential tools to better understand the mechanisms of formation and resistance. Compared to *in vitro* models, *in vivo* models can better take into account the host immune system and are indispensable for the study of medical device related infection. The aim of this review is to summarize information related to the reported *in vivo* models of *C. albicans* biofilms, analyze the operating process and application of them, and compare their advantages and limitations. A literature search was performed from databases in Medline (PubMed), Web of Science, Science Direct, and Google scholar by applying some related search terms. The articles related to agriculture, ecology, and synthetic work and those using languages other than English have been excluded. The bibliographies of papers relating to the review subject were also searched for further relevant references. According to the common sites of *C. albicans* infection; three kinds of *in vivo* models are discussed in this review: oral mucosa model, vaginal mucosa model and implanted catheter model. The former two models can demonstrate the structure and composition of biofilms growing on the mucosa, and implanted catheter model represents different kinds of medical devices. To expedite the success of new treatments of infection, further refinement of *in vivo* models is an urgent need.

INTRODUCTION

The incidence of invasive fungal infections has increased significantly in the last several years, which can be explained by the widely using of wide-spectrum antibiotics and immunosuppressant, invasive procedures and medical implant devices. Among the fungal pathogens of humans, Candida represented by *Candida albicans*, is one of the species that are the most frequently associated with biofilm infection, particularly which on medical implants, and this has a significant impact on morbidity and mortalit [1]. invasive procedures and medical implant devices *C. albicans* which can cause both superficial and systemic infections, is one of the main pathogenic microorganisms caused nosocomial infection. Data in 2002 from the US National Nosocomial Infections Surveillance System ranked *C. albicans* as the fourth most common cause of bloodstream infection, behind coagulase-

negative *Staphylococci, Staphylococcus Aureus and Enterococci*, and also ranked third in cause of catheter-associated infection with a high mortality rate and high cost for treatment.

The fact that *C. albicans* commonnly exist in biofilms and attach on mucosal or plastic surfaces of indwelling medcal devices [2] may help to explain why *C. albicans* is the causative agent of most candidiasis. Biofilm is defined as microbial communities encased in a matrix of extracellular polymeric substances [3]. Biofilm is a common mode of growth both in bacteria and fungi as it is currently estimated that as much as 80% of all microbial biomass resides in a biofilm state [4]. The ubiquitous presence of biofilms in the environment was realised about 4 decades ago [5] and it can have a detrimental effect on public health, especially associated with medical device infection and chronic infections [6]. Fungal biofilms typically develop over five sequential steps: (1) the adhesion of a microorganism to a surface (2) cells gathering and discrete colony formation, (3) form hyphae and secretion of extracellular polymeric substances, (4) maturation into a three-dimensional structure and (5) dispersion of cells **(Figure 1).**

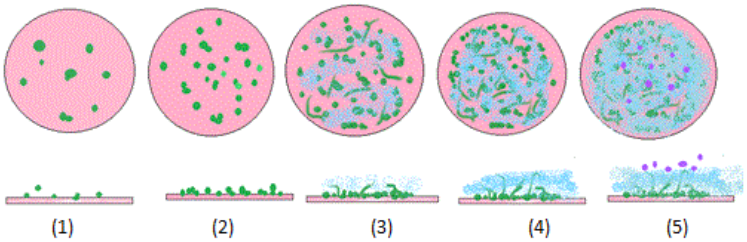

Figure 1. Stages in the formation of *Candida albicans* biofilm. (1) Culture medium surface with an adsorbed conditioning film of host proteins (pink). Initial yeast (green) contact the surface and adhesion to it. (2) Formation of the basal layers of yeast micro colonies. (3) Completion of micro colony formation by addition of the upper, mainly hyphal layer. The extracellular matrix material (blue) was formed. (4) Mature biofilms contain numerous micro colonies with extracellular matrix material that surrounds both yeasts (green) and hyphae (green). They consisted of two distinct layers: a thin, basal region of densely packed yeast cells and an overlying thicker, but more open hyphal layer. (5) Mature biofilms produce new spores (purple) and disperse them.

Biofilms display features that differ from their planktonic counterparts in morphological structure, biological and biochemical features, pathogenicity and antifungal susceptibility. As it is consisted of a dense network of yeasts, hyphae, pseudo hyphae and self-produced extracellular biopolymers [7], fully mature biofilms of *C. albicans* display metabolic cooperation, quorum sensing systems, byproduct influence and some other synergies. These features give them a competitive advantage in antimicrobial agent's resistance. Current researches on the planktonic mode of microbial growth could explain most acute infections, but maybe was inadequate for understanding the cause of chronic infections. While the clinical option of common antifungal agents is mostly designed against microorganisms in planktonic mode, that's another reason why *Candida spp* tends to display highly-resistance and refractory. Now biofilms have been one of the hot spots in the anti-infective study.

C. albicans has become the main experimental fungi for studying fungal biofilms. The availability of well-characterized, reproducible biofilm models is essential to understanding the nature of biofilms and performing studies of biofilm formation and antifungal drug resistance. Over the last decades, many new studies have generated a wealth of fresh insights into the nature of biofilm infection, such as the characterization of the regulating factors and mechanisms that govern the formation and antifungal tolerance of *C. albicans* biofilms. A number of genes involved in all stages of biofilm formation by *C. albicans*, such as in adherence, matrix production, quorum sensing and especially morphogenesis, have been identified and well characterized [8,9,10]. The influence of environmental conditions and regulatory pathways in the expression of these genes is also studied. All these progresses were achieved based on the development of *C. albicans* biofilms models.

In the area of *C. albicans* biofilms model, *in vitro* models have been developed and implemented prosperously. The commonly used *in vitro* models can be classified into several types, among which the microtiter plate based model is the most frequently-used biofilm model systems. *In vitro* models have the advantage of fast, efficient, reliable, and reproducible, with high throughput potential and low cost. But to our great pity, *in vitro* model can only be used to observe how biofilm forming and determinate the antifungal effect of drugs. Clinically biofilm-associated infection is related to the interaction of biofilm and host body. So building *in vivo* model is of great significance. According to the common sites of *Candida albicans* infection in the body, the current *in vivo* models of biofilm have three categories: oral mucosa models, vaginal mucosa models and implanted catheter models. The former two models are utilized to demonstrate the structure and composition of biofilms of *C. albicans* growing on the mucosa. It is worth noting that biofilms formed *in vivo* are influenced by the symbiotic microflora and host cell components, so the composition of biofilms formed on mucosa models are inherently more complex than biofilms formed on abiotic surface. Implanted catheter models have also been developed. Subcutaneous implanted catheters and intravenous implanted catheters represent different kinds of medical devices respectively. This model can be used to characterize the ability of antimicrobial agents to eliminate biofilms, and to evaluate the prophylactic effect of antifungal drugs and biomaterial coatings.

This review focuses on summarizing some widely used *in vivo* models of *C. albicans* biofilms, analyzing the operating process, application and development of them, discussing their advantages and disadvantages **(Table 1).** It also looks forward the research orientation in the future.

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Table 1. Models used to study *Candida albicans* biofilms.

Substrate	Type	Suspension concentration	Research objectives	Intervention medicine	Observation time	Evaluation method(Biofilm ultrastructure/ Quantitative analysis)
Oral mucosa	Oral mucosa model	6×10^8	Structure study		Inoculation procedure: 3 days observation: after 5 days	CSLM
Vaginal mucosa	Vaginal mucosa model	2.5×10^6 2.5×10^8	Structure & formation mechanism study		BF formation:8,24,48,72 h	CM,SEM/CFU
Catheter	Subcutaneously implanted catheters model	5×10^4	Antifungal susceptibility test & formation mechanism study	Fluconazole, anidulafungin, negative control	BF formation:48 h SMICs:7 days BF formation: 0,5,24, 48, 144 h	CSLM,SEM, quantitative real-time PCR/CFU
	Intravenous implanted catheters model	1.0×10^7		Liposomal amphotericin B, fluconazole	Inoculation: 24h; formation: 3days/SMICs: 7days	SEM/CFU

The model of *C. albicans* biofilm formed on oral mucosa

Candida albicans can form biofilm on mucosal surfaces and then cause biofilm-associated infection in immunocompromised patients. The establishment of adequate models of mucosal biofilm is the first step in understanding the mechanisms of biofilm formation on tissue surfaces.

In the study of Dongari-Bagtzoglou et al, researchers used 6-8 week old female C57BL/6 mice and strain SC5314 and GFP-tagged strain MRL51 to establish an *in vivo* model of *C. albicans* biofilms on oral mucosa [11]. Strain SC5314 which displayed a virulent phenotype was used to study *in vivo* and *in vitro* biofilm growth and GFP-tagged strain MRL51 which derived from SC5314 was used in the model for live biofilm observation. One day prior to the operation, mice were immunosuppressed by cortisone acetate (255 mg/kg). After mice anaesthetized, a small cotton pad soaked with *C. albicans* cell suspensions (100 µl, 6×108 yeast per ml) was used to swab the oral cavity. The cotton pad was left underneath the tongue for 2 hours. The operation was repeated for 3 days. During this period, drinking water supplied for the mice was added a daily-fresh suspension *C. albicans* (6×106 yeast per ml) to maintain high oral carriage loads throughout the experiment. Two days after the operation, mice were sacrificed and the tongues were dissected.

The white plaques formed on the dorsal surface of the tongue were examined by confocal microscopy. Confocal imaging followed by 3D reconstruction revealed a lively architecture of mucosal biofilm that followed the epithelial micro anatomical variations of the lingual papillae, forming 'valleys' and higher 'elevations' of stacking fungal cells.

In this study for the first time the structure and composition of biofilms of *C. albicans* growing on the oral mucosa was systematically characterized. By using this *in vivo* model, it was revealed that *C. albicans* forms complex oral mucosal biofilms involving both bacterial and host components. This model is a powerful tool to investigate the potential synergistic relationship between *C. albicans* and the components of oral mucosa surface.

The model of *C. albicans* biofilm formed on vaginal mucosa

A variety of studies suggest that 75% of adult women are affected by vulvovaginal candidiasis (VVC) during their lifetime and VVC has become the most common cause of acute vaginitis in Europe and the United States [12]. The *in vivo* model of *C. albicans* biofilm was developed to confirm the correlation between the forming of biofilm on vaginal mucosa and the pathogenesis of VVC.

The first model of *C. albicans* biofilms formed on vaginal mucosa of mouse was built by Harriott MM et al [13]. In the course of study, the mice were administered 0.1 mg 17-β-oestradiol in 0.1 ml sesame seed oil. Three days later, they were inoculated intravaginally with *C. albicans* 3153A (2.5×106 yeast per ml, 20 µl). Mice were sacrificed at 8, 24, 48 and 72 h postinoculation and their vaginae were cut longitudinally to expose the mucosal surface. Half of the tissue was processed for scanning SEM or CM and the other half was used to determine fungal load.

Microscopic analysis demonstrated that *C. albicans* biofilms were present on the vaginal mucosa which had the architecture similar to that formed in vitro. SEM images showed that the architecture consisted of yeast and hyphae forming a complex network surrounded by ECM which appeared as a thick film covering the cellular portion of the biofilm. This *in vivo* model of *C. albicans* biofilm formed on vaginal mucosal tissue is extremely relevant as it is a newly characterized biotic surface Candida biofilm model in an immunocompetent host.

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The model of *C. albicans* biofilm formed on implanted catheters

The model of *C. albicans* biofilm on subcutaneously implanted catheters

Fungal biofilms can develop on a variety of frequently implanted biomaterials. Almost invariably, an implanted device such as an intravascular or urinary catheter, or endotracheal tube, is associated with fungal biofilms infections. Other devices totally implanted into the body, such as prosthetic heart valves, cardiac pacemakers and joint replacements (eg., hip or knee), are also common sites where Candida infection may occurs [14]. Increased use of medical implant devices is one of important factors contributing to biomaterial- related biofilm infections, and high resistance of biofilm to antimicrobial treatment makes it more difficult to eradicate the infection.

In vivo models, where the biofilms are formed on the surface of catheters in venous system or in subcutaneous tissue of the animals, have been developed in recent years. The benefit of these models is that they also take into account the host immune system and infection site; therefore they are indispensable for our understanding of biofilms infection associated with clinical devices.

Ricicova Marketa et al used polyurethane triple-lumen intravenous catheters to build *in vivo* model [15]. The catheters were cut into segments of 1 cm and incubated in bovine serum at 37 °C overnight. In the next day, the catheters were incubated with *C. albicans* cell suspension at 37 °C for 90 min and washed twice with PBS. After the incubation, catheters were implanted under the skin of rats. The rats kept asleep during the procedure by using mixed gas (enflurane 20% and oxygen 80%). After disinfection with 0.5% chlorhexidine in 70% alcohol, the lower back of the rat was incised to make a 10 mm longitudinal incision. Subcutaneous tissue was dissected to create subcutaneous tunnel. Up to ten catheters pieces were implanted, and surgical staples were used to close the incision. A time course schematic of the experiment is shown in **Figure 2.** The rats were euthanized by CO_2 inhalation to explants the catheters after the biofilm formed on the surface of them. By confocal microscopy observing, biofilm of *C. albicans* was formed by hyphal cells and distributed unevenly along the catheter. This *in vivo* model can be used to evaluate the efficiency of the antifungal drugs in preventing catheter-associated fungal infection.

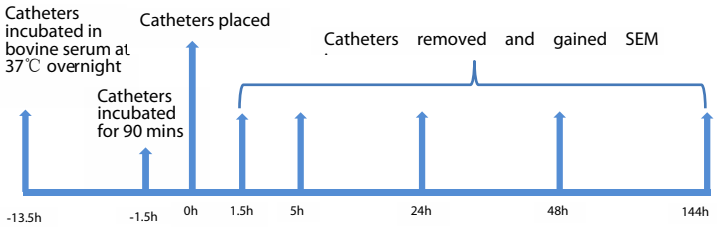

Figure 2. Experimental time line of the *in vivo* rat catheter infection model.

Sona Kucharikova et al established a similar *in vivo* model in the study of the efficacy of anidulafungin against mature *C. albicans* biofilms [16]. They used drinking water added with dexamethasone (1 mg per liter) to immunosuppress the female Sprague-Dawley rats. Polyurethane catheter pieced were incubated overnight in serum and then inoculated with Candida cells at 37 °C for 90 min. After incubation, the catheters were implanted subcutaneously on the backs of the rats for 48 h to make the biofilms mature. Anidulafungin, fluconazole and physiological solution as control was administrated intraperitoneally once a day. The treatment continued for 7 days, and the rats were euthanized by CO_2 inhalation. The catheter pieces were removed and cultured for CFU counting.

This model is a simple tool for the analysis of *in vivo* biofilm formation by *C. albicans*. For future applications, the model can be used to evaluate the biocompatibility of implanted devices (reduction of the foreign body response), the preventive treatment efficacy of new antimicrobial reagents and the potency of chemotherapeutic agents to eliminate biofilms.

The model of *C. albicans* biofilm on intravenous implanted catheters

Central venous catheters (CVCs) are widely used for patients who need total parenteral nutrient (PTN), critically-ill patients and patients whose treatment involves chemotherapy and prolonged period of hospitalization. The long-term retention of catheters may cause biofilm forming, which can then become a potential systemic infection. So far, some *in vivo* models of biofilm on CVCs have been built to study CVCs- associated infection.

Schinabeck et al had developed a novel *in vivo* model of catheter-associated infection caused by *C. albicans* and used the model to assess the effect of antifungal agent lock therapy [17]. Female New Zealand White rabbits were used for all procedures. Rabbits were anesthetized with ketamine (70 mg/kg of body weight) and xylazine (7 mg/kg of body weight). A 4 cm incision was made in the right cervical region to expose the external jugular vein. Using No.11 scalped blade, an incision in the isolated segment of vein was made. The tip of the catheter was placed into the right anterior vena cave. The proximal end of the catheter was tied with the vein; the excess catheter was cut. An 18-gauge Luer stub adapter and a sterile heparin lock device were placed on the external end of the catheter. The operation was shown in **Figure 3.** After catheter placement, the inoculums consisting 107 CFU of *C. albicans*, 100U of heparin and sterile normal saline was locked in the internal lumen of the catheter and dwelled for 24 h. Each catheter was flushed with 300 µl of heparinized (100U) saline and then locked with liposomal amphotericin B or

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fluconazole for 8 h per day. The control group was locked with sterile normal saline. The treatment of the catheters lasted for 7 days. Upon completion of the treatment, all animals were sacrificed and the catheters were removed in a sterile environment. The catheter pieces were observed under SEM.

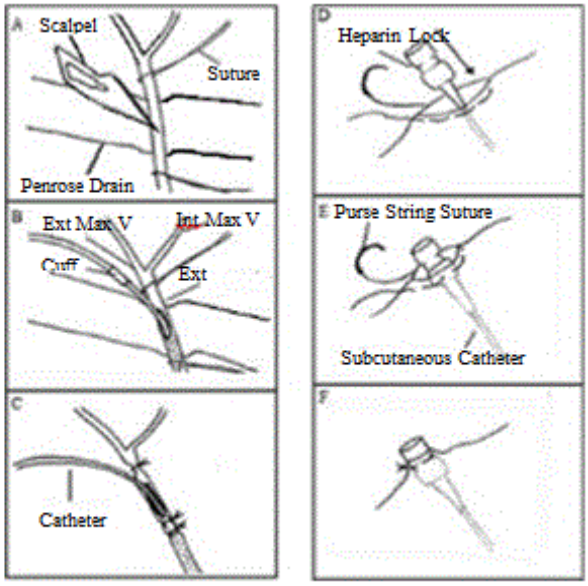

Figure 3. Surgical placement of the intravenous catheter. (A to C) Catheter insertion into the external jugular vein; (D to F) attachment of the heparin lock device to skin. The picture used here comes from the study of Schinabeck.

The results show that this model has utility in preclinical evaluation of antifungal agents and in the study of biofilm pathogenesis. The demonstration of liposomal amphotericin B lock therapy as an effective strategy for treating *C. albicans* catheter-associated infections may have significant clinical implications.

Besides rabbit model, some studied use rats as rodent in the experiments [18-20]. These studies provide a model for further investigation into the molecular mechanisms of *C. albicans* biofilm biology and drug resistance. In addition, the model provides a means to study novel drug therapies and device technologies targeted to the control of biofilm-associated infection.

DISCUSSION

The issues of superbug and hospital- required infection have been paid more and more attention at present. The in-depth study of resistance mechanisms of fungi represented by *C. albicans* are continuously. Biofilms are attracted great attention currently for its significant difference with planktonic cells in drug resistance.

Over the past decades, *in vitro* models of *C. albicans* biofilms have been greatly developed. However, the key limitation of *in vitro* models is that they can only study biofilms in isolation, but not take interactions into consideration. The interactions include which between biofilms and the immune system of host organism, as well as the interaction of different kinds of microorganisms in human body. This limitation of *in vitro* models underscores a need for *in vivo* models to study such issues. The present *in vivo* models can provide a realistic condition of infection site, blood flow, host conditioning protein and the host immunity-biofilm interaction. The current studies showed that factors that predispose organisms to adhere to device surfaces and contribute to the propagation of biofilms *in vitro* may not be the same factors that mediate this process *in vivo* at different sites of infection. This difference represents the significance of developing *in vivo* models which are more closely simulating host environment.

As previously stated, animal models take into account the host immune system which makes it more closely related to the natural pathology. However, the unavoidable reality is that the use of animal models is associated with high costs, more skills required and ethical issues. So the number of animals to be used in the study should be kept to a minimum and intensified attention should be paid to minimize potential pain. Some common used larger animals, such as dogs, sheep is more expensive. With the advantages of relatively economical and easy to infect, small animals including mice, rats, guinea pigs and rabbits are preferred in studies.

Which *in vivo* model is selected mainly depends on the question being addressed. Taking the models of biofilms formed on the implanted catheters as example, the host sites at the subcutaneous location and in the central venous system presumably differ greatly. Whereas in the latter, biofilms are subjected to the blood flow which means more access to nutrients, biofilms developing in catheters implanted under the skin of rats are not exposed to a physiological flow. The subcutaneous model is somewhat more related to biofilm infections that develop in joint prostheses and voice prostheses for example, and may reflect better these host infection sites, in term of environmental conditions and nutrient supplies. And the intravenous model is more related to CVC-associated infections. For now, there is a shortage of systematically further study in the aspect of selecting the

appropriate model of biofilms. This underscores the urgent need for a deeper understanding of the influence of different kind of *in vivo* models on the results in the future.

Despite our progress in understanding *C. albicans* infection, there remains a high mortality associated with it. Exciting advances in adherence, matrix production, quorum sensing and especially morphogenesis, provide new information about the pathogenic mechanism of the Candidiasis and further our understanding of biofilms development, progression, and recurrence. Further refinement of *in vivo* models of *C. albicans* biofilms which including awareness of the limitations each model presents, and taking advantage of the technologies available to study these models will undoubtedly expedite the success of new treatments.

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REFERENCES

1. Kumamoto CA. Candida biofilms. Current opinion in microbiology 2002; 5: 608-611.
2. Hasan F, et al. Biofilm formation in clinical Candida isolates and its association with virulence. Microbes and infection / Institut Pasteur 2009; 11: 753-761.
3. Tobudic S, et al. Antifungal susceptibility of *Candida albicans* in biofilms. Mycoses. 2012; 55: 199-204.
4. Krom BP and Oskam J. Microbial biofilms and wound healing: an ecological hypothesis. Phlebology/Venous Forum of the Royal Society of Medicine 2014; 29: 168-173.
5. Karunakaran E, et al. "Biofilmology": a multidisciplinary review of the study of microbial biofilms. Applied microbiology and biotechnology 2011; 90: 1869-1881.
6. Chandra J, et al. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. Journal of bacteriology 2001; 183: 5385-5394.
7. Hawser SP and Douglas LJ. Biofilm formation by Candida species on the surface of catheter materials in vitro. Infection and immunity 1994; 62: 915-921.
8. Garcia-Sanchez S, et al. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryotic cell 2004; 3: 536-545.
9. Marchais V, et al. DNA array analysis of *Candida albicans* gene expression in response to adherence to polystyrene. FEMS microbiology letters 2005; 245: 25-32.
10. Murillo LA, et al. Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. Eukaryotic cell 2005; 4: 1562-1573.
11. Dongari-Bagtzoglou A, et al. Characterization of mucosal *Candida albicans* biofilms. PloS one 2009; 4: e7967.
12. Sobel JD, et al. Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. American journal of obstetrics and gynecology 1998; 178: 203-211.
13. Harriott MM, et al. *Candida albicans* forms biofilms on the vaginal mucosa. Microbiology (Reading, England) 2010; 156: 3635-3644.
14. Douglas LJ. Candida biofilms and their role in infection. Trends in microbiology 2003; 11: 30-36.
15. Ricicova M, et al. *Candida albicans* biofilm formation in a new *in vivo* rat model. Microbiology (Reading, England) 2010; 156: 909-919.
16. Kucharíková S, et al. *In vivo* efficacy of anidulafungin against mature *Candida albicans* biofilms in a novel rat model of catheter-associated candidiasis. Antimicrobial agents and chemotherapy 2010; 54: 4474-4475.
17. Schinabeck MK, et al. Rabbit model of *Candida albicans* biofilm infection: liposomal amphotericin B antifungal lock therapy. Antimicrobial agents and chemotherapy 2004; 48: 1727-1732.
18. Andes D, et al. Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. Infection and immunity 2004; 72: 6023-6031.
19. Lazzell AL, et al. Treatment and prevention of *Candida albicans* biofilms with caspofungin in a novel central venous catheter murine model of candidiasis. The Journal of antimicrobial chemotherapy 2009; 64: 567-570.
20. Nett J, et al. Beta-1,3 glucan as a test for central venous catheter biofilm infection. The Journal of infectious diseases 2007; 195: 1705-1712.