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Fungal Biofilms: In vivo models for discovery of anti-biofilm drugs

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SUMMARY

During infection, fungi frequently transition to a biofilm lifestyle, proliferating as communities of surface-adherent aggregates of cells. Phenotypically, cells in a biofilm are distinct from free-floating cells. Their high tolerance of antifungals and ability to withstand host defenses are two characteristics that foster resilience. Biofilm infections are particularly difficult to eradicate and most available antifungals have minimal activity. Therefore, the discovery of novel compounds and innovative strategies to treat fungal biofilms is of great interest. Although many fungi have been observed to form biofilms, the most well-studied is *Candida albicans*. Animal models have been developed to simulate common *Candida* device-associated infections, including those involving vascular catheters, dentures, urinary catheters, and subcutaneous implants. Models have also reproduced the most common mucosal biofilm infections, oropharyngeal and vaginal candidiasis. These models incorporate the anatomical site, immune components, and fluid dynamics of clinical niches and have been instrumental in the study of drug resistance and investigation of novel therapies. This chapter describes the significance of fungal biofilm infections, the animal models developed for biofilm study, and how these models have contributed to development of new strategies for eradication of fungal biofilm infections.

SIGNIFICANCE OF FUNGAL BIOFILMS IN INFECTION

Many fungal and bacterial pathogens build biofilms, establishing resilient communities on a variety of clinical surfaces (1, 2). Biofilm formation has become increasingly appreciated as one of the most common modes of growth. Medically, these adherent conglomerates of cells pose a serious obstacle for treatment of infection. Compared to non-biofilm, planktonic cells, they are extraordinarily tolerant to anti-infective therapies and resist killing by host defenses (3). Biofilm formation has been well-described for *Candida albicans*, the most common fungal pathogen in the hospital setting (4, 5). More recently, the majority of clinically encountered fungi have been shown to produce biofilms. This group includes filamentous fungi (*Aspergillus*, *Fusarium*, zygomycetes), *Pneumocystis*, and yeasts (*Blastoschizomyces*, *Saccharomyces*, *Malassezia*, *Trichosporon*, *Cryptococcus*, and numerous *Candida* spp.) (Table 1) (6–16).

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One of the distinguishing traits of biofilm communities is their ability to adhere to a surface. In the medical setting, indwelling devices, such as catheters, provide an ideal niche for biofilm formation (1, 17). As technology advances, the use of medical devices has continued to escalate. At least 35 million devices are implanted yearly in the United States alone and the majority of hospital-acquired infections are device-associated (18). Many types of devices are at risk for biofilm infection, including catheters, dentures, implants, pacemakers, artificial heart valves, and central nervous system shunts (1, 18). Biofilm infections may be catastrophic, resulting in device malfunction or life-threatening, systemic infection (1). Candidiasis in the hospital setting most frequently involves biofilm infection of a medical device. *Candida* spp. are the 4th most common nosocomial bloodstream pathogens and the 3rd most common cause of urinary tract pathogens (19–22). While biofilm infection was initially described for *C. albicans*, the majority of *Candida* spp., including *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis*, have now been shown to cause biofilm infections (23).

Mucosal candidiasis is widespread. Vaginal candidiasis affects approximately 30–50% of women and many suffer recurrent infections (24). The clinical relevance of biofilms on biotic surfaces has become increasingly evident and mucosal biofilms have been described for *Candida*. In an animal model of oropharyngeal candidiasis, *Candida* forms a biofilm of yeast, hyphae, and commensal bacterial within the epithelial surface (25). *Candida* biofilms growing on the vaginal epithelial lining similarly demonstrate a typical biofilm architecture with adherence cells embedded in an extracellular matrix (26). Mucosal biofilms appear to have many similarities to biofilms growing on abiotic surfaces, including sessile growth, protection from environmental factors, and variable access to nutrients (27). However, biofilms on a mucosal surface participate in a dynamic interaction with the adjacent epithelial lining. The host epithelial lining may deliver immune component, nutrients, and antifungal components. Therefore, the basal aspect of a mucosal biofilm would be expected to be exposed to a vastly different environment when compared to a biofilm on an abiotic device surface.

Like *Candida*, other fungal pathogens, including *Cryptococcus* and *Aspergillus*, have been found in biofilms adherent to abiotic device surfaces (15, 28). (For review, see Martinez, Casadevall and Latge, Beauvais chapters in this volume). However, for *Aspergillus*, the biofilm mode of growth is most commonly observed in the absence of a foreign body. Even in the absence of a device, *Aspergillus* has been shown to proliferate as a cellular aggregate encased in extracellular matrix with properties similar to device-associated biofilms (28). Clinical examples of these non-device associated biofilms include fungal sinusitis, pulmonary aspergillosis and aspergilloma (7). As the biofilm lifestyle of *Aspergillus* is a relatively new field of investigation, further investigations are needed to explore this growth mode.

FUNGAL BIOFILM TRAITS

Structure

Fungal biofilms are comprised of adherent cells covered by an extracellular polymeric matrix. The process of fungal biofilm formation *in vitro* was initially described for *Candida*

albicans in three main stages (29). First, during early biofilm formation, *Candida* cells adhere to the biofilm substrate. For *C. albicans*, germ tube formation may be elicited. During intermediate biofilm formation stage, the extracellular matrix begins to appear and covers proliferating fungal colonies. Finally, mature biofilm formation is marked by extracellular matrix encasing the adherent yeast and developing hyphae. It is now recognized that dispersion is a key component of the dynamic nature of biofilms (30). Release of cells from biofilms is a regulated process by which organisms can disseminate throughout the host and establish new sites of infection. This developmental process appears to hold true for *in vivo Candida* biofilms (31, 32).

For *Aspergillus*, aerial, static conditions promote biofilm formation (33). Under these conditions, *A. fumigatus* grows as a cohesive mycelial structure. The cells become progressively encased in a hydrophobic extracellular material over time. Ultrastructure analysis reveals air channels embedded within the conglomerate (33). These multicellular communities, surrounded by acellular matrix material, also develop *in vivo* in a murine model of pulmonary aspergilloma and invasive aspergillosis (7).

Tolerance to antifungals

Biofilms are notoriously difficult to treat in the clinical setting and their physical removal is often required for eradication of infection (34). Available antifungal medications are seldom effective given the high tolerance of biofilms to these commonly used anti-infective therapies. *Candida* biofilms proliferate in the face of antifungal concentrations up to 1,000 fold higher than those needed to inhibit non-biofilm, planktonic cells (29, 35–37).

The biofilm lifestyle of *C. albicans* is associated with resistance to available drug classes compared to activity against planktonic cells (29, 35, 38–42). Resistance to the azoles (fluconazole, itraconazole, voriconazole, posaconazole) is particularly pronounced, while the echinocandins (caspofungin, micafungin, anidulafungin) and liposomal amphotericin are more effective. Various mechanisms have been shown to contribute to this resistant phenotype, including the production of an extracellular matrix, an increase in efflux pump activity, alteration of sterols, production of resistant “persister cells”, activation of stress responses, and an increase in cell density (40, 43–50).

Biofilms formed by *Aspergillus* and *Cryptococcus* also display this multi-drug resistance (8, 33, 51, 52). For *Aspergillus* biofilms, antifungal tolerance has been linked to the presence of an extracellular matrix, as well as an increase in efflux pump activity (52–54). Few investigations examined the mechanisms underlying the antifungal resistance of *Cryptococcus* biofilms, but this phenotype has been correlated with production of melanin (51).

Immune resistance

In addition to the well-described drug resistance, biofilm growth also appears to afford protection from the host immune response (55–58). Compared to planktonic cells, both neutrophils and mononuclear cells are less effective in killing *Candida* biofilm cells. Mononuclear cells become entrapped in biofilms, but do not efficiently activate or

phagocytize fungal cells (57, 59) Neutrophils have impaired function against both *C. albicans* and *C. parapsilosis* biofilms (55, 56, 58). Although most studies involving the leukocyte response to fungal biofilms have been limited to *in vitro* studies, the phenotype appears clinically relevant. An intact immune response is not sufficient to clear *Candida* biofilms. When coupled with antifungal therapy, improved clinical outcomes are observed when *Candida*-infected medical devices are removed (34, 60). Studies examining the innate immune response to *C. neoformans* biofilms also demonstrated a resistant phenotype. *In vitro*, these biofilms are more tolerant of defensins and oxidative stress when compared to planktonic cells (61).

HOST FACTORS INFLUENCING FUNGAL BIOFILMS

Fungal biofilms form in a variety of clinical niches. These sites of infection can vary quite significantly with regard to available nutrients, flow conditions, immune components, pH, and the substrate for cell adhesion and initiation of biofilm growth (Figure 1). Each of these factors is likely to influence the biofilm properties and structure, as discussed below. Models most closely mimicking the clinical niche are necessary to best reproduce the host environmental conditions and ultimately a clinical biofilm infection.

Flow conditions

One of the greatest environmental differences among the sites of common fungal biofilms is the flow conditions. For example, *Candida* can form a biofilm in the face of a low rate of salivary flow (denture stomatitis), a rapid current of blood (endocarditis) or an intermittent flow (vascular and urinary catheter infection) (18). The Douglas group investigated the influence of flow conditions on *C. albicans* biofilm architecture using *in vitro* models (62, 63). Compared to biofilms grown in static conditions, those propagated in a continuous flow device were encased in a higher concentration of extracellular matrix. The continuous-flow biofilms also exhibited increased resistance to antifungals, including amphotericin B and fluconazole. Investigation of continuous flow by an independent laboratory confirmed the drug resistance phenotype, as well as the altered biofilm structure (64). Biofilms formed in the flow environment were more dense and compact. One might expect that this architectural change to greatly impact other aspects of biofilm physiology, such the availability of oxygen and nutrients.

As described above, *Aspergillus* has been shown to form biofilms during invasive pulmonary aspergillosis and pulmonary aspergilloma (7). In the lung, these biofilms proliferate in a static, aerial environment. Unlike *Candida* biofilms, the extracellular matrix production is mostly supported by these low flow conditions (33). The variability in key properties, including matrix production and drug resistance, of biofilms formed under a variety of flow conditions points to the importance of using models that best fit a particular physiologic niche.

Substrates and conditioning

One of the most influential factors for biofilm initiation is the substrate for adherence (4, 65). Although many materials have been shown to support biofilm formation, the

topography and hydrophobicity of the substrate may greatly impact fungal adherence. Indwelling medical devices are often designed to resist microbial adherence. Compared to other plastics, these materials, such as silicone, may even require a preconditioning, or protein coating, for robust biofilm formation *in vitro* (66, 67). However, *in vivo*, medical devices are rapidly conditioned with host factors from the surrounding fluids, such as blood, saliva, urine, or other fluids (68–72). Several of the factors that may coat various devices, influencing adherence and biofilm formation, include fibrinogen, fibronectin, vitronectin, thrombospondin-1, albumin, and von Willebrand factor (VWF) (68–72). As the concentrations of these substances differ among clinical niche sites, *in vivo* models best account for the conditioning of medical devices prior to biofilm initiation. When considering mucosal biofilms, substrate representation becomes even more complex. Here, the epithelial layer of cells provides a surface for microbial adhesion which also involves receptor-ligand interactions (27).

Nutrient composition

Biofilm niche sites vary greatly with respect to availability of nutrients. For example, blood is a fairly nutrient-rich environment, while urine has a lower abundance of sugars and proteins. In addition, the conditions may be altered by both medical illness and diet. Untreated diabetes mellitus raises the glucose content throughout the host while a diet high in sugar primarily raises the glucose content in the oral cavity. The carbon source (galactose or glucose) and abundance can greatly influence biofilm integrity for both *C. albicans* and *C. neoformans* (65, 73). Another factor shown to influence *Candida* biofilm formation is the concentration of metal ions (Ni^{2+} , Fe^{3+} , Cr^{3+}) (74). These variables can impact the rate of biofilm growth, production of extracellular matrix, and the strength of the biofilm. Animal models of biofilm infection which utilize an equivalent anatomical site provide the ideal composition of nutrients and minerals to mimic patient biofilm infections.

Host immune components

Mounting evidence suggests a complex interaction between host immune cells and fungal biofilms. For example, leukocytes, important for controlling fungal infections, have also been shown to promote biofilm growth (59). *In vitro*, *C. albicans* biofilms were observed to proliferate in response to a soluble factor released by mononuclear peripheral blood cells. Ultimately, mononuclear cells became entangled within the basal level of a *C. albicans* biofilm and were not able to phagocytize the biofilm cells. When examining oral mucosal *Candida* biofilms, Dongari-Bagtzoglou et al. observed the migration of neutrophils throughout the biofilm (25). Host immune cells appear to incorporate into biofilm, even augment biofilm growth, but are most often unable to contain the infection. To understand how the immune system impacts the biofilm lifestyle, models encompassing immune components at the site of infection are optimal.

IN VIVO MODELS OF FUNGAL BIOFILMS AND DRUG DISCOVERY

Animal models best integrate the influence of host factors on the formation of biofilms and acquisition of their phenotypic traits. Utilization of animal models incorporates not only the influence of the immune system, but also niche-specific factors, such as the flow conditions,

nutrients in the environment, and the substrate or surface of adherence. As *Candida* has served as a model organism for fungal biofilm infection in this arena, models involving this pathogen will be much of the focus of discussion in this chapter (Table 2 and Figure 2).

Vascular catheter model

Perhaps the most commonly used animal model for *in vivo* biofilm study is the venous catheter model. This model has been adapted for use in a rat, a rabbit, and a mouse and has been instrumental for examining the efficacy of antifungals against biofilms formed *in vivo* (31, 75, 76). As a close mimic one of the most common clinical biofilm infections, biofilms on the luminal catheter surface are exposed to host conditions, flow, serum proteins, and immune components. The model involves surgical vascular catheter insertion (often jugular vein) followed by subcutaneous tunneling and securing with a protective device. Performing the procedure prior to luminal inoculation allows for a period for host protein conditioning of the device surface. The influence of anti-infectives on biofilm growth can be assessed following systemic administration of drug or by instilling in the lumen as a lock therapy. Common techniques include microscopy for evaluation of biofilm extent and architecture or viable burden determination (Figure 3). Results of these studies have corroborated the multi-drug resistant phenotype of *in vitro* *Candida* biofilms and the need for discovery of new strategies and drugs to treat these infections (31).

One approach to circumvent the drug tolerance of biofilms is to directly administer antifungal in the form of lock therapy (75, 77–79) (Table 3). By avoiding the majority of systemic toxicities, significantly higher drug doses may be safely delivered. Vascular catheter animal models have been valuable for analyzing the efficacy of various antifungal lock therapies against *Candida* biofilms *in vivo*. When instilled in the lumen of *C. albicans* infected catheters, several lock therapies were found to successfully treat the biofilm infections. Of the clinically available antifungals, the more efficacious solutions have included liposomal amphotericin B (10 mg/ml), amphotericin B lipid complex (5 mg/ml), and caspofungin (6.67 mg/ml) (75, 77–79). As might be predicted by *in vitro* biofilm susceptibility studies, the azole drugs and lower doses of echinocandin drugs have significantly less activity against vascular catheter biofilms and are not ideal for catheter lock therapy (75, 76). One concern regarding use of available antifungals for lock therapy is the potential for fostering a resistance-promoting environment. As a method to avoid this possibility, studies have also explored the use of alternative agents, such as biocides. Encouraging results have been observed *in vitro* for ethanol, ethylenediaminetetraacetic acid (EDTA), ethanol, and high dose minocycline (3 mg/ml) lock solutions (80–82).

A second tactic to overcome the profound antifungal tolerance of biofilms is delivery of combination drug therapy. The rat vascular catheter biofilm infection model has successfully been used to evaluate the *in vivo* efficacy of several combination therapy lock solutions (48, 49). These studies have explored the impact of adding agents targeting cellular stress responses on azole drug resistance. Uppuluri *et al.* demonstrated the efficacy of combining calcineurin inhibitors and fluconazole for lock therapy treatment of *C. albicans* biofilms (48). An agent inhibiting the calcineurin pathway (tacrolimus) was found to augment the activity of fluconazole against *C. albicans* catheter biofilms. Subsequent investigations

suggest calcineurin inhibitors similarly potentiate the activity of agents in other drug classes, including the echinocandins and amphotericinB (83). Robbins *et. al* also used a rat venous catheter model to test the efficacy of combination lock therapy (49). Combining an inhibitor of the Hsp90 pathway 17-AAG) with fluconazole improved the activity against *C. albicans* biofilms. The mechanism of this action is thought to involve a decrease in biofilm extracellular matrix, limiting the capacity of the matrix to sequester antifungal.

Denture model

Denture stomatitis involves biofilm formation on a denture surface and inflammation of the adjacent oral mucosal surface (84, 85). These infections are common, occurring in up to 70% of denture-wearers, and are often quite painful, even impairing the ability to eat. Biofilms are frequently polymicrobial with *Candida* spp. playing a key role. Several models have been developed to explore the pathogenesis and treatment of denture stomatitis (32, 86, 87). Early models included primarily Macacairus monkeys with custom-fitted acrylic plates and Wistar rats fitted with prefabricated acrylic devices (88–90). The focus of these investigations was examination of the mucosal inflammatory process associated with the infected device. *Candida*-infected animals with oral devices were observed to develop mucosal lesions similar to those seen in patients with denture stomatitis (88–90). Although both models were useful for describing the host response to denture biofilms, the rat model was more suited for drug efficacy studies, primarily related to cost. In these investigations, the incorporation of either chlorhexidine or miconazole to the denture acrylic material prevented the development of mucosal lesions of palatal candidiasis (88, 89). However, the chlorhexidine product was poorly tolerated with rat undergoing weight loss from poor dietary intake.

With the discovery of the role of biofilms in device-associated infections, there has been renewed interest in animal models to mimic denture stomatitis (32, 87, 91). Two models have been developed to replicate this clinical scenario in rats. In the first model, a Sprague-Dawley rat undergoes placement of an acrylic dental device over the hard palate, which is secured in place by orthodontic wire (32). As the device is fitted to the individual rat, there is close approximation of the device with the oral mucosa and this space can be inoculated with *Candida* to produce a biofilm device infection and associated mucosal inflammation over the course of 24–72 hours (Figure 4). This model represents an acute infection in the setting of immunosuppression, as rats are treated with a single dose of cortisone prior to infection. In a second rat model, Wistar rats are custom fitted to palatal acrylic devices retained by orthodontic wires (87, 91). However, a portion of the device is secured by embedded magnets and is easily removable throughout the experimental course. Following inoculation of *Candida*, biofilm develops on the device surface over weeks. In addition, mucosal biofilm infection and inflammation ensue, mimicking clinical infection occurs. As the devices can remain in place for an extended time (8 weeks), this model offers the opportunity to longitudinally follow the course of an individual animal with a chronic infection.

Studies have only just begun to explore the antifungal treatment in the rodent denture models. As might be predicted from clinical scenarios and other infection models, the *C.*

albicans biofilms on the denture surface were found to exhibit high tolerance of both fluconazole and micafungin upon either topical or systemic administration (32). The model has also been helpful for exploring the role of gene products on denture biofilm infection *in vivo*. Chen *et al.* described the importance of the calcineurin pathway for *C. dubliniensis* in the processes of both filamentation and biofilm formation in a rat denture biofilm (92). This suggests that calcineurin inhibitors may be a viable option for treatment of *C. dubliniensis*. As drugs in this class exert synergistic activity with azole and echinocandin drugs, combination therapy is an attractive possibility for treatment of *C. dubliniensis* biofilm infections (92).

Subcutaneous implant model

To study the activity of a novel antifungal formulation against biofilms, Zumbuehl *et al.* developed a murine model of subcutaneous *Candida* biofilm infection (93). Disks containing amphogel, a dextran-based hydrogel loaded with amphotericin B, were inoculated with *C. albicans* and surgically implanted in the subcutaneous flank tissue of BALB/c mice. After 3 days, *Candida* had been cleared from the surface of the disks containing amphogel. In contrast, control disks with hydrogel only were coated with *C. albicans* biofilm and host cells. The amphogel was well-tolerated, eliciting only a minimal or mild inflammatory response. As this antifungal hydrogel maintains efficacy for over 50 days, it is ideally suited for prevention of device-associated infection.

As an alternative, simpler model for study of *Candida* biofilm infections, Riciova *et al.* fabricated a subcutaneous catheter implant model. In this model, a polyurethane catheter segment is inoculated with *Candida* and implanted under the skin of a rat (94). Over the course of 1–6 days, a multi-layer biofilm consisting of both yeast and hyphae forms on the catheter surface. Compared to the vascular catheter model, this procedure is less invasive and requires a shorter period of anesthesia. In terms of mimicking patient infection, the model has similarities to both vascular catheter infections and wound infections. The implanted catheter material is a close mimic of the vascular catheter material used in patients. The model is avascular, so biofilm cells are not subjected to blood flow conditions and not exposed to the same concentrations of serum protein and blood cells. However, the devices can be treated with serum prior to implantation to partially mimic this exposure. The anatomical location of the implantation is most similar to a biofilm wound infection. The model allows for the interaction between host immune components and *Candida* biofilms in the subcutaneous tissue. One clear advantage is that the procedure allows for placement of multiple segments of catheter for potential comparison of several *Candida* strains or conditions within the same animal.

The subcutaneous implant model has also proven to be efficacious for evaluation of anti-biofilm treatments. Bink *et al.* used this model to test the efficacy of combining a non-steroidal anti-inflammatory drug (NSAID) and an echinocandin for treatment of *C. albicans* biofilms *in vivo* (95). NSAIDs impair prostaglandin synthesis by targeting mammalian cyclooxygenases. Agents in this class are available for the treatment of pain and inflammation. However, the activity of this drug class is not limited to mammalian systems, as they have also been shown to disrupt filamentation and biofilm formation in *C. albicans*, likely though

inhibition of prostaglandin E2 synthesis (96, 97). To examine the impact of disrupting this pathway *in vivo*, rats received diclofenac treatment in the setting of subcutaneous catheter implant infection (95). In rats that had been treated with diclofenac prior to development of *C. albicans* biofilm infection, the anti-biofilm activity of caspofungin was enhanced. It is of great interest to identify an available drug class able to potentiate the activity of echinocandins. However, the animals in the study received diclofenac prior to infection and whether or not it would be a helpful adjuvant later in the course of infection is not known.

Urinary catheter model

To investigate catheter-associated urinary tract infections and candiduria, Wang *et al.* developed a murine model representing this clinical scenario (98). In this model, a guide wire is inserted through the urethra of a female mouse and a catheter segment is threaded over a guide wire and into the bladder. The segment is secured by suture through the bladder wall. After 5–7 days, the animal is infected with *C. albicans* intravesicularly. Candiduria is detectable quickly after infection and persists for 28 days. A dense biofilm of adherent yeast and hyphae forms on both the luminal and extraluminal surfaces. To increase the susceptibility to *Candida* infection, mice lacking lysozyme M, an important effector for mucosal innate immunity, can be utilized. The model closely mimics patient *Candida* biofilm formation with regard to the use of biofilm substrate (catheter) and anatomic location (bladder). As only a segment of catheter is in place, the flow conditions are likely less than would be observed for a patient catheter functioning to drain the bladder. The model incorporates the mammalian immune system with the option of using wildtype or immunocompromised animals. To our knowledge, this model has not yet been used for investigating the activity of anti-biofilm therapies.

Mucosal candidiasis models

Biofilms have frequently been described in association with medical devices and abiotic surfaces. However, there is mounting evidence that *Candida* spp. exhibit similar characteristics when growing on mucosal surfaces (25, 26). Murine models of both oropharyngeal and vaginal candidiasis demonstrate that *Candida* produces conglomerates of yeast, hyphae, and extracellular material associated with mucosal surfaces. In an oropharyngeal candidiasis model, the biofilms appear to be complex, involving commensal bacteria, neutrophils, and keratin (25). A murine model of vaginal candidiasis shows *C. albicans* regulators of biofilm formation on abiotic surfaces are similar to those required for development of vaginal biofilms. Although mucosal biofilms share many characteristics with device-associated biofilms, it is not clear they exhibit the same degree of drug resistance. Clinically, mucosal biofilms are most often responsive to antifungal therapies, including azoles (99, 100).

Fusarium keratitis

Outbreaks of *Fusarium* keratitis have prompted interest in models to investigate this contact lens-associated biofilm infection (101). Two murine models have been developed to investigate the pathogenesis of this process (102, 103). Sun *et al.* demonstrated the ability of *F. oxysporum* to form a biofilm on the surface of a contact lens and for this to induce

keratitis on an injured mouse cornea (102). The model has successfully been implemented for study of the host aspects of *Fusarium* keratitis. Zhang *et al.* developed a similar model of murine fungal keratitis employing *F. solani* (103). Although this model also involves inoculation of an injured cornea, the organisms are directly seeded and no contact lens is involved. The model utilizes fluorescently-labeled fungi for visualization of the infective process.

FUTURE DIRECTIONS

Recognition of the importance of animal models for the discovery of anti-biofilm drugs has only recently emerged. Most investigations have focused on *C. albicans* as a model pathogen and the vascular catheter models of biofilm infection have been the most popular. It will be interesting to see how biofilms formed under the conditions of other clinically relevant niches respond to antifungal therapies. The murine urinary catheter model, the rat subcutaneous model, and rat denture models will be of great value for these investigations for *Candida* (32, 87, 94, 98). The ocular lens model should be similarly useful for identification of preventative and therapeutic compounds for fungal keratitis. The models allow for testing of anti-biofilm compounds under physiologic conditions very similar to those encountered clinically. In addition to these animal models of device associated infections, models of mucosal *Candida* biofilms will surely be helpful for study of these common infections (25, 26).

As it is becoming increasingly clear that infections caused by diverse fungal pathogens involve biofilm communities, animal models of these infections will be beneficial for pathogenesis and drug discovery studies. Models are underway for several of the fungal pathogens. One example, discussed earlier, is *Fusarium* keratitis in the setting of contact lens biofilm infection. Murine models have been developed to study both host and pathogen aspects of this process and could be utilized to evaluate novel anti-biofilm therapies in this unique clinical niche (102, 103). Also, *Aspergillus* spp., including *A. fumigatus*, have been shown to produce fungal aggregates during pulmonary infection (7). Models mimicking aspergilloma or invasive aspergillosis will be helpful for exploring the impact of antifungal treatment on this mode of growth.

Although *C. albicans* has been the model pathogen for many *in vivo* biofilm investigations, the *in vivo* biofilm models can likely be adapted to biofilm infections caused by a variety of non-*albicans* *Candida* spp. and other yeasts, such as *Cryptococcus*. Of note, the rat vascular catheter model has been successfully used for study of *C. parapsilosis* and *C. glabrata*, while a rat denture model has been employed for investigation of *C. dubliniensis* (92, 104).

There are many approaches to the discovery of new anti-infectives. One strategy is to screen large libraries of compounds. Using *in vitro* models, the mining of pharmaceutical and natural product libraries has identified novel compounds with antibiofilm activity (105–107). An alternative approach is to determine a mechanism leading to drug resistance and identify or develop an anti-infective that disrupts the process. For *C. albicans*, the biofilm property most closely linked to resistance is the extracellular matrix. Enzymatic degradation of key matrix components, such as extracellular DNA and β -1,3 glucan, has been shown to

enhance antifungal activity, suggesting these as potential drug targets (67, 108). In fact, a therapy directed at extracellular DNA degradation has shown to be a beneficial for patients with cystic fibrosis. It is thought dornase alfa (Pulmozyme), a clinically available inhaled enzymatic treatment, works by degrading extracellular DNA of bacterial biofilms (109). Regardless of the path of drug discovery, animal models will be beneficial for testing the efficacy of compounds against clinical biofilms and establishing safety.

One of the unique aspects of exploring anti-biofilm activity of drugs in animal models is the opportunity to vary the mode of antifungal delivery. For example, compounds may be systemically administered, topically administered, coated on a device, or embedded in a device. Another interesting delivery method is direct administration of a gel with prolonged elution of high antifungal concentrations, such as was developed for amphotericin B (110). Systemic administration is feasible to test in all models, while direct, topical administration of a compound is easily achievable in either the denture models via topical therapy or the venous catheter models via lock therapy. The subcutaneous tissue model may be ideal for exploring the utility of embedding or coating a device with an anti-biofilm compound, as numerous devices can be tested in a single animal. Another potential application is the investigation of vaccine efficacy, such as the NDV-3 vaccine in clinical trials, vaccines found to be efficacious in non-biofilm models of infections, or future vaccines designed specifically to inhibit the biofilm mode of growth (111–113).

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HOST CONDITIONS INFLUENCING BIOFILMS

Figure 1. Host factors influence fungal biofilm formation

This schematic depicts host conditions that may impact fungal biofilm formation and architecture. *In vivo* animal models can closely mimic biofilm infection niches, incorporating many of these host and environmental conditions.

Figure 2. Animal models of fungal biofilm infection

Rabbit venous catheter-associated *Candida* biofilm infection (A) (75). Rat venous catheter-associated *Candida* biofilm infection (B) (31). Mouse contact lens-associated *Fusarium* biofilm model (C) (102). Mouse urinary catheter-associated *Candida* biofilm infection (D) (98). Rat denture-associated *Candida* biofilm infection (removable intraoral device)(E) (87). Rat denture-associated *Candida* biofilm infection (32). Images adapted from prior publications (32, 75, 87, 98, 102, 114).

Figure 3. *C. albicans* biofilm infection of rat jugular venous catheter

C. albicans was instilled in the lumen of a subcutaneously tunneled jugular venous catheter and allowed to dwell for 6 hours. After a growth period of 24 hours, the catheter was harvested, fixed, and dehydrated. Catheter segments were imaged by scanning electron microscopy on a JEOL JSM-6100 at 10 kV (50× and 1000×). The biofilm is composed of both yeast and hyphae encased in an extracellular matrix. Host components, including red blood cells, appear to associate with the biofilm.

Figure 4. *C. albicans* biofilm infection in a rat denture model

C. albicans was inoculated between the hard palate and an acrylic device secured with orthodontic wire. After a growth period of 48 hours, the denture was harvested, fixed, and dehydrated. Oral devices were imaged by scanning electron microscopy on a JEOL JSM-6100 at 10 kV (50× and 1000×). The biofilm is composed of both yeast and hyphae encased in an extracellular matrix. Larger, host cells were observed as well. Microbiologic

evaluation identified a polymicrobial infection consisting of *C. albicans* and various bacteria.

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Table 1

Medically important fungi forming biofilms

<i>Aspergillus</i>
<i>Blastoschizomyces</i>
<i>Candida</i>
<i>Cryptococcus</i>
<i>Fusarium</i>
<i>Malassezia</i>
<i>Pneumocystis</i>
<i>Trichosporon</i>
<i>Zygomycetes</i>

Table 2

Animal models of *Candida* biofilm infection

Central venous catheter	Rat (31) Rabbit (75) Mouse (76)
Urinary catheter	Mouse (98)
Subcutaneous implant	Mouse (93) Rat (94)
Denture stomatitis	Rat (32, 87)
Oral mucosal	Mouse (25)
Vaginal mucosal	Mouse (26)

Table 3

Strategies for treatment of *Candida* biofilms with demonstrated efficacy in animal models

Lock therapies	liposomal amphotericin B (10 mg/ml) (75) amphotericin B lipid complex (5 mg/ml) (77) caspofungin (6.67 mg/ml) (78)
Combination therapies	calcineurin inhibitor (tacrolimus) and fluconazole (48) Hsp90 inhibitor (17-AAG) and fluconazole (49) Diclofenac (NSAID) and caspofungin (95)