Fungal Biofilms in Human Disease

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Craig Williams and Gordon Ramage

1 Introduction

Fungal biofilms are an important clinical problem. The widespread use indwelling medical devices, broad spectrum antibiotics and an aging and more immuno-compromised patient population has created an opportunity for yeasts and moulds to form infections in the form of biofilms. This chapter will discuss the diversity and importance of fungal biofilms in different anatomical areas, provide insights into the management of fungal biofilm infection, explain why biofilms may be difficult to treat with antifungal therapy, and discuss how our current level of knowledge may lead to different treatment interventions.

A biofilm is composed of microorganisms attached to surfaces or one another and enclosed within an extrapolymeric matrix. The biofilm mode of growth is the preferred form of growth of microorganisms and account for up to 65 % of all clinical infections. This mode of growth gives the organism a number of advantages including high level antimicrobial resistance which may cause problems for the clinician attempting to treat such infections (Donlan and Costerton 2002). Over recent years there has been a growing appreciation that pathogenic fungal species both have the ability to form biofilms and that these biofilms may impact clinical practice (Ramage et al. 2009; Sayed et al. 2012; Fanning and Mitchell 2012).

Fungi can be broadly divided into yeasts and moulds and in terms of the number of infections, *Candida albicans*, a normal commensal of human mucosal surfaces and opportunistic pathogen in immunocompromised patients, is the most clinically important of fungi species in terms of the production of clinically relevant biofilms. This dimorphic fungus exists in both yeast and hyphae forms which results in a structurally complex biofilm. This begins with yeast cells attaching to a relevant surface using defined adhesins, such as the agglutinin-like sequence protein Als3p and the GPI anchored cell wall protein Eap1p (Zhao et al. 2006; Li et al. 2007). The next step is the formation of a microcolony with yeast cells undergoing morphological switching to pseudo- and true-hyphae under the regulatory control of Efg1p (Ramage et al. 2002) which results in the rapid formation of a meshwork of hyphae interspersed with budding yeast cells. As the biofilm matures it becomes enclosed in a glucan rich polymeric matrix (Nett et al. 2010a) which provides protection from host defences and treatment with antifungal agents. Within the biofilm there are a range of niches and in hypoxic areas, Tye7p controlled

C. Williams (✉)
Institute of Healthcare Associated Infection, University of the West of Scotland, Paisley, UK
e-mail: Craig.Williams@ggc.scot.nhs.uk

G. Ramage
Infection and Immunity Research Group, Glasgow Dental School and Hospital, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

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up-regulation of glycolytic genes, which influence filamentation, occurs (Bonhomme et al. 2011). Flow of fluids across the surface of the biofilm may then result in the dispersion of daughter cells which attach to a new substrate and the cycle starts again (Uppuluri et al. 2010). This entire process is controlled by transcription factors, such as Bcr1p, Ace2p, Efg1p and Zap1p, which are involved in precisely regulated molecular pathways (Zhao et al. 2006; Finkel and Mitchell 2011; Nobile and Mitchell 2006; Fanning et al. 2012).

From a clinical point of view it is important as an understanding the basis of adherence, proliferation, maturation and dispersal both forms the basis for all other pathogenic fungal biofilm studies and signposts important potential new targets for clinical interventions in these infections. This chapter will review fungal biofilms and their clinical importance, discuss why these infections may be so difficult to treat, and provide evidence for potential novel strategies to improve clinical management.

2 Where May Fungal Biofilms Be Important?

2.1 Oral Cavity

The oral cavity represents one of the major portals of entry for microorganisms, and is a site in which the presence of multispecies microbial biofilms has been widely studied both in the presence and absence of foreign materials (Jakubovics 2010). Within the oral cavity, caries, periodontal disease, endodontic infection and mucosal infections all involve microbial biofilms (Beikler and Flemmig 2011) and *Candida* species are the important fungal pathogen (Rautemaa and Ramage 2011). The oral cavity is a good environment for biofilm growth for a variety of fungal species. In a study of 20 healthy individuals Fungal microbiome analysis of the oral cavity, using a pyrosequencing approach, identified 74 culturable and 11 non-culturable fungal (Ghannoum et al. 2010). *Candida* species were shown to be the most prominent genera in this group (75 %), followed by *Cladosporium* (65 %), *Aureobasidium* (50 %), *Saccharomycetales* (50 %), *Aspergillus* (35 %), *Fusarium* (30 %), and *Cryptococcus* (20 %). Overall 101 species were present and each individual had from 9 to 23 different fungal species. While these results demonstrate the potential diversity of fungi in the mouth there remains the possibility that DNA from these fungal species was ingested or inhaled.

Nevertheless, yeasts clearly exist within the oral cavity and form biofilms. Oral candidosis, best defined fungal biofilm infections of both soft and hard tissue in the mouth forms complex biofilms in association with host components and bacteria (Rautemaa and Ramage 2011; Dongari-Bagtzoglou et al. 2009). *Candida* species have been isolated from periodontal pockets, orthodontic appliances, enamel, dentures and mucosal surfaces (Dongari-Bagtzoglou et al. 2009; Ramage et al. 2004; Sardi et al. 2010; de Carvalho et al. 2006; Arslan et al. 2008). Where *Candida* species are isolated from subgingival mixed species biofilms in patients with severe chronic periodontitis (Canabarro et al. 2012) there is a clear correlation between disease severity and both the quantity and species of yeast cells isolated with *C. albicans* being found in high numbers from those with moderate and severe chronic periodontitis. However a causal relationship has yet to be demonstrated and the presence of *Candida* species may simply represent poor oral health.

Interestingly there seems to be a relationship between Yeasts and other bacteria in oral biofilms. Another metagenomic analysis of elderly patients showed that increased candidal load favoured the presence of oral streptococci (Bamford et al. 2009) a potential mechanism for this has been suggested in mixed *C. albicans* and *Streptococcus gordonii* mixed biofilms where growth is enhanced through specific cell-cell interactions involving both Als3p and the surface protein adhesion SspB (Silverman et al. 2010). In addition these physical interactions chemical interactions have also been shown to influence biofilm formation (Bamford et al. 2009). Complex biofilms are a key mode of survival within the oral cavity and this process may influence both oral and systemic health (Coulthwaite and Verran 2007).

When foreign materials such as dentures are present in the mouth biofilms are also important in the causation of denture stomatitis (Nett et al. 2010b). This is characterized by *Candida* species forming on a denture prosthesis (Pereira-Cenci et al. 2008) usually associated with the upper fitting denture, where the biofilm forms on the surface adjacent to the oral mucosa (Ramage et al. 2004). *C. albicans* is the most frequently isolated yeast from the denture, but *Candida glabrata*, *Candida dubliniensis*, *Candida tropicalis*, *Candida krusei* and a range of other *Candida* species have been isolated (Coco et al. 2008; Williams et al. 2011). The level of inflammation of the palate ranges from localised areas of erythema to diffuse areas of severe erythema (Newton 1962). In this condition again the species of yeast present seems to be important in that those with severe inflammation preferentially cultured *C. albicans* (Coco et al. 2008). Further in vitro analysis of these strains showed a positive correlation between the severity of disease and secreted aspartyl proteinase (Sap) release and expression when the organism was grown as a biofilm (Ramage et al. 2012), which has also been shown with strains isolated from type 1 diabetes patients (Rajendran et al. 2010). These proteolytic enzymes have been shown to be present in various in vivo studies (Naglik et al. 2003, 2004, 2006) but it is not possible to attribute a causal role as no single Sap plays a predominant role in mucosal invasion (Lermann and Morschhauser 2008; Naglik et al. 2008). It is possible however to suggest that Sap proteins play a role in proteolytic cleavage of the mucin Msb2, which activates the Cek1 MAPK pathway and induce filamentation (Puri et al. 2012) which is known to play a key role biofilm development and stabilisation (Ramage et al. 2002) which suggest a physical and regulatory role for proteolytic enzymes in *C. albicans* biofilm production.

It is clear that there is interaction between yeasts and bacteria, however synergistic interactions between different *Candida* species within a biofilm have also been proposed as a pathogenic mechanism. Coco et al (Coco et al. 2008) showed that *C. glabrata* and *C. albicans* are often co-isolated from patients, particularly those with severe inflammation. He suggested that as *C. glabrata* does not produce hyphae and therefore forms relatively structurally poor and unstable biofilms, that it was possible, in mixed yeast biofilms, that *C. glabrata* was using *C. albicans* as a structural scaffold to gain entry to the host. This has now been confirmed in another study where *C. albicans* appears to assist the invasive capacity of *C. glabrata* within an in vitro reconstituted epithelial biofilm model (Silva et al. 2011). Further studies using in vivo models to investigate the pathogenesis of denture stomatitis would be useful in this context (Nett et al. 2010b).

2.2 Upper Airways

Sinusitis (or rhinosinusitis) is defined as an inflammation of the mucous membrane lining the paranasal sinuses. It may be acute or chronic however subacute, and acute exacerbation of chronic diseases have also been described and as all types have similar symptoms it is often clinically difficult to distinguish these. Around 90 % of adults have had some symptoms of sinusitis at some time. There is a growing appreciation that chronic rhinosinusitis is typified by biofilm growth (Foreman et al. 2011; Keir et al. 2011; Ebbens et al. 2009a). While there is increasing evidence for the role of bacterial biofilms in this infection, there role of fungi remains controversial (Ebbens et al. 2009b). Paranasal sinus fungus balls have been described (Grosjean and Weber 2007; Karkas et al. 2012), which share some of the features of fungal biofilms (Harding et al. 2009; Mowat et al. 2009). In a recent study of 118 patients with chronic sinusitis, nasal discharge, headache and visual disturbance, over a 14 year period 23.7 % had a sphenoidal fungus ball in which *Aspergillus fumigatus* and *Aspergillus nidulans* hyphae were observed microscopically (Karkas et al. 2012). Other fungi have also been implicated including *Schizophyllum commune* (Chowdhary et al. 2013; Sa et al. 2012), *Trichosporon inkin* (Janagond et al. 2012), *Mucorales* (Mignogna et al. 2011), and *Fusarium* (Rombaux et al. 1996). In terms of infections associated with foreign bodies *A. fumigatus* infection within

the maxillary sinus associated with a zygomatic implant has been reported (Sato et al. 2010). Experimental studies have shown that *A. fumigatus* biofilms form in a primary human sinonasal epithelial model (Singhal et al. 2011) and in a sheep model of induced sinus biofilms *A. fumigatus* readily forms biofilms often associated with *Staphylococcus aureus* (Boase et al. 2011). These data suggest that fungal biofilms, alone or more likely in mixed species biofilms with other organisms, may play a role in sinus infection however there is little evidence to support the role of fungi in other upper airway biofilm infections such as otitis media (Bakaletz 2007; Martin et al. 2005; Yao and Messner 2001).

When foreign bodies are present, such as head and neck related prostheses polymicrobial biofilms containing *Candida* species have been reported (Ariani et al. 2012). Biofilms including *C. albicans* and *C. glabrata*, have also been extensively described in voice prosthesis biofilms (Buijssen et al. 2012; Ell 1996) where they have been shown to bind to salivary proteins (Holmes et al. 2006), and are often found co-aggregated with bacterial species (Kania et al. 2010). Clinically they restrict airflow (Elving et al. 2001), impede speech, swallowing and respiration (Sayed et al. 2012). Other fungal species such as *Fusarium solani* species complex (Honraet et al. 2005) and *Cryptococcus neoformans* (Bauters et al. 2001) have also been described.

The finding of fungal biofilms on speech prostheses may also be of relevance in terms of the pathophysiology of Ventilator associated pneumonia (VAP). Studies have shown that the isolation of *Candida* species isolated alone or in combination from respiratory secretions in those with suspected VAP are associated with increased mortality compared to those with only bacteria isolated, an unadjusted odds ration of 2.9 (Delisle et al. 2011). In addition *Candida* colonisation has been associated with an increased risk of isolation of multi-drug resistant bacteria (Hamet et al. 2012). The mechanisms for this are unclear but it is possible that yeasts form the basis of multi-species biofilms which effect the pathogenicity of other yeasts or bacteria contained in the biofilm. It is also possible that the incidence of fungi within these VAP samples may be due to previous treatment with broad-spectrum antibiotics, but in VAP following cardiac surgery 30.19 % of patients were culture positive for fungi, including *C. albicans* (16.97 %), *Pneumocystis jirovecii* (3.77 %), *C. glabrata*, *Candida sake*, *C. krusei*, *Geotrichum capitatum* and *Cryptococcus humicola*, (1.89 % each) (Serban et al. 2010).

Care bundles which include measures that may reduce or prevent the growth of biofilms such as oral decontamination with chlorhexidine, have dramatically reduced the rates of VAP in the intensive care setting (Stonecypher 2010; Caserta et al. 2012).

2.3 Lower Airways

Lower respiratory tract infection may be due to biofilm infection, the archetype of which is *Pseudomonas aeruginosa* in cystic fibrosis patients (Singh et al. 2000). It is also now recognised however that fungal biofilms present in the lung may also contribute to infection.

Filamentous fungi, mainly *A. fumigatus,* may cause a spectrum of respiratory disease including from a discrete lesion in a pre-existing cavity, aspergilloma, wheezing mediated by an immune response, allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis (IA) (Denning 1998). A bronchopulmonary lavage (BAL) of these individuals often reveals the presence of numerous intertwined hyphae in the form of a complex multicellular structure when examined histologically (Jayshree et al. 2006), this is indicative of a biofilm phenotype (Harding et al. 2009; Mowat et al. 2009). The recently described Aspergillus bronchitis may also be biofilm associated and is characterized by bronchial casts containing mycelia forming compact masses (Young et al. 1970). It is clear that *Aspergillus* species form medically important biofilms (Gutierrez-Correa et al. 2012; Ramage et al. 2011) and understanding their clinical role in is crucial, as with all biofilms, these structures are highly resistant to antifungal therapy (Mowat et al. 2008; Seidler et al. 2008).

A number of fungal species including *Aspergillus* spp., *Scedosporium* spp. and *Exophiala* spp. have been isolated from different cohorts of CF patients (Blyth et al. 2010; Cimon et al. 2000; Kondori et al. 2011). Given the ubiquitous nature of moulds within the environment, and with thousands of conidia being inhaled every day (Richardson 2009), it is unsurprising that pathogenic fungi can adhere, colonise and form complex multispecies biofilms in lungs with abnormal clearance mechanisms such as CF however their pathogenic role has not yet been fully elucidated. A number of recent studies have reported that lung function declines more rapidly in patients co-infected with *A. fumigatus* and *P. aeruginosa when* compared to single-species infection (Amin et al. 2010; Gangell et al. 2011), this has also been reported with *Candida* species and *P. aeruginosa* (Chotirmall et al. 2010). Evidence is therefore increasing for the improved clinical management of these patients (Delhaes et al. 2012).

There is a suggestion that interactions in mixed eukaryotic prokaryotic biofilms (polymicrobial infections) in the CF lung may lead to adverse clinical outcomes (Leclair and Hogan 2010). It has been shown that *P. aeruginosa* is able to both form biofilms and kill *C. albicans* in the hyphal form but not the yeast form (Hogan and Kolter 2002) possibly through the release of a phenazine toxin (Gibson et al. 2009; Morales et al. 2010). Pseudomonas has also been shown to inhibit the morphological transition of yeast through a 3-oxo-C12 homoserine lactone (Hogan et al. 2004) which has also been demonstrated in studies of *A. fumigatus* biofilms (Mowat et al. 2010). Further evidence of eukaryotic/prokaryotic interaction comes from the fact that the release of farnesol, a quorum sensing molecule of *C. albicans* impacts by inhibiting its quinolone signalling and subsequent pyocyanin production in *P. aeurginosa* (Cugini et al. 2007). These studies highlight potential battles going on within a polymicrobial environment such as the CF lung, which plays a crucial role in the overall pathogenesis of disease (Peters et al. 2012) exemplified by studies in a Drosophila model of polymicrobial infection in which microorganisms from CF showed a different outcome depending on the presence or absence of *P. aeruginosa* (Sibley et al. 2008a, b).

3 Gastrointestinal and Urinary Tract

The mucosa of the gastrointestinal (GI) tract is heavily laden with bacterial microbiota, growing as healthy biofilm communities (Macfarlane and Dillon 2007). Clinically they present a problem, for example when they are located in the stomach of those with percutaneous endoscopy gastronomy (PEG) feeding tubes for enteral nutrition, or in the large intestines in diseases such as ulcerative colitis (Macfarlane 2008). *C. albicans* and *C. tropicalis* have been shown to colonise these PEG tubes and contribute to degradation of the polyurethane (Trevisani et al. 2005; Gottlieb et al. 1993). Clinically this may lead to diarrhoea, or possibly cause translocation of microbes across the epithelial barrier, leading to sepsis.

Candida spp. colonisation of the GI tract is common, accounting for 30–80 % in normal healthy adults (Damman et al. 2012). Chronic colonisation may lead to GI candidisasis, which in immunocompromised individuals may lead to systemic candidiasis. Whilst little direct work has focussed on fungal biofilm in the GI tract per se, this environment is largely a polymicrobial biofilm, and interactions between yeasts and bacteria are likely to exist and play a role in health and disease. In fact, it has been suggested that *Candida* colonization may enhance inflammation in the GI tract (Kumamoto 2011). Investigations of *Escherichia coli* and *C. albicans* co-infection have reported synergistic virulence when grown together (Klaerner et al. 1997). Interestingly, in vitro studies have revealed dynamic population changes of these two organisms within biofilms, with a proposed role for lipopolysaccharide (LPS) modulation of *C. albicans* (Bandara et al. 2009). Recently, experimental murine studies have reported that *C. albicans* are able to modulate the bacterial microbiota composition of non-pathogenic species following antibiotic exposure (Mason et al. 2012), suggesting that in health

there is a bidirectional relationship between bacteria and *C. albicans*, rather than simply competitive inhibition by bacteria.

The urinary tract is also a polymicrobial environment, with a diverse metagenome present that is capable of preventing bacterial vaginosis, yeast infections, sexually transmitted disease and urinary tract infections (Ma et al. 2012). High acidity from lactic acid bacterial metabolism is a key mediator of selective inhibition of other species (Gajer et al. 2012). Therefore, control of candidal biofilms may be best achieved through competitive inhibition by bacterial flora, such as lactobacilli, though no definitive studies have focussed in this area yet (McMillan et al. 2011). Nonetheless, it is suggested that 75 % of woman experience vulvovaginal candidiasis at some point in their life, suggesting that *Candida* species are important in this body site. *Candida* species have been associated with pyelonephritis, cystitis and prostatitis (Kauffman et al. 2011; Sobel et al. 2011). *Candida* biofilms have been detected on ureteral stents and have been shown to grow in this lifestyle on experimentally on vaginal mucosa (Reid et al. 1992; Harriott et al. 2010). Urinary catheters are also a significant risk factor in intensive care units for healthcare associated fungal infections (Yang et al. 2013). Moreover, they are commonly detected on intrauterine contraceptives (Chassot et al. 2008). Whilst relatively rare, reports of an aspergilloma also occurs within the urinary tract (Lee 2010; Muller et al. 2011).

3.1 Wounds

Non-healing wounds, such as diabetic foot ulcers (Seth et al. 2012) represent a significant clinical burden to patients, and are associated with the presence of microbial biofilms. *S. aureus* and *P. aeruginosa* are often isolated together in these patients and have been shown to have a non-random association within the wound site (Fazli et al. 2009). Evidence is emerging that pathogenic fungal species may play a role in these infections (Branski et al. 2009).

Wounds acquired in combat situations especially with persistent evidence of wound necrosis often contain fungi with mould isolates found in 83 % of cases (*Mucorales*, n = 16; *Aspergillus* spp., n = 16; *Fusarium* spp., n = 9), commonly with multiple mould species among infected wounds (28 %). Clinical outcomes included three related deaths (8.1 %), frequent debridements and amputation revisions (58 %) (Warkentien et al. 2012).

A metagenomic approach to venous leg ulcers reveals that *C. albicans, C. glabrata* and *Aspergillus* species are present, but intriguingly the authors report that individuals seem to have unique microbial profiles (Wolcott et al. 2009). A further retrospective molecular analysis of 915 chronic wound infections, pressure ulcers, diabetic foot ulcers, non-healing surgical wounds and venous leg ulcers, showed that 208 (23 %) of these contained pathogenic fungi (Dowd et al. 2011). Yeasts were the most abundant fungi (*Candida* spp.), but *Aureobasidium, Cladosporium, Curvularia*, *Engodontium, Malessezia*, *Trichtophyton,* and *Ulocladium* were also. Overall, fungal species represented over 50 % of the microbial burden in the majority of specimens examined but direct evidence that the fungi were present as biofilms is lacking.

There is a potentially interesting interaction between *Staphylococcus* and *Candida.* In the studies above there was a negative association however previous studies have shown a positive biofilm relationship between these organisms, with *S. aureus* using *C. albicans* hyphal biofilms as a scaffold through Als3p, in an analogous way to *S. gordonni* and *C. glabrata* (Silverman et al. 2010; Coco et al. 2008; Harriott and Noverr 2009). Synergistic interaction between these two organisms has been described with respect to mortality in a murine intraperitoneal model (Carlson 1982) which may be due to *S. aureus* upregulating lactate dehydrogenase (Peters et al. 2010).

3.2 Medical Devices

Broad-spectrum antibiotics, parenteral nutrition, immuno-suppression due to chemotherapy and radiotherapy, and disruption of mucosal barriers due to surgery, are among the most important predisposing factors for invasive fungal infection (Odds 1988). *Candida* species predominate and

are the fourth most common cause of bloodstream infection in patients requiring intensive care and the most common etiologic agent of fungal related biofilm infection.

Indwelling medical devices, such as intravascular catheters, become colonized with *Candida spp*. allowing the development of adherent biofilm structures from which cells can then detach and cause an acute fungemia and/or disseminated infection. Experimental studies have reported that cells detaching from the biofilm have a greater association with cytotoxicity and mortality than equivalent planktonic yeasts (Uppuluri et al. 2010). Investigations have therefore begun to investigate whether the biofilm phenotype does play a defined clinical role. In an initial retrospective investigation using multivariate analysis to analyse the risk factors associated with patients with candidaemia it was reported that inadequate antifungal therapy (OR 2.35, P = 0.03), APACHE III scores (OR 1.03, P < 0.001) and biofilm formation (OR 2.33, P = 0.007) were independent predictors of mortality (Tumbarello et al. 2007). Analysis of mortality with biofilm forming ability demonstrated that both *C. albicans* (P < 0.001) and *C. parapsilosis* (P = 0.007) correlated with increased mortality. In a subsequent prospective case-control study by the same group it was shown that *Candida* bloodstream infections caused by biofilm forming isolates could be independently predicted by the presence of central venous catheters, urinary catheters, total parenteral nutrition and diabetes mellitus (Tumbarello et al. 2012). The hospital length of stay and cost of antifungal therapy were also greater in those with biofilm forming isolates, and these patients had a greater risk of hospital mortality (OR 1.77). However in these studies biofilm formation was defined by XTT and spectrophotometric transmittance, rather than a biofilm biomass and/or dry weight (Taff et al. 2012; Kuhn et al. 2003) which may bias the data towards non-albicans species, such as *C. glabrata*, which do not form hyphae (Kuhn et al. 2002a). In fact, it was reported in this study that *C. albicans* biofilm production was significantly less frequent (26.2 % n = 122) than non-albicans species (61.1 % n = 85) (p < 0.001) (Tumbarello et al. 2012), an observation also reported elsewhere (Pannanusorn et al. 2012).

One of the first documented episodes of biofilm related disease associated with *C. glabrata* was in terminally ill patients with intravenous catheters (Valdivieso et al. 1976). Interestingly, although the patients ultimately died, the candidaemia was treated by the removal of the catheter, a practice reported elsewhere in the same era (Berkowitz et al. 1979). Historically, biofilm associations have also been reported in patients with endocarditis (Heffner and Franklin 1978), prosthetic joints (Goodman et al. 1983), peritoneal dialysis (Cecchin et al. 1984), venous catheters (Paige et al. 1987), cannulation (Komshian et al. 1989), ventriculoperitoneal shunts (Walter et al. 1990), in addition to other indwelling devices. Not surprisingly, the presence of an indwelling catheter was a defined risk factor for the development of *C. glabrata* candidaemia (Fortun et al. 2012).

C. parapsilosis is another important species of *Candida* that has been shown to play an important clinical role in biofilm infections. Biofilm development by these organisms is similar to *C. albicans*, sharing the key transcriptional biofilm regulator Bcr1 (Ding et al. 2011). Indwelling catheters in the neonates patient group are an important risk factor for this organism (Pammi et al. 2013). However, prosthetic knees, hip joints and breast implants have also been implicated (Wada et al. 1998; Fox and Lee 2012; Younkin et al. 1984), in addition to a substantial literature on its role on endocarditis of bioprosthetic valves (Wallner et al. 2012; Garzoni et al. 2007). *C. tropicalis* has also received attention in relation to biofilm formation in vitro and in vivo, having been shown to be important in bioprosthetic heart valves and catheter related disease (Mansur et al. 1996; Negri et al. 2012).

Other yeasts and filamentous fungi biofilm related infections have also been increasingly described, including *Aspergillus* (Escande et al. 2011), *Cryptococcus* (Walsh et al. 1986), *Coccidioides* (Davis et al. 2002), *Zygomycetes* (Singh et al. 2011), *Blastoschizomyces* (D'Antonio et al. 2004), *Malassezia* (Cannizzo et al. 2007). *Aspergillus* species have been reported to cause serious biomaterial related biofilm infections, involving catheters, joint replacements, cardiac pace makers, heart valves, and breast augmentation implants (Escande et al. 2011; Langer et al.

2003; Rosenblatt and Pollock 1997; Jeloka et al. 2011; Golmia et al. 2011). *C. neoformans* has been shown to colonize and subsequently form biofilms (Ajesh and Sreejith 2012), cardiac valves (Banerjee et al. 1997), peritoneal dialysis fistulas (Braun et al. 1994), ventricular shunts (Walsh et al. 1986), and prosthetic hip joints (Johannsson and Callaghan 2009). *Malassezia pachydermatis* has been isolated from patients undergoing parenteral nutrition and upon catheters (Cannizzo et al. 2007; Curvale-Fauchet et al. 2004), *Blastoschizomyces capitatus* has been associated with catheter-related fungemia (D'Antonio et al. 2004), and recurrent meningitis has been associated with a *Coccidioides immitis* biofilm at the tip of a ventriculo-peritoneal shunt tubing (Davis et al. 2002). Finally, *Trichosporon* species can cause biofilm-related infections (Agirbasli et al. 2008; Di Bonaventura et al. 2006; Pini et al. 2005), including cardiac grafts (Krzossok et al. 2004), catheters (Ruan et al. 2009), and breast implants (Reddy et al. 2002).

Fungal biofilms are also associated with building fabrics and hospital infrastructure (Richardson 2009; Short et al. 2011; Siqueira et al. 2011; Anaissie et al. 2002).

3.3 Clinical Management

It is clear from the literature that a wide variety of fungi have the capacity to form biofilms on a range of anatomically diverse sites. Arguably the most important reason for their clinical importance is our inability to manage these infections effectively, leading to unacceptably high rates of morbidity and mortality. The following section discusses conventional and novel methods for effective clinical management of fungal biofilms.

4 Conventional Antifungal Approaches

Undoubtedly the most effective and logical way of dealing with clinically important fungal biofilms is to either inhibit their development, use mechanical force to disrupt them or simply remove and replace an implicated medical device. The European Society for Clinical Microbiology and Infectious Disease (ESCMID) have recently produced guidelines discussing the role of catheter associated infection and their clinical management (Cornely et al. 2012). The guidelines indicate that where possible the catheter should be removed. This is supported by clinical data, such as a prospective randomized trial comparing fluconazole to amphotericin B deoxycholate, where removal of a catheter within the first 24 h of candidaemia resulted in a shorter duration of candidaemia (Rex et al. 1995). Conversely, when comparing echinocandins to liposomal amphotericin B the removal of the catheter showed no improved time to mycological eradication, possibly due to the effectiveness of both antifungal agents against biofilms (Nucci et al. 2010). A recent meta-analysis from seven prospective randomized clinical trials has provided some clarity to this, reporting that removal of central venous catheter is associated with decreased mortality (OR, 0.50, 95 % Cl, 0.35–0.72, $p = 0.0001$) (Andes et al. 2012).

Where removal of the catheter is not possible, the use of antifungal therapy should be considered, though unlike bacterial biofilm infections, there are currently no guidelines for treating *C. albicans* associated biomaterial infections with chemotherapeutic agents (O'Grady et al. 2011). However, limited evidence exists for in situ use of antifungal lock therapy (ALT) in fungi. A recent review has highlighted a limited number of case studies that advocate the potential for ALT where clinically appropriate (Walraven and Lee 2013). This small analysis reported 11 studies (20 cases) where *C. albicans* was the most frequently treated ($n = 9$), followed by *C. parapsilosis* ($n = 5$), *C. glabrata* ($n = 4$), *C. tropicalis* ($n = 1$), *C. guillermondii* ($n = 1$), *C. lipolytica* ($n = 1$), *Rhodoturula* ($n = 1$) and *Malassesia furfur* ($n = 1$). Amphotericin B deoxycholate was used most frequently, and was effective in 76.9 % of cases (Arnow and Kushner 1991; Johnson et al. 1994; Krzywda et al. 1995; Benoit et al. 1995; Viale et al. 2001; Angel-Moreno et al. 2005; Wu and Lee 2007). Liposomal amphotericin B was also effective in 60 % (three of five cases) (Castagnola et al. 2005; Buckler et al. 2008).

Caspofungin was used, but only once and was effective against *C. lipolytica* (Ozdemir et al. 2011). In one case ethanol was used as the solitary ALT solution rather than antifungals, which was shown to be effective (Blackwood et al. 2011). ALT was most commonly used for 14 days when negative blood cultures were observed. These studies collectively provide evidence to demonstrate that antifungal ALT for biofilm associated infections is worth considering, with emphasis on using either echinocandins, liposomal amphotericin B or amphotericin B lipid complexes (Cornely et al. 2012), this recommendation relates to evidence from early in vitro studies, where these compounds were shown to be highly effective against *C. albicans* biofilms (Bachmann et al. 2002; Kuhn et al. 2002b). More recently, a number of important studies have been conducted in vitro with various *Candida* species to test the potential in ALT, which have compared a range of antifungal compounds (Walraven and Lee 2013). In these models, caspofungin and micafungin has been shown to have excellent activity, though complete eradication of the biofilm was not demonstrated (Cateau et al. 2008, 2011). Time-dependant killing analysis has recently reported that liposomal formulations of amphotericin B kill significantly quicker than echinocandins (Ramage et al. 2013). Rather oddly, in an independent in vitro ALT study it was reported that azoles (itraconazole, voriconazole and fluconazole) were more effective than both caspofungin and amphotericin B (Ko et al. 2010). This study highlights how biofilm study design can negatively impact interpretation of data during in vitro studies, as it is universally accepted that azoles have little effect on mature fungal biofilms in vitro (Ramage et al. 2013), and in vivo (Andes et al. 2004; Kucharikova et al. 2010).

Animal studies have shown that *C. albicans* biofilms in implanted catheters respond to both caspofungin and amphotericin B formulations (Lazzell et al. 2009; Schinabeck et al. 2004; Mukherjee et al. 2009). Fluconazole (10 mg/ml) on the other hand was unable to salvage any treated catheters, whereas liposomal amphotericin B (10 mg/ml) led to a 100 % success rate (Schinabeck et al. 2004). Finally, comparison of amphotericin B deoxycholate (3.33 mg/ml) with caspofungin (6.67 mg/ml) produced a 81.3 and 100 % catheter salvage success rate, respectively (Shuford et al. 2006).

This has important implications for other fungal biofilm infections, particularly those associated with indwelling devices or on anatomically 'hard-to-reach locations', such as heart valves and orthopaedic joints (Falcone et al. 2009; Dutronc et al. 2010). For *Candida* endocarditis retrospective data suggest that combined antifungal treatment with surgery gives the best outcomes, with prosthetic valve infections having poorer outcomes than native valve infection (Falcone et al. 2009). Prognosis is poor, with 1-year mortality greater than 50 %, combined with substantial relapse rates (Ellis et al. 2001). Valve replacement should be performed as soon as possible, though if prevented liposomal amphotericin B and caspofungin can be used (Boland et al. 2011). There is however an account of successfully treating *A. fumigatus* prosthetic valve endocarditis with oral voriconazole (Reis et al. 2005). Less evidence is available for the treatment of infected hip joints, though it has been reported that voriconazole and amphotericin B have been used together in bone cement to treat a *C. albicans* infected hip (Deelstra et al. 2013). Amphotericin B and fluconazole have also been used to treat a *C. neoformans* infection of a prosthetic hip joint, but this was unsuccessful due to poor penetration through the biofilm (Johannsson and Callaghan 2009). There are suggestions however that fluconazole may have a role in the treatment of candidal prosthetic joint infection (Kelesidis and Tsiodras 2010).

Wound fungal biofilms are managed with surgical debridement (Warkentien et al. 2012). In severe wounds, such as those occurring from combat trauma, liposomal amphotericin B, voriconazole and posaconazole have been used, often as combinational therapy, although the clinical outcomes were variable. Nevertheless, it has been reported that in the management of a case of fungal osteomyelitis combined use of voriconazole and terbinafine along with surgical debridement was able to successfully control a

Scedosporium inflatum infection and salvage the limb (Cetrulo et al. 2012).

These studies suggest that wound fungal biofilms may have a different structural composition, as they respond to azoles more effectively than other fungal biofilms. Many of these infections are polymicrobial, and undergo repeated debridement with topical antiseptics. Moreover, wound dressings containing antimicrobial molecules are used, so it is not surprising that fungal wound biofilms respond to azole therapy in this context.

5 Concluding Remarks

From review of the available literature it is evident that fungal biofilms do play a significant role in clinical medicine. Over 20 different genera of fungi have been implicated in some way of another in clinical biofilm infections, most notably the *Candida* genera. Fungi have been demonstrated to form biofilms on both hard and soft tissue, and upon implanted medical devices. Diagnosing the presence of a fungal biofilm is difficult, with reliance on clinical skill and judgement, along with some key mycological considerations. Removal and replacement of medical devices, or surgical debridement of soft tissue, where appropriate, represents the first line in clinical management, followed by antifungal management. Treatment outcomes vary to conventional antifungal agents, which are largely dictated on by the accessibility of the infection site. Liposomal formulations of amphotericin B and echinocandin antifungal agents show the greatest efficacy against fungal biofilms, whereas azoles are highly ineffective against mature biofilms. Developing methods to augment antifungal activity have been demonstrated experimentally, such as matrix degrading molecules, natural products and microbially derived molecules. Moreover, our knowledge of the how adaptive resistance within the biofilm has revealed therapeutic targets, potentially through the pharmacological depletion of specific molecules involved in these processes. Collectively, these approaches provide a viable platform to successfully manage fungal biofilms of clinical importance. However further consideration needs to be given to how interactions between prokaryote and eukaryote in polymicrobial biofilm infections impact clinical management.

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