# The Pathogenicity of *Aspergillus fumigatus*, Drug Resistance and Nanoparticle Delivery

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<th>Journal:</th>
<th>Canadian Journal of Microbiology</th>
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<td>Manuscript ID</td>
<td>cjm-2017-0749.R1</td>
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<tr>
<td>Manuscript Type:</td>
<td>Review</td>
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<td>Date Submitted by the Author:</td>
<td>27-Feb-2018</td>
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</table>
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| Is the invited manuscript for consideration in a Special Issue?: | N/A |
| Keyword: | *Aspergillus fumigatus*, fungal pathogen, invasive aspergillosis, antifungal drugs, nanoparticle delivery systems |
The Pathogenicity of *Aspergillus fumigatus*, Drug Resistance and Nanoparticle Delivery

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Running Title: *Aspergillus fumigatus* Pathogenesis and Drug Resistance  

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Review Article  
Abbreviations: invasive aspergillosis (IA) 16 times  
chronic pulmonary aspergillosis (CPA) 5 times
Abstract

The genus *Aspergillus* includes fungal species that cause major health issues of significant economic importance. These microorganisms are also the culprit for production of carcinogenic aflatoxins in grain storages, contaminating crops, and economically straining the production process. *Aspergillus fumigatus* is a very important pathogenic species, being responsible for high human morbidity and mortality on a global basis. The prevalence of these infections in immunosuppressed individuals is on the rise and physicians struggle with the diagnosis of these deadly pathogens. Several virulence determinants facilitate fungal invasion and evasion of the host immune response. Metabolic functions are also important for virulence and drug resistance since they allow fungi to obtain nutrients for their own survival and growth. Following a positive diagnostic identification, mortality rates remain high due, in part, to emerging resistance to frequently used antifungal drugs. In this review, we discuss the role of the main virulence, drug target and drug resistance determinants. We conclude with the review of new technologies being developed to treat aspergillosis. In particular, microsphere and nanoparticle delivery systems are discussed in the context of improving drug bioavailability. *Aspergillus* will likely continue to cause problematic infections in immunocompromised patients, so it is imperative to improve treatment options.

**Keywords:** *Aspergillus fumigatus*, fungal pathogen, invasive aspergillosis, antifungal drugs, nanoparticle delivery systems
Introduction

Fungal metabolism plays an important role in the recycling of many types of organic matter that fungi use for energy and nutrition, allowing metabolic versatility between and within fungal species. Fungi can degrade complex carbohydrates, proteins, lipids, and many other polymers and small molecules (Marzluf 1981). Given the promiscuity of energy source substrates, fungi could colonize and parasitize both plants and animals. Fungi can reduce crop yield, as well as indirectly and directly cause disease in people and animals. Various species within the genus *Aspergillus* are able to colonize grain storages, decomposing the grain and in the process producing a very potent known human carcinogen called aflatoxin. Thus, aflatoxin levels in human and animal food sources are highly regulated by the United States Food and Drug Administration (Ferreira et al. 2013; Techapiesancharoenkij et al. 2015).

Some of the more serious fungal diseases in humans and animals occur as invasive pathogenic infections from a few *Aspergillus* species. On a daily basis, people encounter, via spore inhalation, many airborne ubiquitous *Aspergillus* species that may be quickly destroyed by the innate immune system. Otherwise in immunosuppressed patients, the failure of the immune response can directly lead to disease by colonization of the lungs with a potential spread to extrapulmonary locations (Deigendesch et al. 2017). Benign infections by these pathogenic species may manifest as simple allergic reactions that may lead to rhinitis (Chowdhary et al. 2017). However, severe disease may occur in patients that are immunocompromised due to autoimmune diseases, having major organ transplants, diagnosed with cancer or very young/old individuals. These infections can be very severe, as it is estimated that around 3 million people

Importantly, medical treatment options for other diseases have caused a significant increase in invasive aspergillosis (IA) cases over the past 30 years (Tekaia and Latge 2005). Particularly, immunosuppression for organ transplants and aggressive cancer therapies can leave patients at increased risk to develop IA (Jacob Kizhakedathil et al. 2017). This is a major threat to the health of some hospital patients, as the mortality rate for IA infections in 2005 was estimated to be around 60-90%, depending on the patient’s specific situation (Tekaia and Latge 2005). Currently, in spite of progress in the treatment of fungal and associated bacterial infections by narrow and broad range antibiotics, the mortality still remains high in the range of 30-50% in immunocompromised patients (Bitar et al. 2014; Chowdhary et al. 2017; Verweij et al. 2015). However, similarly to viral infections, fungal diseases are more difficult to treat, as both the pathogen and the host are eukaryotic systems. This situation could be problematic as most antibiotics are not effective in treating fungal infections; and pathogen specificity is more difficult to achieve, leading to increased host toxicity. This review focuses on *Aspergillus fumigatus* infections including diagnosis, treatment options, and the emergence of drug resistance. In addition, we discuss promising developing technologies, such as fungicidal nanoparticles (Shirkhani et al. 2015; Xiang-Gen et al. 2011) that are being developed to control this disease.

**General Features of Aspergillus**
Aspergillus belongs to the phylum Ascomycota. A sexual reproductive spore called an ascospore initially distinguished this diverse phylum, which includes cup fungi, morels, yeasts, and molds. However, many of the species within this phylum have no known sexual lifecycle, which leads to low genetic diversity within the species (Lutzoni et al. 2004). Nonetheless, heterothallic (e.g., mating types in different individuals) life cycles have been elucidated under special cultural conditions for Aspergillus fumigatus (Paoletti et al. 2005) and Aspergillus flavus (Horn et al. 2009). Moreover, the recent isolation of an A. fumigatus supermater pair with opposite mating types (MAT1-1 and MAT1-2) has opened the possibility of in-depth genetic studies (Sugui et al. 2011). Moreover, the full sequence has been determined for each of the eight chromosomes (Nierman et al. 2005) of the A. fumigatus haploid genome (29.4 million bp and 9,840 ORFs) and a well-annotated database is readily available at http://www.aspergillusgenome.org/.

Aspergillus includes molds so named because they resemble the shape of a holy water sprinkler, called an aspergillum. Molds are able to grow in multi-cellular, branched structures called hyphae. Aspergillus spp. are fairly hardy organisms that can grow on many carbon and nitrogen sources, is thermotolerant (37-50°C) due to ribosomal biogenesis proteins, and can withstand high osmotic pressure (Jacob Kizhakedathil et al. 2017; Oliveira and Caramalho 2014). Interestingly, these microorganisms exhibit an alternative phenotype, termed “fluffy” growth, under certain stress environments. This pattern is characterized by aerial hyphae growth and lack of sporulation, and can be induced by stressors like 5-azacytidine, farnesol, divalent cations and hypoxia (McCormick et al. 2012; Tamame et al. 1983). As saprophytic molds, Aspergillus spp.
are able to survive typical compost heap temperatures around 70°C, allowing survival at temperatures found in grain storages where they may produce aflatoxins. These toxins, produced most readily by *A. flavus* and *A. parasiticus*, are very potent mutagens known to cause liver cancer in humans and animals (El-Nagerabi et al. 2012). Additionally, several species are directly pathogenic to humans, especially for immunocompromised hosts. The major pathogenic species in this type of infection is *A. fumigatus*, though there are several species that are also capable of causing similar diseases in humans. These species, which may often simultaneously infect the same tissue in the host, include *A. lentulus*, *A. viridinutans*, and *A. calidoustus*. Though some of these species were previously identified as *A. fumigatus*, they may differ in key virulence factors or antifungal resistance (Pelaez et al. 2013).

**Diseases Caused by Pathogenic Aspergillus**

The general term for an infection caused by *Aspergillus* is aspergillosis, and this disease is well documented in birds, domestic animals, and humans. In addition to *A. fumigatus*, aspergillosis is also caused by *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus* (Latge 1999; Walsh et al. 2008). This disease prevalence is relatively low in most domestic animals, though it is a major cause of morbidity and mortality in birds (Tell 2005). Through DNA sequence and mass spectrometry (MALDI-TOF MS) proteome fingerprint analyses, new species of *Aspergillus* closely related to *A. fumigatus* have been identified, including *A. lentulus*, *A. viridinutans*, *A. felis*, *A. pseudofischeri*, *A. fumigatiaffinis*, *A. udagawae*, *A. fumisynnematus*, *A. hiratsukae*, *A. fischerianus*, and *A. novofumigatus* (Almeida and Araujo 2013; Gautier et al. 2016; Nakamura et al. 2017). Though these ‘cryptic’ species are rarely associated with IA, their prevalence is 12% in
clinical samples (Alastruey-Izquierdo et al. 2013). Moreover, due to the high proportion of drug resistant isolates, correct species identification is imperative in clinical practice to avoid incorrect therapy (Alastruey-Izquierdo et al. 2014).

As many of these pathogenic species of *Aspergillus* are found ubiquitously throughout the air, lungs are major colonization sites. *A. fumigatus* is the most commonly encountered pathogenic mold in humans, causing serious complications in susceptible hosts. Upon interaction with Toll-like receptors, especially TLR2 and TLR6 (Wong and Latge 2017), airway epithelial cells ingest and kill the conidia by antimicrobial peptides. Conidia that escape this action are phagocytosed by alveolar macrophages that complete the microbicidal effect. However, melanin on the outer layer of the conidia may suppress macrophage acidification and only after melanin is shed phagosomes are acidified. In addition, the action of neutrophils is also important for pathogen clearance since low numbers of neutrophils render patients at a greater risk for IA. Most individuals are able to quickly eliminate the pathogen by the action of the ciliated epithelium of the upper respiratory tract and pulmonary macrophages that effectively remove conidia that reach the alveoli. Nonetheless, infections due to *Aspergillus* inhalation are still common in immunosuppressed patients as a result of AIDS, and treatments for cancer and organ transplants (Humphrey et al. 2016; Kaur et al. 2017; Kupeli et al. 2015; Latge 1999; Lipovy et al. 2017; Schwartz and Patterson 2018). Even though AIDS deaths overall are slowly decreasing every year, fungal infections overall account for 47% of those deaths (9% for CPA) compared to 30% for tuberculosis in 2015 (Denning 2016; Limper et al. 2017).
The three main manifestations of aspergillosis are the following: allergic bronchopulmonary aspergillosis (Agarwal and Chakrabarti 2013; Agarwal et al. 2010; Tracy et al. 2016), CPA (Agarwal et al. 2012; Huang et al. 2017), and IA (Mayr and Lass-Florl 2011; Tekai a and Latge 2005). For noninvasive allergic bronchopulmonary aspergillosis, the symptoms are usually asthma and possibly bronchiectasis, characterized by hardening and scarring of lung tissue, which may eventually lead to respiratory failure. Understandably, this syndrome is mostly found in asthma, chronic obstructive pulmonary disease, tuberculosis, and cystic fibrosis patients. Fungal infections leading to endophthalmitis have also been reported (Vinekar et al. 2014). CPA progresses slowly and is considered as the phase between invasive and noninvasive disease that can be manifested into several types: aspergilloma, chronic forms (cavitary, fibrotic, and necrotizing), and aspergillus nodules (Jacob Kizhakedathil et al. 2017). An aspergilloma is essentially a clump of Aspergillus found in a body cavity (e.g., sinus) or an organ such as the bronchi or lungs of patients suffering from tuberculosis, cancer, central nervous system diseases, hydatid disease or other chronic infections (Chowdhury et al. 2014; Huang et al. 2017; Kumar et al. 2017; M'saad et al. 2010; McCarthy et al. 2017). The most common symptoms are fatigue, weight loss, breathlessness, and hemoptysis (coughing up blood), but this disease is not usually problematic, as patients frequently show no symptoms and it is difficult to detect. In fact, computed tomography has been used to identify Aspergillus in lung tissue. CPA is often misdiagnosed as pulmonary tuberculosis. IA is frequently regarded as the most deadly form of aspergillosis, usually in the lungs and referred to as invasive pulmonary aspergillosis (Jacob Kizhakedathil et al. 2017). This infection typically begins by
colonizing the lungs and spreads via the bloodstream to other organs anywhere in the body. The symptoms will vary based on the locations where the fungus settles, but typically include fever, chills, blood clots, and breathing difficulties. There are two distinct patterns of IA: discrete nodule necrosis surrounded by hemorrhage and fused lobular consolidation that is similar to bronchopneumonia. As *Aspergillus* is capable of using energy sources from a variety of tissues, it is not surprising that it has been found in the bones and joints, causing severe and difficult to treat conditions. Thus, fungal osteomyelitis is a growing concern for patients with both bone and joint implants (Koehler et al. 2014).

**Virulence Determinants**

*Aspergillus* infections in humans typically occur because of deficient host immunity. However, fungal virulence traits are required for successful pathogenicity (Table 1). First, nutrients need to be obtained as the conidia germinate into hyphae in the lungs. Proteases secreted by these pathogens are usually involved in this nutrient uptake. However, the acquisition of micronutrients, such as zinc, required for enzyme activity, is also necessary. Zinc acquisition through the plasma membrane transporters has been studied, revealing a gene, *zrfC*, shown to be required for the virulence of *A. fumigatus* (Amich et al. 2014).

The genes required for invasiveness have also been studied. Naturally, fungal proteases have been assumed to be the main culprit for *Aspergillus* angioinvasiveness. However, studies have shown that mutants without individual or even multiple proteases have retained their invasiveness. Genetic regulation of these virulence factors may be as relevant. A recent finding has implicated a pH-responsive transcription factor, PacC,
as a positive regulator of both protease secretion and invasion of lung epithelial cells (Bertuzzi et al. 2014). Deletion of pacC drastically decreases these two processes. These types of transcription factors may be useful targets to develop inhibitors for treatment of severe fungal infections.

Once the pathogen is bloodborne, the fungus may find specific microenvironments within various bodily locations. Thus, Aspergillus needs to be able to adapt to these diverse conditions where it may further colonize. Gene products that are expressed at different levels in these microenvironments are then likely to be important for virulence. Though some of these do not fit the traditional definition of virulence determinants, they may still be important for Aspergillus virulence. For example, transitioning from room to body temperature may require distinct gene expression patterns, including a gene coding for the nucleolar ribosomal biogenesis protein CgrA. Inactivation of this gene product leads to impaired growth at 37°C, as the ribosomal machinery cannot assemble fast enough to sustain the levels of growth at this physiological temperature (Bhabhra et al. 2008).

Another potentially important gene is gliP, involved with gliotoxin secretion, a molecule thought to be involved in antagonizing the host immune system. However, removing gliotoxin production by disruption of gliP did not reduce virulence of the isogenic mutant for neutropenic mice (Askew 2008). This suggests that gliotoxin is only a virulence factor when the host mounts an important neutrophilic response, as these phagocytic cells may be targeted by the GliP toxin. Therefore, it is often difficult to determine if these types of virulence factors are absolutely necessary for virulence, or if they simply play some role in the physiology of the microorganism. Nonetheless, these
factors may contribute to the virulence of highly adaptive fungi that may be able to
acclimate in many different bodily environments.

Phospholipases have been implicated as important host membrane disrupting
factors in many pathogenic bacteria and fungi. In A. fumigatus, deletion of the pld gene
greatly attenuated the virulence of the mutant strain in mice immunosuppressed with
hydrocortisone acetate (Li et al. 2012). This finding is consistent with recent reports
demonstrating that the 3,303 bp ORF of the Purpureocillium lilacinum pld gene encodes
a protein of 1,100 amino acids that is orthologous to an ORF from Penicillium oxalicum
and A. fumigatus (Yang et al. 2015). Pld from P. lilacinum was shown to be significantly
upregulated during the infection of eggs from the root nematode Meloidogyne incognita.
In addition, the pld gene is upregulated in media with maltose or L-alanine and certain
environmental conditions, such as acid pH and at temperatures of 27-29°C.

Iron levels are highly regulated in pathogenic microorganisms as iron is both an
essential nutrient and toxic in excess. A. fumigatus SreA is a factor involved in the
repression of ferrous and ferric iron uptake under iron sufficient conditions. Upon iron
starvation, HapX downregulates iron-utilizing pathways such as heme and siderophore
biosynthesis (Haas 2012). Both SreA and HapX are transcriptional regulators acting in
opposite fashion to repress functions that are not needed under either iron sufficient or
deficient conditions. In addition, HapX activates siderophore biosynthesis. Inactivation
of either sreA or hapX genes impairs growth under iron sufficient and deficient
conditions, respectively. However, inactivation of hapX has the major effect on
virulence, as A. fumigatus hapX mutants were shown to be attenuated in a murine
model. Thus, adaptation to low iron environments seems the most important factor for
fungal virulence. This result predicts that mutants deficient in either siderophore synthesis or secretion may also be attenuated. There is also an interesting relation between siderophore and ergosterol biosynthesis (Yasmin et al. 2012). Iron starvation increases demand for siderophore biosynthesis and increases the demand for mevalonate. As this metabolite is also an intermediate in the biosynthesis of ergosterol, iron starvation decreases ergosterol content. However, this effect may be compensated by mutations in the hmg1 gene that may also impinge on voricanozole resistance (see Treatment Options: Antifungal Drugs and Mechanisms of Resistance).

Host factors, such as the pulmonary microbiome, also play important roles in modulating Aspergillus infections (Kolwijck and van de Veerdonk 2014). The predominant view is that colonization of the lung epithelium by diverse bacteria may actually diminish the number of human infections by Aspergillus-related diseases. It still needs to be further explored what is the effect of antimicrobial treatments on the microbiome and how those changes influence the colonization of Aspergillus species.

Host signaling pathways impact infections by various pathogens and have also been shown to be necessary for Aspergillus virulence. In A. fumigatus, the cell wall integrity signaling pathway is mediated by the mitogen activated protein kinase cascade (Valiante et al. 2009; Valiante et al. 2015). This pathway is involved in cell wall maintenance. The gene whose inactivation has the most drastic effect on virulence is mkk2, as the corresponding mutants have increase susceptibility to antifungal drugs and decreased adherence and virulence in mice (Dirr et al. 2010). The cell wall integrity pathway involves protein kinases and it seems intimately connected with glycoprotein receptors that mediate a connection between the cell wall and the cytoplasmic
membrane (Valiante et al. 2015). These receptors may also interact with the Ras proteins (see Treatment Options: Antifungal Drugs and Mechanisms of Resistance) localized to the cell membrane (Al Abdallah and Fortwendel 2015). These proteins may provide the transduction mechanisms to activate the cellular response to external stimuli.

**Diagnosis**

Diagnosis, often determined late, is one of the major challenges with *Aspergillus* infections as CPA has a 15-30% death rate during the first 6 months (http://www.life-worldwide.org/about-us). Computed tomography scans were used for many years, though this method is not very effective for IA diagnosis as the mold is hard to distinguish from other soft tissues (Brown et al. 1998; Caillot et al. 1997). Otherwise, biopsies were performed, though this procedure is difficult to handle for patients who may already be in critical health (Carrafiello et al. 2006). However, there is improved accuracy when combining computed tomography with guided biopsies followed by culturing or additional tests (Lass-Florl et al. 2017). A noninvasive commercial immunoenzymatic assay using a rat monoclonal antibody (Platelia™ *Aspergillus* Ag, Bio-Rad) became available in the United States during 2003, previously available in France in the late 1990’s, for detecting the presence of galactomannan in the *Aspergillus* cell wall in serum and bronchoalveolar lavage fluid from patients at risk for IA (Stynen et al. 1992). Unfortunately, this kit does not work well in solid organ or bone marrow transplant patients and it loses sensitivity in patients treated with certain antifungal agents (e.g., ampicillin, amoxicillin-clavulanate, etc.) which leads to false positives (Bart-Delabesse et al. 2005; Zandijk et al. 2008). Further developments
resulted in a more specific detection assay for *Aspergillus* species using a mouse monoclonal antibody that binds to an extracellular glycoprotein antigen (Chaturvedi et al. 2005; Fenelon et al. 1999; Kumar and Kurup 1993). However, this assay has reduced sensitivity compared to galactomannan, which makes earlier detection difficult. Thus, assays were developed to detect β-1,3-D-glucan in the cell wall, which can be used in combination with the galactomannan assay to increase sensitivity. Several β-1,3-D-glucan commercial kits are available: Fungitell® (Associates of Cape Code), Wako® (Wako Pure Chemical Industries), Fungitec-G (Seikagaku Kogyo), and B-G Star (Maruha) (Hachem et al. 2009; Kawazu et al. 2004; Ostrosky-Zeichner et al. 2005; Theel and Doern 2013). In addition, screening tests to detect anti-*Aspergillus* IgG were developed for rapid and routine diagnosis of IA: ELISA Classic *A. fumigatus* IgG (Serion\Virion), Platelia™ *Aspergillus* IgG (Bio-Rad), and *Aspergillus fumigatus* IgG ELISA (Bordier Affinity Products) (Barrera et al. 2015; Dumollard et al. 2016; Guitard et al. 2012). It is suggested that once a positive sample is found, a Western blot assay, such as the commercialized *Aspergillus* Western blotting IgG kit (LDBio Diagnostics) should be performed to confirm results (Barrera et al. 2015). A new commercial *A. fumigatus* IgM antibody assay (Dynamiker) for the diagnosis of IA did not offer any promising results over current diagnostic methods (Yao et al. 2017). Even though serum galactomannan screening is the most efficient microbiology marker of IA, a pre-emptive bi-weekly monitoring in cancer patients did not result in any significant benefits (Couchepin et al. 2018).

More rapid molecular diagnostic assays became available with the use of PCR; however, results varied depending on quality of DNA/primers, PCR conditions,
machines utilized, sample type such as blood, plasma, bronchoalveolar lavage, etc. (Pham et al. 2003; Spiess et al. 2003; White et al. 2006). More recently, a PCR commercial assay (MycAssay™ Aspergillus, Myconostica) become available for the diagnosis of fungal DNA in the serum and bronchoalveolar lavage fluids of patients at risk of IA. Overall, PCR-based assays have advantages over the galactomannan assay, namely detection at the species level, where timing is critical (hours vs. days), provides higher specificity/sensitivity, and when low fungal levels are present (Guinea et al. 2013; Orsi et al. 2015; Pini et al. 2015; Torelli et al. 2011). Unfortunately, it requires more expensive equipment, false positives and negatives through cross-reactivity can be generated, rigorous standardization and validation methods, and PCR inhibitors can interfere with the results (Morton et al. 2017; Steinbach 2013). Therefore, the PCR assay is recommended to evaluate clinical samples that were previously called positive using the galactomannan assay as two positive tests were needed to confirm diagnosis (Mengoli et al. 2009) and plasma samples are preferred over serum to increase sensitivity (White et al. 2015a).

Other methods under development use mitochondrial single nucleotide polymorphism (SNPs) markers to detect IA in immunocompromised patients (Oliveira and Caramalho 2014; Oliveira et al. 2014). This test has the potential advantage of simultaneous detection, identification, and genotyping of fungal species in samples from patients. Nuclear medicine has also been proposed for diagnosis of IA (Agrawal and Mather 2012). One approach uses radio-imaging technology with radioactively labelled gallium (⁶⁸Ga). Experimental results revealed that A. fumigatus was able to readily take up ⁶⁸Ga siderophores and was effectively used to detect Aspergillus infections via a
positron emission tomography scan. In mouse models, this method could easily detect IA, though it was difficult to diagnose mild infections (Petrik et al. 2012). Further testing and development of this technology could yield a practical method for detecting IA in human patients.

**Treatment Options: Antifungal Drugs and Mechanisms of Resistance**

Further difficulties in IA lie in applying an effective treatment once infections are diagnosed as the optimal therapy is still unknown. Treatment of bacterial agents usually involves finding an antibacterial agent that can be delivered to the site of infection or into the bloodstream before being significantly inactivated. Treatment of pathogenic fungi; however, is more difficult as both pathogen and host are eukaryotic. This leads to a lower specificity and higher host toxicity. Nonetheless, the fungal cell wall, composed of complex carbohydrates (e.g., glucan, chitin, galactofuran, and mannan) and steroids (e.g., lanosterol and ergosterol), provide the basis of selective toxicity for a few drugs that inhibit the biosynthesis of these macromolecules (Valiante et al. 2015). Thus, there are a few antifungal drugs, mechanism-based biologic agents, and small-molecule kinase inhibitors that are available for treating IA and other fungal diseases: corticosteroids, itraconazole, voriconazole, posaconazole, isavuconazole, amphotericin B, caspofungin, micafungin, infliximab, adalimumab, etanercept, certolizumab pegol, ibrutinib, and bevacizumab (Lionakis and Levitz 2017; Sanford et al. 2017). In addition, clinical concerns arise as these agents may not completely eliminate *A. fumigatus* persister cells, or because resistant strains have emerged. Therefore, agent choice is based on *in vitro* susceptibilities and other available data (Sanford et al. 2017). A recent study stressed the importance of routine susceptibility testing to keep up to date on
strain resistance to antifungal drugs on the market (e.g., caspofungin, amphotericin B, itraconazole, voriconazole, and posaconazole) and its impact on therapeutic outcomes (Sabino et al. 2016).

There are currently four classes of compounds to treat IA characterized by their mechanism of action: triazoles (e.g., itraconazole, posaconazole, isavuconazole, and voriconazole), polyenes (e.g., amphotericin B), echinocandins (e.g., caspofungin and micafungin), and allylamines (e.g., terbinafine). Triazoles, the most commonly used antifungal IA drugs, inhibit ergosterol synthesis resulting in fungistatic effects. The lethal effect of amphotericin B has been traditionally thought as inhibiting ergosterol biosynthesis followed by pore formation in the cell membrane (see below in this section for alternative hypotheses). Echinocandins interfere with β-1,3-D-glucan synthesis, leading to a deficient cell wall formation (Buzina 2013). Due to the increased frequency of azole-resistant strains of *A. fumigatus*, allylamines (unsaturated amines) have been recently added as a chemotherapeutic treatment (Oliveira and Caramalho 2014). In susceptible strains, these treatments, particularly the most commonly used drugs like voriconazole and amphotericin B, provide some protection from IA complications.

Untreated IA cases have mortality rates around 80-95%. Patients receiving amphotericin B or voriconazole have mortality rates of 42.1 and 29.2%, respectively, when treated for 12 weeks (Hadrich et al. 2012). These are significant improvements but the mortality rate still remains high. Furthermore, many cases become chronic infections (van de Veerdonk et al. 2017) requiring drug administration for extensive periods. Itraconazole may be useful treating such chronic forms of the disease (Chen et al. 2015), though extensive administration of azoles leads to increased risk of
developing resistance to the already short list of effective antifungal drugs (Howard and Arendrup 2011). If patients cannot endure itraconazole or voriconazole, posaconazole can be taken as a second line treatment (http://www.life-worldwide.org/about-us).

Aspergillus strains with resistant phenotypes/genotypes to the main drugs, especially azoles (Wiederhold 2017), have been isolated within the last 20 years (Table 2). Triazoles target the Aspergillus Cyp51 enzyme, encoded by cyp51A, which is a Cytochrome P450-dependent enzyme involved in the formation of sterols. This inhibition leads to sterol deficiency in the cell membrane and a buildup of toxic sterol intermediates in the fungi, arresting growth. Recent studies have shown that mutations that lead to specific amino acid substitutions in the enzyme target, particularly L98H, render the fungus resistant to triazole antifungal drugs. Snelders, et al. (2011) used structural modeling of the Cyp51 protein to unravel the mechanism through which these amino acid substitutions confer drug resistance. The molecular model of Cyp51 identifies two potential ligand channels important for the docking of triazole antifungal drugs. Though the leucine at position 98 is not located in either channel, it is found in a bridge that is highly conserved in the Cyp51 enzyme family. Furthermore, replacing this leucine with histidine resulted in increased bridge flexibility, decreasing the size of these channels and the binding of these antifungal drugs. A 34 nucleotide tandem repeat (TR34) is also found in the promoter region of the cyp51A gene in Aspergillus resistant strains. This element, especially when combined with L98H, increases the expression of cyp51A significantly, thus conferring resistance by target overproduction (Mellado et al. 2007; Snelders et al. 2011). Similarly, other promoter mutations (TR46) and amino acid substitutions (Y121F and T289A) that may change the shape of the binding channels to
confer resistance to commonly used antifungal drugs (Snelders et al. 2015; Snelders et al. 2010). These mutations have also been associated with resistant isolates from patients in the United States (Wiederhold et al. 2016), Spain (Pelaez et al. 2015), Japan (Hagiwara et al. 2016), Iran (Seyedmousavi et al. 2013), Turkey (Ozmerdiven et al. 2015), and Taiwan (Wu et al. 2015). Likewise, these mutations were related to environmental isolates from flower fields in Colombia (Alvarez-Moreno et al. 2017), greenhouses for vegetables and fruits in China (Ren et al. 2017), and potato and fenugreek fields in India (Chowdhary et al. 2014) that were all treated with fungicides. A few commercial tests have become available to detect A. fumigatus DNA and azole resistance markers. The AsperGenius® (PathoNostics) real-time PCR kit utilizes TR34, L98H, Y121F, and T289A mutations in the cyp51A gene. This kit was validated for sensitive and rapid detection of IA in bronchoalveolar lavage fluid (Chong et al. 2015; Chong et al. 2016) and serum (White et al. 2015b) patient samples. The MycoGENIE® A. fumigatus real-time PCR (Ademtech) assay utilizes 28S rRNA and TR34 and L98H mutations in the cyp51A gene. This kit was validated as having good sensitivity and rapid detection for both serum and respiratory samples from IA patients (Dannaoui et al. 2017; Denis et al. 2018; Morio et al. 2018).

More recently, azole resistance in A. fumigatus has been associated with several target genes in addition to the well-known mutations in cyp51A that nonetheless accounted for about 70% of the drug-resistant isolates (Losada et al. 2015). However, this study did not detect isolates with the tandem repeat in the promoter region as described above. Additional mutations identified included single nucleotide polymorphisms in hmg1 (encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase),
erg25 (encoding C-4 methyl sterol oxidase required for lanosterol biosynthesis), ssc70 (encoding the heat shock Hsp70 chaperone), ganA (encoding a G-protein), and a gene encoding an ABC multi-drug transporter. This study also demonstrated that different fungus mating types do not differ in virulence or drug resistant phenotypes. Further issues on the clinical implications of azole resistance has been recently reviewed (Meis et al. 2016).

Amphotericin B resistance, though prevalent, is not well understood. In Candida albicans, a diploid opportunistic fungal pathogen, overexpression of genes involved in ergosterol biosynthesis (ERG5, ERG6 and ERG25) led to amphotericin B and fluconazole resistance (Barker et al. 2004). Thus, prior studies focused on the role of ergosterol in resistance to amphotericin B in A. terreus, a less prevalent, but now emerging, fungal pathogenic species. Ergosterol content and metabolism has been associated with resistance. One general mechanism in A. terreus may involve the production of accessory conidia. These structures have less ergosterol than the typical phialidic conidia and this lower content have been associated with higher resistance to amphotericin B (Deak et al. 2009). Interestingly, accessory conidia have also been associated with greater adherence and overall pathogenicity (Deak et al. 2011). In contrast, other studies in A. terreus suggested that amphotericin B resistance was not associated with a lack of ergosterol (Dannaoui et al. 2000). Indeed, recent studies with various A. terreus strains seem to indicate that ergosterol content does not play a major role in resistance to this antifungal agent (Blum et al. 2013). These investigators showed that, upon drug treatment, strains susceptible to amphotericin B, as compared to resistant strains, tended to release less amino acids and total protein into the
extracellular medium, and had decreased levels of potassium efflux. This result is contrary to the expectation that the drug main mechanism of action involves membrane pore formation in the mold. In contrast, *A. terreus* resistant strains were able to handle oxidative stress better than the strains that were susceptible to amphotericin B. As this drug was also shown to produce more oxidative damage, and resistant strains of *A. terreus* had significantly higher catalase activity, a potential mechanism for resistance to amphotericin B is the reduction of oxidative damage by the overproduction of enzymes involved in the antioxidant response (Blum et al. 2013).

The mechanism of resistance to echinocandins in *Aspergillus* is even less understood. There has been a reported case of echinocandin resistance in an isolated recovered from a patient receiving therapy for CPA due to *A. fumigatus* (Jimenez-Ortigosa et al. 2017). In this instance, the resistant mutant strain was associated with a point mutation in the fks1 gene that resulted in a glucan synthase highly resistant to the drug. This mechanism was also studied in *C. albicans*, an organism that is more commonly treated with caspofungin than *Aspergillus*. In *C. albicans*, a point mutation in the *fks1* gene involved in the β-1,3-D-glucan synthesis complex is responsible for resistance to caspofungin. Other species of *Candida*, however, have a different mechanism (Kanafani and Perfect 2008). Recent studies indicate that the chaperone Hsp90 is involved in caspofungin resistance in *A. fumigatus* by improving membrane integrity, though the mechanism is not clearly documented. Further research needs to be conducted to determine if targeting Hsp90 is an appropriate strategy for the development of antifungal agents (Lamoth et al. 2014). In summary, the limited choices
of antifungal drugs, along with increasing drug resistance, makes *Aspergillus* a difficult pathogen to treat after diagnosis.

Another interesting recent finding was the discovery of a new glycosyltransferase, encoded by the gene Afu3g03620 (a.k.a. *tft1*), synthesizes unique mixed β-1,3;1-4-D-glucan branches that are added to the core β-1,3-D-glucan and comprise up to 10% of the glucan content (Samar et al. 2015). Its role in glucan formation suggests a function in the mechanisms of action and resistance of caspofungin. Using an improved and elegant system for the creation of unmarked gene deletions in a shorter time span, a *tft1* deletion mutant was generated and characterized (Kieler et al. 2013; Samar et al. 2015). However, the mutant did not reveal any difference in drug susceptibility as compared to the wild type strain, but the mutant displayed a hypervirulent phenotype in worms.

As indicated above (see Virulence Determinants), both the cell wall integrity and Ras pathways are important for virulence and antifungal agent susceptibility. Protein targets in both systems have been proposed for the development of novel antifungal agents (Al Abdallah and Fortwendel 2015; Valiante et al. 2015). Though inhibitors of these pathways may not lead in many cases to fungicidal effects as mutants are of decrease virulence but viable, these compounds may be used in adjuvant therapies, especially if they are synergistic with fungicidal drugs. In this context, the cell wall integrity pathway has been less explored, while the Ras pathway seems to offer a variety of potential targets, especially since inhibitors used in human cancer therapy have been developed. For example, the RasA and B proteins may both be directly targeted (Fortwendel et al. 2008; Fortwendel et al. 2012; Fortwendel et al. 2004). In addition, Ras proteins undergo
complex processes of post-translational modifications including farnesylation, proteolysis, methylation, and palmitoylation that can be targeted for inhibition as well (Al Abdallah and Fortwendel 2015; Norton and Fortwendel 2014). The RhbA Ras-related protein may also provide an additional target as it seems to be important for growth on minimal medium low in nitrogen and virulence in mice, as shown by deletion mutant analysis (Panepinto et al. 2002).

New Frontiers in Treatment: Nanoparticle and Microsphere Delivery Systems

New technologies are being developed to improve outcomes in IA patients. Several issues often compromise the integrity of these treatments. Drugs are generally hydrophobic and degraded in the bloodstream, causing decreased bioavailability. In addition, drugs may not be adequately directed to the sites of infection giving rise to off-target side effects and decreased efficacy. Encapsulating drugs in hydrophilic nanoparticles (≤ 100 nm) may ameliorate these problems. However, there are many challenges in developing nanoparticles for drug delivery that have been reviewed elsewhere (Barnard 2015). These issues include nanoparticle diversity (e.g., carbon, silicon, or diamond based particles), size dispersion, binding properties, biophysicochemical properties (e.g., charge, surface chemistry, and hydrophilicity), and delivery route (e.g., pulmonary vs. parenteral routes). Though several nanomedicines have been approved for human use both in the European Union and the United States, safety issues still remain (Dawidczyk et al. 2014; Etheridge et al. 2013; Zeitoun et al. 2015). One interesting approach to ameliorate toxicity is the green synthesis of nanoparticles using natural polymers such as chitin for nanoparticle coating (Palkhiwala
and Bakshi 2014). Moreover, as this study also suggests, it is not wise to assume that the nanoparticle toxicity equates the toxicity of the bulk form of the product.

Progress has nonetheless been made in the development of nanoparticles for delivery of antifungal drugs. Inexpensive water-soluble polymethacrylic acid nanoparticles may effectively encapsulate amphotericin B for improved drug delivery. Nebulized nanoparticles can be directed to the site of potential infections via inhalation. Recent research has focused on prophylaxis. In mouse models, amphotericin B-polymethacrylic acid nanoparticles were shown to prevent the damaging effects of aspergillosis in the lungs when administered each of the three days prior to infection. Spore germination was effectively halted in mouse models, but this treatment has not been tested in human subjects. Additionally, though this treatment could be effective with solid organ transplant patients, those chronically at risk would not be able to take advantage. However, in cases that it would be effective, this treatment compares favorably to the use of intravenous injections of amphotericin B during several weeks (Shirkhani et al. 2015).

Microspheres are other options to encapsulate chemotherapeutic agents (Rokstad et al. 2014). Even though, microsphere sizes may overlap with large nanoparticles, sizes are in the range of least 500-1000 nm. Thus, though microspheres may share some of the issues regarding nanoparticles, the larger size and the encapsulation procedures tend to lessen these concerns. In the treatment of aspergillosis, significant progress has been done with the use of microspheres. For example, most treatments for aspergillosis recommend using voriconazole (Sanford et al. 2017), as opposed to amphotericin B. In this context, an animal model was developed to test the effect of increasing doses of
voriconazole contained within microspheres. The microspheres were injected into the vitreous humor of rabbits with endophthalmitis and the results indicated that the treatment eliminated the infection and eye inflammation. However, the mechanism for this protective action of these microspheres, was not explored (Xiang-Gen et al. 2011). Further research into this new field of medicine may yield significantly improved treatment for deadly aspergillosis infections in immunocompromised patients.

Conclusions

Aspergillosis, especially by *A. fumigatus* will continue to be a major problem in immunocompromised patients. Future research may explore targeting the virulence factors possessed by *A. fumigatus* to find a selective inhibitor for an effective treatment of IA. Unfortunately, *Aspergillus* species are developing resistance mechanisms to the treatment options that are currently available. Since the mortality rate is as high as 50% in treated individuals, prophylaxis may be a viable option for IA infections (Shirkhani et al. 2015). However, preventative treatment is not always possible. Many patients are constantly at risk of developing aspergillosis, especially in the case of HIV/AIDS or cystic fibrosis patients. Prophylactic treatment cannot be administered constantly, as there may be adverse effects to the patient or resistant strains could develop.

Nanomedicine and microsphere encapsulation are relatively new fields. More research into this area could result in improved treatments, even with the same antifungal drugs that have been used for years. Use of these delivery technologies can increase the bioavailability of the drug, effectively increasing the concentration achieved at the site of the infection. As nanotechnology and encapsulation technologies continue to improve
and concerns on the use of nanoparticles are reduced, it is likely that these treatments may become effective in the therapy of human disease.

Acknowledgements

This review used the database AspGD http://www.aspergillusgenome.org/. In addition, the authors were supported by the United States Department of Agriculture National Institute of Food and Agriculture Hatch/Multi State Project NEB 39-168 and Animal Health Project NEB 39-162. RGB was also supported by the College of Agricultural Sciences and Natural Resources. The authors declare no conflict of interest.
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Table 1. Virulence determinants and metabolic functions that contribute to *Aspergillus* pathogenesis.

<table>
<thead>
<tr>
<th>Gene Encoding Virulence Factor*</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>zrfC (Afu4g09560)</td>
<td>Fungal growth in alkaline and extreme zinc-limiting conditions</td>
<td>(Amich et al. 2014)</td>
</tr>
<tr>
<td>pacC (Afu3g11970)</td>
<td>Transcriptional regulator involved in the activation of virulence factors</td>
<td>(Bertuzzi et al. 2014)</td>
</tr>
<tr>
<td>cgrA (Afu8g02750)</td>
<td>Ribosome biogenesis that contributes to fungal thermal adaptation</td>
<td>(Bhabhra et al. 2008)</td>
</tr>
<tr>
<td>gliP (Afu6g09660)</td>
<td>Potential toxicity to neutrophils</td>
<td>(Askew 2008)</td>
</tr>
<tr>
<td>pld; most likely pld2 (Afu2g16520), based on reported gene size</td>
<td>Host-membrane disrupting phospholipase</td>
<td>(Li et al. 2012; Yang et al. 2015)</td>
</tr>
<tr>
<td>sreA (Afu5g11260)</td>
<td>Negative transcriptional regulator of iron uptake</td>
<td>(Haas 2012)</td>
</tr>
<tr>
<td>hapX (Afu5g03920)</td>
<td>Negative transcriptional regulator of metabolic iron consuming processes; positive regulator of siderophore biosynthesis</td>
<td>(Haas 2012)</td>
</tr>
<tr>
<td>mkk2 (Afu1g05800)</td>
<td>Mitogen-activated protein kinase kinase; cell wall integrity signaling pathway</td>
<td>(Dirr et al. 2010)</td>
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</table>

*Name in parenthesis corresponds to the systematic nomenclature in the AG database: [http://www.aspergillusgenome.org/](http://www.aspergillusgenome.org/).
Table 2. Determinants and metabolic functions that contribute to *Aspergillus* drug-susceptibility and resistance.

<table>
<thead>
<tr>
<th>Gene Encoding</th>
<th>Drug-resistance Determinant†</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cyp51A</em> a.k.a. <em>erg11A</em> (Afu4g06890)</td>
<td>Cytochrome P450-dependent enzyme involved in sterol biosynthesis (14-alpha sterol demethylase); lethal target for triazoles; mutations leading to certain amino acid substitutions reduce drug binding; mutations leading to enzyme overexpression determine resistance; ortholog of ERG11 in <em>C. albicans</em></td>
<td>(Snelders et al. 2010; Snelders et al. 2011; Losada et al. 2015)</td>
<td></td>
</tr>
<tr>
<td><em>erg5</em> (Afu1g03950) <em>erg6</em> (Afu4g03630) <em>erg25</em> (Afu8g02440) (orthologs to ERG5, ERG6 and ERG25 in <em>C. albicans</em>)</td>
<td>Ergosterol biosynthesis: C-22 sterol desaturase (<em>erg5</em>), Delta(24)-sterol C-methyltransferase (<em>erg6</em>) and C-4 methyl sterol oxidase (<em>erg25</em>); <em>C. albicans</em> studies infer that gene overexpression may lead to amphotericin B and fluconazole resistance in <em>Aspergillus</em> (questionable for <em>erg5</em> and <em>erg6</em> gene products)</td>
<td>(Barker et al. 2004; Losada et al. 2015)</td>
<td></td>
</tr>
<tr>
<td><em>hmg1</em> (Afu2g03700)</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme in mevalonate</td>
<td>(Losada et al. 2015)</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Reference(s)</td>
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<tr>
<td><strong>ssc70</strong> (Afu2g09960)</td>
<td>Putative mitochondrial Hsp70 chaperone; a high level of resistance to itraconazole</td>
<td>(Losada et al. 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>ganA</strong> (Afu3g12400)</td>
<td>G-protein involved in cAMP-mediated signaling; multiple azole resistance</td>
<td>(Losada et al. 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>ABC Transporter</strong> (Afu4g14760)</td>
<td>ABC multiple drug transporter; multiple azole resistance</td>
<td>(Losada et al. 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>fks1</strong> (Afu6g12400)</td>
<td>β-1,3-D-glucan synthetase activity and the likely lethal target of echinocandins; SNPs are associated with resistance</td>
<td>(Balashov et al. 2006; Kanafani and Perfect 2008)</td>
<td></td>
</tr>
<tr>
<td>Afu3g03620 a.k.a. <strong>tft1</strong></td>
<td>Putative mixed-linkage glucosyl transferase; hypothesized to be involved in the mechanisms of action of caspofungin, but current evidence suggests otherwise</td>
<td>(Samar et al. 2015)</td>
<td></td>
</tr>
<tr>
<td><strong>hsp90</strong> (Afu5g04170)</td>
<td>Heat-shock chaperone for conidiation and cell wall integrity; enzyme overproduction determines resistance to caspofungin</td>
<td>(Lamoth et al. 2014)</td>
<td></td>
</tr>
<tr>
<td><strong>rasA</strong> (Afu5g11230)</td>
<td>Ras family GTPase proteins</td>
<td>(Fortwendel et al. 2004; Fortwendel et al. 2008; Al</td>
<td></td>
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<tr>
<td>Gene</td>
<td>Activity</td>
<td>Reference</td>
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<tr>
<td>Afu4g10330</td>
<td>Ortholog(s) have protein farnesyltransferase activity</td>
<td>Abdallah and Fortwendel 2015</td>
<td></td>
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<tr>
<td>ram1</td>
<td></td>
<td></td>
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<tr>
<td>Afu3g06470</td>
<td>Zinc ion binding activity; potential palmitoyltransferase activity</td>
<td>Abdallah and Fortwendel 2015</td>
<td></td>
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<tr>
<td>erfD</td>
<td></td>
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<tr>
<td>rhbA (Afu5g05480)</td>
<td>Ras-related signaling protein</td>
<td>Panepinto et al. 2002</td>
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</tbody>
</table>

1 Name in parenthesis corresponds to the systematic nomenclature in the AG database:

http://www.aspergillusgenome.org/.