Chapter 1

Overview of Invasive Fungal Infections

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Abstract

The incidence of invasive fungal infections (IFIs) has seen a marked increase in the last two decades. This is especially evident among transplant recipients, patients suffering from AIDS, in addition to those in receipt of immunosuppressive therapy. Worryingly, this increased incidence includes infections caused by opportunistic fungi and emerging fungal infections which are resistant to or certainly less susceptible than others to standard antifungal agents. As a direct response to this phenomenon, there has been a resolute effort over the past several decades to improve early and accurate diagnosis and provide reliable screening protocols thereby promoting the administration of appropriate antifungal therapy for fungal infections. Early diagnosis and treatment with antifungal therapy are vital if a patient is to survive an IFI. Substantial advancements have been made with regard to both the diagnosis and subsequent treatment of an IFI. In parallel, stark changes in the epidemiological profile of these IFIs have similarly occurred, often in direct response the type of antifungal agent being administered. The effects of an IFI can be far reaching, ranging from increased morbidity and mortality to increased length hospital stays and economic burden.

Key words: Invasive fungal infection, Rapid, Early diagnostics, Antifungal drug resistance

1. Introduction

Fungi are eukaryotic organisms, found worldwide in a wide range of habitats from soil and rotting vegetation to extreme environments such as deserts and deep sea sediments. Although over 100,000 species of fungi have been described, the Kingdom Fungi is estimated to have in the region of 5 million species (1). Classification was traditionally based on morphological characteristics, such as the size and shape of spores or fruiting structures. Species were also distinguished by their biochemical and physiological characteristics. The advancement of molecular tools, such as DNA sequencing and phylogenetic analysis, has greatly enhanced

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our knowledge of the genetic diversity within various taxonomic groups (2, 3). The existence of fungi is crucial to the survival of many organisms with which they form synergistic associations. They can be predators of invertebrates, pathogens of plants and animals and are also of profound importance to man (1). Fungal plant pathogens can destroy crops. For example, in the USA an estimated $200 million is lost due to fungal crop damage annually despite a $600 million yearly spend on fungicides (4). Systemic fungal infections in pets and other animals represent a huge problem for the veterinary clinician, and despite the fact that no precise numbers of incidence are available it is believed that the increase in fungal infections seen in human medicine is actually mirrored in veterinary medicine (5). Fungi are routinely used in basic research as experimental model systems for investigation of animal cell functions, e.g., *Saccharomyces cerevisiae*. Many fungi are manipulated in the food and pharmaceutical industries as major producers of materials such as beer, bread, wine, citric acid and other food additives, and important medicines such as antibiotics (6). For example, *Penicillium chrysogenum* is the primary commercial source of penicillin. Penicillin is the precursor for most ß-lactam antibiotics, which represents one-third of the antibiotic market and $8 billion in annual sales, with annual worldwide production of penicillin estimated to be over 40,000 metric tons (4).

It has become apparent over the decades that a number of fungal species previously considered to be innocuous environmental inhabitants are in fact capable of causing devastating disease in humans. These organisms can be difficult to identify with current diagnostic methods and also have been found to vary greatly in their susceptibility to antifungal agents. This causes major treatment management problems for the clinician (7). The earliest known record of a fungal infection was a mycetoma of the foot in the Indian Atharva Veda (c. 2000–1000 BC), later described in 1714 by the French missionary Ponticharry as “padavalmika” (foot ant hill) (8). There are four recognized types of fungal infections: superficial skin infections (e.g., athlete’s foot, nail infections, and ringworm), superficial mucosal infections (e.g., oral and vaginal thrush), allergic infections (e.g., asthma and chronic sinusitis), and invasive infections (e.g., aspergillosis and fungal pneumonia) (9, 10). Invasive infections are further divided into two, namely, endemic mycoses (usually causing pulmonary disease in otherwise healthy individuals, such as pneumonia) and opportunistic mycoses (the main focus of this chapter, usually nosocomial affecting immunocompromised patients). Examples of endemic mycoses include infection with *Histoplasma capsultum*, *Cocciodiodes immitis*, and *Blastomyces dermatitidis* (11). The importance of diagnosis of endemic fungi is often overlooked and testing is not performed until the patient fails to recover following antibacterial treatment (12).

1.1. Challenges with Fungal Nomenclature

Frequent name changes at all levels of fungal classification, from species level upwards, have been the source of much confusion over the decades. Not surprisingly, this can hinder the accuracy of diagnostics and reporting of species isolated from clinical settings. For example, *Torulopsis* and *Monilia* are both obsolete synonyms for the genus *Candida*. The species *Candida famata* is also known as *Debaryomyces hansenii* and to a lesser extent, *Torulopsis candida* (13–15). Frequently a fungus can have multiple scientific names depending on its lifecycle and sexual state. The sexual form of a fungus is called the teleomorph, and the asexual form is known as the anamorph. Often these forms are physically distinct and have different names, for example, *Candida kefyr* is the anamorph and *Kluyveromyces marxianus* is the teleomorph of this species. An example of a filamentous fungus is *Aspergillus glaucus*, where the teleomorph is called *Eurotium herbariorum* (14). Efforts among researchers are underway to establish the usage of a unified nomenclature in the field of mycology (2, 3). A comprehensive list of medically important fungi, including their synonyms was compiled and published in 1995, and subsequently updated in 1999 for the Journal of Clinically Infectious Disease by McGinnis et al. (13, 14). Web sites such as Index Forum and ITIS compile lists of current names of fungi with reference to older synonyms. A large-scale collaborative project which involved the efforts of many mycologists and taxonomists was published in 2007 (2).

1.2. Challenges with Fungal Diversity and Diagnosis

Recent advances in molecular biology have hugely enhanced our knowledge of fungal diversity and revolutionized fungal taxonomy. Molecular advances, including genome sequencing, provide crucial information on host/pathogen interaction, how the organism reproduces, and how it can persist both in the environment and within the host. It also provides sequence information which can be used to design unique species specific DNA probes for use in new detection systems (4). Molecular biology has lead to different classification systems within the field of mycology. Many challenges have thus been presented pertaining to the emergence of new species, previously thought to be related or part of another species or group. From a diagnostic point of view this presents challenges to the researcher when new species are identified and require differentiation from others. In an attempt to address these classification issues, a comprehensive phylogenetic classification of Fungi has recently been proposed. This was the result of a large multicentre collaborative effort which shows the importance and need for one unified classification system (2).

1.3. Incidence of Opportunistic Fungal Infections

Recent advances in the health care sector have meant that fungal infections are on the increase due in no small part to the expansion of at-risk populations, in addition to the treatment strategies that often result in longer survival rates of these patients (16).

Patients most at risk of opportunistic colonization from an invasive fungal infection (IFI) are those who have undergone solid organ transplant, those in receipt of immunosuppressive or chemotherapeutic agents, those suffering from HIV, pediatric, and elderly patients, and finally, those patients undergoing surgery (17). While bacterial infections are far more prevalent than fungal infections, mortality rates appear to be far higher for fungal infections when compared to bacterial infections (18–20). Filamentous fungi, yeast-like fungi, and dematiaceous fungi are well-known causative agents of IFIs (21); however, *Candida* and *Aspergillus* still remain the most frequently isolated species. The majority of diagnostic tools have been developed with these two organisms in mind (22) as together, *Candida* and *Aspergillus* account for approximately 90% of all nosocomial fungal infections (11). Morbidity–mortality rates for high risk patients suffering from either of these two pathogen groups are in the region of 40–50% for *Candida* while for *Aspergillus* the numbers stand between 80 and 100% (23).

2. Clinically Relevant Fungi

2.1. Medically Important Yeasts

Hippocrates was the first to describe a yeast infection when he wrote of thrush in the fifth century BC (8). Detection of yeast cells in thrush by microscopic techniques did not occur until 1839. It was thought that *Candida albicans* was the only yeast species capable of causing human infection. As advances in the medical field ensued it soon became apparent that other yeast species were also clinically relevant. Despite this, little medical concern was given to them until relatively recently (7). The advent of new cancer treatment regimes, increased use of intravenous catheters, and other medical developments essentially prolonging the lives of immunocompromised patients has resulted in a major shift in the epidemiology of yeast infections and since the 1960s there has been a steady rise in the number of opportunistic yeast species causing severe human infections (7).

2.1.1. Candida Species

Candida species are normal commensals of the skin, mucosa membranes, and gastrointestinal tract of humans and other mammals and are responsible for most nosocomial fungal infections. Of the approximate 100 known species of *Candida*, relatively few (12–14) have been associated with human infection (11, 24). *Candida* species can cause a wide spectrum of disease, from superficial infections such as thrush and nail bed infections to serious life-threatening illnesses such as endocarditis, meningitis, osteomyelitis, and candidemia (11). In the USA, candidemia is the fourth most common bloodstream infection (25).

The route of infection for *Candida* species is typically via intravascular devices or through the gastrointestinal tract (26). Interestingly, one study found that about 70% of hospital personnel harbored yeasts on their hands (27). While there is no doubt that *Candida albicans* is the most frequently isolated *Candida* species from clinical specimens (24), when combined with other *Candida* species, or "*Non-albicans candida*" (NAC) such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, together they account for roughly 99% of all human episodes (28). This is of particular concern since many NAC have intrinsic resistance to current antifungal agents, added to this are the technical difficulties associated with diagnosis as it is thought best to diagnose to species level (11, 29, 30). A retrospective surveillance study conducted in 2000 found that the species responsible for *Candida* bloodstream infections were as follows: *C. albicans* (54%), *C. glabrata* (16%), *C. parapsilosis* (15%), *C. tropicalis* (8%), *C. krusei* (1.6%), and other *Candida* species (4.6%) (25, 30). *C. tropicalis* has been reported to be a common cause of fungemia where risk factors are similar to that of *C. albicans* (24). *C. krusei* has been reported to spread from the gastrointestinal tract of severely immunocompromised patients to cause fungemia and endophthalmitis and is of particular concern because of its intrinsic resistance to some antifungal drugs (11). Although these aforementioned species are the most common NAC species, *C. lusitaniae* and *C. guilliermondii* are emerging as important species causing IFIs (11, 24, 31). *C. rugosa* is a common veterinary pathogen however it has been isolated from wounds of patients in burn units and from both blood and urine of hospitalized patients (11, 32). A number of other NAC species such as *C. famata*, *C. cifferri*, *C. pulcherrima*, *C. utilis*, *C. catenulata*, *C. norvegiensis* have been isolated from clinical samples and account for <1% of candidemia (31).

Research into diagnostics for detection of *Candida* infection has largely focused on systemic disease although methods for diagnosis of vaginal candidiasis have also been evaluated. It is thought that as the use of molecular methods of detection become more widespread that the cost of such tests will reduce over time, increasing their use for diagnosis of superficial infections (33). The diagnosis of invasive *Candida* infections is challenging, symptoms can be nonspecific, and positive cultures may not be obtained until late in the infection (29, 34). The Infectious Disease Society of America recently published guidelines for the treatment of *Candida* infections (35).

2.1.2. Cryptococcus Species

Cryptococcus neoformans is an environmental saprophyte and has a worldwide distribution. An opportunistic human pathogen, it is the most common etiological agent of cryptococcosis. It is found in the environment and clinical setting as budding yeast. Cells are

spherical and are protected by a polysaccharide capsule. This polysaccharide is the diagnostic target for the cryptococcal antigen test. *C. neoformans var gattii* also known as *Cryptococcus gattii* can also cause infection and is found primarily in tropical regions. *C. neoformans* can cause infection in any organ but predominantly infects the CNS and lungs. Pulmonary cryptococcosis, most commonly caused by inhalation of *Cryptococcus* cells, may be asymptomatic or may present with nonspecific symptoms including cough and fever. Cryptococcal meningitis has become widespread in recent times, specifically with the spread of HIV and the use of immunosuppressive drugs. It has become a common opportunistic infection among late stage AIDS patients and solid organ transplant patients and other immunocompromised hosts but also is often reported in individuals who seem to be otherwise immunocompetent. Because of the nonspecific nature of these symptoms, cryptococcal infection should be tested for should symptoms of meningitis occur in the relevant clinical setting. Diagnosis of cryptococcal infection can be through microscopy using Indian ink stain, positive blood or CSF cultures, serology, or histology. Diagnosis is rarely difficult in HIV-associated cryptococcal infection due to the high organism load. But in non-HIV-associated infection diagnosis can be difficult due to false-negative cultures and antigens can result from low organism yield. CT scans, large volume CSF cultures, and lumbar punctures may be needed (36). Rapid and accurate diagnosis is crucial since untreated cryptococcal meningitis is fatal. Treatment is aggressive, usually using a combination of fluconazole and amphotericin B. Unlike *Candida* and *Aspergillus*, enchinocandins have little activity against *Cryptococcus neoformans* (36).

2.1.3. New and Emerging Yeast Pathogens

Since yeast infections are not notifiable diseases, there is no central database available to record isolations of specific yeast species from year to year. As a result of this, it is thought that the actual number or incidence of such diseases is under reported. It has been suggested that case reports in the literature significantly under represent the incidence of infection with emerging yeasts. This is thought to be due to investigators reluctance to publish non-novel data. Nevertheless, organisms emerging as important yeast pathogens include *Malassezia*, *Rhodorula*, *Hansenula*, and *Trichosporon* species. However, this spectrum of organisms is growing (7). *Trichosporon* species normally cause a superficial infection of the hair shaft called white piedra; however, recent reviews have described manifestations of trichosporonosis including severe skin infections, endocarditis, peritonitis, and bloodstream infections. *Malassesia* species are frequently being observed as nosocomial pathogens (11).

3. Medically Important Filamentous Fungi

A number of fungi previously thought to be non-pathogenic, including the *zygomycetes* and haline and dematiaceous molds, are capable of causing opportunistic infections in humans. Although *Aspergillus* species account for most cases of invasive mold infections, a number of other species are being recognized as important pathogens causing devastating and often fatal diseases (17).

3.1. Aspergillus Species

Aspergillus species are ubiquitous in nature, commonly occurring in soil, water, and vegetation. *Aspergillus* species are opportunistic human pathogens and are the most common clinically associated invasive molds, primarily *A. fumigatus*, although *A. flavus*, *A. niger*, and *A. terreus* are increasingly being recognized as important human pathogens (34, 37). Aspergillosis was first described in the 1940s, since then it has become a major problem and is now the leading cause of death among IFIs (38). This enormous increase in invasive aspergillosis (IA) cases has been linked to the ever increasing immunocompromised host population which has resulted from immunosuppressant therapies and advances in medical procedures, previously unheard of. Risk factors associated with aspergillosis include prolonged granulocytopenia, development of graft-versus-host disease, immunosuppressive therapy, use of adrenal corticosteroids, diseases such as chronic granulomatous disease, AIDS, cancer, solid organ, and bone marrow transplants (39). *Aspergillus* species can cause invasive aspergillosis, aspergilloma, chronic necrotising aspergillosis, tracheobronchotic aspergillosis, but colonization without infection can also occur (34). Invasive aspergillosis is associated with a mortality rate of approximately 85%. *A. fumigatus* is the most common species to cause invasive aspergillosis, causing in the region of 90% of cases worldwide (37, 40). The primary route of *Aspergillus* infection is thought to be through inhalation of conidia. Dissemination to other organs is thought to occur following invasion of the lung tissue. Exposure of the immunocompromised host to environmental isolates plays a role in the pathogenesis of this disease (39). These fungi have been isolated in hospitals from air ventilation systems, carpets, and dust dislodged during construction (41). Stringent management and control measures should be paramount. Another proposed route of *Aspergillus* infection is through ingestion of contaminated food, although no outbreak of *Aspergillus* infection has been reported. To date some localized infections have been associated with contaminated wound dressing or tape (11). Like *Candida* species, the most useful way to select an appropriate treatment regime comes from identification

to the species level (30). A suitable rapid diagnostic test for *Aspergillosis* is severely lacking. At present, proof of IA infection can only be shown by growth in tissue or culturing of the fungus from the test specimen, and in many patients, proof is only found at autopsy (42). Diagnosis is currently via CT scan, open lung biopsy, microscopy, and culture (34). Early treatment of invasive aspergillosis is essential. Primary treatment involves Amphotericin B; however, surgery may be necessary for localized infections (37, 43).

4. Emerging Molds

The incidence of infection involving *Fusarium* species has dramatically increased in recent years (44–46). *F. solani* is the predominant species isolated (44). The only antifungal agent effective against *Fusarium* is amphotericin B, unfortunately few patients survive disseminated *Fusarium* infections despite treatment (39). Other opportunistic filamentous fungi often involved in cases of fungemia, disseminated infections, and fungal pneumonia include: *Acremonium* species, *Scedosporium* species, the class *Zygomycetes*, *Paecilomyces* species. Some of these mimic the clinical symptoms of aspergillosis and thus accurate diagnosis is essential (39). *Scedosporium* species are emerging as human pathogens among immunocompromised hosts, in particular, *S. apiospermum* and *S. prolificans*. Such infections are usually difficult to treat because of resistance to current antifungal therapies and mortality rates are extremely high (46). *Zygomycetes* are a class of fungi typically found in soils and rotting vegetation and are increasingly seen as opportunistic human pathogens. Routes of infection can be ingestion, inhalation, or through percutaneous inoculation of spores (47). *Zygomycetes* have emerged as important pathogens in immunocompromised patients particularly those belonging to the order *Mucorales* (e.g., *Mucor* species and *Rhizopus* species). Another order of *Zygomycetes*, the *Entomphthorales*, which are principally insect pathogens, are now frequently implicated in human disease, e.g., *Conidiobolus* and *Basidiobolus* species (48). The increase in incidence of human infection with *Zygomycetes* is thought to be linked to more widespread use of the antifungal drug, voriconazole which has no activity in vitro against the *Zygomycetes* (46, 48). Mortality rates are as high as 80% (47). Successful management relies on early detection. Treatment involves urgent surgical debridement of infected tissue and initiation of suitable antifungal therapy (46–48).

5. Diagnosis of IFIs

Proven, probable, and possible are the three levels of classification used to identify the existence of an IFI. The term proven can be applied to all types of patient, be they immunocompromised or not, however, both probable and possible refer to those patients who have been categorized as being immunocompromised (49). The introduction and acceptance of such standard terminology in defining types of IFI is of critical importance, adding credence to the consistency and reproducibility of clinical studies. Not withstanding the importance of correct classification of an IFI the fact still remains that early diagnosis is vital, and therefore it is imperative that treatment is started as soon as an infection is suspected as any delay in treatment leads to increased morbidity and mortality (50).

5.1. Traditional Methods of Diagnosis

Early and accurate diagnosis of life threatening fungal infections is of paramount importance to allow timely initiation of antifungal therapy and to reduce mortality rates. A clinician faces a myriad of challenges when attempting to accurately diagnose and treat an IFI. Historically, the detection and identification of fungi has depended largely on the more traditional based methods such as histology, microscopy, and culture-based techniques. Although considered the cornerstone of proving the presence/absence of a fungal disease, their diagnostic worth is limited. Despite recent advances in diagnostic methods, microscopic examination and cultivation of clinical samples are still considered to be the "gold standard" method of identification. However these methods are not without limitations, they can result in low sensitivity and specificity, and often only give positive results in the later stages of infection (49).

5.1.1. Culture

Culture-based diagnostic methods are fraught with difficulties, for example, in order to obtain a biopsy to be used for either culture or histopathology from a sterile site such as the lung, an already critically ill patient would need to be subjected to an invasive technique. In addition, when a sample has been proven to be positive, doubt remains over whether the result of a biopsy taken from a non-sterile site is actually down to colonization or due to an active infection (51). Due to its transient nature, blood cultures have been shown to have only a 50% and a 10% success rate for diagnosing candidemia and IA, respectively (52). However, due to the longer incubation times required for growth before starting specific treatment, survival is severely impacted. Similarly, when a sputum or bronchoalveolar lavage (BAL) is taken from a patient

with suspected invasive aspergillosis (IA), frequently the organism cannot be cultured (53). One of the most important roles of the clinician is to determine if a patient requires antifungal treatment in the early period of infection. Therefore it is of paramount importance that a rapid test for early diagnosis is developed (22). Frequently, primary identification media such as CHROMagar Candida media for culturing *Candida* can be used for the initial presumptive identification of some of the most medically important species of *Candida* such as *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*. In addition, the majority of yeasts arising from clinical samples can be identified using commercially available biochemical kits such as API 20C AUX, VITEK 2, and RapID Yeast Plus (54). Despite the user friendly nature of these kits, the identification process does not end here, instead, a suite of additional morphologic based tests must be performed to avoid misidentification of those microorganisms showing identical biochemical profiles (53).

5.1.2. Histopathology

Histopathology is an important diagnostic tool and refers to the microscopic examination of infected tissue. It is a rapid and cost effective method for diagnosing the presumptive or definite presence of an IFI (17). While it is not always possible to retrieve a biopsy from a critically ill patient, this method is further compounded by the fact that several different organisms can display similar histopathological profiles, thereby rendering it almost impossible to identify a specific pathogen based on morphological traits alone. For example, many *Fusarium* sp., *Pseudallescheria* sp., and *Penicillium* are all alike in that they share hyaline and narrow septate hyphae making them indistinguishable from *Aspergillus* in tissue biopsies (55, 56). This inability to definitively identify a specific organism can severely affect the outcome for a patient (57).

5.2. Fungal Cell Wall Markers

When a clinician suspects the presence of an IFI often the patient is treated empirically with antifungal therapies that may include the unnecessary administration of potentially harmful and costly drugs (58). Due to the potentially toxic nature of this approach, interest has increased in the use of what is known as pre-emptive antifungal therapy which refers to the deferred treatment until there is sufficient evidence to confirm the presence of an IFI (59). Therefore, intense efforts have been put into the development of laboratory markers in an attempt to reduce the diagnosis time of an IFI (53). These markers are known as galactomannan (GM) and 1,3-beta-D-glucan (BG) and mannan. This in turn has led to a shift in the way that IFIs can be prevented and treated more efficiently (60).

5.2.1. Galactomannan

Definitive diagnosis of invasive aspergillosis is of the utmost importance to allow for early initiation of antifungal treatment (22). Galactomannan is a cell wall polysaccharide released by *Aspergillus*

species into extracellular fluid during fungal growth in tissue (61). For early diagnosis of IA, GM antigen monitoring has proven its importance as a noninvasive diagnostic tool (62). Before clinical manifestation of IA, circulating GM can be detected anywhere between 5 and 8 days before fungal burden is obvious. Not only is GM detectable in serum or plasma (63) but it can also be detected in BAL (64) and cerebrospinal fluid (65) using a sandwich type enzyme linked immunosorbent assay (53). Following analysis it appears that the BAL fluid assay is more sensitive than the serum assay (66). However, as with other methods of diagnosis this assay is not without its limitations, resulting in contradictory results due to a number of factors including: prior treatment with antifungal therapy affecting the levels of circulating fungal components (67), false-positive results in conjunction with use of antibiotic treatments (68), and finally, the range of cut offs of positivity across different studies (60). According to a recent meta-analysis of serum galactomannan in patients with neutropenia and/or hematologic malignancy sensitivity results of 71% coupled with a specificity of 89% were reported for those definitive cases of invasive aspergillosis (69). It is clear that the diagnosis of invasive aspergillosis should not be based on a single test alone and that the BAL fluid galactomannan assay should be used as an adjunct to further tests.

5.2.2. 1,3 Beta-D-Glucan

1,3 Beta-D-glucan is a component of the cell wall of most fungi which can be detected in the blood during an IFI. There are two notable exceptions, namely, the *Zygomycetes* which do not produce BG and *Cryptococcus* species which release such low levels of BG that it cannot be detected in human serum (70). The test is of significance due to its ability to detect infections caused by such species as *Fusarium*, *Trichosporan*, *Saccharomyces*, and *Acremonium*. While these species are undoubtedly not as common as *Aspergillus* and *Candida*, they are extremely dangerous organisms for the immunocompromised patient (71). Limited data exists as to the efficacy of this test as a diagnostic tool; however, it may useful as an early identifier of infection and is reputed to be highly sensitive (72). Of the commercially available assays, two in particular are most commonly used, namely, Fungitec-G and Fungitell, manufactured by Sikagaku Kogyo Corporation and Associates of Cape Cod, respectively. A possible pitfall of the Fungitec-G assay is that, in the absence of an IFI, medical sources of BG can lead to a positive test. For example, filters and dialysis membranes made from cellulose contain BG, as do cotton gauzes and sponges used in surgery, and some drugs. Unfortunately only a limited number of studies have been performed to date so literature is limited. Those studies that are available have reported sensitivities in the range of 70–100% while specificity ranges anywhere from 76 to 83.8%. Again, these results must be viewed with caution, as only a limited number of invasive aspergillosis cases have been subjected to testing

with this assay (72, 73). Thus, further more intensive testing must be performed before this assay is put forward as a viable diagnostic option (74).

5.2.3. Mannan

Mannan is the major *Candida* cell wall antigen and is the substrate for one of the most extensively studied antigen tests for detection of systemic candidiasis which was proposed as far back as 1979 (75). A vast quantity of literature has amassed over the years, all of the similar opinion that a positive mannan test may actually correlate with invasive candidiasis. As detection of the infection at an early stage is critical, the immunoenzymatic Platelia Candida Ag (Bio-Rad) test is performed in parallel with the anti-mannan antibody test (Platelia Candida Ab/Ac/Ak; Bio-Rad) (76). Mannan occurs at low levels and is rapidly cleared from the bloodstream, therefore, one disadvantage of using this marker is the frequency with which tests must be performed and thus the increased cost (77).

5.2.4. Imaging

Chest computed tomograph (CT) scans have proven to be a very useful tool, especially in the early stages of infection (78). More specifically, the "halo sign," which is known as a region of ground-glass attenuation surrounding a pulmonary nodule on CT scan of the chest, is considered to be an early indicator of invasive pulmonary aspergillosis (IPA) (79). However, using the "halo sign" for diagnosis is not without its difficulties, the reason being that the CT scan must be performed within 5 days of the onset of a suspected infection, if not, then approximately 75% of the halo signs disappear within a week (80). The diagnostic use of the subsequent "air crescent" sign is limited by the fact that it only becomes visible in the third week of infection which may be too late to begin treatment of an invasive aspergillosis infection (81). To conclude it is clear that the CT halo sign has more diagnostic potential than the later air crescent sign, as treatment can be started immediately.

5.2.5. Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

In recent years, MALDI-TOF MS has been introduced to the clinical laboratory for rapid species identification (82, 83). The method works by analyzing the mass patterns from crude cell extracts of an isolate and comparing to a database of patterns of reference strains. A recent study evaluated the potential use of two commercially available MALDI-TOF MS systems for their application in clinical diagnostics using over 1,000 yeast and yeast-like clinical isolates from geographically distinct locations. The investigators compared the performance of both kits to classical clinical identification methods using microscopy and biochemical techniques (82). It was reported that the identification of pathogenic yeasts using MALDI-TOF MS in the clinical laboratory will greatly improve fungal diagnostics and hence improve treatment regimes. One of the main findings was that MS could be performed in a fraction of

the time it takes for classical techniques. Another important advantage found was that MS could differentiate closely related yeast species. For example, both methods could differentiate the *Candida* ortho meta parapsilosis cluster, whereas the classical methods could not. This holds significant clinical importance since different antifungal susceptibility profiles have been observed within this cluster (82). Another recent article describes a MALDI-TOF MS method for precise and rapid identification of common clinically isolated *Aspergillus* species as well as newly reported species in the clinical setting (83). The authors suggest that since the procedure only takes a few minutes, introduction of these techniques into main stream clinical practice will not only provide a more rapid diagnostic process but also lead to more accurate identification of fungal species (82, 83).

5.3. Molecular Diagnostics

In theory, an ideal molecular test should be capable of providing absolute sensitivity without adversely affecting specificity and should have the ability to rule infection in or out. However, this is rarely the case, if assays are extremely sensitive then inevitably they will generate false-positive results, and a compromise is needed that will find a balance between the early detection of subclinical infections and at the same time between the early detection of subclinical infections and low level contamination (84). Timing is critical for a patient suffering from an IFI. Due to the changing epidemiology and increase in emerging fungal pathogens, there is a knock on affect, causing an increased demand for more broad-spectrum diagnostic tests. The overall aim of using molecular methods for diagnostics is the hope that they will provide superior specificity, sensitivity, and turn around time. Many published accounts are available, which outline the use of molecular methods for diagnosis, most specifically real-time PCR. While these assays show great promise, being sensitive and specific, their usefulness is limited to single generas, for example, *Candida* (85, 86) or *Aspergillus* (87). In order to address this problem, Landlinger et al. developed a panfungal real-time PCR assay based on the 28S ribosomal RNA multicopy gene, which facilitated the detection of at least 80 pathogenic species (88). Despite its obvious value, PCR assays have not been widely accepted and as such have been side tracked by other diagnostic methods such as antigen detection assays. However as previously discussed, these methods are far from perfect and often fail to detect all of the infection causing fungal pathogens. Nonetheless, PCR assays still offer several advantages over other methods such as imaging, culture, and histopathology. Due to the nature of PCR, it has the supreme ability to detect minute amounts of starting material, especially when targeting a specific gene that has multiple copies (89). In addition, PCR assays have a multitude of options when it comes to the design process, ranging from complete genera down to a single species.

Not only can a PCR assay be designed to be specific but it can also be quantitative, allowing the clinician to determine the fungal load of the particular infection and the ability to ascertain how far the infection has progressed. While there is no denying the importance of molecular methods for fungal diagnostics and its potential for improving patient survival, it is still somewhat thwarted by a lack of standardization (90). This, coupled with the lack of a commercial system that has been rigorously tested means that routine PCR testing is not incorporated into the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria (91). Again, as seen with all other available diagnostic approaches for IFIs, results when using PCR vary, with sensitivities reported as being anywhere from 45 to 92%, with sensitivity being high at greater than 90% (85, 92, 93). Most notably is an analysis performed by Mengoli et al. which assessed the efficacy of the PCR assays in diagnosing IA (94). Sample types included plasma, serum, and blood. Analysis revealed that sensitivity and specificity were 75% and 87%, respectively, for two positive samples, while for a single positive sample the results were 88% and 75%, respectively. Clearly the difference in these percentages was of concern, and obviously a single negative PCR result cannot be considered to exclude the presence of IA. It seems most likely that future diagnostic efforts, for the reliable detection of IFIs, will rely on a combination of diagnostic procedures as opposed to the sole reliance on any single method. This approach will promote more informed decisions on treatment strategies that can be implemented by clinicians.

6. Treatment of IFIs

According to the National Nosocomial Infection Surveillance Program, there has been a decrease in hospital bed numbers in the USA, while conversely, ICU has seen bed numbers increase (95). This increase is attributable to the improvement in supportive medical care in the ICU. This improvement may be viewed as a double edged sword since critically ill patients have prolonged hospital stays and improved survival rates and are as such vulnerable to IFIs (96).

Broadly speaking, there are four main approaches to the timely administration of antifungal agents for the management of a suspected IFI, namely, prophylactic, empiric, pre-emptive, and finally target therapy. The main difference between all four treatment options is the timing, with prophylaxis being the earliest and target being the latest to be given. Prophylactic therapy refers to the administration of antifungals to patients who are considered to be at risk of developing an IFI but have shown no attributable signs or

symptoms, the main goal being prevention (97). Notwithstanding the obvious benefits of this type of therapy, there are however a number of shortcomings including the high cost of treatment not to mention the associated risks of giving drugs to individuals before an IFI has been confirmed or indeed excluded. Those patients deemed to be at a high risk of developing an IFI and have signs and symptoms indicative of infection are given empiric therapy, as with prophylactic therapy an actual organism has not been identified (98, 99). Pre-emptive therapy is that given to a patient who has had early diagnostic tests performed, and evidence suggests that an IFI is likely. Finally, targeted therapy as the name suggests is given once an actual pathogen has been identified by histopathology and/or culture (97–99) and therefore treatment can be very specific. As with all other treatment options these four approaches are not without their advantages and associated disadvantages (100).

6.1. Antifungal Therapy

For some time Amphotericin B, first generation azoles, and flucytosine were the only available treatment for fungal infections. The emergence of antifungal resistance isolates and also the toxic effects observed with some of these agents prompted the development and introduction of new formulations and classes of antifungal drugs. This has greatly improved treatment options available to clinicians. Essentially only four classes of established antifungal drugs are available, namely, polyenes (e.g., amphotericin B), azoles (e.g., fluconazole and itraconazole), allylamines (e.g., terbinafine), and the newly introduced echinocandins (e.g., caspofungin). Of these aforementioned classes, only three are used to treat systemic fungal infections (10). Although the development of new antifungal agents has significantly contributed to the successful treatment of fungal diseases, their effectiveness depends on the fundamental understanding of how these drugs interact with concomitant medications in addition to their associated toxicity. Clearly, for management of IFIs and treatment with such antifungal compounds, an in-depth knowledge of their pharmacokinetic and pharmacodynamic properties is essential (101). In addition, it is a well-known fact that inappropriate antifungal use actually adds to the global increase in antifungal resistance and may in fact lead to a variety of adverse outcomes, including unnecessary exposure to antifungal drugs, continual infections, and an associated increase in hospital costs (102).

6.1.1. Polyenes

Polyenes are by far the oldest category of antifungal agents (103) and while in excess of 200 polyene antibiotics have been identified, amphotericin B and nystatin are the only polyenes that are routinely used in a clinical setting. They have been in use since the 1950s and up until the mid-1980s amphotericin B was the gold standard of antifungal therapy (104), but, alternatives were scarce so it was hard to gauge just how effective it was. Despite its high in vitro activity against a broad spectrum of pathogens, an

important drawback of this antifungal agent lay in the fact that it causes acute toxicity negatively affecting the kidneys (105). In order to moderate the effects of drug toxicity seen when using amphotericin B, newer lipid preparations of amphotericin B, including amphotericin B lipid complex (Abelcet; Enzon), liposomal amphotericin B (Am-Bisome; Astellas Pharma US), and amphotericin B colloidaldispersion (Amphotec; Three Rivers Pharmaceuticals) were developed (106). Despite the fact that administering these newer formulations caused a reduction in the percentage of renal toxicity, none of them proved themselves to be more superior to the original molecule, and all versions still have to be given intravenously.

6.1.2. Azoles

Azoles are effective against a suite of fungal pathogens and act by inhibiting the synthesis of ergosterol of the fungal cell membrane. In the 1980s the first azole antifungals, the imidazoles miconazole and ketoconazole, appeared in the USA (103). In addition, two newer broad-spectrum triazoles (voriconazole and posaconazole) have been added to the collection of antifungal agents available to the clinician in order to combat serious fungal infections (107–110).

6.1.3. Echinocandins

Echinocandins (caspofungin, micafungin, and anidulafungin) are the currently approved agents for clinical use (111) and are a new class of parenteral antifungal agents that target the fungal cell wall (29). Although originally discovered in the 1970s, the echinocandins are the most recent class of antifungal agents to be introduced with caspofungin being approved for use in the USA in 2001. The echinocandins are active against *Aspergillus* and *Candida* species, including azole resistant *Candida* species. They are fungicidal in nature causing rapid lysis in growing cells and have shown fewer drug to drug interactions (112). These three agents are pharmacologically similar and only differ in a number of traits (29). Almost all *Candida* show in vitro susceptibility to the echinocandins and by virtue of this they have been approved for the treatment of candidemia and other forms of invasive candidiasis (113). In case of infection with *C. glabrata* or *C. krusei*, echinocandins are preferred over azoles. One disadvantage associated with the use of echinocandins is the fact that they have insufficient bioavailability for oral use and thus must be administered intravenously. In sum, the echinocandins remain the treatment of choice when it comes to *Candida* species; however, their role in the treatment of invasive aspergillosis remains an unknown quantity.

6.2. Alternative Treatment Strategies to Antifungal Treatment

In addition to the dispensing of antifungal agents, urgent debridement of infected tissue, where possible, can significantly improve the patients' chance of survival, particularly in the case of filamentous fungal infections (46, 48, 114). In addition, it is

also recommended that intravenous lines and catheters which may be the source of infection be removed (46, 114). Minimizing or reversing immunosuppression and neutropenia by reducing the use of immunosuppressive drugs, e.g., steroids, is thought to improve the patients outcome. Some studies have suggested that the use of immunostimulatory drugs (e.g., granulocyte transfusions, cytokines (such as G-CSF, GM-CSF, and INF-γ) in combination with antifungal therapy) is useful and enhances the patients chance of recovery (46, 115, 116).

7. Antifungal Resistance

The increased use of antifungal drugs in recent years has lead to the development of antifungal resistance amongst fungal clinical isolates. This is a worrying concern and has been the focus of much research in recent times. There are various aspects of antifungal resistance which many researchers have recently focused on, understanding the mechanisms of antifungal resistance, alternative treatment options for infections caused by resistant organisms, methods to detect resistance, and strategies to prevent and control the spread of resistance (117). Antifungal resistance can be either intrinsic (i.e., "natural" resistance) or acquired (develops following exposure to the antifungal agent in question). A third type of resistance has also been described referred to as clinical resistance. This type of resistance is observed in severely immunocompromised patients with consistent relapse of infection with an isolate which when tested in vitro appears to be susceptible to the antifungal agent. It is thought that a possible explanation for clinical resistance is due to suboptimal concentrations of the antifungal agent in blood due to interference of other drugs used to treat the patients condition (30, 118). Mechanisms of resistance can include overexpression of efflux pumps to essentially prevent intracellular accumulation of the antifungal drug and genetic modification or overexpression of the antifungal targets. Understanding the specific resistance mechanisms is crucial to finding alternative treatment drugs for resistant strains.

The most prevalent antifungal resistance is that of *Candida* species to azoles (119). Accurate identification of *Candida* to species level is invaluable since it can infer the likely antifungal susceptibility and therefore helps in the selection of a suitable antifungal treatment. For example, *C. glabrata*, *C. krusei*, and *C. rugosa* frequently show resistance or reduced susceptibility to fluconazole, whereas *C. albicans*, *C. parapsilosis*, and *C. tropicalis* are reliably susceptible (32, 46). Identification to species level is also recommended for *Aspergillus* since various species also exhibit different resistance/susceptibility profiles (30).

There is an ever increasing need for standardized drug susceptibility testing, and a method which is fast, accurate, reproducible, and inexpensive is desired. Standard microdilution susceptibility test methods have been developed by the Clinical Laboratory Standards Institute, (CLSI, formerly the NCCLS) for yeasts (*Candida* and *Cryptococcus* species; NCCLS, M27-A2) and molds (*Aspergillus*, *Fusarium*, *Rhizopus*, *Pseudallescheria*, and *Sporothrix* species; NCCLS, M38-A). A standard antifungal disk diffusion susceptibility testing method was also developed for *Candida* species (CLSI, M44-A). European Committee for Antimicrobial Susceptibility Testing (EUCAST) subsequently developed a broth dilution test for susceptibility of yeast species (120). Antifungal susceptibility testing should be routinely performed as this can provide valuable clinical information and aid greatly in treatment decisions, particularly as new resistance isolates come to light (46). In order to prevent and control antifungal resistance, a number of measures should be introduced, including the prudent use of antifungal agents, avoidance of low dose therapy, encouraged use of combination therapy, diagnosis of the etiological agent, treatment with the appropriate antifungal drug, and finally, regular testing and surveillance of antifungal resistance (117, 118).

8. Conclusions and Future Perspectives

IFIs will continue to be a major challenge for the clinical sector, and regrettably, the optimal approach to diagnosing IFIs still remains uncertain. Timing appears to be the single most critical factor if a patient is to survive the ravages of such an infection. Traditional approaches such as direct microscopy, cultivation, and histopathological evaluation still remain the gold standard for diagnosis of IFIs. Great strides have been taken in an attempt to improve the accuracy and speed of diagnosis of these devastating infections, allowing anti-mycotic treatment to begin as early as possible. A number of molecular methods have been developed in recent years; however, have not yet been standardized and have thus far been used only in experimental studies. It has been reported that the most convenient non-culture-based methods for diagnosis of IFI and monitoring antifungal treatment are commercial systems which detect fungal cell wall antigens galactomannan and 1,3 B-glucan (22). DNA and RNA methods appear to hold great promise for improved specificity and sensitivity; however, these methods need to be validated and standardized before they can be employed in routine clinical laboratory testing. Further, an important factor which cannot be ignored when selecting a new diagnostic test for IFIs is the cost. Efforts among researchers continue in order to improve the outcome of fungal disease. Many resources

are currently available, for example, the WHO Collaborating center for the Mycoses "An international center of excellence, developing and promoting cost effective strategies for the diagnosis, prevention and control of mycotic diseases." Other useful resources which provide guidelines for diagnosis and treatment of fungal infections include the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy fungus. Other useful resources which provide guidelines for diagnosis and treatment of fungal infections include the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group.

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