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REVIEW ARTICLE

Immunodetection of fungal and oomycete pathogens: Established and emerging threats to human health, animal welfare and global food security

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Abstract

Filamentous fungi (moulds), yeast-like fungi, and oomycetes cause life-threatening infections of humans and animals and are a major constraint to global food security, constituting a significant economic burden to both agriculture and medicine. As well as causing localized or systemic infections, certain species are potent producers of allergens and toxins that exacerbate respiratory diseases or cause cancer and organ damage. We review the pathogenic and toxigenic organisms that are etiologic agents of both animal and plant diseases or that have recently emerged as serious pathogens of immunocompromised individuals. The use of hybridoma and phage display technologies and their success in generating monoclonal antibodies for the detection and control of fungal and oomycete pathogens are explored. Monoclonal antibodies hold enormous potential for the development of rapid and specific tests for the diagnosis of human mycoses, however, unlike plant pathology, their use in medical mycology remains to be fully exploited.

Abbreviation: mAb, monoclonal antibody; pAb, polyclonal antiserum; Ig, immunoglobulin; scFv, antibody single chain variable fragment; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; LFD, lateral-flow device; POCT, point-of-care test; ABPA, allergic broncho-pulmonary aspergillosis; AFRS, allergic fungal rhinosinusitis; IPA, invasive pulmonary aspergillosis; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; GM, galactomannan; PCR, polymerase chain reaction; NPV, negative predictive value; PPV, positive predictive value

Keywords

Allergens, *Aspergillus*, *Fusarium*, hybridoma technology, monoclonal antibody, mycotoxins, phage antibody display, *Pythium*, recombinant antigen, *Scedosporium*, zygomycetes

History

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Introduction

Diagnostics for fungi and fungal-like organisms: an historical perspective

Fungi and fungal-like organisms are the cause of serious and often intractable diseases in humans and animals. Some of the organisms encountered as opportunist human and animal pathogens are also economically important plant pathogens, accounting for significant losses to global food production not only as destructive root-infecting organisms (*Fusarium* and *Pythium* species), but also as post-harvest storage rots (*Geotrichum*, *Mucor* and *Rhizopus* species) (Thornton, 2010) and producers of mycotoxins and allergens (*Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Stachybotrys*) (Hedayati et al., 2007) that cause respiratory diseases and contaminate food stuffs destined for human and animal consumption. Other fungi, like the emerging human pathogens *Pseudallescheria boydii* and *Scedosporium prolificans* have no reported capacity to infect plants, but are capable of causing a wide range of diseases both in healthy and immunocompromised individuals and animals (Cortez et al., 2008). Recent natural disasters such as the Asian and Japanese earthquakes and tsunamis have shown that species in the *Pseudallescheria–Scedosporium* complex are also capable of causing severe pulmonary and central nervous system infections in near-drowning victims who have aspirated dirty water containing soil and plant debris (Nakadate et al., 2012; Nakamura et al., 2011). Other seemingly benign fungi such as *Trichoderma* are infrequent human pathogens, and are better known as beneficial soil-dwelling organisms that display biocontrol and plant growth-promoting properties (Harman et al., 2004; Ryder et al., 2012). Nevertheless, breeches of the levee system in New Orleans following Hurricanes Katrina and Rita in 2005 led to extraordinary mould growth in flooded homes, dominated by *Trichoderma, Aspergillus*, *Penicillium* and *Paecilomyces* species, whose spores and endotoxins led to detrimental health effects in returning residents (Chew et al., 2006; Rao et al., 2007).

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As groups of organisms, fungi and oomycetes are a major threat to global food security and sustainable agriculture (The Royal Society, 2009). It has recently been estimated that 30–50% (or 1.2–2 billion metric tonnes) of all food produced on the planet are lost before reaching a human stomach, largely due to poor practices in harvesting, storage and transportation and market and consumer wastage (Aggidis et al., 2013). Pre- and post-harvest pathogens contribute significantly to this loss, and current assessments indicate that they destroy at least 125 million tonnes of the top five food crops (rice, wheat, maize, potatoes and soybeans) annually (Fisher et al., 2012). The damage caused by fungi to rice, wheat and maize alone costs global agriculture a staggering $60 billion per year. Fungi are also responsible for significant rates of mortality and morbidity in patients with heightened risks of infections by opportunistic pathogens, especially those with hematological malignancies whose immune systems are impaired. Recent statistics of the 10 most significant invasive fungal pathogens show the number of life-threatening infections by opportunistic invasive mycoses and endemic dimorphic mycoses exceeds two million cases per year (Brown et al., 2012). Invasive fungal diseases represent a substantial financial burden to healthcare providers, with a single hospitalization for invasive pulmonary aspergillosis (IPA) typically costing more than $60 000 (Dodds et al., 2006).

Organisms previously considered environmental contaminants or weak pathogens have emerged over recent years as lethal agents of plant diseases, such as the oomycete *Phytophthora ramorum*, that has devastated large swathes of Tan Oak, Japanese Larch and woody ornamentals in California and in the West of the United Kingdom (Grünwald et al., 2008), and a virulent strain of the fungus *Chalara fraxinea* that has caused widespread destruction of ash trees in mainland Europe and the United Kingdom. Animals are not immune to serious fungal diseases. The chytrid fungus *Batrachochytrium dendrobatidis* has led to a decline in nearly half of all amphibian species globally (Stuart et al., 2004), and the microsporidian fungus *Nosema ceranae* is responsible, at least in part, for colony-collapse disorder in bees (Cameron et al., 2011), an insect of major importance to global ecosystems services as a pollinator of >66% of the world's 1500 crop species (Kremen et al., 2002).

The historical importance of fungi as plant pathogens meant that extensive use of antigen-based diagnostics for plant-infecting species preceded similar diagnostic procedures for human mycoses by several years (Dewey & Thornton, 1995). This lag in development of immunodiagnostic assays for diagnosis of human mycoses is explained, at least in part, by the relatively recent appearance of fungi as serious human pathogens, precipitated by the advent of HIV/AIDS in the 1980s, by the use of more potent immunosuppressants in the ever-expanding population of transplant recipients and by the implementation of more aggressive anti-cancer therapies.

Despite this, a common thread runs through the development of diagnostics for human and plant-infecting pathogens. The poor specificity of polyclonal antisera hampered the accurate diagnosis of human and plant infectious agents. The advent of hybridoma technology in the mid 1970s (Kohler & Milstein, 1975), and the more recent development of phage display technologies (Arap, 2005), allowed the generation of highly specific monoclonal antibodies (mAbs) or single-chain antibody variable fragments (scFvs) that are able to discriminate not only between groups of organisms, but also between different genera and species of pathogenic fungi. This resulted in a rapid expansion of mAb-based diagnostic procedures for plant pathogen detection followed by mAb-based diagnostics for human mycoses.

The development of polymerase chain reaction (PCR) led to the swift displacement of antigen-based diagnostics with a myriad of nucleic acid-based technologies that have the capability for same-day detection of fungal DNA in a broad selection of specimen types. These techniques promise greater specificity and sensitivity and have, to a large extent, replaced the use of antigen-based diagnostics for plant disease detection. However, in the field of medical mycology, the uptake of PCR as a diagnostic tool has been constrained by the lack of standardization, such that PCR in not an accepted diagnostic criterion for the detection of human fungal diseases according to 2008 EORTC/MSG guidelines (De Pauw et al., 2008). Nevertheless, nucleic acid-based technologies have found wide applicability and have enjoyed extensive use in the detection and differentiation of human pathogenic fungi (Babady et al., 2011). These tests are expensive to perform, requiring sophisticated machinery, costly reagents and skilled personnel and are generally restricted to laboratories well rehearsed in such tests, although PCR technologies are now used in the field to detect plant pathogens (Tomlinson, 2012).

Lateral-flow technology incorporating mAbs specific to different fungi allows the development of quick, cheap and unsophisticated diagnostic tests that can be performed at the near bedside or in the case of plant pathogens or in the field. Their ease-of-use and relative cheapness, compared to more complex laboratory-based technologies, mean that multiple, sequential, samples can be tested for diagnostic antigens at a fraction of the cost of plate-based enzyme immunoassays (EIAs) and PCR. As semi-quantitative diagnostic assays, lateral-flow devices (LFDs) can be used as front-line tests to rapidly identify fungal pathogens, with subsequent confirmation or quantification of infections being undertaken in the diagnostic laboratory with more sophisticated detection procedures. They have the potential to aid in the rapid screening of patients at risk for invasive fungal diseases and to facilitate timely intervention with appropriate anti-fungal drug treatments. In plant pathology, they have been used to rapidly identify devastating and notifiable diseases such as sudden oak death, and to guide the quarantine of infected plant material (Thornton, 2012). Where samples require testing in reference laboratories they have the capacity to reduce the time from specimen acquisition to final diagnosis and treatment.

The aim of this review is to examine the types of antigens that have been used to track human and plant pathogenic moulds that threaten human health and global food production. Furthermore, it aims to investigate their success as diagnostic markers, and to identify mAbs and antigens that could be used in the development of immunoassays such as LFDs, that have found use as field tests for plant pathogens and which hold promise for the rapid point-of-care diagnosis

of human mycoses. With the exception of the post-harvest storage rot pathogen *Geotrichum candidum*, a yeast-like fungus that is also capable of causing human infections, mycoses caused by yeast species (*Blastomyces*, *Candida*, *Coccidioides*, *Cryptococcus*, *Histoplasma, Malassezia, Rhodotorula, Trichosporon*) will not be covered in this review as they have been comprehensively described elsewhere recently (Hsu et al., 2011; Miceli et al., 2011).

Fungi as human and plant pathogens

Of the 5.1 million species of fungi that are believed to exist (Blackwell, 2011), only a handful cause human and plant infections. Those that do, have a significant impact not only on food security as pathogens of crops and as food spoilage organisms, but also as life-threatening pathogens of humans and animals. The disciplines of plant pathology and medical and veterinary mycology have long histories. The origins of plant pathology date to the mid nineteenth century, due to the pioneering work of the Reverend Miles Joseph Berkeley who identified *Phytophthora infestans* as the etiologic agent of potato late blight disease, cause of the Irish potato famine (Berkeley, 1846; Lucas et al., 1991). While *P. infestans* was subsequently shown to be a heterokont (Kingdom Chromista) (Cavalier-Smith & Chao, 2006), it nevertheless established fungal-like organisms as having the potential to cause widespread economic damage to food crops. Since then, the related oomycete *P. ramorum* has emerged as a devastating pathogen of trees. Fungi, such as the rice blast pathogen *Magnaporthe oryzae*, which accounts for 10–35% loss of the global rice crop annually (Wilson & Talbot, 2009), and the emerging hyper-virulent strain Ug99 of the wheat stem rust pathogen *Puccinia graminis*, capable of causing up to 100% crop loss, highlight the threat of fungi to global food security.

Plant infecting fungi and oomycetes are true pathogens that have specialized infection structures and secrete virulence factors and effectors that modulate plant defence mechanisms (Oh et al., 2009; Wilson & Talbot, 2009). With the notable exception of endemic mycoses caused by the dimorphic fungi *Blastomyces*, *Histoplasma, Coccidioides* and *Paracoccidioides* that are capable of infecting seemingly immunocompetent individuals, fungi that cause mould infections in humans are predominantly opportunistic, taking advantage of a weakened immune system. Few, well-characterized, virulence factors have been identified in human pathogenic moulds, but certain cellular components such as hydrophobins, melanins and gliotoxins have been shown to modulate host immunity or to protect infectious propagules from destruction by phagocytic cells (Chai et al., 2010; Kuhn & Ghannoum, 2003; Paris et al., 2003). Melanins and hydrophobins similarly play a role in plant infection by foliar pathogens (Chumley & Valent, 1990; Talbot et al., 1993).

Certain moulds have long been known as pathogens of immunocompromised humans, and historical accounts of human and animal infections (Ainsworth, 2002) show that a number of these prevail today. Arguably, *Aspergillus* species are the best well known of these, but other species have emerged in recent years as the cause of life-threatening infections in humans, causing a wide range of diseases ranging from chronic infections of the joints to fatal, disseminated, diseases. Most notable among these are the infections caused by members of the *Pseudallescheria–Scedosporium* complex (*P. boydii*, *Scedosporium apiospermum* and *Scedosporium aurantiacum*), *S. prolificans*, *Fusarium* species and fungi in the phylum zygomycotina (Pfaller et al., 2006; Walsh & Groll, 1999; Walsh et al., 2004). These pathogens are not only limited to humans, but also have started to appear with increasing frequency as pathogens in other organisms. Examples include *Aspergillus sydowii* aspergillosis of the sea fan coral *Gorgonia ventalina* (Rypien et al, 2008), *F. solani* infestations of loggerhead turtle (*Caretta caretta*) eggs (Sarmiento-Ramírez et al., 2010) and *Scedosporium* infections in wild and domesticated animals (Haulena et al., 2002; Elad, 2011). The reasons for these increases are unclear, but are likely, in some instances, to be due to immunosuppression compounded by environmental change (Fisher et al., 2012) since, in the case of coral aspergillosis, epizootics are associated with temperature anomalies (Harvell et al., 1999).

Pathogenic organisms

Yeast-like fungi

G. candidum. G. candidum is a filamentous yeast-like fungus. It is a ubiquitous organism found in a range of habitats including air, water, silage and soil (Butler & Eckert, 1962; O'Brien et al., 2005) and also in foodstuffs such as milk, cheese and fermented milk products (Boutrou & Guégen, 2005; Marcellino et al., 2001; Mistry, 2004; Pottier et al., 2008; Ruas-Madiedo et al., 2006; Wouters et al., 2002). As a plant pathogen, *G. candidum* causes sour-rot of citrus fruit, tomatoes, carrot and other fruits and vegetables (Pitt and Hocking, 2009). Ripe or overripe fruits and vegetables are most susceptible to infection, particularly those kept in moisture-holding packaging (Skavia et al., 2004) and wounded tissue (Moline, 1984). Decay spreads rapidly, resulting in a sour-smelling watery mass. As a component of the normal microbiota of the human digestive tract, it has been reported, albeit infrequently, as a cause of infection in immunocompromised patients (Andre et al., 2004; Farina et al., 1999; Henrich et al., 2009; Sfakianakis et al., 2007; Vasei & Imanieh, 1999; Verghese & Ravichandran, 2003).

Moulds

Aspergillus and Penicillium species. Arguably, *Aspergillus* species are the best well known of the moulds or filamentous fungi capable of causing infections in humans and account for greater than 200 000 life-threatening infections per year with an associated mortality rate of 30–95% (Brown et al., 2012). In the United States alone, it is estimated that more than 10 000 hospitalizations per year are attributable to IPA, totalling 0.03% of hospital discharges overall. They are abundant saprotrophs of decaying organic matter and are found in a wide range of habitats including soil, self-heating compost heaps, manure and hay. As opportunist pathogens, *Aspergillus* species cause a spectrum of diseases in humans including aspergilloma (fungus ball), IPA, chronic necrotizing aspergillosis, and are contributing factors to allergic fungal

rhinosinusitis (AFRS; Karpovich-Tate et al., 2000). The most common species of *Aspergillus* causing invasive disease are *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus* and *Emericella* (*Aspergillus*) *nidulans*, with *A. fumigatus* accounting for ~90% of all cases of IPA (Denning, 1998). As well as being infectious pathogens, the *Aspergilli* produce numerous allergens that are implicated in asthma and allergic bronchopulmonary aspergillosis (ABPA). ABPA is an allergen-induced immunological disease of the lung characterized by increased IgE and IgG serum titers, enhanced peripheral blood eosinophilia and transient pulmonary infiltrates. The *A. fumigatus* allergens Asp f 2 (a 37 kDa fibrinogen-binding antigen), f 3 (a 19 kDa peroxisomal protein) and f 6 (a 26.5 kDa Mn superoxide dismutase) together react with IgE from patients with asthma and ABPA (Kurup, 2005), suggesting a role for these allergens in disease exacerbation. In addition, *Aspergillus* species (especially *A. niger*), along with *Penicillium* species and a number of other fungi are the cause of otomycosis, a superficial mycotic infection of the outer ear canal (Gurr et al., 1997; Karpovich-Tate et al., 2000).

Aspergillus infections occur in domesticated and wild animals also (Stanley et al., 2010; Whitney et al., 2012). In companion birds and broilers, aspergillosis is considered a major respiratory pathogen, causing diseases of the upper and lower respiratory tract. Certain species, such as African grey parrots (*Psittacus erithacus*), Amazon parrots (*Amazona* spp.) and cockatoos appear to be more susceptible to fungal rhinitis (Tully & Harrison, 1994). In the trachea, aspergillosis causes plaques and granulomas and particularly affects macaws, raptors, Amazon parrots and African grey parrots (Morrisey, 1997). Avian aspergillosis is a serious problem in poultry production and accounts for significant economic losses, particularly in turkey production (Arné et al., 2011). Bovine aspergillosis is also a serious problem in cattle production and, in addition to systemic aspergillosis, can cause mycotic abortions. In systemic infections, the main portal of entry is the respiratory tract, whereas the main site of infection in abortions appears to be the gastrointestinal tract, since infections of aborted calves are caused by maternally derived strains of *A. fumigatus* (Sarfati et al., 1996). This indicates the contamination of feed material with fungus.

Aspergillus species are generally not regarded as aggressive pathogens of plants, but certain species, most notably members of *Aspergillus* section *Nigri* (*A. niger*, *A. carbonarius*, *A. japonicus*), are able to infect grape vines causing vine canker (Vitale et al., 2012). *Aspergillus* species are more widely known as noxious spoilage pathogens of foodstuffs such as nuts, dried fruits, seeds and unpolished rice (Dewey et al., 1990; Mphande et al., 2004; Sakai et al., 2005), and as opportunist colonisers of damaged grapes or developing berries (Kazi et al., 2008). As producers of carcinogenic mycotoxins such as Ochratoxin A (OTA) (Kazi et al., 2008), they are considered a significant threat to human health when ingested. Mycotoxins (aflatoxins and OTA) are responsible for damaging up to 25% of the world's food crops, resulting in huge economic losses in developed countries (Abbas, 2005) and, in developing countries, where surveillance for aflatoxin contamination is poor, cause acute aflatoxicosis (hepatic necrosis, cirrhosis and carcinoma of the liver). Aflatoxin production varies substantially in natural strains of *A. flavus* and some are atoxigenic (Kato et al., 2011; Murakami et al., 1967; Rank et al., 2012). Indeed it is hypothesized that the closely related species *A. oryzae* (Gibbons et al., 2012; Kurtzman et al., 1986), used in the production of the Japanese food products soy sauce, miso and sake (Nout & Aidoo, 2010), was domesticated from an atoxigenic clade of *A. flavus* (Gibbons et al., 2012).

In addition to the *Aspergilli*, many species of *Penicillium* are also known to produce mycotoxins, and pose a serious threat to human health as contaminants on staple foods such as rice. *Penicillium islandicum*, a cause of one type of yellowing in stored milled rice, has been associated with toxicity, particularly in south-east Asia (Dewey et al., 1990; Tsunoda, 1953), with symptoms including liver lesions, cirrhosis and primary liver cancer (Moreau, 1979). *Aspergillus* and *Penicillium* species are also present as contaminants in grape juice destined for wine production (Dewey & Meyer, 2004). Both groups of fungi were also responsible for adverse health effects in residents returning to mouldy houses in the aftermath of the Hurricanes Katrina and Rita in New Orleans in 2005. As producers of mycotoxins (Kuhn & Ghannoum, 2003), these fungi, and others such as *Alternaria*, *Cladosporium*, *Stachybotrys* and *Trichoderma*, together contributed to the extraordinary mould growth and associated respiratory diseases and symptoms in returning residents.

Of more than 270 species of *Penicillium*, *P. marneffei* is the only species considered to be a pathogen of humans (Cao et al., 2011). Uniquely, *P. marneffei* is a dimorphic fungus that is able to switch from filamentous growth to intracellular macrophage-associated fission yeast at 37 °C. It is an endemic opportunistic pathogen in south-east Asia, and has emerged, along with *Cryptococcus neoformans*, to become a defining co-morbidity of AIDS (Duong, 1996). Indeed, prior to the HIV pandemic in Asia during the early 1990s, human infection by *Penicillium* species (penicilliosis) was extremely rare (Vanittanakom et al., 2006). Rodents in the genera *Rhizomys* and *Cannomys* are an important reservoir of the pathogen which was first isolated from the liver of a bamboo rat (*Rhizomys sinensis*) in 1956 (Capponi et al., 1956). A study by Cao et al. (2011) showed that bamboo rats trapped during June 2004 and July 2005 across Guangxi Province, China demonstrated 100% prevalence of infection.

The Pseudallescheria–Scedosporium complex. *P. boydii*, *S. apiospermum* and *S. aurantiacum* are members of the *Pseudallescheria-Scedosporium* complex (Gilgado et al., 2005). *P. boydii* is often referred to incorrectly as the sexual or teleomorphic state of the asexual anamorph *S. apiospermum*, but has been shown to be a separate species with its own asexual or anamorphic state, *Scedosporium boydii*. Notwithstanding this, all three fungi are capable of causing disease in immunocompromised humans (Cortez et al., 2008). *P. boydii* has long been known as an agent of white grain mycetoma in immunocompetent humans (Cortez et al., 2008) but has, along with *S. apiospermum* and *S. aurantiacum*, emerged over recent years as the cause of fatal disseminated infections in individuals with neutropenia, AIDS, diabetes, renal failure, bone marrow or solid organ transplants,

systemic lupus erythematous, Crohn's disease, those undergoing corticosteroid treatment, and leukemia and lymphoma patients (Torres & Kontoyiannis, 2011). *P. boydii* is the most prevalent species after *A. fumigatus* in the lungs of cystic fibrosis patients (Defontaine et al., 2002) where it causes allergic broncho-pulmonary disease (Cimon et al., 2000) and chronic lung lesions simulating aspergillosis (Koga et al., 2005). Near-drowning events and recent natural disasters such as the Indonesian tsunami in 2004 and the Tōhoku (Great East Japan) earthquake and tsunami in 2011, have shown *P. boydii* and the related species *S. apiospermum* and *S. aurantiacum* to be the cause of fatal central nervous system infections and pneumonia in immunocompetent victims who have aspirated polluted water (Nakadate et al., 2012; Nakamura et al., 2011). Their significance as potential pathogens of disaster evacuees has led to their recent inclusion in the Centres for Disease Control and Prevention list of infectious etiologies in persons with altered mental status, CNS syndromes or respiratory illness.

In animals, most *P. boydii* and *S. apiospermum* infections are localized, manifesting as nasal infections, mycetoma, keratomycosis, osteomyelitis and discospondylitis (Coleman et al., 2005; Hugnet et al., 2009; Smedes et al., 1992). The few reports of disseminated infections have been restricted to dogs, particularly German Shepherds (Elad et al., 2010), and may reflect an increased likelihood of exposure of dogs to the pathogen in urban environments.

There are no reports of these three species as plant pathogens, but their prevalence in soils impacted by human activity in the form of pollution and agricultural intensification may explain their occurrence in human dominated environments (Berzina et al., 2011). However, their occurrence in natural saline habitats such as estuarine sediments (Thornton, 2009b), and reports of infections by *Pseudallescheria* and the related species *Petriella setifera* in marine mammals (Haulena et al., 2002; Lackner & de Hoog, 2001; Poelma et al., 1974), and in victims of near-drowning events, suggest they are equally adept at colonizing aquatic environments.

S. prolificans. The dematiaceous (melanized) fungus *S. prolificans* is taxonomically related to *P. boydii*, *S. apiospermum* and *S. aurantiacum*. First described as a human pathogen in 1984 (Malloch & Salkin, 1984), it is a truly emerging pathogen of humans and animals (Salkin et al., 1988). Localized infections of immunocompetent individuals by *S. prolificans* are rare (Greig et al., 2001), but disseminated infections of immunocompromised patients are increasing in frequency and now account for approximately 25% of all non-*Aspergillus* mould infections in organ transplant recipients (Husain et al., 2003). The fungus causes debilitating diseases in a broad spectrum of patients at risk for invasive fungal infections including AIDS patients (Hopwood et al., 1995), allogeneic bone marrow and solid organ transplant recipients (Bouchara et al., 2009; Lionakis et al., 2004; Pihet et al., 2009; Vagefi et al., 2005), and patients with acute lymphoblastic leukemia and hematological malignancies (Alvarez et al., 1995; Maertens et al., 2000; Song et al., 2009). In neutropenic cancer patients and transplant recipients, disseminated infections are rapid and invariably fatal (Alvarez et al., 1995; Husain et al., 2005), with mortality rates of up to 75–80% (Rodriguez-Tuela et al., 2009). Along with *P. boydii*, the fungus is recovered from the sputum samples of cystic fibrosis patients, but its involvement in disease exacerbation has yet to be established (Bouchara et al., 2009; Pihet et al., 2009).

In animals, *S. prolificans* causes localized infections including osteomyelitis and arthritis in dogs and horses (Salkin et al., 1988), and has been isolated from a draining sinus in a cat and eye scrapings from horses (Salkin et al., 1988). As with aspergillosis and fusariosis, disseminated *S. prolificans* infections in dogs appear to be particularly prevalent in the German Shepherd (Haynes et al., 2012), further highlighting the apparent high susceptibility of this breed to invasive fungal infections.

The natural habitats of *S. prolificans* are practically unknown (Cortez et al., 2008; Husain et al., 2005; Solé et al., 2003), but it is a soil-borne organism, first isolated from a greenhouse soil in Belgium in 1974 (Hennebert & Desai, 1974). Since then, it has been recovered from soil in Australia (Cooley et al., 2007), but its environmental distribution in other countries is poorly described. In a recent survey of *Pseudallescheria* and *Scedosporium* species in soils from human-dominated (urban and agricultural) and natural environments in Austria and The Netherlands, *S. prolificans* was not isolated, despite an abundance of related species including *P. boydii*, *P. minutispora*, *S. apiospermum*, *S. dehoogii* and *S. aurantiacum*. In addition, attempts to identify the source of infection following a nosocomial outbreak in leukemia patients failed to recover the fungus in dust samples from hospital floors, ceilings and walls of rooms and corridors (Alvarez et al., 1995). Despite this, a single isolate of the fungus was reported in soil from a hospital pot plant (Summerbell et al., 1989), and indoor plant soils have been linked to other cases of invasive fungal diseases such as aspergillosis caused by *Aspergillus* species (Staib et al., 1978; Summerbell et al., 1989). More recently, Thornton used a selective isolation technique (Rainer et al., 2008) to recover *S. prolificans* and the related fungus *P. boydii* from naturally infested pot plant soils including a palm and an ornamental banana (Figure 1), showing that pot plants are a potential reservoir of infectious propagules for community acquired infections. The recovery of *S. prolificans* and *S. apiospermum* from the bronchoalveolar lavage (BAL) fluid of a near-drowning victim of the 2011 Japanese tsunami who had aspirated water contaminated with soil, suggests that *S. prolificans* spores are a component of the soil microbiota (Nakadate et al., 2012).

Zygomycetes

Fungi in the phylum Zygomycota are fast growing saprotrophic fungi, with a cosmopolitan distribution (Richardson, 2009). They are minor pathogens of crops plants, but are serious post-harvest storage pathogens, causing soft rots of fruits and vegetables during storage and shipping (Agrios, 2005). *Rhizopus* and *Mucor* are also used in the preparation of the Chinese and Indonesian fermented soybean foods sufu and tempe (Han et al., 2001; Nout & Aidoo, 2010). Two major orders of zygomycetes contain pathogens of humans

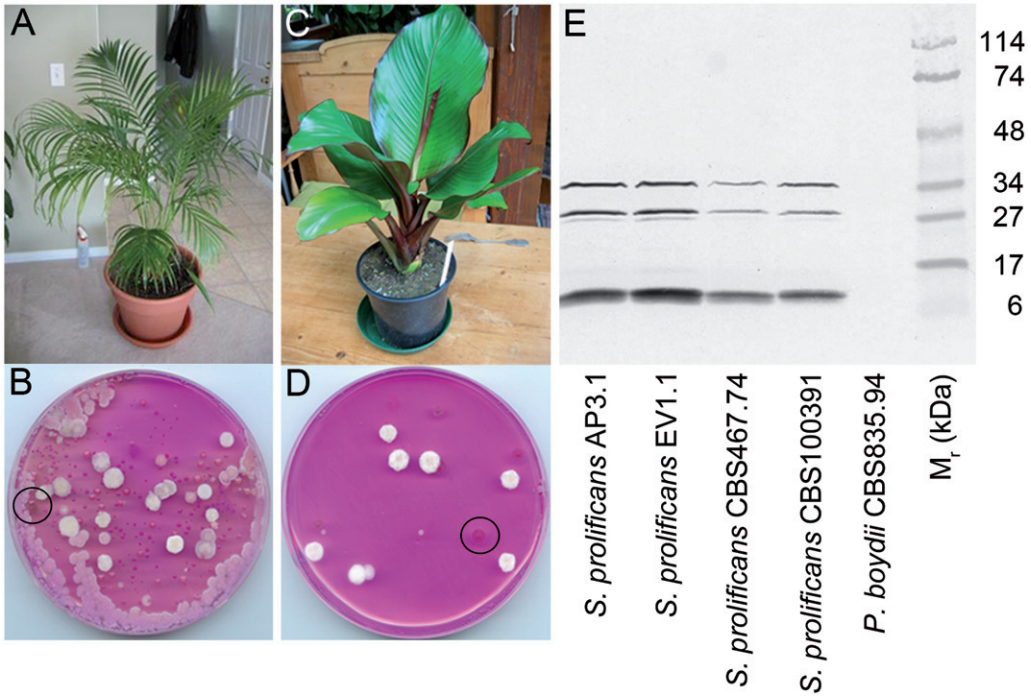

Figure 1. Recovery of *Scedosporium prolificans* from pot plant soils and detection using monoclonal antibody CA4. Soil samples were taken from an Areca palm (*Dypsis lutescens*) (A) and from an Abysinnian banana (*Ensete ventricosa*) (C) and water suspensions spread on the surface of a selective medium (B and D, respectively). After 7 d growth at 37 °C, colonies with dematiaceous hyphae and spores characteristic of *S. prolificans* (black circles in B and D) were visible. Colonies of yeast and zygomycete species and numerous colonies of the related fungus *Pseudallescheria boydii* (white colonies) were also present, confirmed in ELISA tests by using the *P. boydii*-specific mAbs GA3 and HG12 (Thornton, 2009a). A western blot (E) of antigens from the two *S. prolificans* soil isolates (AP3.1 and EV1.1), two clinical isolates of the fungus (CBS467.74 and CBS100391) and a single clinical isolate of *P. boydii* (CBS835.94) was probed with the *S. prolificans*-specific mAb CA4, followed by goat anti-mouse IgG alkaline phosphatase conjugate and binding visualized with NBT/BCIP substrate. Note specific binding of the mAb to immunoreactive bands of ~34, 27 and 10 kDa in the antigen extracts from the four *S. prolificans* strains, but lack of binding with *P. boydii* antigens.

and animals. The first order, the Entomophthorales, contains human opportunistic pathogens that cause chronic subcutaneous and mucocutaneous infections known as entomophthoramycosis in immunocompetent patients in tropical and subtropical climates. Human pathogens in this order include the genus *Basidiobolus* that produce chronic subcutaneous infections, and the genus *Conidiobolus* causing infections of the nasal sub-mucosa and subcutaneous tissue of the nose and face (Prabhu & Patel, 2004). The second order, the Mucorales, contains human and animal pathogens causing infectious diseases traditionally known as mucormycosis, which are caused by fungi in six separate families (Mucoraceae, Cunninghamellaceae, Mortierellaceae, Saksenaceae, Syncephalastraceae and Thamnidaceae). Species belonging to the Mucoraceae family are isolated more frequently from patients with mucormycosis than any other family and the most important species in terms of infection frequency is *Rhizopus oryzae* (formerly *arrhizus*) (Bouza et al., 2006; Roden et al., 2005; Spelberg et al., 2005). Inhalation of airborne infectious propagules (sporangiospores) is the most common pathway of infection by *R. oryzae*, followed by percutaneous inoculation or ingestion (Bouza et al., 2006; Rogers, 2008). As a consequence, the major manifestations of mucormycosis are rhino-orbito-cerebral, pulmonary, cutaneous, disseminated and gastrointestinal infections. *Mucor*, *Rhizopus*, *Absidia* and *Mortierella* species have also been implicated in bovine abortions and, together with *A. fumigatus*, account for 2–30% of all infectious abortions in cattle.

Mononuclear and polymorphonuclear phagocytes of healthy hosts are capable of killing zygomycete spores and hyphae through the generation of oxidative metabolites and the cationic peptide defensin (Bouza et al., 2006; Prabhu & Patel, 2004; Roden et al., 2005; Spelberg et al., 2005). Immunocompromised patients on the other hand, are extremely susceptible to opportunistic zygomycete infections. Most at risk are patients with hematological malignancy, neutropenia, diabetes mellitus, those undergoing corticosteroid therapies, diabetic ketoacidosis, organ transplantation, deferoxamine therapy, intravenous drug users, trauma and burns (Lee, 1999; Prabhu & Patel, 2004; Roden et al., 2005; Rogers, 2008; Spelberg et al., 2005). Recently, the use of broad-spectrum antifungal agents such as voriconazole in prophylaxis or therapy, has led to a number of institutions encountering increased incidences of zygomycete infections (Ambrosioni, 2009; Clark et al., 2011; Imhof et al., 2004; Kontoyiannis et al., 2005; Malani, 2007; Oren, 2005; Ritz et al., 2005).

The most distinctive feature of these infections is hyphal invasion of blood vessels, resulting in hemorrhage, infarction and necrosis of tissue. This angio-invasion is associated with the ability of the organism to disseminate from the original site of infection to other target organs (Rogers, 2008; Spelberg et al., 2005; Sugar, 1992). Rapid diagnosis, reversal of predisposing conditions, and aggressive surgical debridement remain the treatment strategy for infections that are typically associated with mortality rates of up to 80% (Prabhu & Patel, 2004; Ribes et al., 2000; Spelberg et al., 2005; Webster & Weber, 2007).

Fusarium species. The genus *Fusarium* currently includes over 100 species of ubiquitous soil fungi and contains two species, *F. graminearum* and *Fusarium oxysporum*, ranked fourth and fifth, respectively, in terms of scientific and economic importance in a 'Top 10' list of fungal plant pathogens (Dean et al., 2012). Some species cause root and stem rots (for example *F. solani*), while others cause vascular wilts (e.g. the devastating Panama disease of cultivated banana caused by tropical race 4 of *F. oxysporum*), fruit rots (numerous including *F. culmorum*, *F. equiseti* and *F. moliniforme*) or ear diseases (e.g. *F. graminearum* head blight of wheat). In some species, host-specific pathogenic strains are distinguished as *formae speciales* (e.g. the root and stem rot pathogen of pea *F. solani* f.sp. *pisi*) which otherwise do not differ from saprotrophic strains of the same species. *Fusarium* species are not only important as plant pathogens, but also as producers of mycotoxins (Jelinek et al., 1989). *Fusarium* head blight (FHB) is one of the major cereal diseases, responsible for significant reductions in grain yields of wheat, barley and oats (Rudd et al., 2001). From 1998 to 2000 the Midwestern US suffered $2.7 billion in losses following a FHB epidemic. In addition to reducing grain yields, the fungus produces a toxin deoxynivalenol (DON), sometimes referred to as vomitoxin, that, as a contaminant of flour, can cause nausea, fever, headaches and vomiting in humans and alimentary toxic aleukia in animals (Groll & Walsh, 2001; Nelson et al., 1994). *Fusarium verticillioides* is one of the most prevalent seed-borne fungi associated with ear and kernel rot of maize, and is a producer of carcinogenic fumosins, which are highly hepatotoxic and cardiotoxic (Hu et al., 2012). While approximately 15 different fumonisins have now been described, fumonisin B_1, B_2 and B_3 are considered the most important.

Of the 100 or so species of *Fusarium*, roughly 12 are considered human pathogens, but represent a considerable emerging threat to patients with hematological malignancies (Boutati & Anaissie, 1997; Dignani & Anaissie, 2004; Nucci & Anaissie, 2007; Zhang et al., 2006). The most common pathogen is *F. solani* accounting for 50% of reported cases of human infections, and is also responsible for emerging diseases in wild and domesticated animals (Jain et al., 2011), illustrated by recent reports of turtle egg infections (Sarmiento-Ramírez et al., 2010), pulmonary hyalohyphomycosis in a Kemp's Ridley sea turtle (Oros et al., 2004), and intracranial fusariosis in a German Shepherd dog (Evans et al., 2004). Other species capable of causing infections are *F. anthophilum*, *F. oxysporum*, *F. verticillioides* (*F. moliniforme*) and *F. proliferatum*. A handful of other species have been reported as human pathogens, but their occurrence is extremely rare (Letscher-Bru et al., 2002).

Trichoderma species. Species of the genus *Trichoderma* are cosmopolitan soil and compost-borne saprotrophic fungi and plant root endophytes (Romão-Dumaresq et al., 2012). They have found use in the commercial production of enzymes (Cullen & Kersten, 1992), in the biological control of plant diseases caused by economically important plant pathogens (Romão-Dumaresq et al., 2012; Whipps, 1997, 2001) and as plant-growth-stimulants (Harman et al., 2004; Ryder et al., 2012). In addition, certain strains of *Trichoderma aggressivum* and *T. harzianum* are noxious compost-borne pathogens of cultivated mushrooms (Samuels & Dod, 2002; Seaby, 1987). A number of thermo-tolerant species, most notably *T. longibrachiatum*, have emerged as medically important opportunistic pathogens of healthy humans and of immunocompromised, anemic and transplant patients, causing allergic fungal sinusitis and frequently fatal disseminated infections and perintonitis (Chouaki et al., 2002; Esel et al., 2003; Furukawa et al., 1998; Gautheret et al., 1995; Guarro et al., 1999; Guiserix et al., 1996; Hennequin et al., 2000; Jacobs et al., 1992; Munoz et al., 1997; Myoken et al., 2002; Richter et al., 1999; Robertson, 1970; Rota et al., 2000; Seguin et al., 1995; Tang et al., 2003; Tanis et al., 1995; Walsh & Groll, 1999). Species of *Trichoderma*, notably *T. viride* and *T. koningii*, were among the predominant fungi isolated from mouldy buildings in the aftermath of Hurricanes Katrina and Rita and, along with *Aspergillus*, *Penicillium*, and *Stachybotrys* species, were associated with adverse health effects in repatriated residents who were exposed to spores and endotoxins (Chew et al., 2006; Rao et al., 2007).

Alternaria, Cladosporium and Stachbotrys species. The ascomycete genera *Alternaria* and *Cladosporium* contain mycotoxin and allergen-producing dematiaceous fungi which, along with toxinogenic *Stachybotrys* species, cause a range of human diseases including pulmonary, immunologic, neurologic, and oncologic disorders (Breitenbach & Simon-Nobbe, 2002; Kuhn & Ghannoum, 2003; Schmechel et al., 2008; Tasic & Tasic, 2007). The genus *Alternaria* contains numerous plant pathogens that are mostly host-specific and often seed-borne. A few species are ubiquitous soil-borne saprotrophs, the commonest being *A. alternata* that occurs on many types of plants, foodstuffs and textiles (Ellis, 1971). *Alternaria alternata* is also recognized as an important aeroallergen both indoors and outdoors, and exposure to the fungus has been identified as a risk factor for asthmatics (Schmechel et al., 2008). It is the most frequent *Alternaria* species encountered in clinical practice, responsible for a variety of diseases including osteomyelitis, sinusitis and cutaneous infections (Morrison & Weisdorf, 1993; Pastor & Guarro, 2008; Viviani et al., 1986; Wiest et al., 1987). The fungus also causes phaeohyphomycosis in horses (Genovese et al., 2001).

Because *Cladosporium* species are frequent environmental contaminants and are most commonly isolated from nonsterile body sites, their involvement in human disease is debatable and can only be established if isolated from sterile tissues with accompanying evidence of tissue invasion by direct microscopy. Where proven, *Cladosporium* species cause phaeohyphomycosis, cutaneous infections, onychomycosis, sinusitis and pulmonary diseases (Tasic & Tasic, 2007). However, such infections are rare, and they pose the greatest hazard to human health in the form of allergens and mycotoxins. *Cladosporium cladosporioides*, for example, produces the mutagenic and cytotoxic compounds cladosporin and emodin, while *Cladosporium* conidia can cause allergic reactions on respiratory mucosa, followed by edema in acute asthma, or pulmonary emphyzema in chronic asthma (De Hoog et al., 2000). As plant pathogens, their parasitic capabilities are poor and while species such as *C. herbarum*

and *C. fulvum* are able to penetrate healthy leaves through stomata, direct penetration of healthy epidermal cells is generally unsuccessful. *Cladosporium* species and *A. alternata* are major components the sooty mould complex of fungi on cereal heads, which decreases both grain and seed quality (Dickinson, 1981).

Stachybotrys species are common saprotrophic fungi found in soil, on decaying plant material and in the rhizosphere of many plant species. They are not plant pathogens and similarly do not infect humans. However, the most common species in the genus, *Stachybotrys chartarum*, produces highly toxic compounds including roridin E, satratoxin H, sporidesmin G, trichoverrins and verrucarol (Kuhn & Ghannoum, 2003), that cause severe allergies and neurologic, immunologic and oncologic disorders. As a common component of the microbiota of damp buildings, *Stachybotrys* species, most notably *S. chartarum*, are implicated in sick building syndrome, a term given to severe illnesses resulting from indoor mould exposure. Stachybotryotoxicosis (mycotoxicosis caused by *S. chartarum* trichothecenes) has also been reported in horses (Ciegler & Bennett, 1980; Ueno, 1977).

Oomycetes

The fungal-like oomycetes comprise a large number of soil-dwelling organisms capable of causing serious economic damage to crop plants, problematic infections in wild and domesticated animals and humans. The most frequently described oomycete plant pathogens are the foliar pathogens (*Phytophthora* species) and root-infecting pathogens (*Pythium* species). *P. infestans* is a serious foliar pathogen of potato which accounts for losses of approximately €1 billion (cost of control and damage) to the almost 6 Mha of potatoes grown annually in the European Union (Haverkort et al., 2008), while the related species *P. ramorum* and *Phytophthora kernovii* have emerged as destructive pathogens of commercially logged Larch trees and woody perennials, such as Rhododendron and Camelia (Thornton, 2012).

Aphanomyces species have long been known as destructive plant pathogens, causing root rots in a wide range of crop plants including pea, beans, and clovers. They have also recently emerged as pathogens of sea otters (Jessup et al., 2004), and are responsible for declines in freshwater brown crayfish due to *A. astaci* crayfish plague (Holdich et al., 2009), and epizootic ulcerative syndrome of Tilapia fish caused by *A. invadans* (Andrew et al., 2008).

Of the numerous species of *Pythium* that are pathogens of plants, *P. ultimum* is arguably the best well known and is one of the commonest species of the genus isolated from temperate soils. It is a plurivorous plant pathogen causing pre- and post-emergence damping-off of seedlings and root rots of a wide range of plant species.

Pythium insidiosum is unique amongst the genus in that it is a pathogen of humans and animals, causing pythiosis, which manifests as cutaneous, subcutaneous, vascular or ophthalmic disease (Heath et al., 2002). *Pythiosis* has long been recognized as a veterinary pathogen, mainly of horses and dogs (Gaastra et al., 2010). It is now recognized as an emerging human pathogen (Kaufman, 1998), with less than 150 cases reported to date, and confined, for the most part, to tropical and sub-tropical regions of the world (Rivierre et al., 2005; Salipante et al., 2012).

The need for differential diagnosis

The recent push for sustainable agriculture with reduced inputs of costly and environmentally damaging pesticides (Royal Society, 2009), combined with evidence-based prescription of harmful antifungal drugs in medicine, will boost the demand for diagnostics that allow pathogenic fungi to be tracked quickly and accurately and at minimal cost to the user. Fungicide usage in modern agriculture, informed by accurate predictions of crop damage using disease forecasting, is now well established (Beest et al., 2008, 2009; West et al., 2003). The picture is less clear-cut in medicine where the danger of not administering anti-fungal drugs to a patient at high risk for life-threatening fungal infections (for example hematological malignancy and alloBMTx patients) far exceeds the financial gains and health benefits of not treating a sick patient. However, given the wide spectrum of susceptibility of human pathogenic moulds to currently available antifungal drugs (Denning & Hope, 2010), it is clear that diagnostics could play a more important role in guiding decision making in the future, allowing timely and rational administration of mould-active drugs, rather than prophylatic use which has become commonplace, and which increases healthcare costs and may lead to resistance (Azoulay et al., 2012; van der Linden et al., 2011).

Ironically, the prolonged use of azole-based crop protectants in agriculture, and prophylactic use of systemic antifungal drugs in human and veterinary medicine, in the absence of invasive infections (Azoulay et al., 2012), may be a contributing factors to the selection and emergence of multiple-azole-resistant (MTR) strains of *A. fumigatus* and other resistant fungal pathogens in humans and in poultry (Beernaert et al., 2009; Torres & Kontoyiannis, 2011; van der Linden et al., 2011; Verweij et al., 2007).

Diagnosis of mould infections

For human fungal infections, detection must conform to European Organisation for Research and Treatment of Cancer/Mycology Study Group (EORTC/MSG) diagnostic criteria (De Pauw et al., 2008) and comprises supporting data obtained from clinical, radiological, serological and mycological sources. At present no ‘gold standard’ tests exist for the diagnosis of mould infections, the exception being IPA, where serological detection of *Aspergillus* galactomannan (GM) is an accepted diagnostic criterion within EORTC/MSG guidelines.

Agents of aspergillosis and, more widely, hyalohyphomycoses caused by other hyaline moulds such as *Scedosporium*, *Pseudallescheria, Fusarium* and *Trichoderma* species, are extremely difficult to discriminate in histological samples based solely on hyphal morphology and require characteristic spore-bearing structures to be present to allow a definitive identification. However, these are frequently not present, and so attempts must be made to recovery the fungi from tissues using selective media. However, fungi such as the zygomycetes are often unculturable from biopsy samples, and identification relies on the identification of ribbon-like

hyphae in tissue specimens. Because of this, and the time-consuming nature of fungal culture and need for identification by skilled medical mycologists, techniques have been developed to more rapidly detect the organisms in biopsy samples or in body fluids. Some pathogens such as *Fusarium* disseminate hematogonously by sporulation *in vivo* and can be cultured directly from spores present in the bloodstream (Boutati & Anaissie, 1997). However, most invasive pathogens cannot be recovered from blood cultures. Nevertheless, fungi such as *Aspergillus*, *Fusarium* and the zygomycetes are highly angiotropic and angioinvasive, which presents opportunities for tracking invasive infections by detecting signature molecules (antigens) circulating in the bloodstream or in other fluids.

In plant pathology, detection of pathogens is less problematic, and different diagnostic tests can be used to identify the causal agents of disease including microscopy, selective media, immunoassays and nucleic acid-based procedures. Unlike plant pathology, PCR is not an accepted method for diagnosis of human mycoses and is not included in EORTC/MSG diagnostic criteria.

Antigens and antisera

Early immunological attempts to detect circulating antigens in humans and animals were based on rabbit or goat antisera raised against antigens present on the surface of spores and hyphae (Latgé et al., 1993) and which are shed into the bloodstream during angioinvasion. While some success in diagnosing human fungal diseases using antisera has been reported (Chan et al., 2002; Chumpitaza et al., 2000; Woo et al., 2002), attempts to differentiate fungal pathogens in infected plant material using antisera are uniformally unsuccessful due to the poor specificity of polyclonal antibodies (pAbs). Fungi share conserved glycoprotein antigens that are highly immunogenic (Notermans & Soentoro, 1986; Vojdani, 2004), which means that while highly sensitive immunoassays can be developed for the detection of different animal and plant pathogens, the cross-reactivity of the antisera employed meant that they were largely unable to differentiate the target fungus from non-target species (Kaufman et al., 1995, 1997; Schmechel et al., 2008; Vojdani, 2004). Attempts were made to improve the specificity of antisera using cross-absorption and immunological tolerance (Del Sorbo et al., 1993), but the limited success with these methods negated the widespread used of pAb-based immunodiagnostics in human and plant disease diagnosis. Despite this, these early studies demonstrated the concept of using fungal antigens as diagnostic markers of infection (Richardson et al., 1979), and the advent of hybridoma technology in the mid-1970s, that allowed the production of highly specific mAbs, revolutionized the use antibodies in fungal immunodiagnostics.

Hybridoma technology and mAbs

Immunization of rabbits, goats or mice with fungal antigens elicits a humoral immune response that results in the production of antibody from multiple plasma B cell clones. Antiserum from these animals therefore consists of pAbs targeted against specific and against conserved (non-specific) antigens or epitopes. Hybridoma technology, pioneered by Köhler and Milstein in the mid-1970s (Köhler & Milstein, 1975) allows the identification and isolation of individual plasma B cell clones secreting antibodies of pre-defined specificities. Immortalization of the specific B cells by fusion with myeloma cells (cancerous B cells) allows the production of hybrid cells (hybridomas) that retain the characteristics both of the B cell (antibody production) with that of the myeloma (indefinite growth in vitro). This ground-breaking technology led to the production of murine mAbs capable of differentiating fungi at the genus- (Meyer & Dewey, 2000; Thornton et al., 2002, 2010), species- (Thornton et al., 1993), near-isolate (Meyer & Dewey, 2000) and even isolate-level (Schmechel et al., 1997), and has been used to develop highly specific and sensitive tests for the detection of plant pathogens in soil and in planta (Dewey & Meyer, 2004; Thornton, 2008a; Thornton & Talbot, 2004; Thornton et al., 1993, 2002, 2004) and for the diagnosis of human mycoses (Thornton, 2008b, 2009a).

Phage display and single-chain variable antibody fragments

The advent of hybridoma technology revolutionized fungal immunodiagnostics. However, it has its limitations, as many mAbs generated using this approach often recognize carbohydrate residues on abundant immunodominant glycoprotein molecules. While such molecules can make excellent targets for diagnostic purposes, they are often conserved among related and unrelated species of fungi. This means that many hundreds, and sometimes thousands, of hybridoma cells lines must be screened to identify mAbs that bind to species-specific epitopes. Because of this, attention has turned to the production of mAbs using recombinant DNA or phage display technology. Phage antibody display allows the rapid selection of antigen-binding phage from 10^9 different phages each displaying a potentially novel arrangement of variable heavy and light chain antibody fragments (Barbas et al., 2001; George, 1995). This vast pool of antibody combinations and specificities can allow the identification of scFvs that bind to rare and possibly specific antigens or epitopes, as well as abundant, conserved molecules. Despite this, scFvs derived from phage display have not been used to any great extent in fungal disease diagnosis (Hu et al., 2012). However, a fascinating application of phage antibody display is in plant disease prevention, where protective phage antibodies have been used to genetically engineer wheat and other crop plants with increased resistance to *Fusarium* infections (Brar and Battacharyya, 2012; Hu et al., 2008; Peschen et al., 2004).

Immunodiagnostics

Yeast-like fungi

G. candidum. Detection and enumeration of fungal contaminants in food rely on microscopic and cultural methods. These procedures are laborious and, in the case of the Howard Mould Count (Potts et al., 2001), a technique for enumeration of heat-resistant moulds in processed tomatoes and fruit juices, they are non-specific and provide counts of total fungal burden only (Downes & Ito, 2001). As an alternative to mould counts, an EIA using polyclonal antiserum was

developed to quantify mould content of tomato paste, but the antibodies cross-reacted with all of the major tomato spoilage organisms (Robertson & Patel, 1989). Recently mAbs have been developed for the detection of spoilage fungi in processed tomatoes. Thornton (2010) reported the development of an IgM mAb (FE10) specific to *G. candidum* and the closely related teleomorphic species *Galactomyces geotrichum* and anamorphic species *Geotrichum europaeum* and *Geotrichum pseudocandidum* in the *G. geotrichum*/*G. candidum* complex. The mAb did not cross-react with a wide range of unrelated fungi, including zygomycetes (*Mucor* and *Rhizopus* species) and ascomycetes (*Aspergillus*, *Penicillium* and *Fusarium* species) likely to be encountered during crop production and processing. The mAb binds to an immunodominant high molecular weight (~200 kDa) extracellular polysaccharide (EPS) antigen that is present on the surface of arthroconidia and hyphae of *G. candidum*. The mAb was used in a highly specific enzyme-linked immunosorbent assay (ELISA) to accurately detect the fungus in infected tomato fruit and juice.

Moulds

Aspergillus species. The widespread conservation of antigens between fungi (Hearn & Mackenzie, 1989) means that antisera raised against an *Aspergillus* species invariably cross-reacts not only with antigens from other members of the same genus, but also with unrelated fungi, most notably *Pencillium* species (Kaufman et al., 1997; Schmechel et al., 2003; Vojdani, 2004). The cross-reactivity of *Aspergillus* antisera with different species of fungi makes specific detection of *Aspergillus*-related infections difficult, particularly where mixed infections are suspected (Hirschi et al., 2012; Koga et al., 2005; Lam et al., 2008). Despite this, some success in detecting IPA has been reported using antisera raised against somatic antigens and recombinant cell wall galactomannoprotein from *A. fumigatus* (Chan et al., 2002; Chumpitaza et al., 2000; Woo et al., 2002).

Chumpitaza et al. (2000) developed an inhibition EIA to monitor the occurrence of IPA in 45 immunocompromised individuals by using patient sera. The test employed rabbit polyclonal antiserum raised against a mixture of somatic antigens from *A. fumigatus* containing three predominant antigens with molecular weights of 18, 33, and 56 kDa. Circulating antigens were found in five of seven proven cases of IPA due to *A. fumigatus*, while no antigens were detected in sera from two patients with proven IPA due to *A. flavus* and *A. terreus*, or in sera from four patients with probable IPA. Circulating antigens were not detected in the sera of 30 healthy blood donors, but 4 patients with no evidence of IPA gave a positive result in this test.

Using guinea pig antiserum raised against recombinant Afmp1p, Chan et al. (2002) developed an ELISA specific to *A. fumigatus*. Anti-Afmp1p antiserum did not react with antigens from other *Aspergillus* species, or with antigens from *P. marneffei*, *Candida albicans*, *C. neoformans*, *Blastomyces dermatitidis* and *Histoplasma capsulatum* by Western blotting. However, clinical evaluation of sera from known IPA patients showed that only 8 (53%) of 15 were Afmp1p antigen positive in ELISA tests, due either to infections caused by *Aspergillus* species other than *A. fumigatus*, or lack of assay sensitivity. To increase the sensitivity of the ELISA, an additional immunoassay was required that detected the recombinant antigen using sera from the same patients.

A drawback of this technique and that of Chumpitaza et al. (2000) is their inabilities to detect infections caused by species other than *A. fumigatus*, importantly *A. flavus*, *A. niger* and *A. terreus*. For genus-specific tests, hybridoma technology can be used to develop mAbs that detect all species of *Aspergillus*, but which do not cross-react with non-target (unrelated) fungi. Nevertheless, very few mAbs have been developed for the specific purpose of diagnosing IPA. While a number of mAbs have been raised to *Aspergillus* species (Candlish et al., 1997; Dewey & Meyer, 2004; Gurr et al., 1997; Karpovich-Tate et al., 2000; Momany et al., 2004; Schmechel et al., 2005), only a handful have been used to detect invasive *Aspergillus* infections in humans (Fenelon et al., 1999; Fratamico et al., 1991; Hao et al., 2008; Jensen et al., 1996; Ste-Marie et al., 1990; Stynen et al., 1992; Thornton, 2008a). However, some of these mAbs have been used in immunofluorescence (IF) tests of ear swabs to detect *A. fumigatus* and *A. niger* in patients with otomycosis (Gurr et al., 1997), and in IF and ELISA tests to differentiate *A. niger* from other fungi (*Alternaria alternata*, *Cochliobolus lunata*, *Penicillium expansum* and *Cladosporium* species) present in sinus samples from patients with AFRS (Karpovich-Tate et al., 2000).

One of the best-characterized diagnostic markers of invasive *Aspergillus* infections is the cell wall component GM. Rat mAbs have been successfully used in the detection of *Aspergillus* GM, and they form the basis of commercial laboratory-based tests such as the Pastorex Aspergillus (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France) latex agglutination test (Hamilton & Gomez, 1998; Warnock et al., 1991), and the Bio-Rad Platelia *Aspergillus* GM-EIA that incorporates rat mAb EB-A2 directed against tetra (1→5)-β-D-galactofuranoside, the immunodominant epitope in the antigen (Morelle et al., 2005; Stynen et al., 1992, 1995). The GM-EIA has been used for many years to detect IPA both in humans (Barton, 2013) and in animals (Whitney et al., 2012), and is included in the EORTC/MSG criteria for diagnosis of IPA (De Pauw et al., 2008).

Murine mAbs have subsequently been generated that detect surrogate markers of IPA including the *Aspergillus*-specific mAb (JF5) that has been used to develop a rapid immunochromatographic test (LFD) for point-of-care detection of IPA. The mAb detects an extracellular glycoprotein antigen (an α-1-3-, α1-6-linked mannoprotein) secreted during active growth and so it is able to discriminate between invasive hyphal infection and dormant spores in the lungs. mAb JF5 does not cross-react with purified *Aspergillus* GM, indicating that it binds to a novel antigen(s) within the *Aspergillus* mannoprotein super-family. The LFD has been used to accurately detect IPA in a guinea pig model of disease (Wiederhold et al., 2009, 2013) and to detect infection in hematological malignancy and solid organ transplant patients by using serum and BAL fluids (Hoenigl et al., 2012; Thornton et al, 2012). Comparison of the LFD with the Platelia GM-EIA and Fungitell β-glucan tests, show that is

has a 100% negative predictive value (NPV) for IPA diagnosis using BAL (Hoenigl et al., 2012) and a 100% positive predictive value (PPV) when combined with PCR tests of human serum (White et al., 2013).

While the GM-EIA and the JF5 LFD are genus-specific tests, mAbs have been developed that detect single species. Species-specific mAbs have been developed to recombinant *A. fumigatus* galactomannoproteins (Afmp1p and Afmp4p) produced in a *Pichia pastoris* expression system (Wang et al., 2012). Twenty mAbs were produced against recombinant Afmp1p (rAfmp1p) and a further 13 against recombinant Afmp4p (rAfmp4p). Using the mAbs, two antigen-capture ELISAs (which employed mAbs as both the capture and the detection antibodies for rAfmp1p and rAfmp4p) were developed. The two immunoassays specifically detected Afmp1p and Afmp4p in cultures of *A. fumigatus* and had no cross-reactivity with the other pathogenic fungi tested, including *P. marneffei* and other pathogenic *Aspergillus* species. The two antigen ELISAs were able to capture circulating or excreted antigens during the acute phase of IPA in an animal model of disease.

Of 23 IgG1 mAbs recently raised against *Aspergillus terreus* hyphal exoantigens, 16 were shown to be species-specific (Nayak et al., 2011b). While serum spiking experiments demonstrated that human serum components do not inhibit the epitopes bound by the mAbs, the mAbs have not been used to detect *A. terreus* infections in either animal models or during human infections, and so the ability of the mAbs to track the fungus *in vivo* has yet to be established. mAbs have also been raised against a recombinant hemolysin from *A. terreus*, terrelysin (Nayak et al., 2012). Sixteen hybridomas of various isotypes were generated to the recombinant protein, of which seven demonstrated reactivity to the native protein in hyphal extracts. Three of the mAbs were species-specific, with two of the mAbs (13G10 and 10G4) recognizing the same epitope, PSNEFE and the third (15B5) recognizing the epitope LYEGQFHS. The observation that terrelysin was detected in *A. terreus* culture filtrates suggest that the hemolysin may be a useful candidate biomarker for diagnosis of infection, although this remains to be shown in animal models or in humans.

Attempts to generate mAbs to the mycotoxin-producing fungus *A. versicolor* using homogenized spores failed to produce species-species antibodies (Schmechel et al., 2005). Of the 46 mAbs developed (35 IgM, 9 IgG3, 2 IgG1), all cross-reacted strongly with *Aspergillus*, *Penicillium*, and *Eurotium* species and some also cross-reacted with *Paecilomyces variotii* and several *Cladosporium* and *Stachybotrys* species. This work showed that antibody responses in mice against spores of the fungus were dominated by cross-reactive mAbs of the IgM isotype.

mAbs have also been produced to spores of the toxigenic fungus *A. flavus* (Hetherington et al., 1994). A panel of five IgG isotype mAbs, raised against *A. flavus* conidia, all recognized a 97 kDa extracellular antigen present in conidia, swollen conidia, and hyphae of the fungus. Cross-reactivity of the mAbs was examined by ELISA tests of fungal culture filtrates. Four mAbs reacted with 10 of 11 *A. flavus* isolates, and the fifth one reacted with 9 of them. One mAb also reacted with *A. fumigatus*, two reacted with *A. niger*, *A. wentii* and *A. nidulans* and all five reacted with *A. ochraceus*. None reacted with *A. terreus*, *A. glaucus*, *A. versicolor* or a *Penicillium* species. Each mAb bound to *A. flavus* hyphae in formalin-fixed paraffin sections of a muscle biopsy from a confirmed human case of invasive aspergillosis.

Alternative strategies have been used for tracking toxigenic fungi by raising antibodies to their mycotoxins or allergens themselves (Meulenberg, 2012; Morgan, 1995). Immunodetection of ochratoxins, mycotoxins produced as secondary metabolites by *Aspergillus* and *Penicillium* species in particular, has received prolonged attention (Muelenberg, 2012). Ochratoxin A, ochratoxin B, ochratoxin C and ochratoxin α are teratogenic, mutagenic, hepatotoxic, nephrotoxic and immune suppressive, and therefore pose serious health risks for exposed humans and animals as contaminants of food and feed commodities. Numerous pAbs and mAbs have been developed for OTA (the ochratoxin considered to be most toxic) for ELISA detection in cereal grains and other foodstuffs and also in human serum and plasma (Muelenberg, 2012). These have been used in combination with mAbs against other fungal toxins such as aflatoxin B1, T-2 toxin and fumonisin B1, to detect different mycotoxins in food and feed (Anderson et al., 2010; Lacey et al., 1991).

A tube immunoassay has been developed for the detection and quantification of *Aspergillus* and *Penicillium* antigens in grape juice contaminated with fruits with Bunch rot (Dewey & Meyer, 2004). The assay, which is rapid, user-friendly and does not require laboratory facilities was designed and tested for on-site use at wineries during harvest. The assay incorporates a mouse mAb (AF-CA2) that recognizes all species of *Aspergillus* and *Penicillium* and water-soluble fungal antigens present in juice from infected grapes. The assay is highly sensitive detecting between 1% and 3% levels of rot in grapes. No issues were encountered with tests carried out on the testing stands at wineries at harvest time by personnel with no scientific background.

Penicillium species. Many cases of penicilliosis caused by *P. marneffei* are misdiagnosed as tuberculosis, due to their similarities in symptomology (Duong, 1996). Patients suffering from penicilliosis often present with non-specific symptoms, such as low-grade fever, anemia and weight loss. Diagnosis is often made by identifying fungal cells in bone marrow, spleen, lymph nodes and skin biopsies, but these are invasive procedures that significantly delay diagnosis. As with IPA diagnosis, early attempts at *P. marneffei* immunodiagnostics were based on pAbs and, as with *Aspergillus* species, cross-reactivity of *P. marneffei* antisera with other human pathogenic fungi meant that diagnosis of penilliosis marneffei using pAb raised against somatic antigens was not feasible (Fuhrmann et al., 1989). However, as was found with *Aspergillus* immunodiagnostics, the use of recombinant antigens (in particular recombinant cell wall mannoproteins) appeared to improve substantially the specificity of *P. marneffei* antisera. To this end, Cao et al. (1998a) cloned the *P. marneffei MP1* gene, which encodes an abundant antigenic cell wall mannoprotein. Rabbit anti-Mp1p antiserum was generated using recombinant Mp1p protein

purified from *Escherichia coli*. Western blot analysis with anti-Mp1p antibody revealed that the antigen belongs to a group of cell wall proteins that are present in the cell walls of the yeast, hyphal and conidial phases of the fungus, with yeast phase-specific mannoproteins revealed subsequently (Pongpom & Vanittanakom, 2011).

It was observed that infected patients develop a specific antibody response against Mp1p, suggesting that this protein was a suitable cell surface target for host humoral immunity. Cao et al. (1998b) developed a sensitive ELISA for Mp1p detection using guinea antiserum raised against the recombinant Mp1p antigen. The anti-Mp1p antiserum was shown to be specific, failing to cross-react with *Candida albicans*, *H. capsulatum* or *C. neoformans* by Western blotting. Preliminary clinical evaluation of sera from penicilliosis patients indicated that 17 (65%) of 26 patients were Mp1p antigen-test positive using the guinea pig antiserum ELISA. When combined with a serological assay using sera from immunocompetent penicilliosis patients and from penicilliosis patients with HIV, the diagnostic capabilities of the *P. marneffei* immunodiagnostic tests were improved substantially.

More recently, Wang et al. (2011) have generated mAbs and rabbit polyclonal antisera against Mp1p expressed in *P. pastoris*. Two Mp1p antigen capture ELISAs were developed which employed mAbs for both the capture and detecting antibodies (mAb–mAb pair) or pAbs and mAbs as the capture and detecting antibodies (pAbs–mAb pair), respectively. The two Mp1p antigen ELISAs detected Mp1p specifically in cultures of *P. marneffei* yeast phase and had no cross-reaction with other pathogenic fungi tested. The sensitivities and specificities of the two antigen assays were found to be 55% (11 of 20) and 99.6% (538 of 540) for mAb–mAb Mp1p ELISA, and 75% (15 of 20) and 99.4% (537 of 540) for pAbs–mAb Mp1p ELISA performed using 20 sera with culture-confirmed penicilliosis, and 540 control sera from 15 other mycosis patients and 525 healthy donors. An anti-Mp1p IgG antibody ELISA with an evaluated sensitivity of 30% (6 of 20) and a specificity of 98.5% (532 of 540) using the same sera improved the sensitivity of penicilliosis diagnosis to 100% (20 of 20).

Few antibodies have been developed for the detection of *Penicillium* species in plant material. A notable exception is the work of Dewey et al. (1990) that used hybridoma technology to generate an IgG1 mAb for tracking *P. islandicum* in infested rice. The mAb, PI01, was specific to *P. islandicum* and did not cross-react with antigens of other *Penicillium* species, *Eupenicillium* species or with *Aspergillus* species. The mAb was used to develop ELISA and dip-stick assays for detection of the fungus in individual grains, with approximately 90% of grains in naturally infested samples from Indonesia testing positive, by ELISA, for *P. islandicum*. A tube immunoassay incorporating mAb AFCA2 has also been used by Dewey & Meyer (2004) to detect *Penicillium* antigens in grape juice.

As with *Aspergillus* immunodiagnostics, mAbs have also been used to detect *Penicillium* toxins directly (Meulenberg, 2012; Morgan, 1995; Ramakrishna et al., 1990), while Schmechel et al. (2003) attempted to raise mAbs specific to the allergenic fungus *P. brevicompactum* directly. However, of the five mAbs generated against spores, all of them cross-reacted with several of 31 related *Aspergillus*, *Penicillium* and *Eurotium* species, but cross-reactivities with 21 unrelated species were rare. This work demonstrates the close antigenic relationship shared between members of the order Eurotiales.

Pseudallescheria and Scedosporium species. *P. boydii* is thought to be an under-diagnosed fungus (Walts, 2001) and mis-identification is one of the reasons that the mortality rate due to invasive pseudallescheriasis is high. Detection of invasive *P. boydii* infections, based on cytopathology and histopathology, is problematic since it can occur in tissue and bronchoalveolar and bronchial washing specimens with other hyaline septated fungi such as *Aspergillus* and *Fusarium* (Tarrand et al., 2003) which exhibit similar morphological characteristics on microscopic examination (Walts, 2001).

Immunological diagnosis of *Pseudallescheria* infections has focused on the detection of antigens by counter-immunoelectrophoresis and by immunohistological techniques using polyclonal fluorescent antibodies, but cross-reactions with antigens from other fungi such as *Aspergillus* species occurs (Cortez et al., 2008; Jabado et al., 1998; Kaufman et al., 1997). Pinto et al. (2001, 2005) isolated a peptidorhamnomannan from hyphae of *P. boydii* and proposed the antigen as a diagnostic marker for the pathogen. Cross-reactivity with *Sporothrix schenckii* and with *Aspergillus* have however been noted (Pinto et al., 2001). Furthermore, it is uncertain whether a similar antigen is present in the related pathogenic species *S. prolificans*, an important consideration in patient groups susceptible to mixed *Scedosporium* infections (Cooley et al., 2007; Husain et al., 2005), or coinfections with unrelated fungi (Hirschi et al., 2012).

Recently, Thornton (2009b) reported the development of mouse IgG1 and IgM mAbs that are highly specific to *Pseudallescheria* species and the related fungi *S. apiospermum* and *S. aurantiacum*. They do not cross-react with the related species *S. dehoogii* or *S. prolificans*, or with a wide range of clinically relevant yeasts and moulds including *Aspergillus*, *Candida*, *Cryptococcus*, *Fusarium* and *Rhizopus*. The mAbs HG12 (IgG1) and GA3 (IgM), which bind to carbohydrate epitopes on the same 120 kDa extracellular glycoproteins, were used in a double-antibody-sandwich ELISA to track the fungus in estuarine sediments, a potential environmental reservoir of infective propagules. Furthermore, mAb HG12 was used in IF to accurately differentiate mixed cultures of *P. boydii* and *A. fumigatus*. The assays have yet to be tested on human samples for diagnosis of scedosporiosis or pseudallescheriasis.

No antibodies are currently available for the detection of *S. prolificans* in human and animal disease diagnosis, but Thornton has recently developed a mAb (CA4), and used it to track the fungus in naturally infested soil samples (Figure 1). ELISA and Western blotting studies show that the mAb is species-specific and does not cross-react with related species in the *Pseudallescher*ia–*Scedosporium* complex or with a wide range of unrelated fungi. Similarly, mAb CA4 has yet to be evaluated as a diagnostic reagent for detection of *S. prolificans* infections.

Fusarium species. The importance of *Fusarium* mycotoxins to human and animal health is demonstrated by the numerous reports of mycotoxin-specific polyclonal antisera and mAbs in the literature. Monti et al. (1999) used rabbit antiserum to detect fusaproliferin, a toxic metabolite of *F. proliferatum* and *F. subglutinans*, found in contaminated maize. However, the unsatisfactory sensitivity of indirect and competitive ELISAs and immunoblotting assays using the antiserum meant that the immunoassays were not competitive with traditional ultraviolet high-performance liquid chromatography methods. mAb-based immunoassays specific to fusarins, T-2 toxin, zearalenone (F-2 toxin) and DON, have been developed for tracking *Fusarium* mycotoxins in contaminated crops (Barna-Vetró et al., 1994; Casale et al., 1988; De Saeger & Van Peteghem, 1996; Maragos et al., 2008). These assays have improved the sensitivity, specificity and speed at which the mycotoxins can be detected in contaminated food through the development of rapid on-site immunoassays for use in farms, storehouses and factories (De Saeger & Van Peteghem, 1996).

Crop disease symptoms such as FHB are not always correlated with mycotoxin production, and so attempts have been made to develop immunoassay that allow quantification of fungal load in the growing plant (Hill et al., 2006). Early attempts to detect *Fusarium* were conducted using chicken antibodies. Antiserum raised against mycelial-soluble antigens from *F. sporotrichioides*, *F. poae* and *F. graminearum* were found to cross-react with antigens from related and unrelated fungi, whereas antiserum raised against immunodominant extracellular antigens with molecular weights >28 kDa were genus or species-specific (Gan et al., 1997). A murine mAb capable of differentiating the banana pathogen *F. oxysporum* f.sp. *cubense* race 4 from other races in the same *Fusarium* formae speciales, was reported by Wong et al. (1988), but most murine mAbs raised against plant pathogenic *Fusarium* strains using hybridoma technology display genus- or species-specificity, and all appear to bind to extracellular immunodominant antigens (Arie et al., 1991, 1995; Banks et al., 1996; Hayashi et al., 1998). More recently, single-chain variable antibody fragments to *Fusarium verticillioides* have been developed using phage display (Hu et al., 2012). Chicken-derived scFvs against cell wall proteins from the fungus were isolated from an immunocompetent phage display library. A highly sensitive scFv antibody (FvCA4) was shown, by IF, to bind to the cell wall of conidia and hyphae. This antibody was able to detect 10^{-2} mg of contaminating mycelium/g maize. This is the first report on scFv antibodies derived from phage display being used to accurately monitor a fumosinin-producing pathogen in agricultural samples.

Few *Fusarium* mAbs have been developed for the specific detection of opportunistic infections in humans and animals. However, Jensen et al. (2011) recently reported the development of *Fusarium*-specific mAbs for immunohistochemical diagnosis of fusariosis. The IgM mAbs, which recognize 51 kDa and 63 kDa antigens, reacted strongly with fungal elements in both experimentally infected animals and biopsy samples of patients with fusariosis sepsis and dissemination to the skin. The mAbs did not cross-react with tissue sections containing heterologous (aspergillosis, candidiasis and scedosporiosis) fungal elements. Thornton has also used a human pathogenic strain of *F. solani*, isolated from blood, to develop a genus-specific mAb (ED7) that binds to a major, extracellular, 120 kDa antigen that localizes, in immuofluorescence, to the spore and hyphal cell walls of *Fusarium* species (Figure 2). Using mAb ED7, a highly specific ELISA has been developed for rapid detection of root rot of pea caused by *F. solani* f.sp. *pisi* (Figure 2). The mAb is specific to *Fusarium* species and does not cross-react with unrelated fungi.

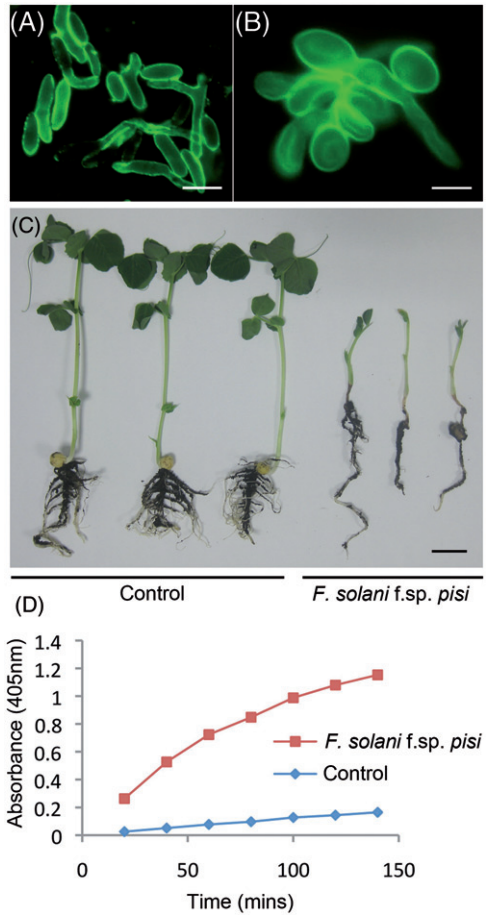

Figure 2. Immunodetection of *Fusarium* species. Spores of the plant pathogen *F. oxysporum* f.sp. *lycopersici* (A) and a human pathogenic strain of *F. solani* (B) were tested by immunofluorescence using the *Fusarium*-specific mAb ED7. The spores were probed with mAb ED7 followed by goat anti-mouse polyvalent FITC conjugate and viewed under ultraviolet light using an epifluorescence microscope. Intense fluorescence of spore cell walls and germ tubes was noted. An ELISA was used to detect the ED7 antigen in the roots of pea plants inoculated with the root pathogen *F. solani* f.sp. *pisi* or in control (uninoculated) plants. (C) Plants were grown for 14 d in compost infested with spores, the roots excised and shaken in phosphate-buffered saline for 1 h, and solubilized antigens were immobilized to the wells of plastic microtiter plates for ELISA assay (D). The wells were probed with mAb ED7 followed by goat anti-mouse alkaline phosphatase conjugate and *p*-nitrophenol substrate. Absorbance at 405 nm was monitored over time. Noted increase in absorbance values with root extracts from infected plants indicates the presence of the ED7 antigen.

Zygomycetes

As with all invasive fungal infections in humans, confirmation of zygomycete infections (zygomycosis) relies on culture of the infectious agent from a sterile site or biopsy sample and

production of spore-bearing structures for species identification. However, biopsy is often precluded by the unstable condition of many at-risk patients (Bouza et al., 2006; Maertens et al., 2001; Sugar, 1992). Furthermore, recovery rates of zygomycetes from biopsy tissue are generally poor, and so direct visualization of the characteristic broad 'ribbon-like' hyphae in histology sections is required. However, this is a laborious process and does not allow accurate discrimination between species. The advent of molecular diagnostics heralded new opportunities to accurately detect zygomycete infections using fresh and formalin-fixed, paraffin-embedded tissues (Hata et al., 2008) and to rapidly identify cultured fungi to species level (Dannaoui, 2009).

Early serological assays for zygomycosis in humans relied on detection of crude antigen preparations using human immune serum in ELISA (Kaufman et al., 1989; Pierce et al., 1982). However, non-specific reactivity with *Rhizopus* and *Rhizomucor* antigens was particularly evident with sera from patients with aspergillosis and candidiasis, which meant that human immune serum could not be used to identify systemic zygomycosis.

To date, no studies have been undertaken to develop diagnostic tests for zygomycosis based on the detection of extracellular circulating antigens in humans. The production of rabbit hyperimmune antisera and mAbs to immunodominant intracellular antigens of Mucorales has previously been reported by Jensen et al. (1994, 1996). Jensen et al. (1994) demonstrated that heterologous absorption rendered antisera raised against *R. oryzae* somatic antigens monospecific by indirect IF, whereas heterologous absorption of *Lichtheimia corymbifera* (previously *Absidia corymbifera*) antiserum did not abolish reactivity with *R. oryzae*. The reactivity of the heterologously absorbed antisera, and a murine IgG_1 mAb ($1A_7B_4$) raised against *L. corymbifera*, enabled zygomycetes within bovine lesions to be identified by immunohistochemistry. In a subsequent study, Jensen et al. (1996) showed that a mAb (WSSA-RA-1), raised against water-soluble somatic antigens from *R. oryzae*, similarly bound to an intracellular antigen. The high degree of specificity exhibited by mAb WSSA-RA-1 allowed them to use immunohistochemistry to detect the pathogen in situ in placenta, lymph node and gastrointestinal tract samples of cows with suspected systemic bovine zygomycosis.

Approaches have been used to identify extracellular antigens specific to the Mucorales (Hessian & Smith 1982). Procedures have been developed that allow the identification and isolation of immunogenic EPSs that appear, using polyclonal IgG antibodies, to be specific to this group of fungi (De Ruiter et al., 1991a,b, 1992a,b, 1994; Notermans & Soentoro, 1986). Fractions of the excreted EPS of Mucorales were found to possess antigenic properties (Miyaki & Irino, 1972; Miyaki et al., 1980), and these fractions were subsequently characterized as fucomannopeptide and mannoprotein (Yamada et al., 1982, 1983). Success in diagnosing other invasive fungal pathogens such as *C. albicans* and *A. fumigatus* using mAbs raised against mannans and galactomannoproteins, respectively (Prella et al., 2005; Thornton, 2008, 2010), suggests that Mucoralean EPS mannoproteins would be good candidates for the development of mAbs specific to zygomycete fungi.

Trichoderma species. Given the importance of *Trichoderma* and related *Gliocladium* species as environmentally friendly and sustainable alternatives to chemical pesticides in the control of plant diseases, there is renewed interest in their use as biological control agents in crop protection. Surprisingly, however, there have been few reports of simple diagnostic procedures that allow precise tracing of these beneficial biocontrol and plant-growth-promoting organisms in the environment (Breuil et al., 1992; Thornton et al., 2002). Arguably, this is one of the reasons why widespread take-up of commercial *Trichoderma* formulations has failed to occur, since the inability to quickly and accurately determine the efficacy of biocontrol agents means that the only real measure of biological disease control is the extent of crop damage. To date, few attempts have been made to precisely determine whether *Trichoderma* biocontrol formulations containing quiescent spores become activated in the soil and initiate antagonism of pathogens in the plant rhizosphere. While nucleic acid-based techniques have been developed for precise tracing of *Trichoderma* biocontrol agents released into the environment, such techniques are restricted to individual strains of the fungus (Abbasi et al., 1999) and do not measure their activity. Using hybridoma technology, Thornton et al. (2002) developed a mAb (MF2) that allows detection of all *Trichoderma* species and used the mAb to develop highly specific diagnostic immunoassays for detecting *Trichoderma* species in the soil, compost and plant rhizosphere (Thornton et al., 2002; Thornton & Talbot, 2004), and for quantifying the effects of biocontrol strains on the population dynamics of human and plant pathogens during antagonistic interactions in soil (Thornton, 2004, 2008a). The mAb is highly specific and detects an extracellular antigen secreted from the hyphal tip during active growth. This ensures that only actively growing and therefore potentially antagonistic propagules are detected following soil application of *Trichoderma* biocontrol formulations. A rapid and user-friendly LFD incorporating mAb MF2 has been developed that allows specific and sensitive detection of active propagules of *Trichoderma* species in soil samples within 15 min of antigen extraction (Thornton, 2008a). The LFD can also be used to detect human infections. Indeed, the device was used to accurately detect *T. longibrachiatum* isolated from the posterior ethmoid cavity of a 47-year-old male with facial pain and nasal obstruction (Thornton and Johnson, unpublished).

Alternaria, Cladosporium and Stachybotrys species. The use of antibodies to detect *Alternaria* species in the indoor environment and in plant material has received prolonged attention. Schmechel et al. (1997) used homogenized spore germlings of the oilseed rape pathogen *A. brassicae* to develop two mAbs, 34/7VG (IgG1) and 73/11/D (IgM) that were found to be species- and isolate-specific, respectively, when tested against 19 isolates of related and non-related fungi representing 13 fungal genera. Using an inhibition ELISA, Schmechel et al. (2008) showed that commercially available pAbs used for *A. alternata* detection in homes were highly cross-reactive recognizing a wide range of unrelated species including *Ulocladium*, *Stemphylium*, *Epicoccum*, *Drechslera* and *Exserohilum*. Consequently, they concluded

that the prevalence data reported for *A. alternata* in American homes (95–99% of homes with detectable *Alternaria* antigens) was biased. This ambiguity has since been mitigated by the development of *A. alternata*-specific rat mAbs that react with *Alternaria* and the closely related genus *Ulocladium* only (Denis et al., 2012).

Attempts to generate mAbs specific to *Cladosporium* species have been limited. However, Karpovich-Tate et al. (1998) using, as immunogens, germinating and non-germinating spores or surface antigens from hyphae of *C. herbarum* race 4, succeeded in raising genus-specific mAbs. Very few cell lines secreting mAbs were generated using the spore immunogen and, of those that were developed, all were non-specific. However, with hyphal antigens, four mAbs, OX-CH1, OX-CH8, OX-CH10 and OX-CH11 reacted strongly with *Cladosporium* species, but did not cross-react with a range of fungi including *Alternaria*, *Aspergillus*, *Penicillium* and *Scopulariopsis* species. mAb OX-CH1 was used successfully to quantify the biomass of *C. fulvum* in infected tomato leaves. Using sera from bronchial asthmatic patients, Chou et al. (2008) demonstrated IgE binding against extracts from the important allergenic fungus *C. cladosporioides*. Among these, sera 55% and 51% showed IgE binding against 36 kDa and 20 kDa proteins of the fungus, respectively. Both proteins reacted with FUM20, a mAb specific to fungal serine proteases. IgE cross-reactivity was detected between purified 36 kDa Cla c 9 allergen and serine protease allergens from *A. fumigatus* and *Pencillium chrysogenum*.

Elevated IgG and IgE antibody titers against *Stachybotrys* antigen extracts have been reported in allergic patients and residents of water-damaged houses. Sera from 50 mould-exposed humans were tested for IgG and IgE antibodies against *Stachybotrys* hemolysin and proteinase-Stachyrase A (Vodjani, 2005). Significantly elevated titers were present in the majority of sera indicating that hemolysin and Stachyrase-A are two major antigenic components of *S. chartarum*. However, detection of these antibodies in blood was partially attributable to cross-reacting proteins from other fungi. Indeed, Wartenberg et al. (2011) have recently shown that hemolysin is also a major secreted protein of *A. fumigatus*. The study of Vodjani (2005) showed that while hemolysin and Stachyrase-A were suitable diagnostic markers for detecting exposure to *Stachybotrys* species, a more specific method for detecting these antigens was needed. Previously Gregory et al. (2002) had used rabbit pAb in immunohistochemical and immunocytochemical localization studies to show that stachylysin was localized in *S. chartarum* spores and mycelia suggesting that it was constitutively produced. Van Emon et al. (2003) subsequently used pAb-based ELISAs to detect stachylysin in human and rat sera and environmental samples. Stachylysin was detected in rat pups that received nasal installations of *S. chartarum* conidia but not in control rat serum. Stachylysin in the serum of five human adults exposed to the fungus in water-damaged environments was 371 ng/ml but none was detected in the control serum. Stachylysin was also detected in wallboard and dust samples, showing that the antigen was a useful indicator for assessing human exposure to *S. chartarum* and in determining its presence in the indoor environment.

Using sera of atopic patients, Xu et al. (2008) identified an extracellular protein from *S. chartarum* that was antigenic in humans. mAbs raised against the protein were used to detect the antigen in house dust with a sensitivity of ~0.2 ng/ml dry weight dust, a detection limit comparable with those of house dust mite allergen and allergens of *A. fumigatus*. The mAbs were specific and did not cross-react with *Aspergillus*, *Eurotium*, *Penicillium*, *Paecilomyces* or *Scopulariopsis*.

In addition to purified protein, species-specific mAbs have been developed against *S. chartarum* propagules. Using spore walls as immunogen, Schmechel et al. (2006) generated a single species-specific IgG1 mAb that reacted only with spore preparations but not mycelium of *S. chartarum* or propagules of 60 other species of fungi tested. Five other mAbs, all belonging to the immunoglobulin class M, cross-reacted with non-related genera including several species of *Cladosporium*, *Memnoniella*, *Myrothecium* and *Trichoderma*. In a subsequent study, Nayak et al. (2011a) developed IgM mAbs against a semi-purified cytolytic *S. chlorohalonata* preparation prepared from hyphae. Cross-reactivity of the mAbs was tested against crude hyphal extracts derived from 9 *Stachybotrys* species as well as 39 other environmentally abundant fungi. All of the mAbs reacted strongly with *S. chlorohalonata* and *S. chartarum* but not with the other 39 unrelated fungi. However, four of the mAbs cross-reacted with antigens from the phylogenetically related genus *Memnoniella*.

Oomycetes

The oomycete genus *Pythium* contains plant, human and animal pathogens. As such, mAbs have been developed for their detection in planta and in humans. Genus- and species-specific mAbs were raised against zoospores and cysts of *Pythium* (Callow et al., 1987; Estrada-Garcia et al., 1989) and rapid ‘on-site’ assays for pythium blight detection in turfgrasses were introduced commercially in 1987 (Miller et al., 1992; Rittenburg et al., 1988). While the assays demonstrated good reactivity with a number of pathogenic *Pythium* species, reactivity was also demonstrated with some *Phytophthora* species (Miller et al., 1992). More recently, Thornton (2010) reported the development of a *Pythium*-specific mAb (GE6) that binds to an extracellular, constitutively expressed, antigen produced by *P. sylvaticum* and a number of other *Pythium* species. It does not cross-react with fungi or *Phytophthora* species in ELISA tests. N-terminal sequencing showed that the 10 kDa antigen bound by mAb GE6 is sylvaticin, an alpha-elicitin-like protein implicated in the induction of the hypersensitive response during plant infection (Lascombe et al., 2007; Ponchet et al., 1999).

Early studies of the antigenic relatedness of *Pythium* species showed that soil and plant isolates share at least one antigen with the human and animal pathogen *P. insidiosum*, but that animal isolates shared six antigens (Mendoza et al., 1987). Using *P. insidiosum* rabbit antiserum in immunodiffusion tests, Mendoza and co-workers showed that animal isolates could be distinguished from plant and soil species based on precipitin reactions and that adsorption of the rabbit pAb with cellular components of *P. diclinum* eliminated all

precipitin activity with the antigens of the non-*insidiosum* *Pythium* species. Garcia et al. (2007) used sera from bovine, canine, equine, feline and human hosts infected with *P. insidiosum* to map hyphal antigens using transmission electron microscopy and showed the presence of multiple immundominant antigens, the majority of which were located within the inner layers and the cell wall structures. This work confirmed previous studies of immunodominant antigen localization using sera from different host species (Leal et al., 2005; Mendoza & Newton, 2005; Pérez et al., 2005) and identified potential diagnostic markers of infection in humans and animals including a novel 74 kDa immunodominant antigen recognized by sera from human patients with pythiosis (Krajaejun et al., 2006). In-house diagnostic assays employing ID, ELISA or Western blotting have been developed for human pythiosis (Imwidthaya & Srimuang, 1989; Krajaejun et al., 2002; Mendoza et al., 1997), and while they demonstrate high degrees of sensitivity and specificity, they require lengthy turnaround times which delays treatment. Speed of diagnosis has now been improved substantially by the development of an LFD that uses culture filtrate of *P. insidiosum*, human anti-*P. insidiosum* antibodies and a sheep anti-human immunoglobulin G-colloidal gold conjugate to detect infections (Krajaejun et al., 2009). The LFD was compared to a standard serological ID test using sera from patients with vascular, ocular and cutaneous pythiosis, healthy blood donors and patients with a variety of infectious and non-infectious diseases. The turnaround time for the LFD was less than 30 min, while the ID test took ~24 h. Based on the results for all sera of pythiosis patients and the control groups, the LFD showed 88% sensitivity and 100% specificity compared to ID sensitivities and specificities of 61% and 100%, respectively. Consequently, the LFD is a rapid, user-friendly and reliable serological test for the early diagnosis of vascular and cutaneous pythiosis.

Unlike *Pythium*, there are no known human or animal pathogens within the genus *Phytophthora*. They are, however, significant pathogens of crops, ornamentals and trees and mAbs have been developed for their detection (Hardham et al., 1986; Wycoff et al., 1987). Over the last decade, a rapid *Phytophthora* LFD has been used to great effect in tracking the spread of *P. ramorum* infections of trees and woody ornamentals in the field (Thornton, 2012).

Bioavailability of antigens for diagnosis of human IFDs and affects of antifungal treatments

While detection of fungal antigens in soil and in plant material has its challenges, biological amplification methods can be used to improve assay sensitivities (Thornton et al., 1993). No such opportunity exists in human and animal disease detection, where delays in diagnosis impact severely on patient survival. Different samples have been used for detecting diagnostic antigens in humans and mAbs can be deployed to visualize infectious propagules in biopsy tissues by using immunohistochemistry (Jensen et al., 1996, 2011). However, detection of pulmonary infections involves invasive capture of infected material from lungs, which is often not possible in very sick patients. For this reason, less-invasive methods that detect circulating markers of disease are preferred but, as is the case with BALs fluids, some still require invasive bronchoscopic procedures. For this reason, most immunodiagnostic assays have focused on the identification of signature molecules in the bloodstream and their detection in serum.

For many years, the mainstay of IPA diagnosis has been serum detection of GM using the Platelia EIA (Beirão & Araujo, 2012), but specimens of other body fluids are increasingly used for detection of GM, including BAL, cerebrospinal fluid (CSF) and urine. This reflects both the underlying nature of the disease, for example a pulmonary infection or infection of the central nervous system, and the ease of sample collection. For rapid diagnosis of fungal pulmonary infections, tests that detect diagnostic antigens in BAL would likely provide earlier identification of disease than ones that rely on antigenemia. Furthermore, imprecise diagnosis might result from rapid clearance of circulating antigens from serum (De Repentigny, 1992) or from antibody cross-reactivity with interfering serum compounds (Verweij & Mennink-Kersten, 2006).

The utility of BAL and bronchial washings for IPA diagnosis was demonstrated over 20 years ago (Levy et al., 1992), but the accuracy of the diagnostic procedures available at the time, based on the presence of characteristic *Aspergillus* hyphae in BAL and washings, now has limited diagnostic credibility and would not currently be accepted as proof of fungal disease unless accompanied by culture of the fungus from a biopsy specimen and evidence of necrosis in the infected tissue. However, such observations, when combined with the immunodiagnostic markers, increase the likelihood of invasive fungal disease especially when accompanied by host factors and other EORTC/MSG certified diagnostic tests such as the colorimetric 1,3-β-D-glucan assay.

Detection of GM in BAL fluids as a method of diagnosing IPA was demonstrated by Park et al. (2010), and subsequent trials in hematological malignancy and solid organ transplant patients have shown the effectiveness of the *Aspergillus* LFD in diagnosing the disease using BAL samples (Hoenigl et al., 2012). Increased accuracy may also be afforded using BAL where the diagnostic antigen is detected during active hyphal growth only (McCulloch et al. 2012; Thornton, 2008; Thornton et al., 2012; Wiederhold et al., 2013). With both the GM EIA and the *Aspergillus* LFD, mould-active drugs have been shown to reduce serum sensitivities, while sensitivities using BAL are less affected (Wiederhold et al., 2013). In contrast to antigen tests, the diagnostic performance of PCR using BAL from hematological malignancy patients was reduced if patients had received antifungals prior to BAL sampling (Reinwald et al., 2012). CSF is a relevant specimen where infections of the central nervous system are implicated and the detection of *Aspergillus* GM in CSF may be diagnostic of cerebral aspergillosis (Klont et al., 2004; Viscoli et al., 2002). However, GM diagnosis of aspergillosis using CSF samples remains experimental with limited data on its clinical worth (Walsh et al., 2008).

Urine has also been investigated as a fluid with diagnostic potential and the easy and non-invasive nature of its capture means it is an attractive alternative to blood and BAL specimens. However, given that the kidney filtration cut-off is ~45 kDa (Anderson & Anderson, 2002), any diagnostic

tests for fungal antigens (particularly those that detect high molecular weight glycoproteins) would need to detect immunoreactive antigenic fragments of <45 kDa. Consequently, urine tests for IPA have focused on the detection of low molecular weight antigens. Latgé et al. (1991) showed that a major antigen secreted in vitro by *A. fumigatus* was an 18 kDa basic protein and was also the major circulating antigen found in urine of patients with IPA. Monospecific rabbit antiserum raised against the protein was used in Western blotting studies to probe a single urine sample from a patient with IPA. A single 18 kDa immunoreactive band was evident, showing the potential of the antigen as a diagnostic marker of invasive disease. This confirmed previous work using rabbit antisera raised against cell wall extracts of *A. fumigatus* which showed that the 18 kDa antigen is one of a number of immunogenic proteins found in the urine of IPA patients (Haynes et al., 1990) and which, in addition to GM, is present in the serum and urine of cattle with systemic aspergillosis (Jensen et al., 1993). GM antigenuria in humans and guinea pigs was recently investigated by Dufresne et al. (2012) using an IgM mAb (mAb476), which recognizes GM-like antigens from *Aspergillus* and other moulds, and demonstrated the feasibility of exploiting lateral-flow technology for the detection of urinary excreted GM in humans.

Conclusions and future prospects for immunodetection

Lateral-flow technology and point-of-care testing (POCT) hold enormous potential for the rapid diagnosis of established and emerging fungal pathogens. The effectiveness of POCT in forestry and agriculture is amply attested by the *Phytophthora* LFD that has been used as a front-line diagnostic test enabling the Fera Plant Health and Seed Inspectorate to rapidly monitor the spread of the oomycete pathogens *P. ramorum* and *P. kernovii* in the United Kingdom and to more effectively manage disease through quarantine and containment measures. Similar devices would enable inspectors to quickly and accurately identify other emergent human, animal and plant pathogens in the natural environment and to screen larger numbers of imported food commodities and animals for exotic pathogens, which represent a growing biosecurity threat to the United Kingdom from international trade (Brasier, 2008). POCT can also optimize diagnosis, triage and patient monitoring during natural disasters (Kost et al., 2006) and to limit the impact of fungal diseases in resource-limited settings. For example, many deaths from HIV-associated cryptococcal meningitis (CM) may be preventable through early diagnosis and treatment. Jarvis et al. (2011) recently demonstrated that an inexpensive POC assay which uses urine or a drop of blood facilitated early diagnosis of cryptococcal infection in resource-limited settings. The novel *Cryptococcus* glucuronoxylomannan test markedly improved early diagnosis of CM in South Africa, enabling testing of urine in patients presenting to healthcare facilities in which lumbar puncture, or even blood sampling, was not possible.

The development of specific and sensitive immunodetection assays relies on the availability of high quality mAbs that detect abundant extracellular antigens. Immunoproteomics and immunosecretomics analysis of fungal pathogens enable the identification of abundant or immunogenic proteins (Gautam et al., 2007; Kumar et al., 2011; Shi et al., 2012; Wartenberg et al., 2011), but many of the most useful and highly immunogenic diagnostic fungal antigens are carbohydrate in nature including *Aspergillus* GM (Hsu et al., 2011) and mannoproteins (Thornton, 2008), *Candida* mannans and glucomannoproteins (Cassone et al., 1988; Farahnejad et al., 2005; Gil et al. 1991; Moragues et al., 2003), *Cryptococcus* glucuronoxylomannan (Brandt et al., 2003; Percival et al., 2011), *P. marneffei* mannoproteins (Cao et al., 1998a,b, 1999), mucoralean immunogenic polysaccharides (De Ruiter et al., 1991a, 1992a, 1994; Jensen et al., 1996), and carbohydrate antigens of *Fusarium* (Arie et al., 1991, 1995; Jensen et al., 2011), *Geotrichum* (Thornton et al., 2010) and *Pseudallescheria* (Thornton, 2009). Consequently, searches to identify new and useful diagnostic markers should include glycomics and glycoproteomics.

New and novel antibody diagnostic methods are on the horizon. Tests that harness the power of antibody and nucleic acid techniques enable the development of sophisticated but highly specific and sensitive in vitro diagnostics, and state-of-the-art in vivo imaging and therapeutic technologies. These include the proximity ligation assay (Weibrecht et al., 2010), a refinement of immuno-PCR (Sano et al., 1992), and the use of mAbs in bioimaging and radioimmunotherapy of fungal infections (Bryan et al., 2012; Dadachova et al., 2003, 2004; Dadachova & Casadevall, 2005, 2011; Nosanchuk & Dadachova, 2012).

Declarations of interest

The authors report no declarations of interest.

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