Laboratory epidemiology and mechanisms of azole resistance in *Aspergillus fumigatus*

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ABSTRACT

Although *A. fumigatus* strains are generally susceptible to azoles, recently**,** acquired resistance to a number of antifungal compounds has been reported, especially to triazoles possibly due to widespread clinical use of triazoles or through exposure to azole fungicides in the environment. The significant clinical problem of azole resistance has led to study the antifungal resistance mechanisms for developing effective therapeutic strategies.

Of 230 clinical *A. fumigatus* isolates submitted during 2008 and 2009 to the Mycology Reference Centre Manchester, UK (MRCM), 64 (28%) were azole resistant and 14% and 20% of patients had resistant isolates, respectively. Among the resistant isolates, 62 of 64 (97%) were itraconazole resistant, 2 of 64 (3%) were only voriconazole resistant and 78% were multi-azole resistant.

The gene encoding 14-α sterol demethylase (*cyp51A*) was analyzed in 63 itraconazole resistant (ITR-R) and 16 ITR-susceptible clinical and environmental isolates of *A. fumigatus* respectively. Amino acid substitutions in the *cyp*51*A,* the commonest known mechanism of azole resistance in *A. fumigatus*, were found in some ITR-R isolates. Fifteen different amino acid substitutions were found in the *cyp51A* three of which, A284T, M220R and M220W, have not been previously reported. In addition, several mutations were found in the *cyp51A* gene in one of the *A. fumigatus* environmental isolates. Importantly, a remarkably increased frequency of azole-resistant isolates without *cyp51A* mutations was observed in 43% of isolates and 54% of patients. Other mechanisms of resistance must be responsible for resistance.

In order to assess the contribution of transporters and other genes to resistance, particular resistant isolates that did not carry a *cyp51*A mutation were studied. The relative expression of three novel transporter genes; *ABC11, MFS56* and *M85* as well as *cyp51A, cyp51B, AfuMDR1, AfuMDR2 AfuMDR3, AfuMDR4* and *atrF* were assessed using real-time RT-PCR in both azole susceptible and resistant isolates, without *cyp51A* mutations. Interestingly, deletion of *ABC11, MFS56* and *M85* from a wild-type strain increased *A. fumigatus* susceptibility to azoles and these genes showed changes in expression levels in many ITR-R isolates. Most ITR-R isolates without *cyp51A* mutations showed either constitutive high-level expression of the three novel genes or induction of expression upon exposure to itraconazole. One isolate highly over-expressed *cyp51B*, a novel finding. Our results are most consistent with over-expression of one or more of these genes in ITR-R *A. fumigatus* without *cyp51A* mutations being at least partially responsible for ITR resistance. Multiple concurrent possible resistance mechanisms were found in some isolates. My work probably explains the mechanism(s) of resistance in *A. fumigatus* isolates with *cyp51A* mutations. Other ITR resistance mechanisms are also possible.

To determine taxonomic relationships among *A. fumigatus* clinical and environmental isolates, the sequences of the ITS, β-tubulin, actin and calmodulin gene of 23 clinical and 16 environmental isolates were analyzed phylogenetically. Actin and calmodulin sequences proved to be good for species differentiation of *A. fumigatus* while both ITS, β-tubulin regions did not, in this dataset. Many cryptic species of *A. fumigates* (complex) were found. All environmental *A. fumigates* complex isolates were ITR susceptible and no cross resistance was found.

DECLARATION:

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institute of learning.

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DEDICATION

I would like to dedicate this PhD to my mother and my wife. Their continued support, encouragement and love for me have made this possible. I am truly grateful to them for everything they have done for me

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Oral presentation

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Posters

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Papers

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ABPA	Allergic bronchi pulmonary aspergillosis
AFLP	Amplified fragment length polymorphism
AIDS	Acquired immunodeficiency syndrome
AMB	Amphotericin B
ABC	ATP binding cassette
ABPA	Allergic bronchi pulmonary aspergillosis
AFLP	Amplified fragment length polymorphism
AIDS	Acquired immunodeficiency syndrome
AMB	Amphotericin B
atrF	ATP binding cassette transporter F
BLAST	Basic Local Alignment Search Tool
BMT	bone marrow transplant
Bp	Base pair
CADRE	Central Aspergillus Data Repository
CASP	Caspofungin
Cfu	colony forming units
CIE	Counterimmunoelectrophoresis
CLSI	Clinical Laboratory Standard Institute
CBPA	Chronic bronchi pulmonary aspergillosis
CF	Cystic fibrosis
CMB	Catalytic molecular beacons
CPA	Chronic pulmonary aspergillosis
Ct	Cycle of threshold
CYP51A	Cytochrome P 450 subfamily 51A
CYP450	Cytochrome P450
DD	Double diffusion
DMSO	Dimethyle sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme- Linked Immunosorbent Assay
EUCAST	European Committee for Antibiotic Susceptibility Testing
FDA	Food and drug administration
FRET	Fluorescence Resonance Energy Transfer
FUN-	Membrane-permeant fluorescent probe
HIV	Human immunodeficiency virus

IA	Invasive pulmonary aspergillosis
ID	Immunodiffusion
Ig G	Immunoglobulin G
Ig E	Immunoglobulin E
Ig M	Immunoglobulin M
IPA	Invasive aspergillosis
ITR	Itraconazole
ITR-R	Itraconazole resistance
MDR	Multidrug resistance
MFS	Major facilitators
MIC	Minimum inhibitory concentration
Min	Minutes
MLEE	Multilocus enzyme electrophoresis
MLP	Microsatellite length polymorphism
MLST	Multilocus sequence typing
Mm	Milmeter
MOPS	Morpholinopropanesulfonic acid
NCCLS	National Committee for Clinical Laboratory Standards
NCBI	National centre for biotechnology information
NFA	Indirect fluorescent antibody
ORF	Open reading frame
PCR	Polymerase chain reaction
POS	Posaconazole
PT	Pyrithiamine
RAPD	Random amplified polymorphic DNA
RFLP	Restrection fragment length polymorphism
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
RVC	Ravuconazole
SAB	Sabouraud agar media
Sec	Seconds
SSRs	Single sequence repeats
UPGMA	unweighted pair group method with the arithmetic mean
VOR	Voriconazole
UK	United Kingdom

Chapter 1

Introduction

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1. Introduction

1.1 Epidemiology of *Aspergillus* infections

Aspergillus is a genus of fungi distributed worldwide. This genus is found extensively in nature, and grows and survives on organic debris (Mullins et al. 1984;Debeaupuis et al. 1997;Zander 2005). Although there are more than 300 species of Aspergillus, only a few are described and known to cause humans and animal illness (Femenia et al. 2009). Aspergillosis is a life-threatening infection in immunocompromised patients (Anaissie et al. 2002). *Aspergillus fumigatus* is the most common cause of invasive aspergillosis (IA)(Balajee et al. 2006) followed by *A. flavus, A. niger*, *A. terreus* and *A. nidulans* (Hope et al. 2005b).

Aspergillus fumigatus is a saprophytic ubiquitous fungus with airborne conidia (Mullins et al. 1984). It has become one of the most pathogenic fungi in industrialized countries (Debeaupuis et al. 1997). Daily, humans inhale at least several hundred *A. fumigatus* conidia. The small size of conidia (2 to 3 µm) allows them to reach the alveoli of the human lungs (Goodley et al. 1994;Hedayati et al. 2007). *A. fumigatus* is harmless to individuals whose immune system has not been compromised by disease. However, fungal infection depends on exposure to sufficient inoculum of organism and the competence of the host defence mechanism (Zander 2005). Exposure to *A. fumigatus* spores may cause an inflammation in sensitive individuals. In recent years there have been an increasingly large number of opportunistic fungal infections. These infections may present in a wide spectrum, varying from local involvement to dissemination and invasive secondary, to other diseases (Rippon 1982). Recently, acquired resistance to a number of antifungal components have been predominately reported especially triazole due to the widespread use of triazole antifungal agents (Calderone 2002;Nascimento et al. 2003;Howard et al. 2009) or through exposure of isolates to azole fungicides in the environment(Van Der Linden et al. 2011).

1.2 History

Aspergillus was first described in 1729 by Micheli, the Italian biologist, who was the first to distinguish stalks and spore heads. He was reminded of the shape of an aspergillum (holy water sprinkler), and named the genus *(Aspergillus*) accordingly (Hedayati et al. 2007;Rippon 1982;Kurup and Kumar 1991). Mayer in 1815 described an infection (aspergillosis) in the lung of a jay (*Corvus glandarius*) (Rippon 1982;Denning et al. 1998). John Hughes Bennett in 1842 recognized human fungal infection and first described pulmonary aspergillosis in a person from Edinburgh (Denning et al. 1998;Rippon 1982;Ross 1951). Sluyter in 1847 described *Aspergillus* pneumomycosis (Rippon 1982;Kurup and Kumar 1991). Virchow in 1856 described *A.fumigatus* as the etiologic agent for bronchial and pulmonary disease (Rippon 1982). *A. fumigatus* as a species was described by Fresenius in 1863 (Denning 1998). Allergic disease of the lungs was recognised by Popoff in 1887. In 1890, the first fatal invasive case was in a 3-year-old child with *Aspergillus* disease (Denning et al. 2002a). Cleland in 1924 found aspergillosis in necrotic lung tissue. Lapham in 1926 described Aspergillosis as an infection secondary to tuberculosis (Rippon 1982). In 1926 Thom and Church first classified the genus (Denning 1998). Deve in 1938 defined aspergilloma (fungal ball). Henrici in 1939 described two endotoxins from *Aspergillus* (Denning et al. 2002a;Tilden et al. 1961). By 1965 more than 150 species were classified in different groups. Samson and Pitt further refined the species using the new technologies such as DNA hybridization and thin-layer chromatography of secondary metabolites (Denning 1998).

However, the number of patients at risk of developing invasive aspergillosis has been increased for many reasons, including AIDS, solid organ transplant recipients, increased use of immunosuppressive regimens for autoimmune diseases and using an intensive chemotherapy regimens for solid tumors (Denning et al. 1998;Richardson 2005). Currently, invasive aspergillosis is the leading infectious cause of death in immunocompromised patients such as bone-marrow-transplant patients and those with leukaemia (Denning et al. 2002a).

1.3 Distribution of Aspergillus

Aspergillus species are commonly found in soil and plant debris throughout the world (Debeaupuis et al. 1997;Rippon 1982), in air and water (Warris et al. 2003;Dillon et al. 2007), in almost all oxygen-rich environments because they are strictly aerobic grow as moulds on the surface of a substrate. Fungi commonly grow on carbon-rich substrates such as glucose (Rippon 1982;Evans 1989). Even the lowly pillow contains substantial quantities of *A. fumigatus* and other species (Woodcock et al. 2006).

1.4 Laboratory identification

Identification and typing of fungi is one of the main issues in medical microbiology (Evans 1989). Some species of *Aspergillus* are resistant to certain antifungals for example *Aspergillus terreus* is an amphotericin B resistant mould Therefore, rapid identification of cultures is essential for treatment of these infections. Unfortunately, many laboratories have little or no experience of the specialized methods required for diagnosis of these infections (Evans 1989).

Several methods have been used for phenotypic identification of *A. fumigatus* strains, primarily methods based on cultural and morphologic characteristics (Rippon 1982;Evans 1989). Currently protein profiling, detection of antibodies (humoral response) or the detection of fungal antigens and metabolites such as enzymatic substrates in body fluids or tissues using serological tests do not distinguish species reliably. Other research techniques such as 2D protein electrophoresis and immunoblotting can be more reliable but are not routinely available (Rippon 1982;Lin et al. 1995;Latge 1999).

Unfortunately, these methods have disadvantages; they are labour intensive, time-consuming, and may lack sufficient discriminatory power to distinguish phenotypic variability. Therefore, these disadvantages limit the use of phenotypic identification for

routine typing of isolates from clinical and epidemiologic studies (Lin et al. 1995). In contrast, genotypic methods are faster, more accurate and reliable for fungal identification than phenotypic methods. However, in some circumstances pre-molecular methods can be useful for analysis at subspecies level.

1.4.1 Phenotypic characterization/ Diagnosis

The morphological characteristics of the conidia and conidiophores of *A. fumigatus* is the common method used in identification and classification. The presence of *Aspergillus* hypae and or conidiophores on microscopical examination, in addition to positive culture, enhances and provides a definitive diagnosis (Rippon 1982;Evans 1989). There are some closely related species which are best identified using molecular tools (see http://www.ncbi. nlm. nih.gov/ pubmed/ 21510879).

1.4.1.1 Colony morphology

A. fumigatus commonly grows on almost all laboratory media (Rippon 1982), and usually grows rapidly on defined media such as Sabouraud Agar or Czapek-Dox media (25^{o}C to 36 ^{o}C) producing a flat white colony. The colony size can reach 4 ± 1 cm within a week, and quickly becomes grey- green with production of conidia (Latge 1999). Morphological features of *A fumigatus* hyphae are described as septate with abundant dichotomous branching (Evans 1989). Septate hyphae and conidiophores may be seen under the microscope using a wet preparation stained with lactophenol cotton blue (Rippon 1982).Spore heads are rarely found on direct microscopy of tissue specimens, and usually only when the fungus is growing within air space such as the bronchial lumen or a pulmonary cavity during initial colonization (Evans 1989).

Under the microscope, the short, smooth-walled conidiophores (up to 300 µm in length and 5 to 8 µm in diameter) gradually enlarge from a foot cell towards the apex, terminating in a flask-shape vesicle. The conidiophores have hyaline green or brownish coloration especially at the tip near the vesicle. The vesicle (20 to 30 um in diameter) is fertile over its upper half, giving rise to peg like, conidium producing cell phialides (6 to

8 by 2 to 3 µm in size, the phialides bend upward). The sterigmata (phialides) produce unbranched chains of conidia. The conidial chains are produced in a columnar mass with rough conidia (2.5-3.0 µm) appear green in mass (Rippon 1982;Evans 1989). A few isolates of *A. fumigatus* are pigmentless and produce white conidia (Latge 1999). *A. fumigatus* is a thermophilic species. Rinyu et al. examined some strains at different temperature and different media and found that macromorphologies of these strains are variable in colony morphology, pigment production, and growth rate (Rinyu et al. 1995).

1.5 Clinical manifestations

The respiratory tract, which is the main site of infection for *A. fumigatus*, and the immunological status of the host play an essential role in pulmonary diseases caused by *A. fumigatus* (Zmeili and Soubani 2007;Kousha et al. 2011). *A. fumigatus* has been associated with a wide spectrum of diseases in humans. Exposure to *Aspergillus* antigens in normal hosts can lead to allergic conditions, ranging from asthma to allergic bronchopulmonary aspergillosis (ABPA) (Kurup and Kumar 1991). Normal hosts rarely develop invasive disease.

The incidence of invasive aspergillosis has increased during the last decade (Vassiloyanakopoulos et al. 2006). *A. fumigatus* is the most common aetiological agent causing aspergillosis in humans (Li et al. 2011). Invasive aspergillosis (IA) and aspergillomas may develop in preexisting pulmonary cavities. However, severe infection can also occur in patients with chronic diseases such as diabetes, alcoholism, and cancer(Kurup and Kumar 1991).

The major predisposing factors for infection include prolonged neutropenia, chronic administration of adrenal corticosteroids, the insertion of prosthetic devices, and tissue damage due to prior infection or trauma. The organism is capable of invading across all natural barriers, including cartilage and bone. It has a propensity for invading blood vessels causing thrombosis and infarction (Bodey and Vartivarian 1989).

1.5.1 Allergic Bronchopulmonary Aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) is a lung hypersensitivity disease mediated by an allergic late-phase inflammatory response to certain antigens of *A. fumigatus* (Almeida et al. 2006). Clinically, ABPA manifests as bronchial asthma with transient pulmonary infiltrates (Agarwal 2009). The primary criteria for ABPA described by Rosenberg and colleagues for a definitive diagnosis are: Personal history of atopic disease such as asthma, food allergy as well as a history of pulmonary infiltrates and supporting serological tests; elevated blood eosinophilia; immediate skin reactivity to *A. fumigatus* antigene; precipitating antibodies (IgG and IgM) and IgE against *Aspergillus* antigen and elevated levels of serum IgE (Almeida et al. 2006;Rosenberg et al. 1977).

ABPA occurs in patients suffering from atopic asthma or cystic fibrosis (CF). The long term treatment with antibiotics is one of the predisposing factors for the colonization of the respiratory tract with *A. fumigatus* in CF patients (Laufer et al. 1984).

1.5.2 Aspergilloma

An aspergilloma, also known as fungus ball, consists of a mass of fungal hyphae and compacted mucolage. In 1938, Deve coined the term aspergilloma to describe the mycetoma like lesion caused by *Aspergillus* (Kurup and Kumar 1991), which exists in a body cavity such as the pulmonary cavities especially those typically caused by sarcoidosis in black patients or by tuberculosis (Hope et al. 2005a;Israel et al. 1982).

A pulmonary aspergilloma is a rounded conglomerate of hyphae, mucus, and cellular debris contained within a fibrotic and thickened wall and occupying a pre-existing cavity (Hope et al. 2005a). Non-invasive aspergillomas may form following repeated exposure to conidia and target preexistinglung cavities such as the healed lesions in tuberculosis patients (Dagenais and Keller 2009).

A common symptom of aspergilloma is hemoptysis (Gupta et al. 2010) from lesions in blood vessels leading to thrombosis, hemorrhage, and eventual dissemination. High antibody titters (precipitins) are detected in patients with aspergillomas and ABPA (Kurup and Kumar 1991;Tomee et al. 1994). Pulmonary aspergillomas can often be diagnosed by their characteristic appearance in chest X rays (Kurup and Kumar 1991).

1.5.3 Invasive Aspergillosis (IA)

A.fumigatus is the most common species associated with IA, causing around 90% of all cases (Li et al. 2011), although this varies geographically for unclear reasons. IA has become a leading cause of death, mainly among hematology patients. IA following solid-organ transplantation is most common in lung transplant patients (19 to 26%). Opportunistic fungal infections seen in transplant recipients caused by *Aspergillus* and yeasts are associated with the highest mortality rates (Promper et al. 1993). IA is recognized today as the main fungal infection in cancer patients. Fungal infections represent a common complication in cancer patients, especially in patients with leukemia (Weinberger et al. 1992;Patel and Paya 1997;Chai et al. 2011).

1.6 Immunology of Aspergillosis

A. fumigatus conidia (spores) are ubiquitous in the atmosphere. Human beings are exposed to spores every day(Debeaupuis et al. 1997). Natural immunity plays an important role in the elimination of spores in immunocompetent people and prevents the establishment of infection(Schaffner 1989) by macrophages (Kurup and Kumar 1991). *A. fumigatus* can cause different diseases like allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis (Debeaupuis et al. 1997;Zmeili and Soubani 2007). The latter can occur in immunocompromised patients such as those with AIDS (Denning 1998). The small size and ability of inhaled conidia to germinate and grow at temperatures higher than 37°C without any specific nutritional requirement are important to cause aspergillosis (Debeaupuis et al. 1997). The lack of efficiency of the

normal phagocytic is contributory to the pathogenicity of opportunistic *A. fumigatus* (Debeaupuis et al. 1997).

The major innate defense mechanisms against *Aspergillus* are the barrier formed by alveolar macrophages (Kurup and Kumar 1991;Steinbach and Stevens 2003), complements and phagocytic cells (Brown 2011). The conidia of *A. fumigatus* are also eliminated from the lungs through the ciliary action of the lung epithelium.

A. fumigatus produces several toxic secondary metabolites such as gliotoxin(Amitani et al. 1995) and protease. These toxins can damage the epithelial tissue and might inhibit spore clearance from the lung. Gliotoxin not only inhibits macrophage phagocytosis but it also blocks T- and B- cell activation and the generation of cytotoxic cells (Latge 1999). The lung surfactant proteins A (SP-A) and D (SP-D) play a protective role against fungal infections caused by *A. fumigatus.* SP-A and SP-D enhance agglutination, phagocytosis, and the killing of conidia of *A. fumigatus* by alveolar macrophages and neutrophils (Madan et al. 1997).

The complement system is a biochemical cascade that contributes to humoral defense against microorganisms. The biochemical pathways that initiate the cascade occur via the classical or alternative pathway (Kozel 1996). C-reactive protein activates the complement cascade as a protection against *Aspergillus* (Latge 1999;Kozel 1996). Kozel reported that all pathogenic fungi examined have the ability to initiate the complement cascade. The complement system plays a role in the pathogenesis of aspergillosis; one study found that C5-deficient mice are more susceptible to infections than are C5-sufficient mice (Cenci et al. 1997). Generally activation of the alternative complement pathway helps phagocytes in the killing of fungus.

Phagocytic cells include macrophages and neutrophils which play an important role of protection against *A. fumigatus.* Alveolar macrophages are the major resident cells of the lung alveoli. Both macrophages and neutrophils are the major cells involved in the

phagocytosis of *A. fumigatus* via inhibition of germination and spore killing. Several studies reported that killing of conidia starts several hours (3-6h) after phagocytosis (Schaffner et al. 1983). Nonoxidative mechanisms are essential for the killing of conidia. No study has reported a 100% killing of inhaled conidia by alveolar macrophages (Latge 1999). After phagocytosis, germination of the inhaled conidia into the invasive hyphal form is inhibited and killed inside the phagolysosome (Steinbach and Stevens 2003). Conidia that are not killed by macrophages can be transformed into invasive *Aspergillus* hyphae which become susceptible to neutrophil killing through the release of toxic oxygen radicals (Steinbach and Stevens 2003).

1.6.1 Humoral immune responses

High levels of *A. fumigatus* specific circulating antibodies of the IgG and IgE isotypes were found in sera from patients with ABPA (Kurup and Kumar 1991;Knutsen et al. 2002). Elevated *Aspergillus*-specific IgE and total IgE levels and eosinophilia were reported in ABPA (Knutsen et al. 2002). Kurup et al. showed that the levels of subclasses of IgG, particularly IgG1 and IgG2, are also elevated in ABPA patients. These subclasses of IgG are also the predominant antibody in patients with aspergilloma (Kurup and Kumar 1991).

1.6.2 Cellular immune response

Lymphocytes (T cells) are involved in the cellular immune response and play a major role in host defense against *Aspergillus* (Grazziutti et al. 2001). Several studies reported that a cellular immune response to *Aspergillus* antigens in ABPA, as well as in allergic asthmatic and cystic fibrosis patients, is characterized by a Th2 CD4+ T-cell response (Kurup and Kumar 1991;Knutsen et al. 2002). ABPA is associated with a pulmonary eosinophilia, a Th2 cytokine profile manifested by the production of IL-4, IL-5, and IL-10, and an increase in levels of total and specific IgE, IgG1, and IgA, reflecting the Th2 humoral response to *A. fumigatus* antigens (Latge 1999).

Immunocompromised people are vulnerable to opportunistic fungal infections because the ability of the immune system to fight infection is compromised. Neutropenia is one major risk factor for invasive aspergillosis, the other is corticosteroid therapy. Generally, corticosteroids suppress the ability of macrophages to kill conidia through inhibition of nonoxidative processes and impairment of lysosomal activity, whereas cytotoxic chemotherapy decreases neutrophil number and function (Steinbach and Stevens 2003).

1.7 Diagnosis of fungal infection

1.7.1 Biochemical and molecular characterizations used in species

Some secondary metabolites including antibiotics and mycotoxins are produced by *A. fumigatus* and are similar to those produced by other species. Therefore, most are insufficient for species classification, with the possible exception of fumigaclavine C, festuclavine and fumagillin (Evans 1989;Latge 1999;Matsuda et al. 1992).

1.7.1.1 Serological diagnosis

The simplest serological test is double diffusion (DD) or immunodiffusion (ID), which is cheap and easy to read but relatively insensitive and time consuming. Ig M antibodies migrate more slowly than IgG and are not detected in a 24 h test. These techniques are useful to diagnose aspergilloma and chronic bronchopulmonary aspergillosis (CBPA). In contrast, most patients with allergic bronchopulmonary aspegillosis (ABPA) and invasive aspegillosis (IA) have weak positive serum levels (Evans 1989). Therefore, other serological tests for example, passive haemagglutination, counterimmunoelectrophoresis (CIE) e.g immunoelectrophoresis, indirect fluorescent antibody (IFA), ELISA, and radioimmunoassy, should be used with IA and ABPA especially for the detection of antigens because IA patients are often immunocompromised and may be unable to produce antibodies as a response to fungal infection (Evans 1989). IgG against *A. fumigatus* in individuals with CF with or

without ABPA was detected (Barton et al. 2008). ABPA is associated with high total serum IgE levels,specific IgE and IgG against *A. fumigatus* antigen (Kraemer et al. 2006) that may affect the specificity of diagnosis while detection of IgG4, a specific subclasses of IgG, may improve the specificity of diagnosis (Barton et al. 2008).

1.7.1.1.1 CIE

Counterimmunoelectrophoresis (CIE) has advantages and disadvantages. Detection of *A. fumigatus* serum precipitins by counterimmunoelectrophoresis is the method most commonly used in the clinical laboratory due to its sensitivity, rapidity, simplicity, and economy. But precipitins will form only if antigen and antibody equivalence become established in the area of the gel between the wells. In addition, precipitins will not form if antigens migrate in the same direction with antibodies (Evans 1989;Latge 1999;Dee 1975). Indirect Fluorescent Antibody (IFA) is rapid and sensitive. Titres greater than 1:20 to *A. fumigatus* are uncommon in healthy subjects, and in patients with non-invasive forms of aspergillosis (ABPA, aspergilloma) titres usually range from 1:40 to 1:640 or higher (Evans 1989;Gordon et al. 1977)

1.7.1.1.2 ELISA

The diagnosis of invasive aspergillosis (IA) is challenging in immunocompromised patients. Conventional techniques like culture are most commonly used for the diagnosis of (IA) and may take several days (Khot et al. 2008). Early diagnosis and therapy has been shown to improve outcomes (von Eiff et al. 1995) but it can difficult to reach a definitive diagnosis quickly. Detection of Galactomannanis (in serum and BAL fluid) and beta-glucan (in serum) are alternatives diagnostic assay. Recently, Enzyme-Linked Immunosorbent Assay (ELISA) technique that detects galactomannan has been approved for use in the USA. However it has been used in Europe for many years (Aquino et al. 2007). Enzyme-Linked Immunosorbent Assay (ELISA) is sensitive, cheap and safe with stable reagents, and may be used to detect not only antibodies but also antigenaemia. Moreover, sophisticated equipment is not required (Evans 1989;Richardson et al. 1982). Galactomannan is a heteropolysaccharide soluble antigen of the cell wall of the *Aspergillus* released during hyphal growth in tissues (Aquino et al. 2007). Many studies found that detection of galactomannanis by ELISA in high-risk patients with invasive Aspergillosis (IA) is more sensitive than culture and allows IA to be diagnosed before clinical manifestations occur. But rear false positive and false negative results remain problematic for such assay (Khot et al. 2008;Adam et al. 2004;Kedzierska et al. 2007).

1.7.1.2 Molecular identification and typing

Invasive aspergillosis (IA) has now become a major cause of death in immunocompromised patients with mortality rates up to 90% (Li et al. 2011). Early and accurate diagnosis of IA is important for several reasons; to investigate the source of infections and to control the progress of fungal infection by using appropriate antifungal therapy (Tuon 2007).

Aspergillus antigen (galactomannan) detected by ELISA and 1, 3-ß-D-glucan is useful for early diagnosis of IA in patients with hematological malignancy or hematological

transplant recipients. but not for solid-organ transplant recipients (Pfeiffer et al. 2006). It may result in improved outcomes (Mengoli et al. 2009) for the diagnosis of IA *Aspergillus* antigens detected by EIA and shows lower sensitivity than PCR tests. However, *Aspergillus* infection is difficult to confirm by blood culture and the immune status of patients such as severe thrombocytopenia patients, may effect the detection of antibodies (Tuon 2007). Isolation of fungus from IA patients samples (sputum and bronchoalveolar lavage (BAL) fluid) are relatively low (50%) and can take several days. Detection of *Aspergillus* DNA in BAL using a molecular diagnostic technique such as PCR is more a sensitive and rapid technique for the diagnosis of IA (Spiess et al. 2003) (Khot et al. 2008;Tuon 2007;Spiess et al. 2003) . PCR has a higher specificity and sensitivity than culture (Tuon 2007) but PCR assay is unable to differentiate *Aspergillus* conidial colonization from true tissue invasion.

Early diagnosis of IA remains a challenge, and some diagnostic tools such as PCR, are being investigated to improve the diagnosis of invasive aspergillosis (Mengoli et al. 2009). Molecular technologies have been utilized for microorganism identification. Molecular approaches such as Polymerase Chain Reaction (PCR) have emerged as diagnostic methods which are more accurate and reliable for microbial identification than phenotypic methods (Petti et al. 2005). Recently, PCR tests have been developed for the detection of the circulating genomic sequences. Since *A. fumigatus* is responsible for about 80% of invasive aspergillosis, typing assays have become required to differentiate between isolates, and to investigate the source of infections (Bertout et al. 2001) for epidemiological studies. In addition, PCR can be used to show variations in the virulence and antifungal resistance of individual strains within a species. For rapid diagnosis of IA a Real-time PCR has been used in the identification of Aspergillus in bronchoalveolar lavage (BAL) and blood samples (Mengoli et al. 2009) (Tuon 2007;Baxter et al. 2011).

Molecular technologies have been invested for sequencing of *Aspergillus.* The complete sequence of Aspergillus helps identify new targets for antifungal drugs and enables

investigative study of the basic biology of fungi. Direct identification of repeated sequences such as microsatellites, which are useful for strain typing, will allow a better understanding of the epidemiology of aspergillosis. The information derived from the first complete genome sequence of a human pathogenic *A. fumigatus* will help for early diagnosis of IA (Denning et al. 2002a).

Studies have used PCR to detect fungal nucleic acids and found that the PCR assay has reasonable sensitivity and specificity when used to test samples from patients at high risk for IA (Tuon 2007). Several methods based on genomic differences have been used for fingerprinting *Aspergillus,* (Anderson et al. 1996), including specific DNA primer (SSDP), Multi Locus Enzyme Electrophoresis (MLEE), fingerprinting with moderately repeated DNA sequences (Bertout et al. 2001), Random Amplified Polymorphic DNAs (RAPD)(Anderson et al. 1996;Loudon et al. 1993), and microsatellite methods.

RAPD and microsatellite techniques are based on PCR and use different primers and different amplification protocols (de Valk et al. 2007b;de Valk et al. 2007a). Restriction fragment length polymorphism (RFLP) is the other method used for typing. Fragments are visualized after cutting with a restriction enzyme (Latge 1999;Anderson et al. 1996). Other methods include Southern hybridizations with various repetitive sequence-based probes (Anderson et al. 1996). Each of these methods has advantages and disadvantages.

1.7.1.2.1 RAPD

Random Amplified Polymorphic DNAs (RAPD) was first described by Welsh and McClelland in 1990. RAPD is the most common method used for fingerprinting *A. fumigatus* (Anderson et al. 1996) (Lin et al. 1995;Anderson et al. 1996;Loudon et al. 1993;Aufauvre-Brown et al. 1992;van Belkum et al. 1993;Buffington et al. 1994;Leenders et al. 1996;Mondon et al. 1997), using different primers. The R108 primer (GTATTGCCCT) is used in the RAPD genotyping with *A. fumigatus* to give the

best discrimination between isolates (Anderson et al. 1996;Aufauvre-Brown et al. 1992;Raclasky et al. 2006). It utilises a single short primer at low annealing temperature and can be performed quickly for a large number of isolates (Bertout et al. 2001).

RAPD typing is easy to perform, rapid, with good typeability, reproducibility and is less labour-intensive than phenotypic techniques (Loudon et al. 1993). However, RAPD patterns are difficult to interpret due to the mismatches between the templates and the primers during the low temperature annealing in PCR (Latge 1999). Furthermore, the distance of migration of bands is very short (Loudon et al. 1995;Verweij et al. 1996). Factors such as buffer composition, primer concentration, thermal cycler performance and gel electrophoresis time may play an important role in the band pattern and affect the discriminatory power of the primer (Verweij et al. 1996). According to some observations, a combination of RAPD, Southern hybridization and RFLP gave better a discrimination than a single procedure (Anderson et al. 1996).

1.7.1.2.2 Microsatellites length polymorphism

Microsatellites are short segments of DNA that have a specific repeated sequence such as CACACACA, (known as CA4) from two to up to ten base pairs long (tandem repeats) (de Valk et al. 2007b), there are also called simple sequence repeats (SSR), short tandem repeats (STR), or variable number tandem repeats (VNTR). Microsatellites are numerous and found throughout the genome of organisms, in coding and non-coding regions, ranging from yeasts through to mammals (Latge 1999;Legendre et al. 2007). Microsatellite methods widely used for the strain identification and discrimination in a wide variety of microorganisms (van Belkum et al. 1997;van Belkum et al. 1998) displayed excellent discriminatory powers and showed 100% typeability (de Valk et al. 2007b). This technique has been successfully applied to *A. fumigatus* (Bart-Delabesse et al. 1998). The method is rapid and highly reproducible

(Lair-Fulleringer et al. 2003). In contrast to RAPD, it uses unique primers and specific sequences flanking the microsatellite (Bart-Delabesse et al. 1998).

In order to obtain stable and reproducible results, important for any successful typing technique, highly standardized reaction conditions are needed for the RAPD technique in contrast to microsatellite methods, which are less sensitive to variation with varying conditions (de Valk et al. 2007b).

One other fingerprinting system that has been widely used is Southern blot hybridization. Southern blot hybridized with various repetitive sequence-based probes; ribosomal or mitochondrial DNA (Wills et al. 1985;Magee et al. 1987) or with nonmitochondrial genomic sequence, have been demonstrated to be effective in many fingerprinting studies (Girardin et al. 1993). Hybridization with moderately repetitive DNA probes gave species specific banding patterns (Girardin et al. 1993). Southern blot hybridized with phage (λ3.9) probe which contains a species-specific repeat sequence provided unique and highly discriminative patterns for each strain tested (Girardin et al. 1993).

1.7.1.2.3 RFLP

Restriction fragment length polymorphism (RFLP) analysis offers an simple, rapid and inexpensive way to identify several microorganisms (Aravindhan et al. 2007), and has been used worldwide for species identification (Aravindhan et al. 2007). The restriction endonucleases recognise specific DNA sequences and cut the DNA in the recognition site. To obtain adequate power to discriminate among isolates it is necessary to use other target genes (Aravindhan et al. 2007).

In spite of the RFLP technique showing high reproducibility and reliability among a large population of *A. fumigatus* strains (Debeaupuis et al. 1997), it has disadvantages; it shows a limited degree of discrimination among different strains (Burnie et al. 1992) and interpretation of outcome results on stained gels is difficult (Latge 1999). Another study has shown RFLP patterns did not effectively provide discrimination among strains of *A. fumigatus* of the same species even with EcoRI digested DNA (Girardin et al. 1993). Denning et al. used RFLP with digested DNA (XhoI and SalI) to distinguish among strains but band patterns were variable and not enough to differentiate large group of isolates in epidemiological studies (Denning et al. 1990). Additionally, Girardin et al. found that RFLP patterns of EcoRI-digested DNA do not adequately discriminate among strains of *A. fumigatus* isolated from diverse sources (Girardin et al. 1993).

1.7.1.2.4 AFLP

Amplified fragment length polymorphism (AFLP) is DNA fingerprinting technique which, described in 1995, is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al. 1995). The main advantage of the AFLP is no prior sequence knowledge is required when using a limited set of generic primers. Other advantages of the AFLP methods are that it is rapid, easy to use, and can generate hundreds of highly replicable markers from the DNA of any organism (Mueller and Wolfenbarger 1999). Therefore, AFLP markers are a major genetic marker with broad application in DNA fingerprinting, population genetics, and quantitative trait loci (QTL) mapping (Mueller and Wolfenbarger 1999). However, AFLP method has successfully been used with a large variety of microorganisms with relatively large genomes including fungi (Warris et al. 2003).

1.7.1.2.5 MLEE

Multilocus enzyme electrophoresis (MLEE) was first used for *Candida albicans* (Bertout et al. 2001). MLEE is one of many typing methods developed for investigating

the diversity of *A. fumigatus*. It has good discriminatory power and allows assessment of the structure and differentiation of *A. fumigatus* populations, including their genetic diversity (Bertout et al. 2001).

1.7.1.2.6 MLST

Multilocus sequence typing is a tool used for differentiating isolates; it compares nucleotide polymorphisms within regions of five to seven genes. MLST was developed to investigate bacterial and pathogenic fungi populations including *A. fumigatus* (Bain et al. 2007).

1.7.1.2.7 Sequence based identification

Determining the order of the nucleotide bases; adenine, guanine, cytosine and thymine, in a DNA oligonucleotide, is called DNA sequencing. This is useful in diagnostic research and for future understanding of the fungal ecology in infections (Ronning et al. 2005). Many projects have generated the complete DNA sequences of microbial genomes. Initially, the first DNA sequencing (1968), was carried out with the chain-termination method (Hutchison 2007) and in 1975 was developed using the Sanger chain technique(Sanger and Coulson 1975;Maxam and Gilbert 1977).

However, the first complete cellular genome sequences from bacteria, appeared in 1995 and eukaryotic genomes were subsequently sequenced (Hutchison 2007). The first eukaryotic genome sequence for the yeast *S. cerevisiae* was completed in 1996 (Hutchison 2007). The genome of *A. fumigatus* Af293 was sequenced by the whole genome random sequencing method in 2005. Af293 contains eight chromosomes. There are 9,926 predicted protein-coding genes. About one-third of these predicted genes (3,288) are of unknown function. Additionally, there are approximately 12 mitochondrial genome copies per nuclear genome (Ronning et al. 2005).

1.7.1.2.8 Probe labelling

The basic mechanism for DNA hybridization based diagnosis occurs when a single strand of oligonucleotide probes finds its complement. In order to increase sensitivity and specificity of hybridization, Tyagi and Kramer developed novel labelled nucleic acid probes (stem-loop oligonucleotide) that recognize and report the presence of specific nucleic acids in homogeneous solutions (Tyagi and Kramer 1996). Labelling of primers, probes or amplicon is essential for the monitoring of the accumulation of amplicon in a real time PCR(Mackay et al. 2002). Several different conventional methods of probe labelling exist some of which are radioactive/ nonradioactive chemicals. Recently, fluorescent probes labelling has been used. Radioactive chemicals encountered difficulties in a real-time detection of nucleic acid amplification both *in vivo* and *in vitro* besides the toxic effects (Basich et al. 1981;Kostrikis et al. 1998). Therefore, several real-time PCR detection chemistries have been produced with advantages associated with each method (Wong and Medrano 2005).

1.7.1.2.9 Real-time PCR detection chemistries

DNA Binding Dye is one of the chemistries used in real-time PCR detection, which emits a fluorescent dye when bound to dsDNA (Figure 1.1A). The fluorescence intensity is proportional to the dsDNA concentration. These assays give false positives as a result of non-specific PCR products binding to DNA binding dyes (Simpson et al. 2000). Hybridization probes are oligonucleotides that are labelled with a reporter fluorescent dye at the 5' end and a quencher fluorescent dye at the 3' end (Gibson et al. 1996). The fluorescence resonance energy transfer occurs when the donor and acceptor fluorophores are in close proximity (Bernard and Wittwer 2000). The nucleolytic activity of DNA polymerase cleaves the hybridization probe and releases the reporter dye during the extension phase of the PCR cycle from the probe (Gibson et al. 1996). There is three or four oligonucleotide methods used. The four oligonucleotide method consists of two specific probes and two primers that bind adjacent to each other (Figure1.1B) (Simpson et al. 2000). The downstream probe is used to detect a mutation (Lay and Wittwer 1997). Hydrolysis Probes is similar to hybridization probes (Figure

1.1C). The quencher reduces the reporter fluorescence intensity by FRET when the probe is bound, while FRET increases intensity in hybridization probes when annealed to the target sequence. Probe degradation by DNA polymerase's 5′ nuclease allows for separation of the reporter from the quencher dye, resulting in increased fluorescence emission(Gibson et al. 1996).

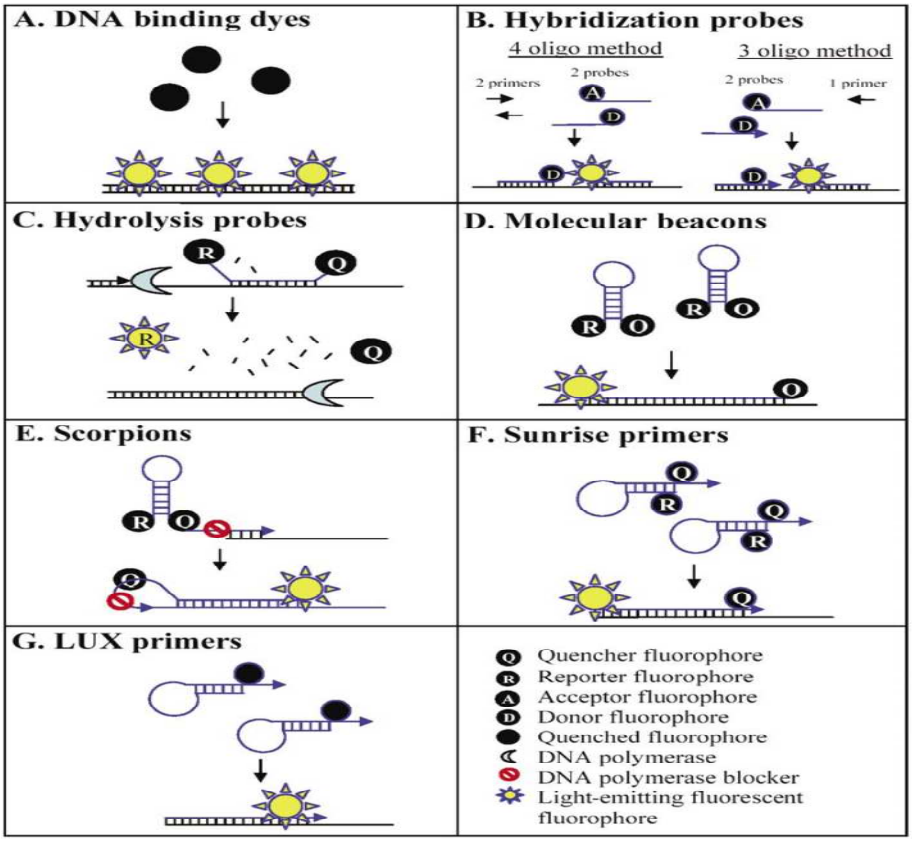


Figure 1.1 Real-time PCR detection chemistries. Probe sequences are shown in blue while target DNA sequences are shown in black. Primers are indicated by horizontal arrowheads (Wong and Medrano 2005).

1.7.1.2.10 Molecular beacons

Since a double-stranded complex is thermodynamically more stable than a probe hairpin structures, hairpin probes tend to have greater specificity (Bonnet et al. 1999). (''Molecular beacon'' fluorescent probes that produce fluorescence on hybridization with the target sequence of nucleic acids have been constructed (Goel et al. 2005).

Molecular beacons are an alternative to TaqMan for the detection of polynucleotides. Structurally, they are single–stranded stem loop oligonucleotide molecules (Figure 1.1D). The loop portion of the molecule is a probe sequence for a complementary target. The stem consists of two self annealing complementary arm sequences, unrelated to target sequence, at the ends of probe sequence (Tyagi and Kramer 1996).

A fluorophore is covalently attached to one end (at 3') of the oligonucleotide, and a nonfluorescent quencher is covalently attached to the other end (5' end of the arm) (Tyagi et al. 1998). The annealing of stems causes an intramolecular energy transfer from fluorophore to quencher. The energy transfer is much more efficient at shorter distances (Goel et al. 2005) in contrast, fluorescence when unwind stem occurs consequent of the probe binds to its target (Tyagi et al. 1998). As the stem keeps these in close approximately to each other fluorescence cannot occur in the absence of a target (Tyagi and Kramer 1996).

There are two synthesized forms available for molecular beacons; soluble (Tyagi et al. 1998) or glass bound probes, which are prepared as biotinylated or controlled pore glass bound probes (Tyagi and Kramer 1996).

The most significant factors that affect the melting temperature of molecular beacons are the length of the stem hybrid, percentage GC (guanine and cytosine) content of arm sequence and concentration of salt(Tyagi and Kramer 1996). Generally, the length of the probe sequence should range from 15 to 35 nucleotides with 5 to 8 nucleotide long arm sequences respectively. Probes should not form any further secondary structure. Tyagi and Kramer found the probe sequence should be at least twice the length of each arm sequence to maximize the separation between the reporter and quencher when hybridization occurs (Tyagi and Kramer 1996)The length and sequence of probe and stem of molecular beacons can exist in three different states in stem-loop, bound-to-target, and random-coil (Bonnet et al. 1999).

Specificity is enhanced by thermodynamic analysis of the transitions between these states (Bonnet et al. 1999). Increase in the probe length results in improved affinity but leads to reduced specificity (Tyagi and Kramer 1996;Kostrikis et al. 1998) and leads to disruption of unimolecular stem-loop conformation (Goel et al. 2005) .Molecular

beacons with short stem length have faster hybridization kinetics but suffer from lower signal to background ratio (Goel et al. 2005). The rigidity of the probe-target sequence forces the hairpin stem to unfold, leading to fluorophore and quencher separation and thus emitting fluorescence of a characteristic wavelength (Goel et al. 2005). Molecular beacons open up when the temperature is higher than their melting point and vice versa. As the temperature in the PCR reaction used is favorable for hybridization, the molecular beacon will interact with the target strands, resulting in fluorescence (Bonnet et al. 1999).

Recently, some probes have been modified to enhance the specificity and sensitivity of conventional probes. Whitcombe et al. developed the Scorpion technique for allele discrimination. Scorpion primers are bi-functional molecules which function simultaneously as a PCR primer and a beacon probe. Scorpions perform better than bimolecular methods under conditions of rapid cycling such as the Light Cycler because priming and probing is a unimolecular event (The upstream PCR primer is covalently linked to the probe at the 5' end. The molecules structurally contain a fluorophore, a quencher and a PCR blocker (HEG, nonamplifiable, monomers) at the start of the hairpin loop (Figure 1.1E). In the initial PCR cycles, the primer hybridizes to the target and extension occurs due to the action of polymerase (Whitcombe et al. 1999). The PCR stopper prevents the hairpin loop opening in the absence of the specific target sequence (Thelwell et al. 2000).

Sunrise primers are commercially called AmplifluoTM hairpin primer, which work similarly to scorpion primer (Figure 1.1F)(Mackay et al. 2002), acting as the PCR primer. When unbound, the hairpin is intact, causing reporter quenching via FRET. Upon integration into the newly formed PCR product, the reporter and quencher are held far enough apart to allow reporter emission (Wong and Medrano 2005).

Light upon extension (LUX) technology represents a new type of primer design and labelling. One primer is a self-quenched single-fluorophore similar to Sunrise primers (Figure 1.1G); the second one is not marked. However, rather than using a quencher

fluorophore, the secondary structure of the 3′ end reduces initial fluorescence to a minimal amount and relies on only two oligonucleotides for specificity (Wong and Medrano 2005).

Catalytic molecular beacons (CMBs) as modular deoxyribozymes are another variant that can detect the target sequences without amplification. In this design, the molecular beacon module interacts with a target oligonucleotide and a deoxyribozyme module amplifies this signal. Catalytic molecular beacons can discriminate between target and mutant oligonucleotides that differ in a single base (Stojanovic, de Prada et al. 2001). PNA is a DNA analogue with a polyamide backbone instead of a sugar phosphate backbone. Specificity has been demonstrated in the detection of ribosomal DNA from *Entamoeba histolytica* (Egholm et al. 1993). PNA molecular beacons were also reported to be superior to conventional molecular beacon probes because of their faster hybridization kinetics, In addition, PNA probes can hybridize to targets at a very low salt concentration, high signal to background ratio and achieve much better specificity (Xi et al. 2003).

1.7.1.2.10 .1 Applications of molecular beacon technology

It has become a very powerful tool in different applications in human, animal and plants aspects. Some important applications of this technique are summarized in the (Table 1.1).

Table 1.1 Application of molecular beacons

Application	Examples	References
Real-time PCR monitoring	rDNA from *Entamoeba histolytica*	(Ortiz et al. 1998)
Genetic analysis	the rpoB gene for rifampin resistance in *Mycobacterium tuberculosis*	(Piatek et al. 1998)
Molecular beacon and array technology	molecular beacon DNA biosensors	(Liu and Tan 1999)
Detection of pathogens	HPV type *Aspergillus fumigatus*	(Nascimento et al. 2003;Takacs et al. 2008)
Nucleic acid–protein interactions	interactions between Single Strand Binding (SSB) proteins and DNA	(Tan et al. 2000)
In vivo RNA detection	mRNA transcripts	(Matsuo 1998)

1.8 Genetic diversity among clinical and environmental isolates of *A. fumigatus*

A. fumigatus is a saprophytic fungus and a soil inhabitant. Its ubiquitous spores released into the atmosphere (Debeaupuis et al. 1997) are easily inhaled and can cause infection in an immunocompromised host. The airborne spores can cause nosocomial invasive aspergillosis (Warris et al. 2003). Some studies reveal an association between aspergillosis infections and hospital sites (Goodley et al. 1994). A genetic relationship

between *A. fumigatus* airborne strains and those causing invasive disease has been shown (Warris et al. 2003). On the other hand, a lack of genetic relatedness was also observed(Chazalet et al. 1998). Thus the role of local airborne spores in nosocomial disease is unknown.

Morphologically, *A. fumigatus* is a variable species. A slight variation was observed when 21 *A. fumigatus* isolates from 8 patients with an aspergilloma were analyzed by Immunoblot fingerprinting (Burnie et al. 1989) and slight variations were also observed when analyzed by restriction fragment length polymorphism (Burnie et al. 1992), amplified fragment polymorphism, random amplification of polymorphic DNA (RAPD)(Loudon et al. 1993) and mitochondrial cytochrome b gene sequence analysis (Wang et al. 2000).

Continuous genetic exchange of the environmental *A. fumigatus* population (Chazalet et al. 1998) may explain the different genotypes from the same geographic area (Debeaupuis et al. 1997). Nowadays, a polyphasic taxonomy that combines morphological and molecular phylogenetic analyses is used to characterize A. *fumigatus* and analyse genetic variation of strains or closely related species. Recently, Balajee et al 2005 found a new clinical species, *Aspergillus lentulus*. A phylogenetic analysis based on multilocus sequence typing was used in analysis (Balajee et al. 2005). In addition, soil-borne *A. lentulus* was isolated from Korean soil (Hong et al. 2005).

Several studies have found that *A. fumigatus* has many characteristics of a sexual species. 215 genes implicated in sexual development were found to be present (O'Gorman et al. 2009). The presence of sexual stage genes suggested that such a cryptic sexual stage exists in the human pathogen *A. fumigatus* (Debeaupuis et al. 1997). This finding may explain the observed genetic variation.

A number of different algorithms have been used to analyse the phylogenetic relationships of *A. fumigatus* strains. These include the unweighted pair group method

with the arithmetic mean (UPGMA), neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood. Hong et al., 2005 used neighbor joining and maximum parsimony methods to determined taxonomical position of the *A. fumigatus* strains. The amino-acid-based neighbor joining tree showed nearly the same topology as those of the DNA-based trees obtained by maximum parsimony (Hong et al. 2005).

1.9 Therapy

Over the past decade, new opportunistic pathogenic fungi have been increasing especially in immunocompromised patients (Nascimento et al. 2003). *A. fumigatus* is the most common species of *Aspergillus* that causes life-threatening pulmonary disease in severely immunocompromised patients (Nascimento et al. 2003). Treatment of aspergillosis depends on the type of the disease and immunological status of the patient. Currently, the effective antifungal drugs used to fight fungal infections are polynes such as amphotericin B, echinocandins and azoles such as itraconazole Table 1.2 (Calderone 2002). Until recently amphotericin B and ITR were the main antifungal drugs used for the treatment of invasive aspergillosis.

Recently, the FDA approved several new compounds against *Aspergillus* species and they are now licensed for treatment of invasive aspergillosis: D-AMB and its lipid formulations (AMB lipid complex [ABLC], L-AMB, and AMB colloidal dispersion [ABCD]), ITR, VOR, POS, and CASP (Walsh et al. 2008). In the United States only voriconazole is licensed for the primary treatment of invasive aspergillosis. ITA, POS, CASP, L-AMB, ABLC, ABCD are all licensed in both the United States and the European Union for second line treatment of invasive aspergillosis. The two classes of echinocandins; micafungin and anidulafungin, have been licensed in Europe for other indications, but micafungin is approved in Japan for aspergillosis (Walsh et al. 2008). There are two classes of anti-*Aspergillus* triazoles active against invasive aspergillosis; (VOR, ravuconazole, and isavuconazole) and (ITR and POS). Both of them vary in their pharmacology and mechanisms of resistance. Fluconazole, which is also an antifungal triazole and similar to VOR, is not active against invasive aspergillosis

The antifungal triazoles inhibit the cytochrome P450–dependent enzyme lanosterol 14-α demethylase, a key reaction in ergosterol biosynthesis pathway resulting in altered cell membrane function and cell death or inhibition of cell growth and replication (Walsh et al. 2008;Osherov et al. 2001).

In spite of the toxicity of amphotericin B, which causes nephrotoxicity, it has been the 'gold standard' in antifungal chemotherapy for many years (Calderone 2002;Nascimento et al. 2003;Petrikkos and Skiada 2007). However, newer more azole potent compounds have recently been synthesized. Azole agents activities and properties against *Aspergillus* strains *in vitro* and *in vivo* were first reported in 1984 (Van Cutsem et al. 1984). In 1990, the first generation of triazoles (fluconazole and itraconazole) were introduced and offered the medical field new alternatives to amphotericin B effective treatment of invasive aspergillosis with less toxic side effects (Petrikkos and Skiada 2007). ITR was the first oral antifungal available for the treatment of invasive aspergillosis (Denning et al. 1997b;Howard et al. 2006).

VOR is now the drug of choice for invasive aspergillosis (Herbrecht 2004). CASP was also recently licensed for the treatment of invasive aspergillosis (Maertens et al. 2004). However, despite availability of these drugs, invasive aspergillosis is often associated with significant morbidity and mortality. The failure of treatment of invasive aspergillosis is unclear so the morbidity and mortality rate is still high. One of the major barriers to unsuccessful treatment of aspergillosis is delay and improper diagnosis (Paterson et al. 2003).

1.10 Antifungal drugs used in treatment

1.10.1 Amphotericin B

Amphotericin B (AmB) is a polyene antifungal agent, which has been used against a wide range of fungal pathogens yeast; mould and some diatomaceous fungi (Loeffler and Stevens 2003). It has been used as standard therapy for treating life-threatening fungal infections for decades. Its basic mechanism of action is the interaction with the membrane sterol, results in the production of pores in the membrane leading to altered permeability and consequently to depolarization and cell death (Traunmuller et al. 2011). Some fungal species are intrinsically resistant to AmB, such as *Candida albicans* (Sterling and Merz 1998). However, it has side effects including nausea and nephrotoxicity. The lipid formulation of amphotericin B has the advantages of reduced nephrotoxicity and better delivery to the lungs, liver, and spleen(Steinbach and Stevens 2003). Sodium deoxycholate is an emulsifying agent for AmB that gives (D-AmB). A review by Denning of the scientific literature showed that the efficacy of conventional D-AmB given for ≥14 days exhibited response rates that varied highly with the nature of the underlying disease. Renal and heart transplant recipients with pulmonary IA showed average response rates of 83%, whilst liver transplantation was associated with poorer outcome (20%). Patients with bone marrow transplantation, haematological malignancies or AIDS responded by 33, 54 and 37%, respectively. None of the immunocompromised patients with cerebral aspergillosis survived, while non-immunocompromised patients from the same case series had a better prognosis (response rate 33%) (Traunmuller et al. 2011;Denning 1996). In many hospitals, conventional D-AmB, despite its lower cost, is largely abandoned as a therapeutic agent against IA (Kleinberg 2006). Despite its unfavourable safety profile,AmB still represents the best proven and most important therapeutic option in salvage situations and in the management of breakthrough infections (Traunmuller et al. 2011). Aerosolization of amphotericin B has recently gained favour in transplant centres, and studies have demonstrated this strategy to be relatively safe (Alvarez et al. 2007).

1.10.2 Azoles

The ergosterol biosynthetic pathway is the target of azoles. The mode of action of all azoles antifungal is the same (Petrikkos and Skiada 2007) inhibition of the enzyme cytochrome p450 lanosterol 14α-demethylase, the gene product of *ERG11* in yeast (*cyp51A* in *Aspergillus*). This enzyme catalyses an intermediate step in lanosterol into ergosterol that is required in fungal cell membrane synthesis (Calderone 2002;Howard et al. 2006).

Ergosterol is the predominant component and the main sterol of the fungal cell membrane (Kelly et al. 1997). The function of ergosterol has been examined in several studies. It is important for modulating the fluidity and permeability of the plasma membrane and is responsible for maintaining cell integrity and control of the cellular cycle (Ghannoum and Rice 1999;Munn et al. 1999). The absence of ergosterol in human cell and its functions make it and its biosynthetic pathway a useful target for antifungal agents(Alcazar-Fuoli et al. 2008).

The reduction of the intracellular levels of ergosterol, the main sterol of fungi, without removal of the 14α-demethyl group results, in growth arrest (Kelly et al. 1995). Kelly, showed cell growth arrest correlates with the accumulation 14α-demethylase-erogosta-8, 24 (28)-dien-3β,6α-diol in a yeast strains with a sterol 14α-demethylase gene disruption(Kelly et al. 1995).

1.10.3 Itraconazole

Two formulations of ITR were used; commercially available ITR oral solution and amorphous nanostructured SFL ITR (Alvarez et al. 2007). ITR is a broad-spectrum triazole antifungal agent with good *in vitro* activity against *Aspergillus spp* , Several studies have shown that *in vitro* activity of ITR against *A. fumigatus* is high, and in some of these studies no itraconazole-resistant (ITR-R) isolates have been identified (Dannaoui et al. 1999). ITR has been successfully used to treat patients with invasive aspergillosis, aspergilloma and chronic necrostising aspergillosis (Dannaoui et al.

2001). Inadequate absorption of ITR in AIDS and neutropenic patients can limit the use of this drug (Oakley et al. 1998). However, it has been available as oral capsules or oral solution for a long time. ITR is available for oral and intervanous administration for clinical use against *A. fumigatus* (Howard et al. 2006).

Azole is an inhibitor of the cytochrome P-450 (cyp51A). However, ITR continues to play a role in antifungal prophylaxis in the early phase after allogeneic HSCT and is recommended in the therapy of allergic bronchopulmonary aspergillosis and allergic *Aspergillus* sinusitis because of its corticoid-sparing effect in these conditions (Traunmuller et al. 2011). New formulation, aerosolized nano-structured ITR was reported for *in vitro* efficacy in preventing pulmonary aspergillosis due to *A. fumigatus* (Alvarez et al. 2007).

1.10.4 Voriconazole

Voriconazole (VOR) is a triazole antifungal agent, similar to Fluconazole, with a broad spectrum of activity against the majority of human fungal pathogens even those resistant to AmB and ITR (Traunmuller et al. 2011). It has also proved clinically useful in patients with both acute and chronic invasive aspergillosis (Oakley et al. 1998;Felton et al. 2010). Anti-*Aspergillus* activity of VOR was reported in 1996 (Petrikkos and Skiada 2007;Oakley et al. 1998). It was first marketed in 2002 after approval by the US, FDA (Greer 2003), and can be given both intravenously and orally. VOR inhibits the fungal enzyme 14-α-lanosterol demethylase, which catalyses a key step in the membrane synthesis, namely the conversion of lanosterol to ergostero (Traunmuller et al. 2011).

Because of its efficacy and relative safety, VOR has become the first-line therapy (Petrikkos and Skiada 2007) for invasive aspergillosis especially given *A. terreus* and *A. nidulans* resistance to amphotericin B (Espinel-Ingroff et al. 2001). It has broad inhibitory activity against filamentous fungi and yeast (Johnson et al. 1998).

The drug has been shown to have good efficacy *in vitro* activity against *Aspergillus spp*., and it has shown encouraging clinical efficacy in animal models of IA (Herbrecht 2004). Consequently, between1994 and 1996, Denning et al. conducted an open, non-comparative clinical trial on VOR treatment of histopathologically or microbiologically proven or probable IA. In a total of 116 eligible patients with various underlying conditions, the proportion of patients with complete or partial response at their respective end of VOR therapy was 48%. 2 out of 16 patients showed complete response (Denning et al. 2002b). It has been proven to treat bone infection due to *Aspergillus* (Petrikkos and Skiada 2007;Mouas et al. 2005) and as prophylactic, empirical therapy in severe immunosuppression and bone marrow transplant patients (Traunmuller et al. 2011), it has also been successfully used to treat patients with invasive aspergillosis (Calderone 2002). Broad activity of VOR was reported against other pathogenic fungi including *Blastomyces dermatitidis, Histoplasma capsulatume, Fusarium* and *Cryptococcus neoformans* (Li et al. 2000;Paphitou et al. 2002).

In animal model, VOR was evaluated by Kirkpatrick et al., 2000 in an immunosuppressed-guinea pig model of invasive aspergillosis. They reported that VOR was more effective than amphotericin B or similar doses of ITR in the clearance of Aspergillus from tissues (Kirkpatrick et al. 2000). The efficacy of VOR in the treatment of patients with acute invasive aspergillosis (Denning et al. 2002b;Grau et al. 2006), and cerebral aspergillosis (Stiefel et al. 2007) has been demonstrated.

1.10.5 Posaconasole

Posaconazole (POS) is a broad-spectrum triazole agent against *Aspergillus* species (Felton et al. 2010). Whilst European Commission had approved the use of POS as a prophylactic agent for invasive aspergillosis for many years (Petrikkos and Skiada 2007) it was not approved by the FDA until 2006.

Posaconazole is currently used for the treatment and prevention of invasive fungal infections. POS is a derivative of ITR, which blocks ergosterol synthesis through

inhibition of lanosterol 14-α-demethylase (CYP51). POS has a narrow drug interaction profile (Petrikkos and Skiada 2007;Pfaller et al. 2002). Posaconazole is a safe and effective agent and the most active triazole against filamentous fungi (Felton et al. 2010). It is more active than ITR against invasive moulds, including *Aspergillus* (Kontoyiannis and Lewis 2002). A certain degree of cross-resistance between POS and ITR has also been reported for *Aspergillus fumigatus* isolates (Mosquera and Denning 2002). Point mutations in the *cyp51A* gene, specifically in codon 54, are associated with POS resistance in *A fumigatus* (Mann et al. 2003).

1.10.6 Echinocandins

Recently, Scientists developed novel antifungal agents known as echinocandins that were approved for use in fungal infection treatment (Ostrosky-Zeichner 2004). The echinocandins are a novel class of semisynthetic amphiphilic lipopeptides composed of a cyclic hexapeptide core linked to a variably configured N-acyl side chain (Walsh et al. 2008). Echinocandin antifungal agents kill *Aspergillus* by inhibiting the (1, 3) - β-D-glucan synthase, a polysaccharide in the cell wall of many pathogenic fungi. The end result of inhibition of (1, 3)-β-D-glucan synthase is cell death because 1, 3-b-glucan and chitin are important in maintaining the osmotic integrity of the fungal cell and play a key role in cell division and cell growth.

Both *in vitro* and *in vivo*, echinocandins are fungicidal against most *Candida* spp. and fungistatic against *Aspergillus* spp (Shao et al. 2007). There are three types of echinocandin available for intravenous use: CASP, micafungin and anidulafungin (Petrikkos and Skiada 2007). All current echinocandins are only available for IV administration (Walsh et al. 2008). Caspofungin is the most common echinocandins agent used for treatment of invasive aspergillosis (Petrikkos and Skiada 2007).

Micafungin has the same spectrum of antifungal activity as CASP. Micafungin blocks the synthesis of a major fungal cell wall component, 1-3- β- D-glucan. Several case reports have reported success with micafungin in severely compromised hosts with refractory aspergillosis (Petrikkos and Skiada 2007). The mode of action of anidulafungin is the same as the other echinocandins. Anidulafungin is highly active in

vitro against a wide range of *Candida* spp. as well as against *Aspergillus spp* (Zhanel et al. 1997).

Table 1.2 the sites of action of antifungal drugs and mechanisms of resistance

Anti fungal drugs	Site of action	Mechanism of action	Mechanism of resistance
Echinocandins	Cell wall	Inhibits 1-3-β-glucan synthase	Mutation in enzyme encoding 1-3-β-glucan synthase
Amphotericin B	Plasma membrane	Interact with ergosterol	Alteration in ergosterol (membrane lipid)
Azoles	Ergosterol biosynthetic pathway	Inhibits P450 14α-demethylase	1.Alteration in the target enzyme 2.Alterations in enzyme of ergosterol pathway 3.Overexpression of genes encoding drug extrusion pumps

1.11 Azoles Resistance

Some fungal species are intrinsically resistant to antifungal drugs such as *Aspergillus terreus* which is intrinsically resistant to AmB (Blum et al. 2008). Primary resistance to amphotericin B is well recognized in some *A. terreus, A. flavus* and *A. ustus* isolates (Howard and Arendrup 2011), while others acquired resistance due to transient genotypic alteration. Unfortunately, the widespread use of triazole antifungal agents to fight these infections has resulted in increased of resistance to a number of these components (Kelly et al. 1995;Nascimento et al. 2003). Azole-resistant *Aspergillus* has been isolated in azole naïve patients, in azole exposed patients and in the environment (Howard and Arendrup 2011).

Higher minimum inhibition concentrations (MICs) have been recorded for some clinical isolates (Denning et al. 1997b). In spite of the uncommon azole drug resistance, there have recently been several articles demonstrating clinical and cellular aspects of multi

drug resistance (MDR) in human pathogenic fungi for analysis (Calderone 2002;Mellado et al. 2006).

Efforts are being made to identify the antifungal resistance mechanisms for developing effective therapeutic strategies. Therefore, azole antifungal resistance has been studied in several other species of fungi. The first report of azole resistance in *A. fumigatus* was published in 1997 (Denning et al. 1997b). ITR-R in *A. fumigatus* has been reported for both clinical isolates and laboratory mutants (Verweij et al. 1998), and resistance to VOR was described in laboratory mutants (Manavathu et al. 2001) and clinical isolates (Bueid et al. 2010). Resistance has currently been reported in Belgium, Canada, China, Denmark, France, Norway, Spain, Sweden, the Netherlands, UK and the USA. Where reported, the frequency of resistance differs significantly. However, the UK (Manchester) and the Netherlands (Nijmegen) have described particularly high frequencies (5 and 6% respectively), and a significant increase in azole resistance in recent years (Howard and Arendrup 2011). There are many factors including penetration, distribution of the drug into the target area immunological state of the host and antifungal resistance that lead to treatment failure.

1.12 Antifungal susceptibility testing

1.12.1 Susceptibility testing methods and *in vitro in vivo* correlations

In vitro susceptibility of fungi to antifungal agents was rarely reported before 1990 (Manavathu et al. 2000;Denning et al. 1992). Three principal methods have been used to determine MIC values for *Aspergillus* spp.; macrodilution broth, microdilution broth, and agar dilution tests. However, many other methodologic variations have been used that included inoculum size, PH, medium composition, temperature of incubation, duration of growth and end-point determination. All of these may effect antifungal activity and MIC (Denning et al. 1992).

Several laboratories have used a number of different methods of determining *in vitro* susceptibility, and they documented that most isolates of *A. fumigatus* are susceptible to ITR (Denning et al. 1997b). Increased numbers of invasive fungal infections and reports of resistance to antifungal drugs in *A. fumigatus* have highlighted the need for reproducible methods of *in vitro* testing with clinical relevance.

The minimum inhibitory concentration (MIC) is the lowest concentration of an antifungal drug that will inhibit the visible growth of a microorganism within a defined period of time usually 48 hours incubation for *Aspergillus*. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm the resistance of microorganisms to an antifungal drug. However, isolates can be reported as susceptible, inhibited by an antifungal agent, or resistant when they exhibit viable growth against an antifungal agent.

Two published standardised methods; the CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) were used for *in vitro* susceptibility testing for filamentous fungi. Both are microdilution but differ with respect to glucose concentration in the medium (0.2 vs. 2% for CLSI and EUCAST respectively), fungal inoculum size (0.4– 5×10^4 vs. 2– 5×10^5 CFU/ml) and shape of the microtitre wells (U-shaped vs. flat bottom) (Howard and Arendrup 2011).

Table 1.3 The CLSI M38 and EUCAST method for testing the susceptibility of *A. fumigatus* to Azoles

CHARACTERISTICS	EUCAST	CLSI M38-A (NCCLS)
Test medium	RPMI-1640 medium with 2% glucose	RPMI-1640 medium
Inoculum concentration	2-5 x 10^5 cfu/ml	0.4-5 x 10^4 cfu/ml
Spores counting	Haemocytometer	Spectrophotometrically at 530 nm
Incubation time	48h	48h
Filter	450nm	490nm
End point	Visual, no growth	Visual, no growth

The *in vitro* activity of antifungal agents, posaconazole, itraconazole, and voriconazole, against 200 clinical isolates of *A. fumigatus* recovered from 26 immunocompromised patients with invasive aspergillosis or bronchial colonization were used. This study concluded that the emergence of resistance in *A. fumigatus* during antifungal therapy with ITR is an uncommon phenomenon (Dannaoui et al. 2004).

A large number of yeasts and moulds of high MICs have been reported in one study which used 19,000 clinically important strains of yeasts and moulds. Susceptibility tests were performed in 45 laboratories using Clinical and Laboratory Standards Institute broth microdilution methods using RPMI 1640 medium. However, 1119 *A. fumigatus* isolates were used. The MIC90 values for *A. fumigatus* isolates were 1.0mg/l for ITR, 0.5 mg/l for VOR, and 0.5 mg/l for POS (Sabatelli et al. 2006).

As a result of emergent ITR resistance in *A. fumigatus*, it is important to perform *in vitro in vivo* correlations to guide therapy (Denning et al. 1997a). Resistance testing might be a more accurate term for describing *in vitro* methods used to assess drug activity (Kontoyiannis and Lewis 2002). Standard bacterial susceptibility methods have been used by clinical laboratories as a result of a good correlation between *in vitro*

results and *in vivo* outcome (Aviles et al. 2001). Recently a good correlation has been demonstrated between *in vitro* susceptibility data and the clinical outcome of antifungal treatment in aspergillosis (Velez et al. 1993;Gehrt et al. 1995).

Investigators have used animal models (mice, rabbits) to establish invasive pulmonary aspergillosis and then to study the efficacy of antifungal drugs in the treatment of aspergillosis (Verweij et al. 1998). For example, animal studies have been used to determine the efficacy of amphotericin B in rabbits with pulmonary aspergillosis (Allende et al. 1994) and the efficacy of itraconazole-cyclodextrin solution against *A. fumigatus* (Patterson et al. 1993). Animal studies were also used to evaluate diagnostic methods such as (ELISA) (Weiner and Coats-Stephen 1979;Patterson et al. 1988). These issues are especially important for immunocompromised patients who have immune deficits that may vary over time in addition to infection of different organ systems and may respond differently to antifungal agents (Denning et al. 1992;Dixon et al. 1989;Jensen and Schonheyder 1993;Richard et al. 1996).

In vitro in vivo correlations are beginning to emerge in yeast infections (Powderly et al. 1988;Galgiani 1990). Standardized methods were developed for *Candida* spp.(Wanger et al. 1995) and one study documented for the first time the *in vivo* relevance of *in vitro* susceptibility to an azole antifungal for *Cryptococcus neoformans* (Velez et al. 1993). There was good evidence of correlation of MIC with clinical outcome in patients and in animal models of yeast infection (Denning et al. 1997a). Johnson et al. were unable to correlate between susceptibility test results and *in vivo* outcome for amphotericin B in *A. fumigatus* (Johnson et al. 2000;Mosquera et al. 2001)

In vitro resistance to ITR (confirmed in a neutropenic murine animal model) has only recently been described in *A. fumigatus.* This finding was used to establish the validity of the MICs. Denning et al. observed animals survived (90 to 100%) with the susceptible isolate (AF210) whereas the control animals had 90% mortality with AF72 that was treated with the ITR (25 mg/kg) and 20% mortality with the ITR (75 mg/kg). The control mice injected with AF90 had a 100% mortality when treated with 25 mg/kg

and 80% mortality for those treated with 75 mg/kg (Denning et al. 1997b). Rabbits treated with POS showed a significant improvement in survival and significant reductions in pulmonary infarct scores (Mosquera et al. 2001).

Denning et al. were able to identify and differentiate the resistant strains from the susceptible ones and the correlation ITR *in vitro* testing results with *in vivo* outcome for *A. fumigatus in vitro* test, with either an agar dilution method with RPMI medium or a broth microdilution method with RPMI medium with added glucose (Denning et al. 1997a).

1.12.2 Other antifungal susceptibility testing methods for moulds

The most useful alternative methods for antifungal susceptibility testing are agar diffusion and Etest strip. For *Aspergillus spp*., good correlations of amphotericin B and ITR with Etest and MICs by the M38 method have been demonstrated. In one study RPMI-based agars gave 96% correlation with the reference microdilution method (Rex et al. 2001). In one study, the susceptibilities of 25 clinical isolates of *Aspergillus* species, to itraconazole and amphotericin B were determined. The results were compared with those obtained by an agar diffusion-dilution method (the Etest method) and a colorimetric broth microdilution method (the Sensititre method) with the CLSI document M38-A. Low levels of agreement between the NCCLS and the Etest methods using the recommended MIC endpoints were found for most *Aspergillus* species, especially after 48 h of incubation (<50%), when the MICs obtained by the Etest method were up to 9 twofold dilutions higher than the corresponding MICs obtained by the NCCLS method. Overall, better agreement was found when MIC-0 was used as the MIC endpoint for the NCCLS method for both drugs and when the MICs by the Etest method were determined after 48 h of incubation for ITR and after 24 h of incubation for amphotericin B (Meletiadis et al. 2002). Etests have been evaluated against CLSI methodology and show excellent correlation for VOR (Howard and Arendrup 2011).

Flow cytometry has also been used for antifungal susceptibility testing. Lass-Florl, Nagl et al. 2001 developed a cell viability assay to assess the activities of antifungal against fungal hyphae using the halogenated dye FUN-1, a membrane-permeant fluorescent probe. This dye is converted to a red fluorescent agent in actively metabolizing fungal cells. Cells with impaired metabolism do not form red fluorescent. The use of FUN-1 is a simple and rapid way to assess the viability of *Aspergillus* spp. under antifungal treatment. This method can distinguish *Aspergillus* isolates susceptible to amphotericin B from those resistant (Lass-Florl et al. 2001;Balajee and Marr 2002). All of these alternative methods (colorimetric microdilution, Etest and Disk diffusion) correlate more or less with the standard method (Lass-Florl C 2006).

European Committee on Antimicrobial Susceptibility Testing (EUCAST) was set up to standardise antimicrobial breakpoint and susceptibility testing methods in Europe. AFST-EUCAST is a subcommittee on Susceptibility Testing that aims to standardise susceptibility testing methods and determine antifungal breakpoint for antifungal drugs (Kahlmeter et al. 2006). AFST-EUCAST has developed the Clinical and Laboratory Standards Institute (National Committee for Clinical Laboratory Standards) reference method for mould.

The minimum inhibition concentration (MIC) of the isolate is determined using the broth microdilution method with modifications details in table1.3. All drugs were tested in a RPMI-1640 medium with 2% glucose.

1.13 Mechanisms of resistance in *Aspergillus fumigatus*

Intrinsically antifungal resistant isolates may be found in nature or develop through the exposure of isolates to azole fungicides in the environment (Van Der Linden et al. 2011) or the development of azole resistance during azole treatment. Azole-resistant *Aspergillus* has been isolated in azole naïve patients, in azole exposed patients and in the environment (Howard and Arendrup 2011;Arendrup et al. 2010;Snelders et al. 2008). Recently, ITR-resistant *A. fumigatus*, with elevated minimum inhibitory concentrations for VOR, POS and ravuconazole, were increasingly detected in clinical and environmental specimens in distinct European countries and China (Verweij et al.

2009b;Lockhart et al. 2011). There is evidence that azole fungicide agents used in agriculture may be in part responsible for this trend (Verweij et al. 2009b).

The primary mechanism of triazole resistance in *A. fumigates* is a mutation of the *cyp51A* gene encoding 14 α -demethylase at the target site and decreased intracellular drug accumulation or efflux (Xiao et al. 2004), Although in some centres the number of resistant isolates without *cyp51A* -mutations is increasing(Bueid et al. 2010). Some *Aspergillus* species less commonly causative for IA show an intrinsically reduced susceptibility to triazoles and AmB, e.g. *A. lentulus and A.viridinutans* (Van Der Linden et al. 2011).

Other mechanisms that decrease the intracellular accumulation of triazole compounds, such as overexpression of the efflux pumps and decreased cellular permeability may also play a role in resistance (Denning et al. 1997b;Manavathu et al. 1999;Cannon et al. 2009;Lockhart et al. 2011). Other molecular mechanisms of antifungal resistance in *A. fumigatus* are described below in Figure 1.5. In 2001, two 14α-demethylase genes (*cyp51A* and *cyp51B*) were identified in pathogenic *Aspergillus* spp. Figure 1.2 shows the structure of the homology model of AF-CYP51A (Howard et al. 2006;Mellado et al. 2001).

Ergosterol is a major and essential component of the cell membrane in most fungi. Minor changes in sterol composition were reported in three isolates (AF72, AF90, and AF91) of *A. fumigatus*, a mutation in *cyp51A* gene being responsible for resistance. This was the first published case of ITR-resistant *A. fumigatus* (Denning et al. 1997b), although this has also been shown to be a mechanism of resistance in *Ustilago maydis* (Joseph-Horne et al. 1995), *S. cerevisiae* and *C. albicans* (Denning et al. 1997b).

Mutation at codons 54, 98 and 220 have reported frequently in *A. fumigates* resistant isolates while mutations in codons 46, 172, 248, 255, and 427 have also been found in both azole susceptible and resistant strains(Howard et al. 2009). Expression of the

cyp51A gene in a resistant isolate is more than the azole-susceptible isolates (Arendrup et al. 2010) and decreasing the intracellular antifungal concentration by upregulation of efflux pumps genes was found and characterized azole resistance mechanisms in yeasts (Sanglard et al. 2009). Few reports have been investigated efflux pump expression in *Aspergillus* strains (Howard and Arendrup 2011).

1.13.1 Mutation in the *cyp51A*

A single mutation at codon 138 (G138C) was detected in the target gene (*cyp51A*) of two strains. The mutant was altered from GGC to TGC, substituting the amino acid glycine for cysteine (138C) (Howard et al. 2006). This residue is located near the heme cofactor.

Replacing G138 with Arg and G448 with Ser would be predicted to disturb the heme environment. Specifically, the large charged side chain of Arg at position 138 would clash with one of the heme's vinyl side chains and with the side chains of neighboring residues (Xiao et al. 2004). The G138C mutation which disturbs the heme environment (near the heme cofactor of *cyp51A*) reduces drug binding affinity (Howard et al. 2006). Other point mutations reported include G138 and G448 and are associated with VOR and RVC cross-resistance, with a lesser reduction in susceptibility to ITR and POS (Howard et al. 2006).

Another point mutation has been identified in the *A fumigates cyp51A* gene from a laboratory strain that was resistant to azole drugs. Alterations at (G54) in *cyp51A* have been described both in clinical ITR-resistant *A. fumigatus* strains and in laboratory mutants (Nascimento et al. 2003;Diaz-Guerra et al. 2003;da Silva Ferreira et al. 2004;Chen et al. 2005) (Howard et al. 2006). ITR-resistant isolates induced the (G54E) (G54K) (G54R) mutation. The G54R mutation is located in a very conserved region, which is demonstrated to induce structural changes in the enzyme molecule (Xiao et al.

2004;Diaz-Guerra et al. 2003). Replacing G54 with either Glu or Arg resulted in a 30-fold increase in the POS MIC.

In fact, both ITR and POS occupy the same binding site (Figure 1.3). Consequently, the substitutions at G54 also resulted in large increases in the ITR MIC (Xiao et al. 2004). This alteration was associated with cross resistance to ITR and POS (Howard et al. 2006;Mellado et al. 2006) but not VOR or Ravuconazole (RVC). This is probably because the VOR lacks a long side chain that would span channel 2 (Figure 1.3), and therefore substitutions at G54 would be predicted to have no impact on VOR binding. This was shown to be the case in *A. fumigatus* isolates with substitutions at G54 that remained susceptible to VOR (Xiao et al. 2004). Alterations at glycine 54 (G54) in *cyp51A* have been described both in clinical ITR-resistant *A. fumigatus* strains and in laboratory mutants (Nascimento et al. 2003;Howard et al. 2006;Diaz-Guerra et al. 2003;da Silva Ferreira et al. 2004). However, the exact approach by which substitutions at M220 and G54 of CYP51A interfere with the binding of azole antifungals is uncertain (Chen et al. 2005).

Methionine 220 (M220) may also be a hot-spot for substitutions conferring resistance in *Aspergillus* (Chen et al. 2005;da Silva Ferreira et al. 2004). Mutations in *cyp51A* result in the replacement of methionine at residue 220 by valine, lysine, or threonine (Mellado et al. 2004) and therefore reduction in the susceptibility (elevated MICs) to all azole (ITR, POS, RVC and VOR) drugs associated with amino acid substitutions at methionine M220 (Howard et al. 2006;Mellado et al. 2006). Mellado et al. reported that M220 in the *A. fumigatus cyp51A* gene is a hot-spot for substitutions conferring resistance to azole antifungal drugs in *Aspergillus in vitro* (da Silva Ferreira et al. 2004;Mellado et al. 2004). The relationships of G54 and M220 modifications with resistance have both been confirmed by transformation of the altered *cyp51A* genes into wild-type strains.

Cross-resistance to all azole drugs related to the presence of Cyp51A substitutions at leucine 98 for histidine (L98H) linked to duplication in tandem of a 34 bp repeat in the *cyp51A* promoter region. Mutation in leucine 98 linked to tandem repeated of 34 bp in the *cyp51A* promoter seem to be responsible for increased *cyp51A* gene expression (Mellado et al. 2006).

The second azole-resistance mechanism results from alteration in cellular content of azole by altered uptake or efflux mechanisms (Denning et al. 1997b), or azole resistance mechanism mediated by increasing in the expression of cytochrome P-450 mediating sterol 14a-demethylase (Denning et al. 1997b). There are two classes of efflux transporters; ABC and MFS (major facilitator superfamily). In *A. fumigatus*, four genes which encode ABC type transporters have been detected, the mdr1, mdr2, atrF and mdr4 genes and also a gene which encodes a protein of the MSF class, the mdr3 gene (Rementeria et al. 2005).

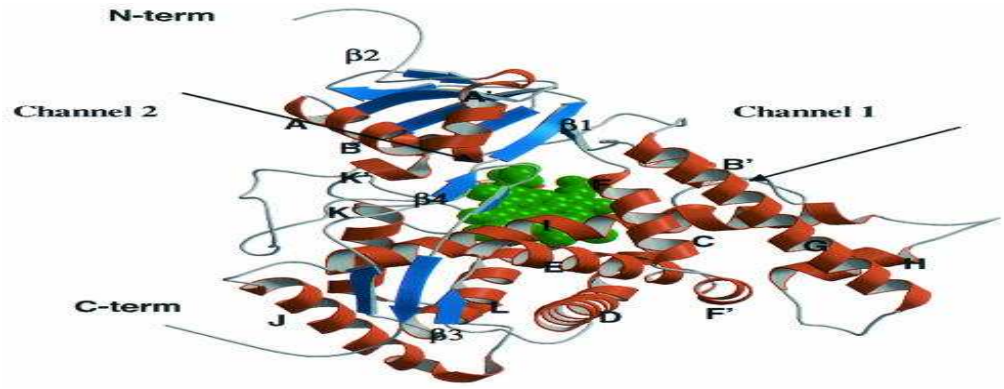


Figure 1.2 Overall structure of the homology model of AF-CYP51A. Helices and strands are shown in brown and blue. The heme cofactor is highlighted in green (Xiao et al. 2004)

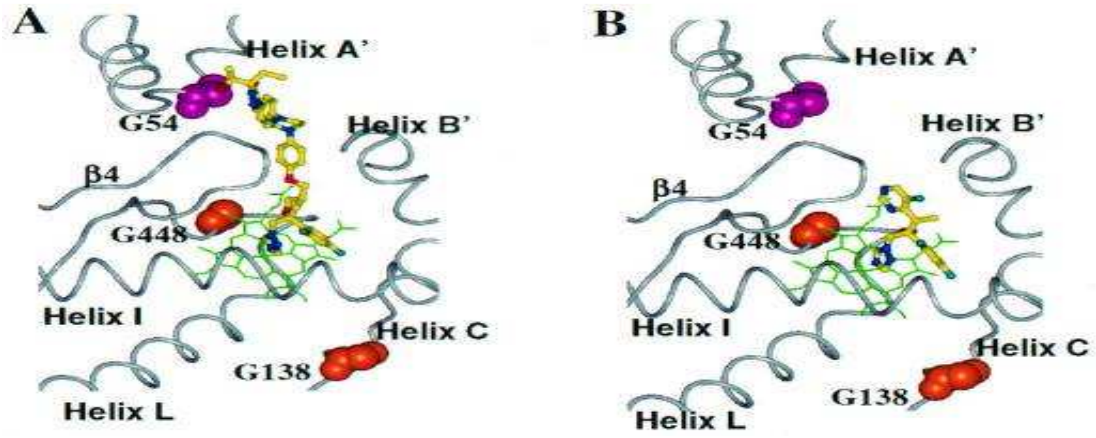


Figure 1.3 Mapping of substitutions in AF-CYP51A that cause azole resistance. The heme cofactor is highlighted in green. POS (A) and VOR (B) are shown as ball-and-stick models with their carbon atoms in yellow. The atoms of the residues at the sites of substitutions are depicted using space filling models (Xiao et al. 2004)

1.13.2 Drug import

Most drugs are hydrophobic and enter the cell by passive diffusion(van den Hazel et al. 1999). Membrane alterations have been related to resistance to antimicrobial agents in bacteria. These alterations may reduce drug penetration (Hernandez-Alles et al. 2000). Reduced accumulation of drug was possible due to reduced penetration, by a great permeability barrier in the resistance strains to ITR (Manavathu et al. 1999). The lipid phase of the plasma membrane plays an important role in MDR (Ferte 2000). Alterations in the lipid composition by impairing the plasma membrane appear to cause the increased drug sensitivity. For example deletion of *PDR16* from *Saccharomyces cerevisiae* resulted in hypersensitivity of yeast to azole inhibitors of ergosterol biosynthesis (van den Hazel et al. 1999)

1.13.3 Drug efflux

The second major mechanism of antifungal drug resistance is the overexpression of efflux pumps. Overexpression of the ABC transporter and major facilitator superfamilies confer resistance to many antifungal drugs in like fungi such as *Candida albicans* and *Candida glabrata*. *.A fumigatus* MDR pumps have been shown to be associated with increased resistance to ITR in several studies. Bioflm is another azole resistance mechanism, which can increase resistance *A. fumigatus* as in shown by Morschhauser (Morschhauser 2010;Rajendran et al. 2011) *A. fumigatus* has 278 different MFS and 49 ABC transporters according to sequence analysis. Some of which have been investigated in clinical resistance (Rajendran et al. 2011).

Expression of atrF, an ABC transporter, was found to be induced in the presence of ITR in an ITR-R isolate, but not in other isolates. itraconazole-inducible expression of the major facilitator MDR3 and the ABC transporters MDR1, MDR2, MDR4, and atrF was observed in several in vitro generated ITR-Rstrains (Morschhauser 2010)

Overexpression of genes encoding drug efflux pumps is one of the major mechanisms responsible for development of multiple drug resistance not only in human beings but also in bacteria and fungi. An overexpression of P-glycoprotien, ABC transporter, has been found to be associated with treatment failure in humans (Higgins 1992;Ambudkar et al. 1999), bacteria (Escribano et al. 2007), yeast (Sanglard et al. 1997) and *Aspergillus* (Andrade et al. 2000).

1.13.3.1. ABC drug transporter of *Aspergillus*

ABC transporters are involved in the export or import of a wide variety of substrates. However, the ABC transporters are characterized by presence of nucleotide-binding domains (NBDs) and multiple transmembrane stretches (TMs) (Walker et al. 1982). However, The NBDs of ABC-transporters utilize the energy of ATP hydrolysis to facilitate drug extrusion, while TMs traverse the membranes (Prasad et al. 2002).

The NBDs of ABC-transporters contain Walker A and the Walker B motifs (Walker et al. 1982). *A. fumigatus* encodes more than 40 ABC transporters compared to 30 ABC transporters in *S. cerevisiae* (Mellado et al. 2007) and *A. fumigatus* has more than 100 major facilitator superfamily (MFS) proteins.

Two genes (AfuMDR1 and AfuMDR2) encoding proteins of the ABC transporters were identified in *A. fumigatus* (Tobin et al. 1997). The ABC transporters proteins AfuMDR1 in *A. fumigatus* and the ABC transporters proteins in yeast contain 12 putative transmembrane regions and two ATP-binding sites (NBD-TMs6)2 (Figure 1.4) (Prasad et al. 2002;Tobin et al. 1997). The AfuMDR2 in *A. fumigatus* has four putative transmembrane domains and an ATP binding domain (Tobin et al. 1997).

The AFUMDR1 gene has been expressed in *S. cerevisiae* conferring resistance to cilofungin (LY121019), an echinocandin B analog (Tobin et al. 1997). Recently, overexpression of atrF gene, encoding proteins of the ABC transporters was found to be correlated with ITR-R *A. fumigatus* (Slaven et al. 2002). Overexpression of one or both of two efflux pump genes, *AfuMDR3* and *AfuMDR4*, of *A. fumigatus* are linked to high-level itraconazole resistance (Nascimento et al. 2003).

1.13.3.2 MFS drug transporter of *Aspergillus*

The major facilitator superfamily (MFS) represents one of the two largest families of membrane transporters. They are present in bacteria and eukaryotes (Pao et al. 1998). These transporter proteins are involved in drug transport; sugars, oligosaccharides, amino acids, nucleosides, organophosphate esters uptake, uptake of Krebs cycle metabolites, and a large variety of organic and inorganic anions and cations (Pao et al. 1998;Prasad et al. 2002). Proteins of the MFS lack the NBDs of ABC transporters and utilize a proton motive force for drug efflux but they possess multiple transmembrane stretches that traverse the membrane (Figure 1.4) (Pao et al. 1998;Prasad et al. 2002). MFS transporters do not hydrolyze ATP (Del Sorbo et al. 2000). AfuMDR3 is the first fungal MFS transport gene identified which can be linked to drug resistance (Nascimento et al. 2003) *Aspergillus* spp. have proportionally more MFS transporter encoding genes than *S. cerevisiae*, *S. pombe*, and *Neurospora crassa* (Ferreira et al. 2005). ABC and MFS transporters can also play a major role in fungicide sensitivity and resistance (Del Sorbo et al. 2000).

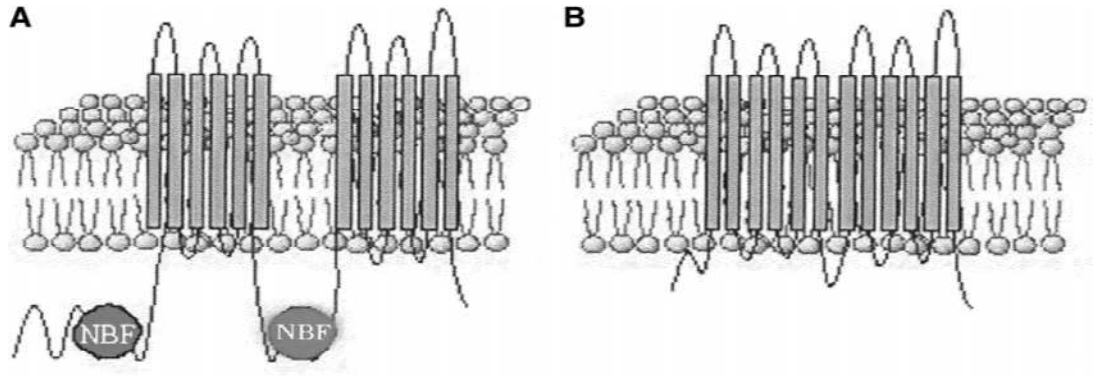


Figure 1.4 Representation of ABC transporters with a (NBF-TMD6)2 topology (A) and a MFS transporter with 12 TMDs (B) (Del Sorbo et al. 2000)

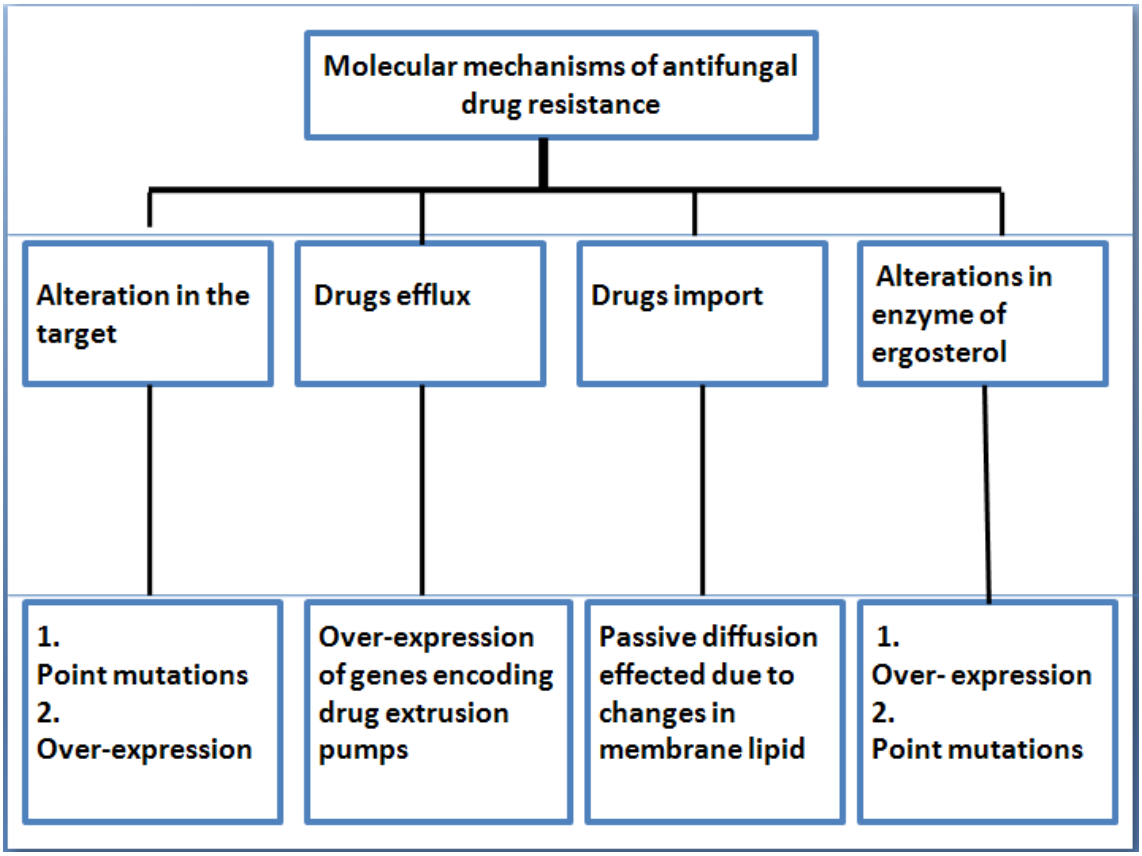


Figure 1.5 A schematic representation of the molecular factors that contribute to antifungal drug resistance in *A fumigatus*

1.14 Ergosterol biosynthesis pathway in *Aspergillus fumigatus*

Ergosterol is the predominant component and the main sterol of the fungal cell membrane (Kelly et al. 1997). The function of ergosterol has been examined in several studies. It is important for modulating the fluidity and permeability of the plasma membrane and is responsible for maintaining cell integrity and control of the cellular cycle (Ghannoum and Rice 1999;Munn et al. 1999). The absence of ergosterol in human cell and its functions make it and its biosynthetic pathway a useful target for antifungal agents such as amphotericin.B (Alcazar-Fuoli et al. 2008).

Ergosterol is essential for fungal growth. Study and understanding of the ergosterol biosynthesis pathway in *A. fumigatus*, especially in resistant strains may lead to alternative mechanisms of antifungal drug resistance. Moreover, it is helpful in the design of new antifungal drugs (Ferreira et al. 2005).The route of the ergosterol biosynthesis pathway has been studied in *S. cerevisiae* (Fryberg et al. 1973) routes have been reported (Ruan et al. 2002). About 20 enzyme encoding genes are involved in the ergosterol biosynthesis pathway (Ferreira et al. 2005). Several of these genes have been studied in other fungi and in plants (Alcazar-Fuoli et al. 2008). The ergosterol biosynthesis steps in the pathway from lanosterol are different in fungi composed with other organisms, depending on growth conditions (Munn et al. 1999).

Efforts have recently been made towards understanding the mechanisms of resistance in *A. fumigatus* from clinical isolates. In order to describe the ergosterol biosynthesis pathway in *A. fumigatus* and the sterol intermediates participating in this pathway, the *S. cerevisiae* ergosterol biosynthesis pathway needs to be considered. Moreover, study of sterol compositions of the *A. fumigatus* wild type strain and strains that are defective in genes encoding enzymes involving in the ergosterol biosynthesis pathway may be particularly useful.

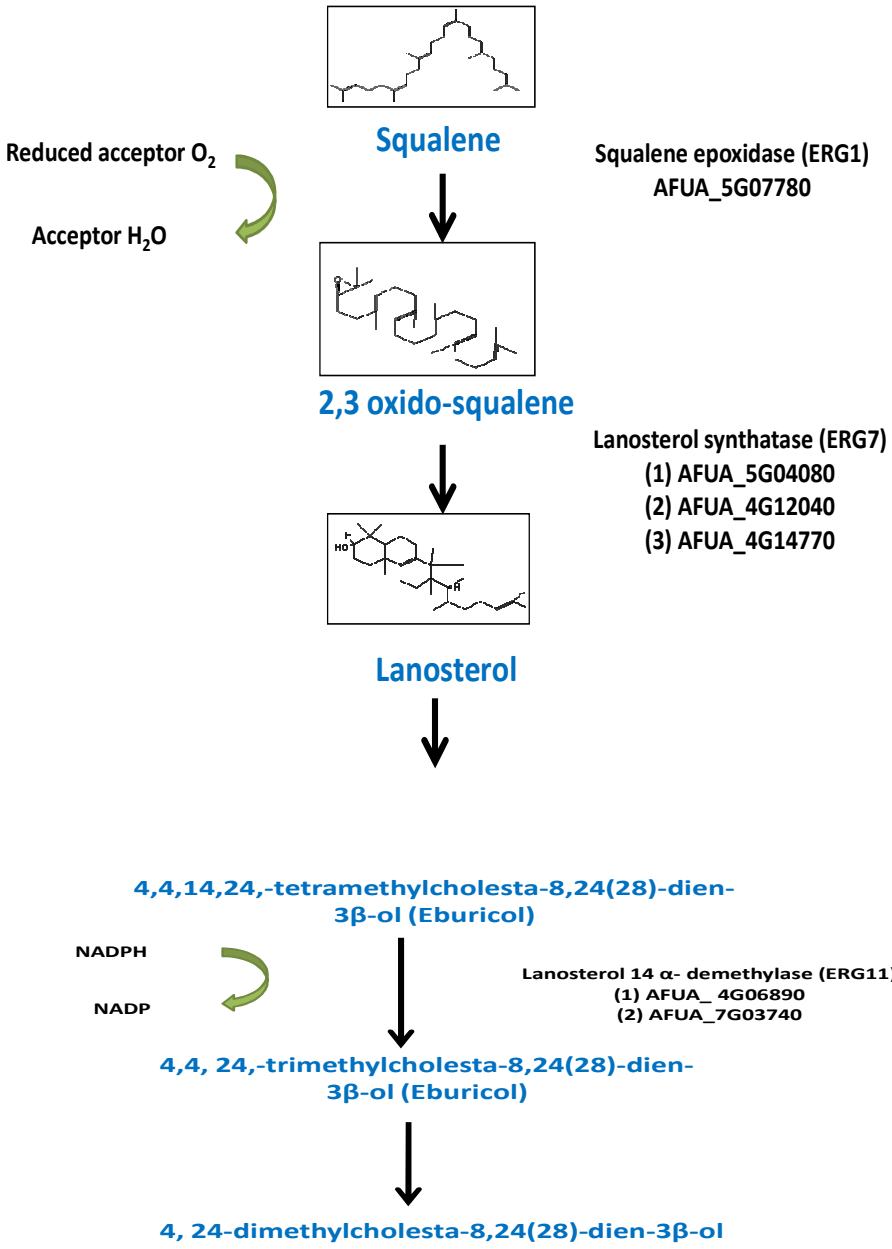
Changes in the sterol composition have been reported to increase or decrease the sensitivity of the yeast cell to certain drugs (Ghannoum and Rice 1999). In *A. fumigatus* few studies exist regarding ergosterol biosynthesis steps and biochemistry of this pathway. For example, two different 14-α sterol demethylases (*cyp51A* and *cyp51B*) (Ferreira et al. 2005) have been identified. However, *cyp51*, a gene that encodes the 14α sterol demethylase that acts half way along the ergosterol biosynthetic pathway, converts the methyl group at position C-14 of precursor sterol (Kelly et al. 1997;Alcazar-Fuoli et al. 2006) Moreover, three C-5,6 sterol desaturases (Erg3) (Alcazar-Fuoli et al. 2006) have been identified. One study has shown that the absence of C-5,6 sterol desaturase or mutation in C-5,6-desaturase leads to an accumulation of ergosta-7-22-dienol, ergosta-7-enol, and episterol in *C. Albicans* (Alcazar-Fuoli et al. 2006) and azole resistance in *S. cerevisiae* and accumulated ergosta-7,24(28)-dienol (episterol) (Kelly et al. 1997). (*ERG3*), a gene that encodes the C-5,6 sterol desaturase

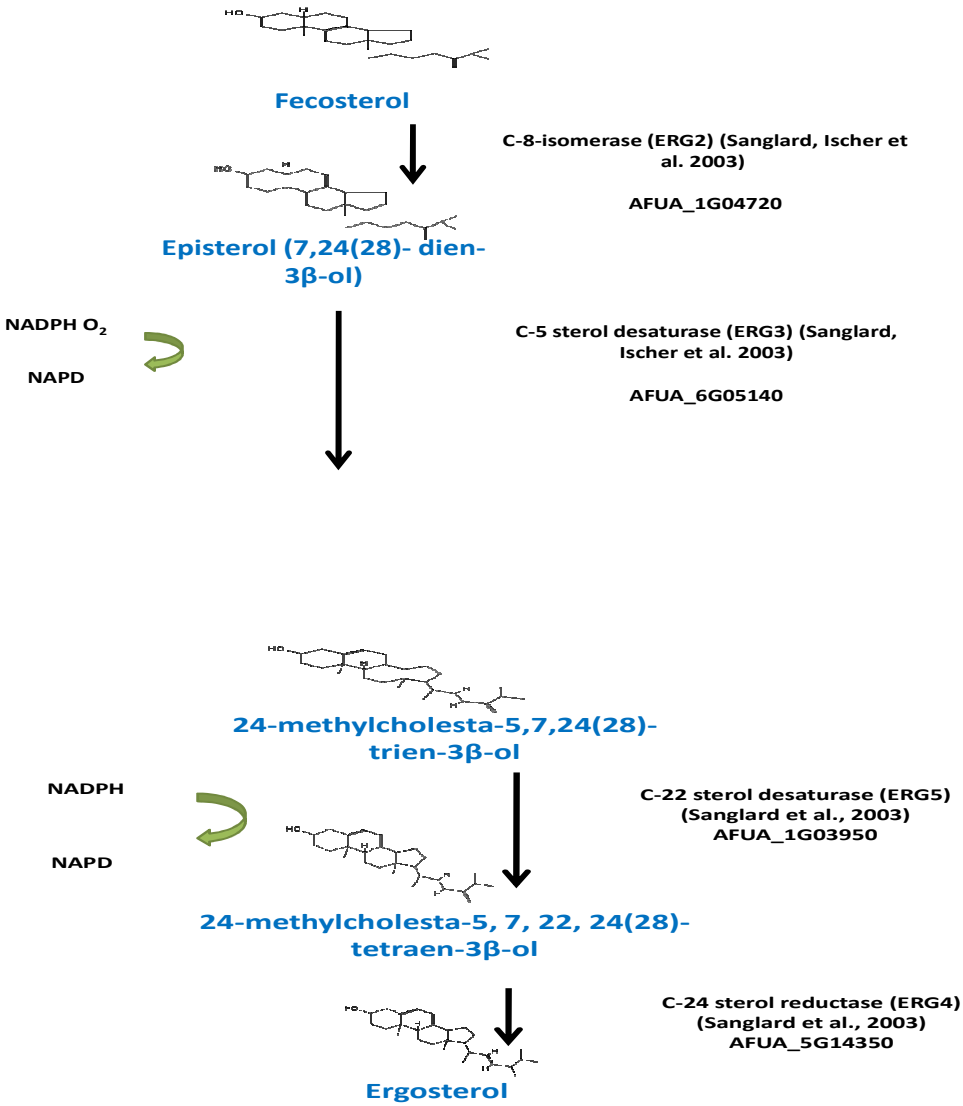
acts at the end of the ergosterol biosynthetic pathway. It catalyzes the introduction of a double bond between C-5 and C-6 in the B ring of the developing sterol molecule, converting episterol to 24-methylcholesta-5,7,24 (28)-trien-3β-ol (Alcazar-Fuoli et al. 2006). Alcazar-Fuoli, et al. have identified different *ERG3* sequences that could encode C-5,6 sterol desaturases proteins in *A. fumigatus*. Moreover, they reported that deletion of the *erg3* genes in *A. fumigatus* does not cause total ergosterol synthesis arrest, whilst deletion of the *erg3* genes in yeast dose leads to growth arrest (Kelly et al. 1997). Therefore, more than one C-5,6 sterol desaturase enzyme has been identified (Alcazar-Fuoli et al. 2006).

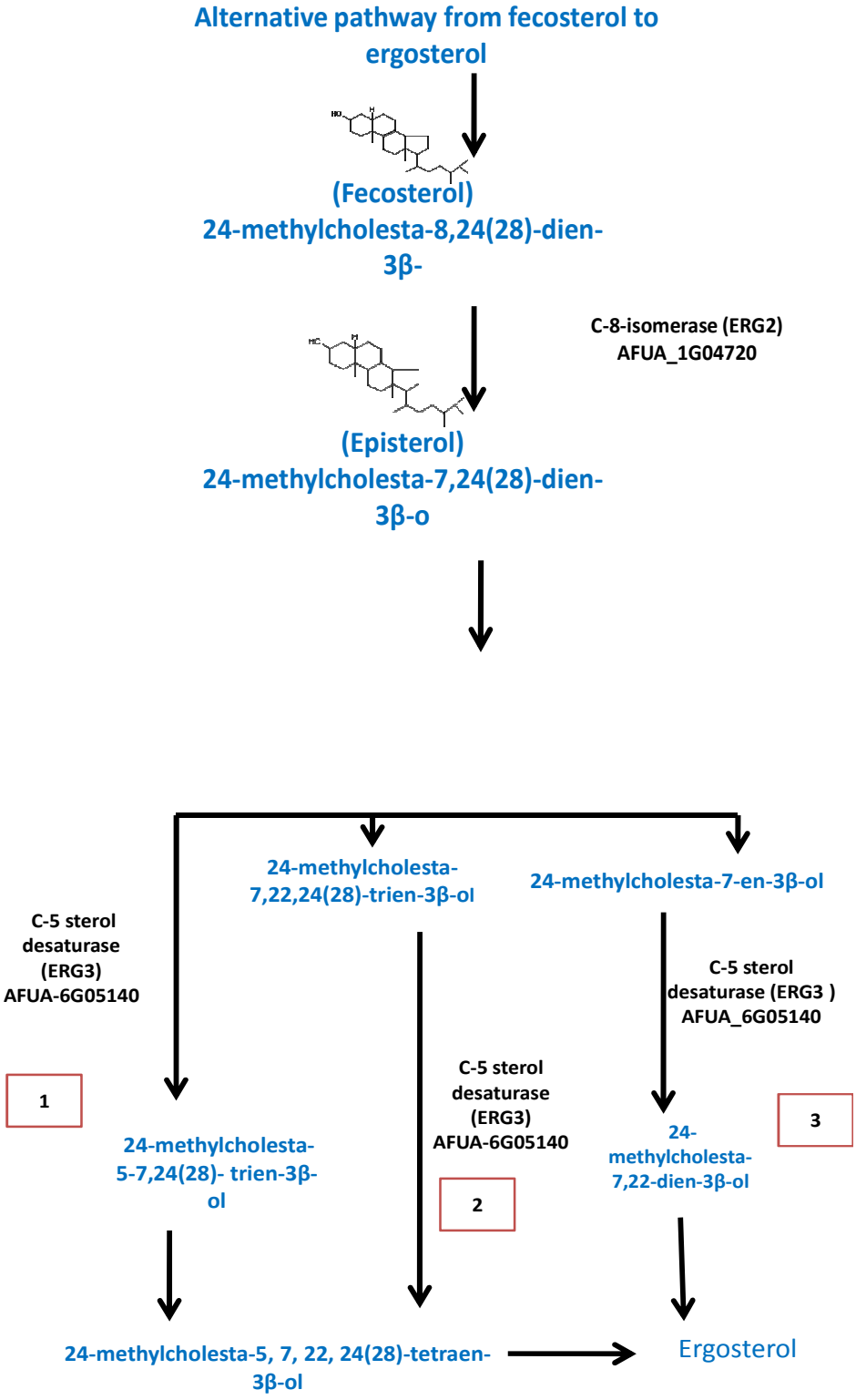
Another enzyme participating in ergosterol biosynthetic pathway is *ERG2*, a gene that encodes the C-8 sterol isomerase that acts in the late part of the ergosterol biosynthetic pathway. Erg2 converts fecosterol to episterol by isomerizing a C-8,9 double bond to a C-7,8 double bond in the B ring of the sterol molecule (Munn et al. 1999). Yeast strains containing mutations in the *ERG2* gene lack the C-8 sterol isomerase activity and are not able to synthesize ergosterol (Munn et al. 1999).

Alcazar-Fuoli et al., 2008, have reported that the sterol composition between the azole sensitive strains and the azole resistant strains were similar, and they suggested that the ITR resistance in *A. fumigatus* is due to single amino acid substitutions in the azole target affecting the azole drug affinity but not the enzyme activity. Therefore, other enzymes might be involved to continue the biosynthetic pathway to ergosterol in different routes. Figure 1.6 illustrates possible routes for ergosterol biosynthetic pathway in *A. fumigatus*.

Figure 1.6 A schematic representation of the ergosterol biosynthetic pathway in *A. fumigatus*.




Fecosterol
C-8-isomerase (ERG2) (Sanglard, Ischer et al. 2003)
AFUA_1G04720
Episterol (7,24(28)- dien-3β-ol)
NADPH O2
NAPD
C-5 sterol desaturase (ERG3) (Sanglard, Ischer et al. 2003)
AFUA_6G05140
24-methylcholesta-5,7,24(28)-trien-3β-ol
NADPH
NAPD
C-22 sterol desaturase (ERG5) (Sanglard et al., 2003)
AFUA_1G03950
24-methylcholesta-5, 7, 22, 24(28)-tetraen-3β-ol
C-24 sterol reductase (ERG4) (Sanglard et al., 2003)
AFUA_5G14350
Ergosterol


Alternative pathway from fecosterol to ergosterol
(Fecosterol)
24-methylcholesta-8,24(28)-dien-3β-
C-8-isomerase (ERG2)
AFUA_1G04720
(Episterol)
24-methylcholesta-7,24(28)-dien-3β-o
24-methylcholesta-7,22,24(28)-trien-3β-ol
24-methylcholesta-7-en-3β-ol
C-5 sterol desaturase (ERG3) AFUA-6G05140
C-5 sterol desaturase (ERG3) AFUA_6G05140
C-5 sterol desaturase (ERG3) AFUA-6G05140
1
2
3
24-methylcholesta-5-7,24(28)- trien-3β-ol
24-methylcholesta-7,22-dien-3β-ol
24-methylcholesta-5, 7, 22, 24(28)-tetraen-3β-ol
Ergosterol

Aims of study

Although the majority of *Aspergillus fumigatus* isolates are highly susceptible to azoles, reports of resistance in clinical *A. fumigatus* have increased. Consequently, treatment of aspergillosis is becoming more difficult. Therefore, new rapid and accurate methods are needed for detection of resistant strains of *A. fumigatus* in clinical samples. Additionally, resistance mechanisms need to be defined more clearly.

The mechanism of azole resistance appears to be due to one or more of the following; membrane alterations, overexpression of genes encoding drug efflux pumps or alterations in the enzyme of ergosterol pathway due to overexpression or mutation. However, the most common known mechanism of azole resistance in *A. fumigatus* is mutation in the drug target enzyme 14-α sterol demethylases (*cyp51A*).

The main aim of the project was to determine whether resistance in *A. fumigatus* isolates in the Mycology Reference Centre Manchester, UK (MRCM) is the result of amino acid substitutions in the drug target (*cyp51A*) or other known mechanisms, and to look for novel resistance mechanisms. To achieve this:

1. Clinical *A. fumigatus* isolates in (MRCM) were assessed for azole resistance using MIC and MFC.
2. The occurrence of *cyp51A* point mutations in azole resistant *A. fumigatus* isolates were evaluated by sequencing the *cyp51A* gene.
3. The presence of *A. fumigatus* in the soil of Wythenshawe hospital was investigated and these isolates were assessed to see whether drugs are azole sensitive or resistant and whether *A. fumigatus* in the environment is linked to *A. fumigatus* clinical isolates. Furthermore, the relationship between environmental and clinical *A. fumigatus* isolates was assessed by sequencing the ITS, β-tubulin, calmodulin, actin and *cyp51A* genes.
4. A number of genes were previously identified to be involved in azole resistance by insertional mutagenesis. In order to assess the contribution of these genes to

azole resistance we characterized possible resistance mechanisms in azole resistant isolates that do not carry any *cyp51A* mutation.

5. Gene expression of *cyp51A, cyp51B, AfuMDR1 AfuMDR2, AfuMDR3, AfuMDR4, atrF, ABC11, MFS56* and *M85* was analysed in susceptible and resistance isolates, both with and without *cyp51A* mutation. The biological activity of efflux pumps in *A. fumigatus* and their differential expression with respect to ITR treatment were investigated using quantitative RT-PCR.

Chapter 2

Materials and mehods

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2. Materials and methods

2.1 Materials

2.1.1 Isolates

2.1.1.1 Clinical isolates

A total of 63 *A. fumigatus* clinical isolates, obtained from the Regional Mycology Laboratory, Manchester, United Kingdom were used throughout this work. These isolates were selected from a total of 230 *A. fumigatus* clinical isolates received between 01.01.2008 and 31.12.2009. Of these, 167 *A. fumigatus* clinical isolates were sensitive to azoles.

The majority of specimens were isolates from sputum of patients. 63 of 230 were azole resistant upon isolation. Four *A. fumigatus* with known *cyp51A* mutations (G138C, G54R, M220T and L98H) from a previous study (F11628 and F12219, F15390 and D1357 respectively) were used as control strains to validate this work.

Candida kruzei ATCC 6258 was included among the isolates as a reference strain for antifungal MIC testing.

2.1.1.2 Environmental (soil) isolates

A total of 9 *A. fumigatus* environmental isolates were used. These isolates were isolated during July 2008 from soil samples obtained from the grounds surrounding Wythenshawe hospital. They were collected from a soil layer of 10cm depth.

2.1.1.3 Environmental (compost) isolates

Eight *A. fumigatus* isolates were isolated during October 2009, from commercial compost (The Compost shop, Orrel Hill lane, UK) and were kindly provided by Urooj Zafar from the University of Manchester.

2.1.2 Chemical reagents

Agarose	Melford, UK
Ammonium nitrate (NH_4NO_3)	BDH England
Citic Acid.1H2O	BDH England / Fisher scientific -UK
CTAB	Sigma- Aldrich Company Ltd,UK
$CuSO_4$. 5H2O	BDH England
D (+) Glucose	BDH England / Fisher scientific -UK
DMSO	Sigma- Aldrich Company Ltd,UK
EDTA	Sigma- Aldrich Company Ltd,UK
Ethidium bromide	Sigma- Aldrich Company Ltd,UK
$FeSO_4$	BDH –England
Glycerol	Sigma- Aldrich Company Ltd,UK
High fidelity polymerase	Invitrogen, Paisley, UK
$H3BO_3$. 1H2O	BDH England
Hyperladder 1kb	Biolab, UK
Hyperladder 100bp	Biolab, UK
Potassium chloride (KCl)	Fisher scientific -UK
K_2 HPO_2	BDH England/ Sigma- Aldrich Company Ltd,UK
Potassium dihydrogen phospate (KH_2PO_4)	BDH England/ Sigma- Aldrich Company Ltd,UK
Magnisum sulphate heptahydate ($MgSO_4$.7H2O)	BDH England / Fisher scientific -UK
Microtiter plates	Appleton Woods –UK
$MgSO_4$.7H2O	Fisher scientific -UK
$MnCl_2$	BDH /England
MOPS	Sigma- Aldrich Company Ltd,UK
NaCl	Sigma- Aldrich Company Ltd,UK
NaOH	Sigma- Aldrich Company Ltd,UK
$(NH_4)MO7$ O_{24}	BDH/ UK
Nutrient broth	Fluka/ Sigma- Aldrich Company Ltd,UK
PEG-6000 sigma	Sigma- Aldrich Company Ltd,UK
Peptone	Appleton Woods –UK
RNas	Sigma- Aldrich Company Ltd,UK
RPMI	Sigma- Aldrich Company Ltd,UK
Sodium acetate	Sigma- Aldrich Company Ltd,UK
Sodium chloride (NaCl)	Sigma- Aldrich Company Ltd,UK
Sucrose	Sigma- Aldrich Company Ltd,UK
TBE buffer	Severn Biotech Ltd
Trisodium citrate dihydrate ($C_6H_5Na_3O7$. $2H_2O$)	BDH- England
Trizma base	Sigma- Aldrich Company Ltd,UK
Tween TM 20	Sigma- Aldrich Company Ltd,UK
Tween TM 80	Sigma- Aldrich Company Ltd,UK
yeast extract	Appleton woods
$ZnSO_4$. 7H2O	BDH- England

2.1.3 Buffers and Solutions

All solutions and buffers were prepared in dH_2O and sterilized by autoclaving at 121°C for 15 minutes at 15 pounds per square inch (psi) or by filtration through a 0.2μM filter (Sartorius) and then stored at an appropriate temperature. The pH was adjusted to the desired value using a bench pH meter (Hanna instruments pH210) and adjusted with 0.1 M NaOH or 0.1 M HCl.

2.1.3.1 Phosphate Buffered Saline (PBS)

NaCl	4.00 g
K_2 HPO2	0.605 g
KH_2PO4	0.17 g

All the ingredients were dissolved in 500ml of distilled water. The pH was adjusted to 7.3 using NaOH or HCl. The PBS buffer was autoclaved at 121°C at 15 psi for 20 minutes.

2.1.3.2 Phosphate Buffered Saline (PBS) with 0.05% Tween 80

0.25 ml of Tween 80 was warmed gently and then added to 500 ml PBS buffer. The pH was adjusted to 7.3 using NaOH or HCl. The PBS-tween 80 was autoclaved at 121°C and 15 psi for 20 minutes.

2.1.3.3 TBE buffer (1L, 10x stock)

EDTA	7.4 g
Tris-base	108 g
Boric acid	55 g
dH_2O up to	1 L

2.1.3.4 TAE buffer (1L, 10x)

EDTA	18.0 g
Tris-base	242 g
Acetic acid	57 ml
dH_2O up to	1 L

2.1.3.5 Tris-HCl pH 8.0

Tris-base	121.1g
HCl	42 ml
dH_2O up to	1L

2.1.3.6 DNA extraction buffer

Dissolve the following in 80ml dH_2O

mMTris-HCL	1.20 g
1.4M NaCL	8.12g
10mM EDTA	0.37g
2% CTAB	2.00g

Volume was then adjusted to100 ml with dH_2O and pH adjusted to 8.0 then autoclaved.

2.1.4. Media

All media were prepared in dH_2O in loosely capped bottles and then autoclaved at 121°C and 15 psi for 15 minutes. They were then allowed to cool to approximately 50°C before adding an appropriate amount of antibiotic or antifungal, 4 mg/l of ITR and 25 µg/ml of chloramphenicol to inhibit bacteria and unwanted fungi and yeast. Once the media was ready it was poured into sterile Petri dishes in a laminar flow hood.

2.1.4.1 Sabouraud dextrose agar (Oxoid)

6.5 g Sabouraud dextrose agar powder (Oxoid) was dissolved in 100ml distilled water. Media was autoclaved and stored at 4°C for not more than 2 weeks before use.

2.1.4.2 Sabouraud dextrose broth (Oxoid)

3 g Sabouraud dextrose broth (Oxoid) was dissolved in 100ml distilled water.

2.1.4.3 Glycerol nutrient broth

This was composed of nutrient broth (Fluka-UK) and glycerol (Sigma-UK). The nutrient broth (used to store fungal spores) was autoclaved and stored at RT.

2.1.4.4 Minimal media

Stock solution 1

$NaNO_3$ 120 g

KCl 10.4 g

KH_2PO_4 30.4 g

Hunter's trace elements 0.1 ml

All the ingredients were dissolved in 1 L of distilled water and autoclaved at 121 °C and 15 psi for 20 minutes.

Stock solution 2

$MgSO_4$. 7H2O (10.4 g) was dissolved in 10 ml of distilled water and autoclaved at 121°C and 15 psi for 20 minutes.

Hunter's trace elements 0.1ml

Citic Acid.$1H_2O$	0.5 g
$ZnSO4$. $7H_2O$	0.5 g
$FeSO_4$	0.1 g
$CuSO_4$. $5H_2O$	0.25 g
$MnCl_2$	0.05 g
$H3BO_3$. $1H_2O$	0.05 g
$(NH_4)MO7\ O_{24}$	0.05 g

All the ingredients were dissolved in 100 ml of distilled water. 1 ml of chloroform was added as preservative. Minimal media was made from 3 stock solutions; 50 ml of stock 1, 1.5 ml of stock 2, and 10% glucose were combined then made up to 1 L with distilled water before autoclaving.

2.1.4.5 Vogel's minimal medium

1x Vogel's medium

50 x salt stock solution	20 ml/l
10% glucose	100 ml/l

Four stock solutions were used to prepare Vogel's minimal medium;

50x stock salt solution, biotin solution (recipe below), Hutner's trace elements (recipe above), and 1 ml chloroform (as preservative) were used to supplement the above solution then stored at room temperature

50x stock salt solution

Trisodium citrate dihydrate ($C_6H_6O_7$ $Na_3.2H_2O$)	125.9 g/l
Potassium dihydrogen phospate (kH_2PO_4)	250 g/l
Ammonium nitrate (NH_4NO_3)	100 g/l
Magnisum sulphate heptahydate ($MgSO_4$.7H2O)	10 g/l
Calcium Chloride diHydrate ($CaCl_2$. H_2O)	5 g/l
Biotin solution	2.5 ml
Hutner's trace elements	5 ml

All the ingredients were dissolved in 900ml distilled water with stirring at room temperature. 1 ml chloroform was used then stored at room temperature

2.1.4.6 Stock biotin solution (0.1mg/ml);

3 mg of D (+) Biotin (Melford) was dissolved in 30 ml of dH_2O, then sterilised by filtration and stored at 4°C.

2.1.4.7 10% stock glucose solution

100 g of glucose was dissolved in 900 ml of dH_2O and adjusted to a final volume of 1 L. The solution was then sterilised by autoclaving and stored at room temperature.

2.1.4.8 RPMI Medium with 2% Glucose

RPMI powder (Sigma, Cat NoR-8758)	10.4 g
MOPS	34.53 g
Glucose	18 g

All the ingredients were dissolved in 500 ml of distilled water for a final volume of double strength media. The solution was stirred until completely dissolved. The pH was adjusted to 7.0 using 10 M sodium hydroxide, then sterilised by filtration and stored at 4°C.

2.1.4.9 Media for transformation

The Aspergillus A1160 strain was prepared for transformation by culture in liquid SAB medium at 37°C for 14-16 hours.

2.1.4.9.1 YPS

YPS medium was used for gene knock-out constructs (gene-GFP-PgpdA-hph-TrpC). It was composed of

Yeast extracts	20.0 g/l
Special peptone	5.0 g/l
Sucrose	342.3 g/l
Agar	15 g/l

This medium was adjusted to pH 8.2 with Tris-HCl (pH 8.8) before autoclaving. 200μg/ml hygromycin B was added to cooled medium at 50 ^{o}C.

2.1.4.9.2 Fungal minimal medium

This was used for gene knock-out constructs (gene–PtrA-gene). It was composed of

$NaNO_3$	6.0 g/l
KC1	0.52 g/l
$MgSO_4$. $7H_20$	0.52 g/l
KH_2PO_4	1.52 g/l
Glucose (dextrose)	10.0 g/l
Agar	15.0 g/l

This medium was adjusted to pH 6.5 with 1 N NaOH before autoclaving. 0.1µg/ml PT was added to cool medium.

2.1.5. Vectors

Plasmids used for gene knock-out and GFP-gene fusion experiments.

2.1.5.1. *pPTRII*

pPTRII (10.0kb) (Kubodera et al. 2002), was kindly provided by Dr Paul Bowyer from the University of Manchester. It was used in this study as a template to amplify a 2.0 kb PtrA cassette. This cassette was combined with gene upstream and downstream flanking regions and used in *A. fumigatus* transformation as a gene knock-out construct.

2.1.5.2. pAN7-1

PAN7-1(6756 pb) Punt et al., 1987(Punt et al. 1987); Gene Bank Z32698.1), was kindly provided by Dr Marcin Fraczek from the University of Manchester. pAN7-1 has a ~2.8kb (PgpdA-hph-TrpC) cassette which was amplified and subsequently combined with gene flanking regions. This cassette was used as a selectable marker in *A. fumigatus* transformation experiments.

2.1.5.3. pFNO3

pFNO3 (6.5kb) was purchased from FGSC (www.fgsc.net/plasmid/vector.html), was kindly provided by Dr Marcin Fraczek from the University of Manchester. It was used in this study as a template to amplify ~0.7kb of green fluorescent protein gene (GFP). The amplified fragment (GFP) was subsquently combined with *A. fumigatus* (upstream of interest gene) flanking regions and used to construct a gene-GFP-hph fusion.

2.1.6 Plasmids used for cloning of PCR amplified DNA fragments

2.1.6.1 PGEM-T easy

The PGEM-T easy (Promega) was used for cloning of taq polymerase-based PCR products of constructs such as gene knock-out construction and gene GFP fusion.

2.1.7 Antibiotics

2.1.7 .1 Ampicillin (Sigma, UK)

100 mg /ml of fresh stock ampicillin solution was prepared in sterile dH_2O filter sterilised and stored at 4^0C. It was used to select *E. coli* containing PGEM-T easy plasmids and for LB broth of transformation of *A. fumigatus.*

2.1.7.2 Hygromycin B (Calbiochem, USA)

A stock solution of 50 mg/ml was obtained from manufacturer and stored stored at 4°C). Only 200 µg/ml of hygromycin B was used in *A. fumigatus* transformation experiments to select transformed colonies of *A. fumigatus.*

2.1.7.3 Pyrithiamine (Sigma-Aldrich Ltd)

A stock powder of 1.0 mg Pyrithiamine was dissolved in 1 ml dH_2O in a dark vial and stored at 4°C until required. It was used as a selectable marker in *A. fumigatus* transformation experiments at a final selective concentration of 0.1 µg/ml.

2.1.8 Antifungal drugs

2.1.8.1 Itraconazole (ITR)

Pure powder with 98% potency was purchased from Sigma-Aldrich Ltd.

2.1.8.2 Posaconazole (POS)

Kindly provided by D. Peter Warn, University of Manchester, with 100% potency, and obtained as standard powder.

2.1.8.3 Voriconazole (VOR)

Pure powder with 98% potency was kindly supplied by Pfizer, Kent.

2.1.9 Oligonucleotide primers

All primers used in this project were obtained from MWG-biotech (Germany).

Table 2.1 Primers used for *cyp51A* amplification and sequencing

Primer	Sequence (5' to 3')	Reference
Afcyp51A1152F	CGTTGACATCATCAATCAGCGC	(Howard et al. 2006)
Afcyp51A1173R	GCGCTGATTGATGATGTCAACG	
Afcyp51A1619F	TCAGCGACGAACACTTCCCCAA	
Afcyp51A668F	GGGAACGAGTTTATTCTCAACG	
Afcyp51A2026R	TACACCTATTCCGATCACACCA	
Afcyp51A2101F	GTCTCTCATTCGTCCTTGTCCT	
Afcyp51A1640R	TTGGGGAAGTGTTCGTCGCTGA	
Afcyp51A709R	CGTTGAGAATAAACTCGTTCCC	

Table 2.2 Primers used for ITS amplification and sequencing

Primer	Sequence (5’ to 3’)	Reference
ITS1	TCC GTA GGT GAA CCT GCG G	White et al., 1990
ITS4	TCC TCC GCT TAT TGA TAT GC	

Table 2.3 Primers used for partial beta-tubulin, calmodulin and actin amplification and sequencing, used in conduct taxonomy work in aspergillus

Gene/region	sequence (5’-3’)	References
bt2a	GGTAACCAAATCGGTGCTGCTTTC	(Hong et al. 2005;Glass and Donaldson 1995)
bt2b	ACCCTCAGTGTAGTGACCCTTGGC	(Hong et al. 2005;Glass and Donaldson 1995)
cmd5	CCGAGTACAAGGAGGCCTTC	(Hong et al. 2005)
cmd6	CCGATAGAGGTCATAACGTGG	(Hong et al. 2005)
act-512F	ATG-TGCAAGGCCGGTTTCGC	(Hong et al. 2005;Carbone 1999)
act-783R	TACGAGTCCTTCTGGCCCAT	(Hong et al. 2005;Carbone 1999)

Table 2.4 *cyp51A* Promoter primer amplification and sequences used in this study

Primer	**Sequence (5' to 3')**	**Reference**
cyp51Apro-1	GTGACAAGCGAAGATTCCACACAT	(Albarrag et al. 2011)
cyp51Apro-2	ACAACAGAAGCGACTTTCTCTTCAG	
cyp51Apro-3	GTCTTTAGATTCGGTGGACGC	
cyp51Apro-4	GCTGCCGCTGAGGAACATATG	
cyp51Apro-5	GTAAGCCATAGCATCGGCACCAT	

Primers listed in Tables 2.5 – 2.7 were used in PCR fusion to make GFP and disruption constructs used in this study.

Table 2.5 ABC11 gene (AFUA_1G14330) primers

Primer	Sequence (5' to 3')
ABC11-F	ACCAAGTGTCATGGCCCAGG
ABC11-R	CACCTGGGTCCGTGACTGGA
Abc11-R nest	GTCTCCAAACCCAAGAGCAG
Abc11-F nest	CAGGACCTTCTTGAGTTGC
PtrA-F	GATCTGACAGACGGGCAATTG
PtrA- R	TCTATCATGGGGTGACGATG
ABC11 PtrA pro R	AATTGCCCGTCTGTCAGATCGATGGCCGATTGAGTGAGAC
ABC11 PtrA ter F	TCATCGTCACCCCATGATAGAAGGGTTCGACGTGACAGCC
GFP-R	ATTTGTATAGTTCATCCATGC
GFP-F	ATGAGTAAAGGAGAAGAAC
ABC11GFP pro R	AGTTCTTCTCCTTTACTCATGATGGCCGATTGAGTGAGAC
ABC11 GFP hph ter F	CATGGATGAACTATACAAATTCGAGTGGAGATGTGGAGTG
hph R	CTATAGAATCATCCTTATTC
hph R nest	ACCCAGAGGGTCATGACTTG

Table 2.6 MFS56 (AFUA-1G05010) primers

Primer	Sequence (5’ to 3’)
MFS56-F	AGTGTTGCCGAAATCGAGGG
MFS56-R	TGCGACCAGGATGTGCAGTG
MFS56 pro nest	CAGAAGCAGGGTGATATCG
MFS56 ter nest	TTGATTTACTCCTGTTATG
PtrA-F	GATCTGACAGACGGGCAATTG
PtrA-R	TCTATCATGGGGTGACGATG
MFS56 PtrA pro R	TGCCCGTCTGTCAGATCCCCCGGAATGCAAGCGCTGTCAG
MFS56 PtrA ter F	AGAGCGGCTCATCGTCACCCCATGATAGATAGCATAGCAAGCATAGAGCAGC
GFP-R	ATTTGTATAGTTCATCCATGC
GFP-F	ATGAGTAAAGGAGAAGAAC
M56 pro GFP R	GTTCTTCTCCTTTACTCATCGGAATGCAAGCGCTGTCAGT
MFS56 GFP hph ter F	CATGGATGAACTATACAAATTCGAGTGGAGATGTGGAGTG
Hph-R	CTATAGAATCATCCTTATTC
hphR nest	ACCCAGAGGGTCATGACTTG

Table 2.7 M85 (AFUA-5G07550) primers

Primer	Sequence (5’ to 3’)
M85-F	ATTTCGAGCTCCTCGGTACG
M85-R	ATCCACCTCGTGTGCTTCCC
M85 pro nest	CCCCAGGATGAAGATGAAGA
M85 ter nest	CGCCTACACGGAACCATACT
PtrA-F	GATCTGACAGACGGGCAATTG
PtrA-R	TCTATCATGGGGTGACGATG
M85 PtrA pro R	AATTGCCCGTCTGTCAGATCATGCCAAAGGGAATGGGGAA
M85 PtrA ter F	TCATCGTCACCCCATGATAGATATCTAGCGTGAGATAGGC
GFP-R	ATTTGTATAGTTCATCCATGC
GFP-F	ATGAGTAAAGGAGAAGAAC
M85 GFP pro R	AGTTCTTCTCCTTTACTCATATGCCAAAGGGAATGGGGAA
M85 GFP hph ter F	CATGGATGAACTATACAAATTCGAGTGGAGATGTGGAGTG
Hph-R	CTATAGAATCATCCTTATTC
hph R nest	ACCCAGAGGGTCATGACTTG

Table 2.8 Gene expression primers

Primer	Sequence (5’ to 3’)	
cyp51 AqPCR- For	TGCAGAGAAAAGTATGGCGA	(Albarrag et al. 2011)
cyp51 AqPCR- Rev	CGCATTGACATCCTTGAGC	
cyp51 BqPCR- For	AGCAGAAGAAGTTCGTCAAATAC	
cyp51 BqPCR- Rev	TCGAAGACGCCCTTGTG	
MDR1-for	TTCCCTTGTTCACAATTCTCTTCG	
MDR1-rev	TGACATAGACTGTGACAAACTCG	
MDR2-for	TTTAGCTCCACCGGGTTTG	
MDR2-rev	TCGAAAGACCGAACATGCTTGA	
MDR3-for	TCTGATGGCGGTCATCACT	
MDR3-rev	ATATCCATCCCCCAGGC	
MDR4-for	TATGGCTTAGTTTGTTTGTGTCACCGA	
MDR4-rev	AGAGCAATTCGTTGCTTCTG	
Atr-F-for	AGAGAAATCGGACAACTGCTGA	
Atr-F-rev	CCTCGTCGCAGATAGTCTTGTA	
Tub6 For	CGACAACGAGGCTCTGTACG	Marcin Fraczek
Tube 6 Rev (792)	CAACTTGCGCAGATCAGAGTTGAG	
ABC 11QPCR-for	GAGTGCGTACGATGTATTCGAC	This study
ABC 11QPCR-rev	GGCAGGACTGGTGAGAGAAG	
MFS56QPCR-for	GGGGGTATGGTAATTGGAGGT	
MFS56QPCR-rev	AAGAAGCGCAGACCATCG	
M85 QPCR-for	GCCAAGCACTATGAGCCTTC	
M85 QPCR-rev	GCCCAGTTCCTTCCAGATAA	

2.2 Methods

2.2.1. Handling and Culturing of Samples and Isolates

Precautions were taken when working with *A. fumigatus* spores to prevent cross-contamination between samples. All work with *A. fumigatus* spore stocks was carried out in a Biological Safety Cabine*t* (BSC) Class II. The work surface of the cabinet, work bench and all materials were decontaminated before and after use with 10% Trigene (Medichem International, UK). Roll tissue paper soaked with 10% Trigene was used as a mat to prevent the spread of spores.

Safety precautions were taken when working with antifungal components, DNA and RNA reagents. Gloves, lab coat, and eye protection were worn to prevent skin contact and prevent inhalation of reagent aerosols and consumption of liquid during use. The work bench and all materials, including all instruments, were decontaminated before and after use with 10% Trigene and RNase.

For long term storage of Aspergillus isolates spore suspension was mixed with 10% sterile glycerol and stored at -80 °C.

2.2.1.1 Growth of clinical isolates

A. fumigatus isolates (clinical culture collection) from stock spore suspensions stored in sterile glycerol nutrient broth at -80°C were sub-cultured on SAB agar (Oxoid) at 37^{o}C for 48 hours.

2.2.1.2 Growth of environmental (soil) isolates

Approximately 20 g of soil from 9 different fields around Wythenshawe hospital were collected from a layer at 10 cm depth. 2 g of each soil were suspended in 8 ml of 0.2 M NaCl 1% Tween 20. 100 µl of this suspension was then plated on Sabouraud dextrose agar containing 4 mg/l of ITR with 25 µg/ml chloramphenicol and 100 µl was plated on

Sabouraud's dextrose agar medium containing 25 μg/ml chloramphenicol (SC), and both were incubated at 37°C for 48 hours.

2.2.1.3 Growth of environmental (compost) isolates

A. fumigatus isolates (culture collection) were kindly provided by Ms Urooj Zafar from the University of Manchester. These isolates had been obtained from compost (The Compost shop, Orrel Hill lane, UK) during October 2009.

2.2.1.4 Growth of wild-type and mutant *Aspergillus fumigatus*

Stock cultures of wild-type (Af293) and mutant A1160 strains were grown on a solid SAB for 48 hours at 37°C. Conidia were harvested using a sterile cotton swab soaked in PBS with 0.05% Tween 80 and spores were counted using an Improved Neubauer haemocytometer (Weber Scientific International Cambridge, UK), then used for experiments such as DNA extraction or transformation.

2.2.1.5 Growth of *Escherichia coli*

Stock *E. coli* culture from -80^{o}C or fresh *E. coli* was grown in LB medium in 37°C in a shaking incubator at 250 rpm.

2.2.1.6 Growth of *A fumigatus* for protoplast transformation

300 μl of a1 x 10^{8} conidia/ml stock of either AF210 or A1160 was inoculated into 100 ml of liquid LB medium supplemented with 100 μg/ml Ampicillin then distributed to at least 5 Petri dishes. Subsequently, the cultures were incubated at 37°C for 14-16 hours without agitation.

2.2.1.7 Growth of transformed (mutant) *A. fumigatus* for MIC and for DNA extraction

SAB agar was inoculated with frozen (-80^{o}C) culture of transformed (mutant) *A. fumigatus* then incubated at 37°C overnight. Fungal mycelia were harvested for MIC as described in (2.2.2) and for DNA as described in (2.2.3.1)

2.2.1.8 Growth of *A fumigatus* for genomic DNA extraction

A. fumigatus isolates were grown in Sabouraud dextrose agar at 37°C, then moistened sterile swabs were used to transfer spores to PBS/ Tween 80. After that *A. fumigatus* spores were counted using a haemocytometer, then all suspensions were adjusted to 1 x 10^6 conidia/ml appropriate for a direct inoculation into minimal media (2.1.4.4) and incubated at 37°C in a shaking incubator at 250 rpm (311DS Labnet) overnight.

2.2.1.9 Growth of *A fumigatus* for genomic RNA extraction

A. fumigatus isolates were grown on Sabouraud dextrose agar at 37°C, then moistened sterile swabs were used to transfer spores to PBS/ Tween 80. After that *A. fumigatus* spores were counted using a haemocytometer, then all suspensions were adjusted to 1 x 10^7 conidia/ml appropriate for a direct inoculation into Vogels medium supplemented with 1% glucose and incubated at 37°C and 240 rpm in a shaking incubator (311DS Labnet) for 14-16 hours.

2.2.1.10 Spore and protoplast counting

Spores were prepared in PBS with 0.05% Tween 80 (2.1.3.1), or 50 mM $CaCl_2$ + 0.6M KCl then counted in an Improved Neubauer haemocytometer (Weber Scientific International Cambridge, UK) and adjusted to 1 x 10^5 colony-forming units (cfu) per ml for MIC and adjusted to 1 x 10^7 cfu/ml for transformation or for cultivation for RNA extraction .

2.2.2 Susceptibility testing

2.2.2.1 Test inoculum preparation for MICs

All isolates were grown in Sabouraud dextrose agar at 37°C, then moistened sterile swabs were used to transfer spores to PBS/ Tween 80. After that *A. fumigatus* spores were counted using Improved Neubauer haemocytometer, then all suspensions were adjusted to 1 x 10^5 (cfu) conidia/ml, appropriate for direct inoculation (100 µl) into the serial drug concentrations (100 µl each). The resulting the microtiter plate was incubated at 37°C for 48 hours.

2.2.2.2 Control inocula

Drug dilutions were controlled using fresh colonies of *Candida krusei*, ATCC strain 6258; a singl colony was suspended in 1 ml of distilled water and the suspension was adjusted to (1 x 10^6) which was diluted to 1 x 10^5 cfu/ml. This control inoculum was used by the EUCAST method for ITR and VOR. A further dilution of 1 x 10^3 cfu/ml was used by the NCCLS M27-A method for POS. Positive (without drug) and negative (without spore) controls were used for each drug dilution series for each isolate. The target range of expected MICs for *A. fumigatus* in the presence of ITR, VOR or POS is given below.

Quality control target range

Antifungal	Target range (mg/l)
Itraconazole	0.03 – 0.125
Voriconazole	0.125 – 0.5
Posconazole	0.06 – 0.25

2.2.2.3 Drugs preparation

Itraconazole (ITR), posaconazole (POS) and voriconazole (VOR) were prepared at 1600 mg/L by dissolving the drugs in 100% dimethyl sulfoxide (DMSO) (Sigma). The stock solutions were diluted (2 fold) in DMSO then every single diluted solution was diluted by 1/100 in a RPMI-1640 medium with 2% glucose. The final drug concentration range was 8 – 0.015 mg/L. Then 96 well flat bottom microtiter plates (Appleton Woods Ltd, Birmingham, UK) were used for the MIC method. The drug dilutions were dispensed into columns 1-10. Columns 11 and 12 were used for positive (without drug) and negative (without spore) controls respectively.

2.2.2.4 Antifungal and susceptibility testing

The minimum inhibition concentration (MIC) of the isolates was determined using the broth microdilution method. The individual MICs were determined by following the Clinical and Laboratory Standards Institute (National Committee for Clinical Laboratory Standards) reference method, with modifications details in (Table 2.9). All drugs were tested in a RPMI-1640 medium with 2% glucose. MICs were read by eye, with a no growth end point.

Table 2.9 the CLSI M38 and EUCAST method for testing the susceptibility of *A.fumigatus* to azoles

Characteristics	mEUCAST	EUCAST	CLSI M38-A (NCCLS)
Test medium	RPMI-1640 medium with 2% glucose	RPMI-1640 medium with 2% glucose	RPMI-1640 medium
Inoculums concentration	2-5 x 10^4 cfu/ml	2-5 x 10^5 cfu/ml	0.4-5 x 10^4 cfu/ml
Spores counting	Haemocytometer	Haemocytometer	Spectrophotometrically at 530 nm
Incubation time	48H	48H	48H
Filter	450nm	450nm	490nm
End point	Visual, no growth	Visual, no growth	Visual, no growth

2.2.2.5 Purity and viability count

Purity and viability count was performed for each isolate by diluting 1 x 10^5 spores/ml 1/10 to 1 x 10^4 spores/ml then 1/5 to 2 x 10^3 spores/ml, then 1/10 to 2 x 10^2 and a further 1/10 dilution to 2 x 10^2 conidia/ml. The purpose of doing dilutions was to more easily count colonies per plate and to ensure accurate inoculum concentration was achieved. The general ranges in a common acceptance for countable numbers of colonies on a plate are 1 to 4 colonies in 100 µl containing 2 x 10^1 spores and 10 to 40 colonies in 100 µl containing 2 x 10^2 spores.

2.2.2.6 Minimum fungicidal concentration

100 µl from each well showing complete growth inhibition in the MIC plate were inoculated onto SAB. After the inocula had dried, plates were streaked and then were incubated at 37 °C for 48 hours.

2.2.3 Extraction of nucleic acids

2.2.3.1 DNA extraction

Two DNA extraction protocols were used in this study

A. The Mycxtra kit (Myconostica Manchester, UK)

A.fumigatus isolates were grown in 20 ml of minimal media in a 50 ml centrifuge tube (Corning) incubated with shaking at 200 rpm at 37 °C for 24 hours. The mycelia from overnight culture were harvested by centrifugation for 15 minutes at 10000 rpm in a Thermo BR4I centrifuge. The mycelial pellets were transferred to bead tubes of the Mycxtra kit (Myconostica Manchester, UK). DNA extraction was done according to the Mycxtra manufacturer's instructions. DNA yield was quantified by gel electrophoresis. 5 µl 1kb (Appleton Woods, UK) markers were used. DNA concentration was also

measured at 260 nm using a spectrophotometer and DNA in the sample was compared to marker bands for verification of quantification.

B. Fungal DNA isolation using Cetyl Trimethyl Ammonium Bromide (CTAB) and glass beads

A. .fumigatus mycelia were transferred to bead tubes containing 1 ml of DNA extraction buffer (composed of 20 mM EDTA, 50 mM tris HCl (pH8), 1% CTAB and 1M NaCl). Tubes were mixed for 10 minutes on a Vortex mixer at maximum speed then incubated for 10 minutes at 60°C. After that, tubes were centrifuged at 13500 xg (-9000 rpm) for 2 minutes at room temperature (RT) and supernatants were treated with a final concentration of 100 µg/ml RNase. Subsequently, 700 µl of phenol chloroform-isoamyl alcohol (25.24.1) was added, inverted several times and was centrifuged at 13500 xg for 2 minutes at RT. The supernatant was transferred to new tubes and an equal volume of chloroform was added and inverted several times. 0.6 volumes of isopropanol were added to the supernatant to precipitate DNA and tubes were centrifuged at 13400 rpm for 5 minutes at RT. The resulting pellet was washed with 0.5 ml 70% ethanol, transferred to a sterile Eppendorf tube and centrifuged for 5 minutes at 3000 xg at RT. The pellet was air-dried for approximately 10 minutes and re-suspended in 50 µl of DNas and RNase free water. Subsequently, DNA was measured using a spectrophotometer and approximately 1 µl of each sample was visualized on an 0.8% ethidium bromide stained agarose gel alongside DNA standards.

2.2.3.2 RNA extraction

A. fumigatus isolates were grown on Sabouraud dextrose agar incubated at 37 °C for 48 hours. Spore suspension was prepared in PBS Tween80. Two baffled conical flasks containing 40 ml liquid medium (Vogel's minimal medium containing1% glucose) were inoculated with spores to a final concentration of 1 x 10^7 spores/ml. Spores were adjusted to a final spore concentration of 10^7 spore/ml using a haemocytometer. Then both flasks were incubated at 35°C with shaking at 250rpm for 14 – 16 hours (mid

exponential phase). ITR was added to one of two flasks to a final concentration of 4.0 mg/l for >8mg/L resistant isolates. The second flask was used as a control and mock inoculated with an equivalent amount of DMSO. Subsequently, fungal mycelia were collected by centrifugation, then ~ 100 mg fungal mycelia were transferred to a red-cap tube containing 1ml of RNA*pro*™ Solution (Lysing Matrix). RNA was extracted using the FastRNA® Pro Red kit (Qbiogene, Europe) according to the manufacturer's instructions. RNA was measured using Eppendorf Biophotometer (Germany).Then two aliquots of RNA were stored immediately at -80°C until used.

2.2.4 Agarose gel electrophoresis

Gel electrophoresis was performed using 1% w/v molecular biology grade agarose (Melford, UK) dissolved in 1 x Tris/Borate/EDTA (TBE) buffer (Severn Biotech Ltd, UK) and stained with ethidium bromide (0.5µg/ml). To estimate the quantity and size of DNA a 5 x loading dye (Bioline, London, UK) or a 6 x bromophenol blue buffer (0.01%of bromophenol blue, 30% glycerol, Tris-HCl, pH7.0) mixed with DNA and 1kb DNA marker (Appleton Woods, UK) were loaded on the gel. The gel was run at 80 V (Biorad, USA) in 1 x TBE buffer for 80 minutes then the DNA was visualised on an UV transilluminator (Universal Hood II imager, Biorad, Italy).

2.2.5 PCR

To prevent cross- contamination, the work bench, which was set up specifically for PCR and all materials were decontaminated with 10% Trigene (Medichem International, UK) before and after use. Gloves and a lab coat were worn to prevent contamination. PCR was set up using a separate set of micropipettes and sterilized filter tips. All PCR reactions were prepared on ice. For qRT-PCR, reactions were prepared in 96 well polypropylene plates (Stratagene, UK) and sealed with optical thermo transparent polyester film (Alpha Laboratories, UK)

PCR for DNA was run on a QB-96 thermal cycler (Quanta Biotech, UK). Negative controls containing the same reaction constituents without a DNA template, was used to check contamination. Post-amplification, products were run on an agarose gel to quantify the product with markers.

QRT-PCR was run in an Mx3005p Real time PCR machine (Stratagene, US). Two negative controls (master mix-SYBR green without RNA template or without reverse transcriptase [RT]) were used to check contamination. After the RT-PCR, 5ul of each sample was analysed by gel electrophoresis on a 1% agarose gel.

One cycle PCR product melting curve programme was run at the end of each PCR cycle for each amplifiled gene to verify single product amplification. This cycle consisted of 1 minute denaturation at 95°C, followed by 30seconds elongation at 55°C and 30 seconds denaturation at 95°C. The fluorescence was read between 55°C and 95°C every 0.5 seconds.

2.2.5.1 Amplification of *cyp51A* amplicons for sequencing

PCR was carried out in a 25 µl reaction mixture, containing 0.5 µM of each forward and reverse primer, approximately 20 ng DNA and PCR Master Mix (Promega). Primers Afcyp51A2101F and Afcyp51A2026R (Table 2.1) were used for *cyp51A* gene amplification. Negative controls containing the same reaction but without DNA template were used to check contamination. PCR was run on a QB-96 thermal cycler (Quanta Biotech, UK) under the following conditions: denaturation at 94°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 30 seconds, then annealing at 60°C for 30 seconds and extension at 72°C for 2 minutes, followed by a final extension step at 72 °C for 10 minutes.

2.2.5.2 Amplification of ITS amplicons for sequencing

A ribosomal internal transcribed spacer (ITS) region was sequenced for the environmental clinical isolates. PCR was performed in 25 µl reaction mixtures, containing 0.5 µM of each primer ITS1 and ITS4 (Table 2.2), approximately 20 ng DNA and PCR Master Mix (Promega, UK). PCR was carried out under the following conditions: 2 minutes at 94^{o}C, followed by 40 cycles of denaturation at 94^{o}C for 30 seconds, then annealing at 44^{o}C for 30 seconds, and extension at 72^{o}C for 1 minute, followed by a final extension step at 72^{o}C for 10 minutes.

2.2.5.3 Amplification of actin amplicons for sequencing

PCR was performed in 25µl reaction mixture, containing 0.5 µM of each primer (ActF and ActR) (Table 2.3), 20ng DNA and PCR Master Mix (Promega, UK). PCR was carried out under the following: 2 minutes at 94^{o}C, followed by 35 cycles of denaturation at 94^{o}C for 1 minute, then annealing at 54^{o}C for 1 minute, and extension at 68^{o}C for 1 minute, followed by a final extension step at 68^{o}C for 10 minutes.

2.2.5.4 Amplification of calmodulin amplicons for sequencing

A partial calmodulin gene was amplified using primers cmd5 and cmd6. (Table 2.3) Then all PCRs were performed in 25 µl reaction mixture, containing 0.4 µM of each primer, approximately 20ng DNA and PCR Master Mix (Promega, UK). PCR was carried out under the following: 94^{o}C at 10 minutes, 35 cycles of a denaturation step at 94^{o}C for 50 seconds, then annealing at 55^{o}C for 50 seconds, and elongation at 72^{o}C for 1 minute, followed by a final elongation at 72^{o}C for 7 minutes.

2.2.5.5 Amplification of beta tubulin for sequencing

PCR was performed in 25 µl reaction mixtures, approximately 20 ng DNA and PCR Master Mix (Promega), containing 0.4 µM of primers bt2a and bt2b (Table 2.3) . PCR conditions were as follows: 2 minutes at 94^{o}C, followed by 35 cycles of denaturation at 94^{o}C for 1 minute, then annealing at 54^{o}C for 1 minute, and extension at 68^{o}C for 1 minute, followed by a final extension step at 68^{o}C for 10 minutes.

2.2.5.6 Amplification of upstream and downstream flanking sequence of ABC11, MFS56 and M85 for construction and cloning

Each upstream and downstream flanking sequence of each gene from genomic DNA of the Af293 strain was amplified separately.PCR was performed in 25µl reaction mixture, containing 0.5 µM of each primer for ABC11 (Table 2.5), for MFS56 (Table 2.6) and for M85 (Table 2.7), 20ng DNA and PCR Master Mix (Promega, UK). PCR was carried out under the following: 2 minutes at 94^{o}C, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, then annealing at 53^{o}C for 0.45 sec, and extension at 68^{o}C for 1.45 minute, followed by a final extension step at 68^{o}C for 10 minutes

2.2.5.7 Amplification of amplicons for gene disruption and fusion constructs

In this technique two different genes are joined to make a new gene construct. Fusion of genes may lead to a gene product with a new or different function from the two fusion partners.

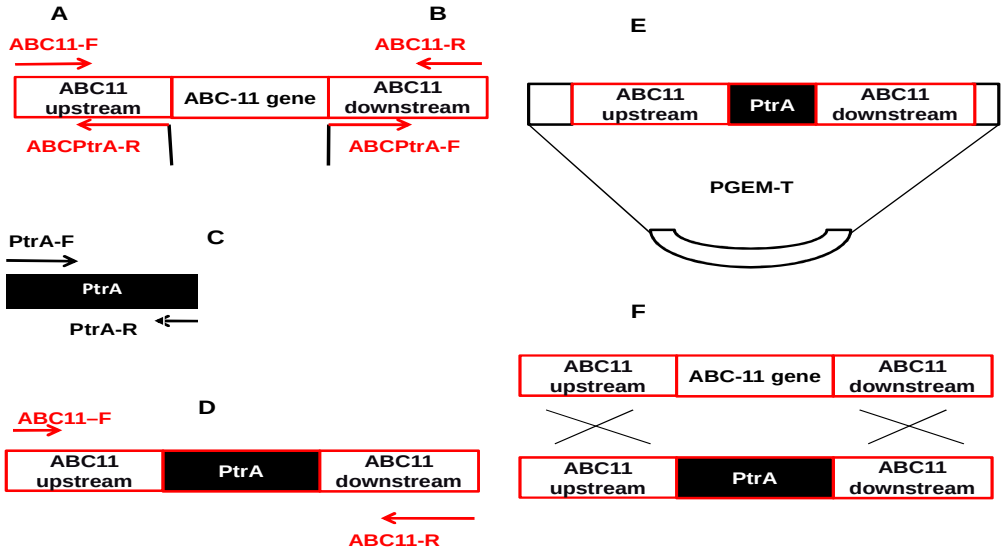

Figure 2.1 Construction of gene replacement constructs using fusion PCR. (A and B) The first PCR amplifies two regions flanking the target gene are amplified with primers (ABC11F and ABCptrA-R) plus (ABCPtrA-F and ABC11-R) as indicated. (C) The second PCR amplifies the pyrithiamine (PtrA) gene which functions as a selectable resistant marker with primers (PtrA-F and PtrA-R). (D) Fusion PCR is completed with the three amplified fragments and gene-specific primers ABC11F and ABC11-R. (E) Cloning in PGEM-T Easy. (F) Transformation and final product of homologous reaction

For the production of fusion PCR products that were used to transform the wild-type *A. fumigatus* to replace target genes, of interest, ABC11 (AFUA-1G14330), MFS56 (AFUA-1G05010) and M85 (AFUA-5G07550) gene fragments were first amplified separately by PCR using primers that produce overlapping endsas (Figure2.1). The selective cassette pyrithiamine resistance gene (ptrA) was amplified by 2 specific primers using plasmid pPTRII. Additionally, 2 nested primers were used to check product specificity and to amplify the final constructs for transformation in the second PCR step.

An outline of the fusion PCR for the ABC11 gene is described here and the two other genes (MFS56 and M85) are similar with corresponding primers.

For the first fragment, an upstream targeting region (Figure 2.1A), consisting of approximately ~1 kb of upstream flanking region of the ABC11 gene, was amplified with primers (ABC11F and ABCptrA-R). Primer ABCptrA-R was complimentary to the 3′ end of the upstream ABC11 flanking region before the start (ATG) codon of the ABC11 gene and has a 5′ 20-bp extension corresponding to the reverse complement of the PtrA gene sequence (Figure. 2.1 A).

For the second fragment (Figure 2.1B), approximately 1 kb downstream flanking sequence of the ABC11 gene was amplified using primers (ABCptrA-F and ABC11R). Primer ABCptrA-F has a 5′ extension of 20 bp identical to the last 20 bp of the PtrA gene before the stop codon (Figure 2.1B). Primer ABC11-R was complimentary to the 3′ end of the flanking region of the gene

For the third fragment (Figure 2.1C), the PtrA cassette is amplified with specific primers (PtrA-F and PtrA-R). The sequence of PtrA-R is complementary to the 5′ extension of ABCptrA-F.

The three purified amplification fragments were fused together using long PCR. 10 ng of the each fragments, 2 μM of each primer (ABC11F and ABC11R), 2.5 ul of 10 × AccuPrime buffer, and 0.2 μl 5U/ μl of AccuPrime (Invitogen, Paisley, UK) and made up to a final volume of 25μl with H_2O.

The PCR cycling conditions were 94°C for 2 minutes and then 10 cycles of 94°C for 20 seconds, 50°C for 50 seconds, and 68°C for 6 minutes, followed by 5 cycles of 94°C for 20 seconds, 50°C for 50 seconds, and 68°C for 6 minutes, then followed by 10 cycles of 94°C for 20 seconds, 50°C for 50 seconds, and 68°C for 6 minutes. The final step is 10 minutes extension at 68°C. The resulting final amplified construct was cleaned by gel purification (QIAGEN, UK,) before cloning and transformation.

2.2.5.7.1 Step 1 amplification of gene flanking sequences

Each segment of disruption or fusion PCR was amplified. Each upstream and downstream flanking sequence of each gene from genomic DNA of the AF293 strain was amplified separately (2.2.5.6).

The pyrithiamine resistance cassette (ptrA) used as a selectable marker in the knock out experiment was amplified from pPTRII (2.1.5.1).

Hgromycin B phsphotransferase gene (hph) with promoter (PgpdA) and terminator (TtrpC) of *A. nidulans* was amplified from pAN7-1 (2.1.5.2). It was used a selective marker in the fusion (upstream- GFP-hph) experiment.

The GFP cassette was amplified from pNO3 (2.1.5.3) and used in the promoter-GFP fusion (upstream- GFP-hph) experiment.

One set of primers was used to amplify each of the above fragments as a described in (2.2.5.7). PCR was carried out in 25 µl Phusion high fidelity DNA polymerase reactions containing 0.5 µM of each primer (Xfor and Xrev) for each segment, for ABC11 (Table 2.5), for MFS56 (Table 2.6) and for M85 (Table 2.7), approximately 20 ng of AF293 genomic DNA, or 10 ng of plasmid DNA. Sterile DNase and RNase free water was then added to a final volume of 25 µl and PCR was carried out for each gene of interest

2.2.5.7.2 Step 2 amplification of gene flanking sequence

To recombine the segments from 1st step PCR, a high fidelity Accuprime Taq DNA polymerase (Invitrogen, Paisley, UK) was used, which has higher fidelity than conventional Taq alone and is effective over a wide range of target sizes up to 20 kb. This enzyme was used for the 2nd step of the gene disruption and tagging PCR. After the 3 segments (upstream and downstream flanking regions of the gene and marker cassette

were amplified in the 1st PCR, the 3 segments were combined in a single PCR reaction as shown in Figure 2.1

For the GFP gene fusion, the GFP, marker cassette (hph) and the upstream flanking region of target gene were amplified in the 1st step. The 3 segments were combined downstream.

PCR was carried out in 25 µl reaction volumes using 0.2 µM of each primer forward of upstream flanking sequence and reverse of downstream flanking sequence for gene knock out. For GFP fusion, the forward primer of the upstream flanking sequence and the reverse primer of the hph cassette were used. Reactions also contained approximately 20 ng of each segment, 2U of AccuPrime and 2.5ul of 10X AccuPrime™ PCR Buffer II and the reactions were adjusted to a final volume of 25 µl with DNase and RNase free water. PCR was then run on a QB-96 thermal cycler (Quanta Biotech, UK). Using the following conditions: 1 cycle of denaturation at 95°C for 2 minutes, 10 cycles of denaturation at 95°C for 20 seconds, then at 70°C for 1 second, before annealing at 50°C for 30 seconds and extension at 72°C for 6 minutes, followed by 5 cycles of denaturation at 94°C for 20 seconds, at 70°C for 1 second, annealing at 50°C for 30 seconds and extension at 72°C for 6 minutes. This was then followed by 10 cycles of denaturation at 94°C for 20 seconds, 70°C for 1 second, annealing at 50°C for 30 seconds and extension at 72°C for 6 minutes and a final step of elongation for 6 minutes at 72°C.

2.2.5.8 PCR set up for fusion and PCR screening of *E. coli* and transformed *A. fumigatus*

To screen plasmid PGEM-T easy in *E. coli* carrying new gene constructs a colony PCR was performed using a proofreading Phusion High Fidelity DNA polymerase (Finnzymes, Finland).

white colonies were chosen from blue/white population then the presence of an insert and its size can be determined by growing each colony in LB liquid for 4hour in a shaking incubator at 37°C and the plasmid purified by a boiling (100°C) for 10 min then

at least 10 minutes at -80°C subsequently 0.5 µl of colony suspention was used for a 25µl PCR reaction.

PCR cycle conditions for colony PCR: 1 cycle of denaturation at 95°C for 2 minutes, 10 cycles of denaturation at 95°C for 20 seconds, 70°C for 1 second, then annealing at 50°C for 30 seconds and extension at 72°C for 6 minutes, followed by 5 cycles of denaturation at 94°C for 20 seconds, 70°C for 1 second, then annealing at 50°C for 30 seconds and extension at 72°C for 6 minutes, followed by 10 cycles of denaturation at 94°C for 20 seconds, 70°C for 1 second, then annealing at 50°C for 30 seconds and extension at 72°C for 6 minutes and a final step of elongation for 6 minutes at 72°C.

2.2.5.9 PCR set up for qPCR

QRT-PCR reactions were performed in 12.5 µl reaction volumes using 50 ng of *A. fumigatus* RNA, 0.2 µM of each forward and reverse primer for each of the genes *cyp51A, cyp51B, MDR1, MDR2, MDR3, MDR4, atrF, beta-tubulin, ABC11, MFS56 and M85* (Table 2.8) and Brilliant II SYBR green PCR Master Mix (Stratagene, USA). PCR was carried out under the following conditions: 50°C for 60 minutes (for reverse transcription), then 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds, 60°C for 1 minute and then extension at 72°C for 1 minute.

One cycle PCR product melting curve programme was run at the end of each PCR cycle for each amplifiled gene to verify single product amplification. This cycle consisted of 1 minute denaturation at 95°C, followed by 30seconds elongation at 55°C and 30 seconds denaturation at 95°C. The fluorescence was read between 55°C and 95°C every 0.5 seconds. The starting amount of cDNA is calculated from the cycle of PCR at which the fluorescence of SYBR green first arises above background (cycle threshold; ct).

2.2.6 Purification of PCR products

PCR products were purified using the QIAquick PCR purification kit (Qiagen, UK) according to manufacturer's instruction. Post purification products were run on an agarose gel to quantify the product using markers.

2.2.7 Sequencing of *A. fumigatus cyp51A* gene DNA

The *cyp51A* gene for all isolates in this study was sequenced (coding sequence and promoter). Eight primers listed in Table 2.1 were used for sequencing to provide overlap between sequenced amplicons produced by the various primer sets. The full coding sequences and promoter of *cyp51A* were amplified in research laboratory and then sequencing was performed by The University of Manchester DNA Sequencing Facility, Manchester. Sequencing analyses were done using the Vector NTI 10 programme (Invitrogen, Paisley, UK)

At least two overlapping complementary sequences were generated for each consensus. Then consensus sequences were aligned to a reference AF293 *cyp51A* sequence and mismatches identified. Sequences of clinical and environmental isolates used in this study were compared to an azole susceptible strain (Genbank accession number AF338659). Basic local Alignment Search Tool (BLAST) (National Center for Biotechnology Information U.S. National Library of Medicine) was used for sequence identity and similarities. Mutations were confirmed by repeat PCR analysis, with forward and reverse primers.

2.2.8 Cloning PCR products

The PCR fragment (final construct) was purified using the QIAgen gel extraction system according to the manufacturer's instructions (Qiagen, UK) and ligated into the

pGEM-T Easy cloning vector (Promega, UK) at a 3:1 insert-vector ratio. A ligation reaction was transferred to competent JM109 *E. coli* or *E. coli* (DH5alpha) and plated into (LB) Agar containing 100 μg/ml ampicillin (sodium salt,), 50 μg/ml 5-bromo-4-chloro-3-indolyl-_-d-galactoside (X-gal) and 0.1 mM isopropyl- -d-thiogalactopyranoside (IPTG). Plates were incubated at 37°C overnight. Then, white colonies were selected and grown overnight in 1.00 ml LB broth and colony PCR was carried out. Minipreps were performed according to manufacturer's instructions (QIAprep Spin Miniprep kit (QIAGEN, UK) to isolate plasmid and restriction analysis was performed to check for the presence of the expected insert with size analysis on 1% (w/v) agarose-TBE gels.

2.2.9 Transforming gene constructs into *E. coli* plasmids

The clean recombinant PCR product (final gene fusion construct) was cloned into the pGEM-T Easy vector according to manufacturer's instructions (Promega, UK). Spores of AF210 and A1160 Ku-80 pyrG^{+} are grown in 100 ml SAB broth containing 100μg/ml ampicillin for 14-16 hours in a static incubator at 37°C, harvested by centrifugation at 10000 rpm at 4°C for 10 minutes. Resulting mycelia were digested in 15 ml sterile 50mM CaCl2 + 0.6M KCl with 6% glucanex (Novo Nordisk Ferment) incubated at 37°C for 3 hours with gentle mixing (100rpm) to form protoplasts. Once protoplasts are ready, the protoplast suspension was filtered using a sterile lens cloth filter then centrifuged at 2000g (3300 rpm) for 10 minutes at 4°C. The pellet was suspended with appropriate volume of 50mM CaCl2 + 0.6M KCl to give a final count of 1 x 10^{7} as assessed by an improved Neubauer haemocytometer (Weber Scientific International, Cambridge, UK) and transferred to ice. A 30 -100μl PCR construct or PGEM-T easy plasmid including target construct, was added to protoplasts followed by incubation in ice for 25 minutes prior to adding 200μl polyethylene glycol 6000. After 10 minutes incubation at RT, the mixtures were transferred to fungal minimal media containing appropriate selective compound and incubated at 37°C. After 2 days actively

growing colonies were picked, and streaked out. Single resulting colonies were picked and used for further analysis.

2.2.10 Phylogenetic analysis of clinical and enviromntal *A. fumigatus* isolates

All clinical and environmental isolates used in this study were characterized for genotype by sequencing the ITS, β-tubulin, calmodulin, actin and *cyp51A* gene. To investigate the relationship of these isolates a phylogenetic analysis was conducted using maximum parsimony bootstrapping with 2,000 replicates (MEGA4) (Tamura et al. 2007). Analysis was performed using single genes or by concatenation of several genes to achieve greater sensitivity. The phylogeny performed on all susceptible and resistant isolates used in this study for classifying the isolates and for an understanding of the evolution of resistance.

All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 522 positions in the final dataset, out of which 63 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

A: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987) The bootstrap consensus tree inferred from 2000 replicates (Felsenstein et al. 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein et al. 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches (Felsenstein et al. 1985).The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site.

B: The evolutionary history was inferred using the UPGMA method (SNEATH 1973). The bootstrap consensus tree inferred from 2000 replicates (Felsenstein et al. 1985) is

taken to represent the evolutionary history of the taxa analysed (Felsenstein et al. 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches (Felsenstein et al. 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004)and are in the units of the number of base substitutions per site.

C: The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff 1966). The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein et al. 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches (Felsenstein et al. 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei 2000) with search level 7 in which the initial trees were obtained with the random addition of sequences (10 replicates).

Chapter 3

Resistance in *Aspergillus fumigatus*

3.1 Introduction

Although *Aspergillus fumigatus* strains are generally susceptible to the azole compounds, (Pfaller et al. 2008) acquired resistance has been documented. Itraconazole resistance reported in *A. fumigatus* for the first time in 1997 (in isolates obtained in the late 1980s). The isolates originally were from the USA (Denning et al. 1997b). Since then, many reports have described resistance in *A. fumigatus* clinical isolates in Belgium, Canada, China, Denmark, France, Norway, Spain, Sweden, India, Japan, the Netherlands, the UK and the USA (Howard and Arendrup 2011). Itraconazole resistance in *A. fumigatus* was a rare phenomenon until 2004 in Manchester, UK and also in the Netherlands (Snelders et al. 2008). Previously, a rising frequency of azole resistance in *A. fumigatus* to the end of 2007 was reported in reference mycology laboratories in Manchester, United Kingdom (Howard et al. 2009). The global frequency is not clearly defined as results from many laboratories do not routinely test the susceptibility of their isolates of *Aspergillus* and they do not use a common standard method for susceptibility.

In vitro susceptibility of fungi to antifungal agents was rarely reported before 1990 (Manavathu et al. 2000;Denning et al. 1992). Three principal methods have been used to determine MIC values for *Aspergillus* spp.; including macro/micro broth dilution, disc diffusion and Etest (Lass-Florl and Perkhofer 2008) but many methodologic variations were used that included inoculum size, PH, medium composition, temperature of incubation, duration of growth and end-point determination. All of these may effect antifungal activity and MIC (Denning et al. 1992). Several laboratories have used a number of different methods of determining *in vitro* susceptibility and they documented that most isolates of *A. fumigatus* are susceptible to ITR (Denning et al. 1997b).

Increased numbers of invasive fungal infections and reports of resistance to antifungal drugs in *A. fumigatus* have highlighted the need for reproducible methods of *in vitro* testing with clinical relevance. Recently there are two standard methods used, the

Clinical Laboratory Standards Institute (CLSI; formerly NCCLS) M38-A (CLSI 2002), and the European Committee for Antibiotic Susceptibility Testing (EUCAST) methods for filamentous fungi (SAST 2007). Several modifications to the standards have been carefully studied and may provide solutions to the limitations of the methods currently available. Improved MIC determinations have led to more reproducible and accurate detection of resistance, which has benefited the study of resistance mechanisms (Verweij et al. 2009a).

In this report chapter we update azole resistance experience to include 2008 and 2009 data, in reference to mycology laboratories in Manchester, United Kingdom using a (slightly) modified EUCAST method.

3.2 RESULTS

3.2.1 Frequency

Since the first case of azole resistance was reported, the percentage of clinical azole resistance *A. fumigatus* has risen (Denning et al. 1997b). Howard et al. (2009) calculated the rate of azole resistance in clinical *A. fumigatus* isolates in the Mycology Reference center Manchester (MRCM) between 1997 to 2007. A rising frequency of 5%, 7% and 17% of azole resistance in *A. fumigatus* in 2005 and 2006 to the end of 2007 (Figure 3.1) has been reported (Howard et al. 2009).

In the current study, we update azole resistance experience in the MRCM to include 2008 and 2009 data. The overall rate of azole resistance in a period from 01/01/2008 to the end of December 2009 was calculated. Of 230 isolates (2008 and 2009), 64 (28%) were azole resistant. In 2008 and 2009, 14% and 20% of patients had resistant isolates respectively (Figure 3.1). Repeat resistant isolates with similar susceptibility profiles from the same patient were removed from the patient analysis. During this period 62 of 64 (97%) were ITR resistant, 2 of 64 (3%) were only VOR resistant and 78% cases were multi-azole resistant.

Although the susceptibility of 230 *A. fumigatus* MRCM culture collection isolates was determined in MRCM, all *A. fumigatus* resistant isolates (64) were re-tested in this study for susceptibility against itraconazole (ITR), posaconazole (POS) and voriconazole (VOR) to confirm the MRCM results. Almost all MIC values for all *A. fumigatus* resistant isolates were consistent with clinical laboratory results with variation within ~ two-fold difference. Identification of all *A. fumigatus* resistant isolates were performed using macro- and micro-morphologic characteristics, with confirmation by ITS sequencing. Resistant *Aspergilli* were sub-cultured onto Sabouraud glucose agar (Oxoid, Basingstoke, UK) for 48 hours at 50°C to eliminate non-*fumigatus* species

3.2.2 Susceptibility testing

Antifungal susceptibilities against ITR, POS and VOR were determined for 64 azole resistant clinical isolates provided by the MRCM. Some susceptible isolates were used for validation. *Candida krusei* ATCC 6258 was tested as a quality control strain for every single MIC plate. All control results (data not shown) were within the acceptable target range. Almost all MIC values for all *A. fumigatus* resistant isolates were consistent with clinical laboratory results with variation within ~ two-fold difference. *In vitro* susceptibility data for all 64 *A. fumigatus* clinical resistant isolates are displayed in Table (3.1, 3.2, and 3.3).

In 2008, 92 *A. fumigatus* isolates were tested for triazole susceptibility, of which 21 (23%) were resistant to at least one azole in Table 3.2. In 2009, 43 of 138 (31%) isolates tested were azole resistant in Table 3.3 and Figure 3.1. Of these resistant isolates, 3 of 21 (14%) and 9 of 43 (21%) were resistant to ITR only in 2008 and 2009 respectively. Only 2 (10%) isolates from 2008 were VOR resistant. The remainders (16 in 2008 and 34 in 2009) were multi-azole resistant. Thus during this period, 62 of 64 (97%) were itraconazole resistant, 2 of 64 (3%) were only VOR resistant and 50 of 64 (78%) cases were multi-azole resistant. Using patients as the denominator rather than isolates (discounting additional isolates from patients with the same susceptibility pattern), the frequency of resistance remains high in the cases referred to our laboratory during 2008-9 at 14% (9 of 64 patients) and 20% (19 of 93 patients) respectively Table 3.1. In 2007 we found a sharp rise from 5 - 7% azole resistance in 2004-2006 to 17% (Howard et al. 2009) (Figure 3.1). This high frequency of resistance continued in 2008 (14%) and 2009 (20%).

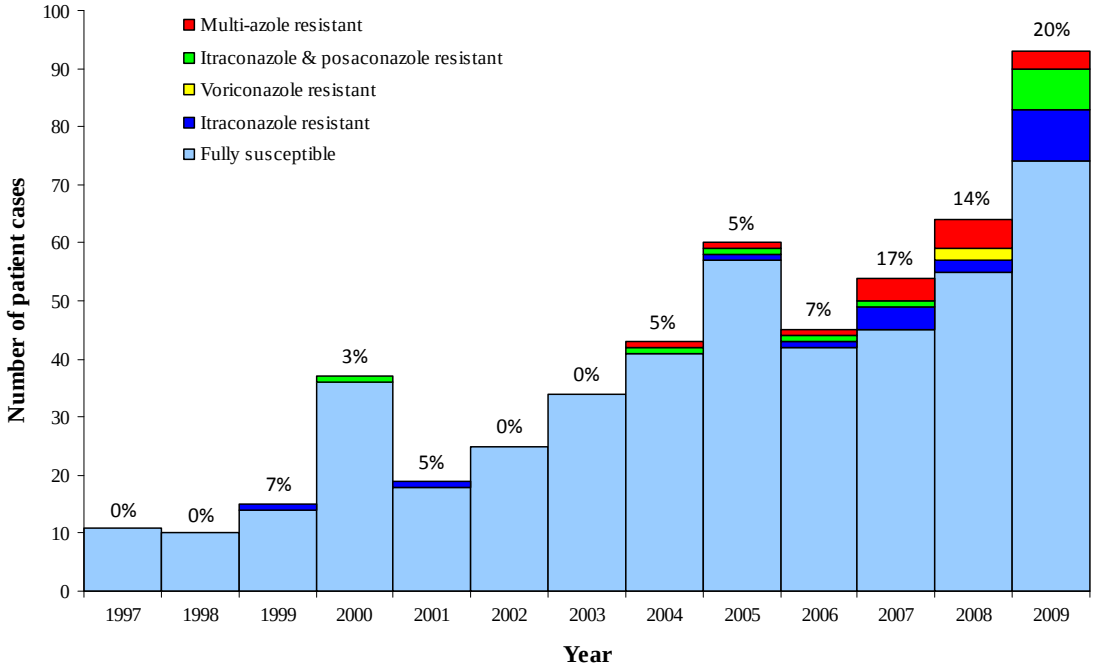

Figure 3.1 Azole resistance frequency in *A. fumigatus* by patient 1997 – 2009. Overall azole resistance for each year is shown above each column as a percentage. The data from 1997 to 2007 have been published previously (Howard et al. 2009).

Table 3.1 In vitro susceptibility data for all (64) *A. fumigatus* clinical resistant isolates

Rate	Year	Resistance
21 of 92 (23%)	2008	at least one azole
43 of 138 (31%)	2009	at least one azole
9 0f 64 (14%)	2008	patients had resistant isolates
19 of 93 (20%)	2009	patients had resistant isolates
62 of 64 (97%)	2008-09	Itraconazole only
2 of 64(3%)	2008-09	Voriconazole only
78% cases	2008-09	Multi-azole resistant

Table 3.2 MIC values for azole resistant *A. fumigatus* clinical isolates in 2008

NO	Isolates	MIC mg/l		
		ITR	VOR	POS
1	F/17582	>8 [R]	1 [S]	0.25 [S]
2	F//17727	>8 [R]	8 [R]	>8 [R]
3	F/17918	>8 [R]	>8 [R]	[R] 2
4	F/17999	>8 [R]	8 [R]	0.5 [I]
5	F/18085	>8 [R]	[R] 4	[S] 0.25
6	F/18149	>8 [R]	>8 [R]	[R] 2
7	F/18205	>8 [R]	>8 [R]	>8 [R]
8	F/18304	>8 [R]	2 [I]	0.125 [S]
9	F/18329	1 [S]	4 [R]	0.25 [S]
10	F/18454	0.5 [S]	>8 [R]	0.06 [S]
11	F/19020	>8 [R]	4 [R]	2 [R]
12	F/19029	>8 [R]	2 [I]	0.25 [S]
13	F/19130	>8 [R]	>8 [R]	2 [R]
14	F/19131	>8 [R]	>8 [R]	2 [R]
15	F/19132	>8 [R]	>8 [R]	2 [R]
16	F/19133	>8 [R]	>8 [R]	2 [R]
17	F/19134	>8 [R]	>8 [R]	2 [R]
18	F/19135	>8 [R]	>8 [R]	2 [R]
19	F/19136	>8 [R]	>8 [R]	2 [R]
20	F/19137	>8 [R]	>8 [R]	2 [R]
21	F/19138	>8 [R]	>8 [R]	2 [R]

Table 3.3 MIC values for azole resistant *A. fumigatus* clinical isolates in 2009

No	Isolates	MIC mg/l		
		ITR	VOR	POS
1	F/19483	>8 [R]	>8 [R]	>8 [R]
2	F19657	>8 [R]	2 [I]	0.125 [S]
3	F19734	4 [R]	1 [S]	0.125 [S]
4	F19736	>8 [R]	>8 [R]	>8 [R]
5	F19755	>8[R]	>8 [R]	>8 [R]
6	F19756	>8 [R]	>8 [R]	>8 [R]
7	F19838	>8 [R]	2 [I]	1 [R]
8	F19896	>8 [R]	1 [S]	0.125 [S]
9	F19980	>8 [R]	>8 [R]	0.5 [I]
10	F20005	>8 [R]	1 [S]	0.25 [S]
11	F20063	>8 [R]	1 [S]	0.5 [I]
12	F20132	>8 [R]	4 [R]	0.5 [I]
13	F20140	>8 [R]	2 [I]	0.5[I]
14	F20141	>8 [R]	>8 [R]	1 [R]
15	F20142	>8 [R]	2 [I]	0.5 [I]
16	F20151	>8 [R]	0.25[S]	1 [R]
17	F20153	>8 [R]	2 [I]	2 [R]
18	F20325	>8 [R]	4 [R]	0.5[I]
19	F20451	>8 [R]	1 [S]	0.25 [S]
20	F20478	>8 [R]	>8 [R]	>8 [R]
21	F20500	>8 [R]	>8 [R]	>8 [R]
22	F20699	>8 [R]	8 [R]	2 [R]
23	F20702	>8 [R]	2 [I]	2 [R]
23	F20720	>8 [R]	2 [I]	4 [R
25	F21019	>8 [R]	0.5[S]	1 [R]

26	F21038	>8 [R]	>8 [R]	>8 [R]
27	F21057	>8 [R]	8 [R]	1 [R]
28	F21116	>8 [R]	0.5[S]	1 [R]
29	F21118	>8 [R]	0.5 [S]	1 [R]
30	F21119	>8 [R]	0.5 [S]	I [R]
31	F21201	>8 [R]	4 [R]	0.5 [I]
32	F21202	>8 [R]	0.5 [S]	>8 [R]
33	F21215	>8 [R]	1 [S]	1 [I]
34	F21294	>8 [R]	>8 [R]	>8 [R]
35	F21295	>8 [R]	>8 [R]	>8 [R]
36	F21407	>8 [R]	0.25 [S]	>8 [R]
37	F21465	>8 [R]	>8 [R]	>8 [R]
38	F21522	>8 [R]	1 [S]	1 [R]
39	F21705	>8 [R]	1 [S]	1 [R]
40	F21732	>8 [R]	2 [S]	0.25 [S]
41	F21746	>8 [R]	2 [S]	2 [R]
42	F21799	>8 [R]	1 [S]	1 [R]
43	F21857	>8 [R]	1 [I]	>8 [R]

Breakpoint	Itraconazole (ITR)	Voriconazole (VOR)	Posaconazole (POS)
Resistant (R)	≥4	≥4	≥0.5
Intermediate (I)	2	2	0.5-1
Sensitive (S)	<1	<1	< 0.25

3.2 Minimum fungicidal concentration (MFC)

MFC is the lowest drug concentration in an MIC plate that showed either no growth or fewer than 5 colonies (approximately 99 to 99.5% killing activity). In this study, the MFC values were re-determined for the 15 of 63 clinical isolates: Table 3.4 and Figure 3.2. Almost all MFC values for all clinical isolates were consistent with clinical laboratory results with variation within two fold difference in azole concentration. Briefly: 13 of 15 (87%) isolates (F17582, F17727, F17999, F18085, F18149, F 18205, F182304, F/19020, F/19029, F/19130, F/19483, F19657 and F19734) showed high MFC values ≥8 mg/ l to ITR. For POS, 6 of 15 (40%) isolates (F17245, F18149, F 18205, F/19130, F/19483 and F19657) showed MFCs ≥8 mg/L. For VOR, 9 of 15 (60%) isolates (F17727, F17999, F18149, F 18205, F/18454, F/19029, F/19130, F/19483 and F19657) showed high MFC values at ≥8mg/L and 2 (13%) (F18085 and F/18329) at ≥4 mg/l.

Table 3.4 MFC results for clinical *A. fumigatus* isolates.

	MFS	ITR	VOR	POS
1	F/17582	>8	2	0.25
2	F//17727	>8	8	>8
3	F/17999	>8	8	0.5
4	F/18085	>8	4	0.25
5	F/18149	>8	>8	>8
6	F/18205	>8	>8	>8
7	F/18304	>8	2	0.125
8	F/18329	1	4	0.25
9	F/18454	0.5	>8	0.06
10	F/19020	>8	4	2
11	F/19029	>8	>8	2
12	F/19130	>8	>8	>8
13	F/19483	>8	>8	>8
14	F19657	>8	>8	8
15	F19734	>8	1	0.125

Figure 3.2 MFC values for 15 *A. fumigatus* clinical isolates

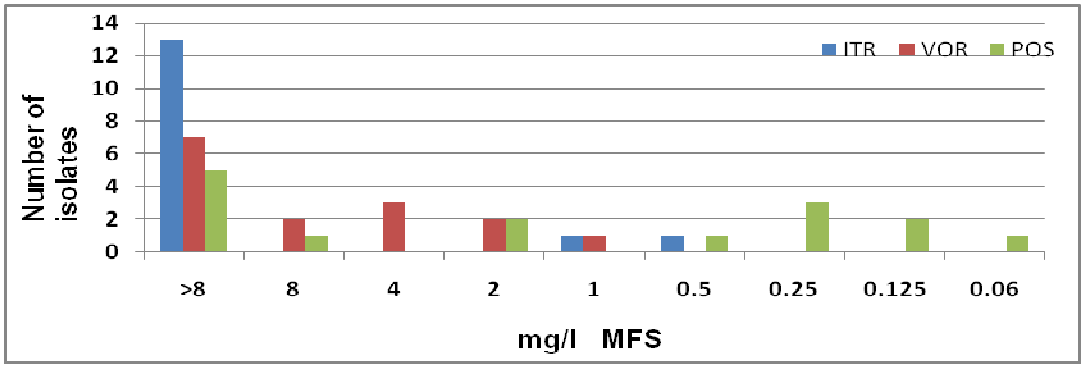

Figure 3.2 shows the distribution ITR, VOR and POS MFCs for 15 clinical *A. fumigatus* isolates. Overall these isolates were shown to have a high MFC value

3.4 Discussion

The incidence of IA has increased during the last decade (Vassiloyanakopoulos et al. 2006). *A. fumigatus* is the most common aetiological agent of aspergillosis in humans. Identification and typing of fungi is the one of the main issues in medical mycology (Evans 1989;Latge 1999).

Treatment of aspergillosis depends on the type of the disease and the immunological status of patients. Until recently, amphotericin B and itraconazole were the main antifungal drugs used for the treatment of invasive aspergillosis. The widespread use of triazole antifungal agents to fight these infections has resulted in development of resistance to a number of these components (Nascimento et al. 2003;Kelly et al. 1995). Higher MICs have been recorded for some clinical isolates (Denning et al. 1997b). The rate of the emergence of resistance to antifungal drug is increasing globally. Denning et al. 1997 reported the first ITR resistance.

The percentage of azole resistance had increased since 2004 (Howard et al. 2009). 64 of 230 *A. fumigatus* isolates were azole resistant to one or more azole compound in 2008 and 2009. In this study, two methods, minimum inhibition concentration and minimum fungicidal concentration, were employed to investigate clinical azole resistant isolates.

3.3.1 Susceptibility

Sensitivity testing was performed for 230 clinical isolates from the Regional Mycology Laboratory Manchester (MRCM) culture collection in 2008 and 2009. The susceptibility test for the resistant isolates was re-tested in this study. The reason behind this replication was that resistance might be lost during storage and to improve accuracy for strains included in this study and also because it is an approximate method and so

repeats are always required to completely characterise isolates. The MIC values achieved here were consistent with the MRCM with variation within a two-fold difference.

According to *in vitro* data, the majority of isolates were POS sensitive. Therefore, POS may be the best option as antifungal treatment. It is not surprising that cross-resistance to azoles in *A. fumigatus* occurs because they inhibit the same target and they both have a similar mode of action. POS resistance was observed in 38 clinical isolates which were also ITR resistant. On the other hand, 18 clinical isolates showed resistance to ITR but not to POS (Table 3.2 and 3.3). Although VOR is a good option for primary therapy of invasive aspergillosis (Petrikkos and Skiada 2007), the rate of VOR resistance is increasing. VOR resistance was observed in 2 clinical isolates with MIC ≥8mg/L. One clinical isolate (F18329) demonstrated variable VOR MICs on repeat testing. Possibly the isolate had been affected by long storage.

The increasing number of isolates submitted reflects referral of patients to the National Aspergillosis Centre and increasing awareness of resistance. The increasing rate of resistance is of concern. There appear to be differences in the geographical distribution of azole resistance in *A. fumigatus*, which cannot be explained by differences in methodology (as excellent concordance has been shown between CLSI and EUCAST methods). Since not all centres monitor the susceptibility of *Aspergilli* to azoles the true incidence is unknown. Nonetheless, resistance has now been reported from many countries in Europe, Asia and N. America, as well as particularly high frequencies from the Netherlands and the north-west of the UK.

3.3.2 Comparison of our method with the EUCAST method

Detection of antifungal resistance in filamentous fungi remains a challenge. ITR MICs increased during the 1990s, although no resistance has been seen before; perhaps because the filamentous fungi susceptibility method has changed more than once. Several laboratories have used a number of different methods of determining *in vitro*

susceptibility, and they documented that most isolates of *A. fumigatus* are susceptible to ITR (Denning et al. 1997b).

The current methodology uses EUCAST (a microtitre method using medium RPMI-1640 with 2% glucose, dimethyl sulfoxide (Sigma, Poole, UK) as drug solvent and a lower final inoculum concentration of 5 x 10^4cfu/mL (Table 1.3 and 2.9) while CLSI method uses 0.2% of glucose. Before 2001 the method used 50:50 acetone/0.2 M HCl as drug solvent and a final inoculum concentration of 5 x 10^5cfu/mL. The EUCAST method may be better than CLSI as result of reducing trailing by increasing glucose concentration to support growth. The CLSI inculam is lower than the EUCAST method. Endpoint determination was problematic for some isolates; trailing growth of filimentous fungi remains a challenge because end point is read visually rather than specrohoptmtically. However as growth is usually in small micro-colonies, optical endpoint reading is likely to be more sensitive than spectrophotometric reading.

In this study, we used a modified EUCAST method (inoculum concentration of 5 x 10^4cfu/mL), which has been used in MRCM since 2001. As a result, detection of the change in frequency of resistance in *A. fumigatus* between 2001 and 2009 was possible. Ongoing comparison of our method with EUCAST method shows equal or 1 dilution higher result with EUCAST, e.g. the resistance rate will be slightly higher if we use the EUCAST method. There was a recent proposal of breakpoints for *A. fumigatus* complex using the proposed EUCAST susceptibility testing methodology: for ITR and VOR, <2 mg/L (susceptible), 2 mg/L (intermediate) and >2 mg/L (resistant); for POS, <0.25, 0.5 and >0.5 mg/L respectively (Verweij et al. 2009a).

Varying methods, inoculum concentration, medium composition, PH, temperature of incubation, duration of growth, end point and breakpoints, contribute to antifungal activity and MIC (Denning et al. 1992).

Trailing growth at higher drug concentrations, especially ITR, can result in end point variability that cause problems for determining MICs, particularly for filamentous

fungi, as end points are determined by eye rather than using the spectrophotometer. As a result, care must be taken with comparisons.

However, a modification of methodology by reduction in inoculum size reduces the appearance of trailing end points that allows a clearer MIC determination.

Increasing specimen numbers received from patients failing therapy or problematic cases in MRCM as a regional and proper clinical diagnostic service have substantially increased the numbers of *Aspergillus* isolates tested. This is another possible reason for the increasing overall rate of resistance found recently compared to other centres. Several modifications to the standards have been carefully studied and may provide solutions to the limitations of the methods currently available. Improved MIC determinations have led to more reproducible and accurate detection of resistance, which has benefited the study of resistance mechanisms (Verweij et al. 2009a).The breakpoint is not yet decided so further *in vitro* studies to determine the optimal conditions for determine MICs are required. Moreover, *in vivo* correlation studies are required to determine the endpoints. Improved MIC determinations have led to more reproducible and accurate detection of resistance, which has benefited the study of resistance mechanisms (Verweij et al. 2009a).

Comparison of the difference between MIC and MFC showed 100%, 93.33% and 80%, of isolates tested for ITR, VOR and POS, respectively were within 2-fold limits. Standardized methods for MFC determination are required and could be a better measure of the efficacy of drug, especially in immunocompromised patients.

Chapter 4

Cyp51A sequence analysis

4.1 Introduction

Invasive aspergillosis is the leading infectious cause of death for immunocompromised patients (Denning et al. 2002a). *Aspergillus fumigatus* is the most common cause of invasive aspergillosis (IA)(Balajee et al. 2006). Early diagnosis and therapy has been shown to improve outcomes (von Eiff et al. 1995). Currently, the effective antifungal drugs used as primary therapy have been largely limited to polyenes such as amphotericin B and the azole compounds ITR and VOR (Calderone 2002;Walsh et al. 2008).

The primary mechanism of triazole resistance in *A. fumigatus* is a mutation of the *cyp51A* gene encoding 14 α-demethylase, which is a component of the ergosterol pathway and the target of the triazoles (Howard et al. 2009;Mellado et al. 2007;Snelders et al. 2008). Specific mutations in *cyp51A* may result in single, multi or pan resistance to azoles (Mellado et al. 2007;Verweij et al. 2009a). Some amino acid substitutions have already been reported: F219C, F495L, G54R, N248K, H147Y, G448S, M220I, M220k M220R, M220V, M220R, M220W, G448S, P216L, F46Y/ M172V/ E427K,and F46Y/ M172V/ N248T/ D255E/ E427K (Nascimento et al. 2003;Howard et al. 2006;da Silva Ferreira et al. 2004;Mellado et al. 2004;Diaz-Guerra et al. 2003;Chen et al. 2005). Mutations at codons 54 and 220 were confirmed to cause resistance (Howard et al. 2009).

In fact, F46Y/ M172V/ E427K/ mutations are likely not to be associated with resistance, as they have been described previously in both susceptible and resistant isolates (Howard et al. 2009;Rodriguez-Tudela et al. 2008). Mutation at L98 with duplication in tandem of 34bp repeated in the *cyp51A* promoter region has been found to be associated with resistance to all triazoles in *A. fumigatus* (Mellado et al. 2007). Resistance to VOR was described in laboratory mutants with mutations at codon 138 (G138) or at codon G488 (Manavathu et al. 2001) and clinical isolates without *cyp51A* mutations (Bueid et al. 2010).

A recent study found that mechanisms other than *cyp51A* mutations may be becoming more common, i.e., 39% of resistant *A. fumigatus* from 2006–2009 demonstrated a wild-type *cyp51A* sequence compared to 0% prior to 2006. Harrison E, Howard SJ, Buied A, *et al.* The changing prevalence of azole resistance mechanisms in *A. fumigatus* Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco USA, September 2009, Abstract M1720). Up-regulation of the *cyp51A* gene has also been implicated in azole resistance (Mellado et al. 2007). A recent report described a four- to six-fold increase expression of the *cyp51A* gene in a resistant isolate without mutations in the target gene (Arendrup et al. 2010). Decrease in the intracellular accumulation of triazole compounds, such as overexpression of the efflux pumps and decreased cellular permeability, are mechanisms which may play a role in resistance in *A. fumigatus* (Howard and Arendrup 2011).

This chapter reports on our attempt to sequence *cyp51A* from a panel of all resistance and some susceptible clinical isolates and find the association between mutations in the *cyp51A* and reduced azole susceptibility in the isolates 2008 to 2009 in *A. fumigatus* MRCM culture collection.

4.2 Results

The DNA of all isolates of *A. fumigatus* was extracted using the MycXtra kit (Myconostica, Manchester, UK) and the *cyp51A* gene was amplified and detected by PCR using primer Afcyp51A2026R and Afcyp51A2101F Table 2.1. Both strands of the entire coding region of the *cyp51A* genes of all 64 isolates of *A. fumigatus* were sequenced as previously described (2.2.7) and compared to an azole susceptible strain (Genbank accession number AF338659) (Mellado et al. 2001) using the software tool AlignX (VectorNTI; Invitrogen, Paisley, UK). We used the amino acid found in the reference sequence to represent the substitution. Mutations were confirmed by repeating DNA extraction, PCR and sequencing of both strands using four forward primers and four reverse primers listed in Table 2.1(2.2.7).

Repeat resistant isolates from the same patient were removed from the patient analysis (but not isolate analysis), even if isolated 1 year apart, unless their susceptibility profiles differed by interpretative breakpoint for at least one azole. The two previously characterised clinical *A. fumigatus* (F11628 and F12219) isolates (Howard et al. 2006) were sequenced and traces were examined. F11628 and F12219 showed two mutations, G138 and G54 respectively, as expected (data not shown). The sequences of all azole sensitive isolates were identical to that of the wild type (AF338659) (data not shown).

Fifteen different amino acid substitutions were found in the *cyp51A*. Twelve have already been reported: F219C, F495L, G54R, N248K, H147Y, G448S, M220I, M220k M220R, M220V, M220R, M220W, G448S, P216L, F46Y/ M172V/ E427K and F46Y/ M172V/ N248T/D255E/ E427K/ (Nascimento et al. 2003;Howard et al. 2006;da Silva Ferreira et al. 2004;Diaz-Guerra et al. 2003;Mellado et al. 2004;Chen et al. 2005), but the others, A284T, M220R and M220W, have not been previously reported. Table 4.1 shows mutations found, with consensus susceptibilities. Most remarkable is the increasing frequency of azole-resistant isolates without *cyp51A* mutations. These isolates have rarely been reported elsewhere (Howard et al. 2009;Bellete et al. 2010). Prior to 2007 very few resistant isolates in our centre had a wild-type *cyp51A* sequence

(Howard et al. 2009). In 2008, of the 13 resistant isolates studied, 1 had a M220K mutation, 3 had the F46Y/ M172V/ E427K combination (which is probably not linked to resistance) and the remaining 9 isolates had no *cyp51A* mutations. In 2009, 10 of 31 (32%) isolates tested had a wild-type *cyp51A* sequence (Figure 4.1). For patients, the frequency of mutations found in at least one isolate was 22% and 58% in 2008 and 2009, respectively (Figure 4.1). Thus 43% of isolates and 54% of patients did not have a *cyp51A* mutation known to confer resistance (including two isolates that were VOR resistant only). Interestingly, three patients had serial resistant isolates, some with *cyp51A* mutations, and others with wild-type sequences.

Figure 4.1 Percentage frequencies of mutations conferring resistance in 2008 and 2009. Overall percentage of mutation found [G54R, H147Y, M220 (I, K, V, R, W), P216L, A284T, G448S] in isolates and patients for each year is shown above each column. The percentage figure above each column refers to those with demonstrated mutations (single nucleotide polymorphisms)

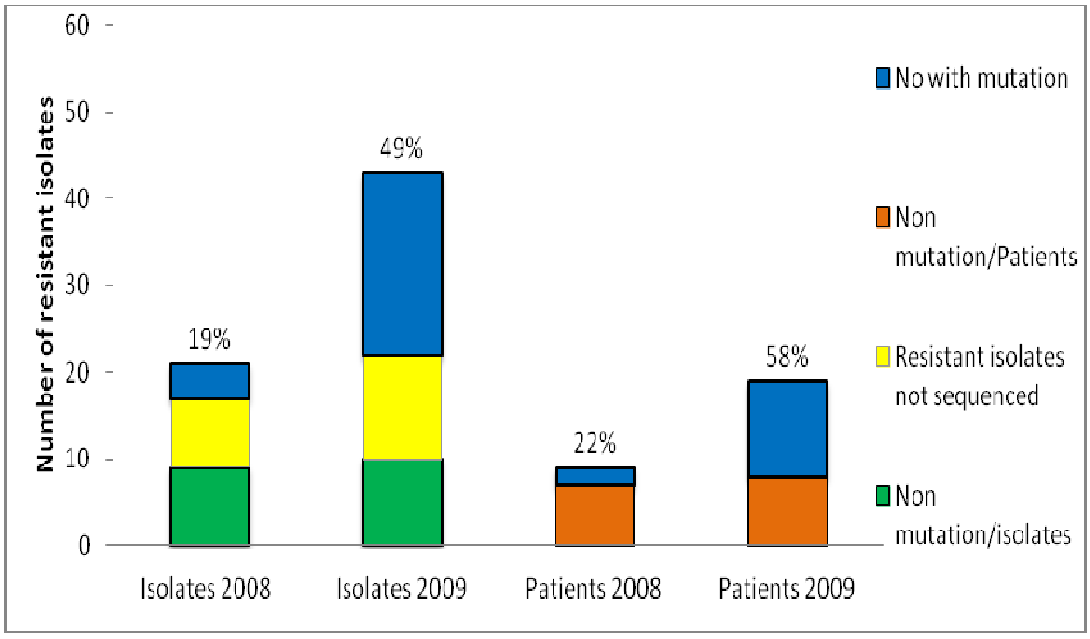

Table 4.1 A summary of mutation in the *cyp51A* gene and MICs for 64 *Aspergillus fumigatus* resistant isolates from MRCM culture collection, 2008–2009

Patients	Resistant isolates	MIC µg/ml			Mutation in the *cyp51A* gene
		ITR	VOR	POS	
2	3	>8	1-4	0.5	A284T
1	1	>8	2	0.5	F219C
1	1	>8	2	0.25	F495L
1	1	>8	0.5	>8	G54R,N248K
1	1	>8	0.5	1	H147Y,G448S
1	1	>8	1	1	M220I
2	6	>8	2->8	1->8	M220k
1	1	>8	1	1	M220V
2	3	>8	0.5-1	0.5-1	M220R
1	1	>8	0.5	1	M220R, Q259Q
1	1	>8	>8	>8	M220W, G448S
1	1	>8	0.25	1	P216L ,T215T
2	2	>8	2->8	0.125-2	F46Y, M172 V, , E427K,
3	4	4->8	1-2	0.125-0.25	F46Y, M172V, N248T, D255E, E427K,
	18	0.5->8	1->8	0.06->8	NMF
	sensitive isolates	0.5-2	0.5-2	0.6-.05	NMF
	19 Resistant isolates				ND

MRCM = Mycology Reference Centre Manchester; ND = not done. .

Breakpoint	Itraconazole (ITR)	Voriconazole (VOR)	Posaconazole (POS)
Resistant (R)	≥4	≥4	≥0.5
Intermediate (I)	2	2	0.5-1
Sensitive (S)	<1	<1	< 0.25

4.3 Discussion

Many reports have found that the common mechanism of resistance for *A. fumigatus* clinical isolates is mutation in the *cyp51A* gene, which is the target of azoles. Mutations at codons 54 and 220 have been confirmed to cause resistance (Howard et al. 2009). Other mutations in the *cyp51A* gene have been reported but have yet to confirm resistance. In this study, we investigated the frequency of *A. fumigatus* ITR resistance (ITR-R) in MRCM culture collection during 2008 and 2009 (see chapter 3) and identified mutations in the *cyp51A* gene. Most remarkable is the increasing frequency of azole-resistant isolates without *cyp51A* mutations so efflux-mediated resistance could be responsible, as it is a common mechanism in yeasts, although it has rarely been mooted in *Aspergillus* (Moore et al. 2000;Slaven et al. 2002) regulation of the *cyp51A* gene has also been implicated in azole resistance, but has yet to be found in isolation (Mellado et al. 2007). It is not certain whether all the *cyp51A* mutations found in Manchester confer resistance, so other mechanisms could also be contributory in these isolates. Despite the influence of other mechanisms, cross-resistance patterns appeared to remain closely linked with the *cyp51A* amino acid substitution. Isolates with G54R, P216L and G448S mutations are all associated with ITR and POS resistance, whilst remaining susceptible to VOR.

We found isolates with five different amino acid substitutions at position M220, namely isoleucine (I), lysine (K), valine (V), arginine (R) and tryptophan (W), of which M220R and M220W have not been previously reported to our knowledge. All alterations at codon 220 are associated with ITR and POS resistance, but result in variable VOR MICs (typically raised). A novel finding is that two patients had one isolate each with a *cyp51A* A284T mutation (alanine to threonine substitution), conferring reduced susceptibility to ITR, VOR and POS. Two patients yielded an isolate each with F46Y/ M172V/ E427K mutations, one of which also had the mutations N248T and D255E. However, it is likely that these mutations are not associated with resistance, as they have been described previously in both susceptible and resistant isolates (Howard et al. 2009;Rodriguez-Tudela et al. 2008). These results highlight the continuing increasing

frequency and evolution of resistance mechanisms in *A. fumigatus*, in both azole-naive and azole-treated patients (data not shown). The increasing rate of resistance is of concern. Furthermore, the emergence of alternative mechanisms of resistance other than *cyp51A* mutations, including isolates resistant only to VOR with no target mutations detected, implies a quite distinct mechanism compared with previously reported resistant isolates.

Chapter 5

Taxonomy in clinical and environmental *Aspergillus fumigatus*

5.1 Introduction

Aspergillus fumigatus is a saprotrophic fungus and a soil inhabitant, and can cause infection in the immunocompromised host (Debeaupuis et al. 1997). Invasive aspergillosis acquired from the environment of the hospital has been reported with genetic relatedness between *A. fumigatus* isolates (Warris et al. 2003;Balajee et al. 2007b;Verweij et al. 2009a).

In another study, a lack of genetic relatedness was observed (Chazalet et al. 1998). The 1:1 ratio of MAT1-1:MAT1-2 in *A. fumigatus* found in the environment and although the species is only known to reproduce by asexual means, *A. fumigatus* possesses a fully functional sexual reproductive cycle as the presence of mating-type genes and expression of sex-related genes in the fungus (O'Gorman et al. 2009). However the recently discovered sexual cycle function in *A. fumigatus* is consistent with substantial genetic change in the environment. Several studies have found that *A. fumigatus* has many characteristics of a sexual species (O'Gorman et al. 2009). The presence of sexual stage genes suggested that such a cryptic sexual stage exists in the human pathogen *A. fumigatus*. This may explain the multiple genotypes from the same geographical area and may explain the observed genetic variation.

Nowadays, a polyphasic taxonomy that combines morphological and molecular phylogenetic analyses is used to characterize *A. fumigatus* complex and analyse genetic variation of strains or closely related species.

Various molecular methods have been used to classify *Aspergilli*; including multilocus sequence typing (MLST), restriction fragment length polymorphism (RFLP), polymorphic microsatellite markers (Balajee et al. 2007b) and internal transcribed spacer (ITS), calmodulin (calm), actin and partial beta-tubulin regions (Balajee et al. 2007a;Hong et al. 2008).

Unfortunately, misidentification of *Aspergilli* is common as a result of unstable morphological characteristics and some clinical *Aspergilli* are slow to sporulate (Balajee et al. 2007a). Although recently some isolates have been previously identified as *A. fumigatus,* they have been reclassified as different species according to genotypic classification (Alcazar-Fuoli et al. 2008). This study and others demonstrated several cryptic species within the *Aspergillus* Section *fumigati* which are indistinguishable on morphological characters alone. For this reason, clinical laboratories may report out isolates of apparent *A. fumigatus* as *A. fumigatus* complex.

In recent years, azole resistant *A. fumigatus* pathogenic fungi have increased, some limited to certain geographical regions (Verweij et al. 2009a). Recently, azole resistance was frequently observed in clinical isolates (Chapter 4, (Bueid et al. 2010), which may be partly due to azole treatment (Arendrup et al. 2010) or azole exposure in the environment (Snelders et al. 2008). Although *Aspergillus* species are generally susceptible to azoles, intrinsic and acquired resistance has been documented including azole cross-resistance in patients (Verweij et al. 2009a).Therefore, identification of *Aspergillus* clinical isolates to species may be important for the choice of appropriate antifungal therapy and epidemiological studies; different species of *Aspergillus* have variable susceptibilities to multiple antifungal drugs. For example, *A. nidulans* isolates are resistant to amphotericin B (19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 19th) Peláez T., Guinea J., Gama B., Flores R., Recio S., Torres-Narbona M., Muñoz P., Bouza E. 2009 P 1297: Is *A. nidulans* susceptible to all antifungal agents? *In vitro* activity of an updated panel of antifungal agents against 63 clinical isolates). *A. terreus* and *A. ustus* appears be resistant to azoles; *A. lentulus* and *Petromyces alliaceus* have exhibited decreased susceptibility to anti-fungal drugs, especially the azoles and echinocandins and amphotericin B (Balajee et al. 2007a). These species may be separated from *A. fumigatus* by phylogenetic analyses based on sequence typing.

In the Netherlands and some EU countries genetic relatedness has been found between clinical and environmental *A. fumigatus* isolates (Snelders et al. 2008). Therefore, the main purpose of this study was to see whether *A. fumigatus* environmental isolates are azole sensitive or resistant and to investigate the relationship between environmental (around hospitals) and clinical isolates of *A. fumigatus* from patients with invasive aspergillosis.

A number of different algorithms have been used to analyse the phylogenetic relationships of *A. fumigatus* strains. These include the unweighted pair group method with the arithmetic mean (UPGMA), neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood. Hong et al. (2005) used neighbor joining and maximum parsimony methods to determined taxonomical position of the *A. fumigatus* strains. The amino-acid-based neighbor joining tree showed nearly the same topology as those of the DNA-based trees obtained by maximum parsimony (Hong et al. 2005).

In this study, we performed a polyphasic analysis of *A. fumigatus* from clinical and environmental sources in order to examine the variability within the species and determine relatedness of the strains.

5.2 Results

5.2.1 Mutations in the *cyp51A* in *A. fumigatus* environmental isolates

The full coding sequence of the *cyp51A* gene was determined by PCR amplification and subsequent sequencing (Bueid et al. 2010) . For analysis, the *cyp51A* sequence under accession number AF338659 in GenBank was used for comparison to detect mutations.

Twenty-three clinical isolates from MRCM clinical culture collection and 16 environmental isolates were investigated. (These samples were collected in July 2008 from different areas around Wythenshawe hospital.) Initially, all isolates were identified as *A. fumigatus* according to macro- and micro-morphological characteristics.

Antifungal susceptibilities against ITR, POS and VOR were determined for all isolates. Susceptibilities were determined using a modified European Committee on Antimicrobial Susceptibility Testing (EUCAST) method, as previously described (Howard et al. 2009). The MIC results for clinical isolates mentioned in Tables (3.1, 3.2 and 3.3).

The susceptibility of 16 *A. fumigatus* environmental isolates (9 were collected from around Wythenshawe Hospital and 7 were from commercial compost samples) was determined. All isolates were tested for susceptibility against ITR, VOR and POS. Values are shown in Table 5.1. Commercial compost *A. fumigatus* isolates showed higher MICs than the MICs of isolates from hospital soil.

Table 5.1 MIC results for environmental *A. fumigatus* isolates and CYP51A amino acid substitutions in *A. fumigatus* environmental isolates

Cyp51A codon	No. isolates	MIC, mg/L		
		ITR	VOR	POS
F46Y,M172V,N248T, D255E, E427K ,	1	0.125-0.5	0.5-2	0.03-0.06
No mutation	8[b]	0.125-0.5	0.5-2	0.03-0.06
No mutation	7[c]	0.25-0.5	1-4	0.125

a F46Y found with M172V N248T, D255E and E427K in 1 isolate with 3 silent mutations, b isolates around Wythenshawe Hospital, c commercial compost**.** Breakpoints used for resistance was ≥4 mg/L for ITR and VOR, and ≥5.0 mg/L for POS.

5.2.2 Taxonomy

Molecular methods were employed to identify and classify these isolates, including internal transcribed spacer (ITS), calmodulin (cal), actin and partial beta-tubulin regions.

23 clinical and 16 environmental isolates from MRCM clinical culture collections, environmental isolates and isolates from compost were molecularly identified as *A. fumigatus*. Both strands of each PCR amplicon were sequenced as previously described (Bueid et al. 2010). Primers used for sequencing and for PCR are listed in Table 2.1. Two primers per isolate per target were used for sequences. Sequences were aligned with ClustalW or MEGA 4.0. (Tamura et al. 2007). Both maximum parsimony (MP) and the neighbor-joining (NJ) methods were used for the phylogenetic analysis. To determine the support for each clade, bootstrap analysis was performed with 2000 replications. Neighbor-joining, NJ tree of phylogenetic relationships are displayed in all cases. Isolates resistant to ITR are in the red box, susceptible strains green: bootstrap values are shown above branch length. In this study, the nucleotide sequence of the reference strains of *A. fumigatus* AF293 was used for comparison.

Sequence alignment for each gene was conducted separately. The ITS region was sequenced for each isolate using primers ITS1 and ITS4 (Table 2.2) Figure 5.1 shows an NJ tree constructed from around 500 base pair alignment of ITS sequences. Two clades, one with 65% bootstrap values therefore ITS data was not useful in determining species in these isolates.

A region of the beta-tubulin gene was amplified by PCR and sequenced using the primers bt2a and bt2b (Table 2.3).The beta-tubulin data suggested the existence of a new clade of 1 isolate (soil). Beta-tubulin sequences were aligned and the resulting NJ tree is shown in Figure 5.2. Beta-tubulin was not better than ITS at discriminating between this group of isolates.

The actin gene was amplified with primers act-512F, act-783R (Table 2.3) and the actin sequences were aligned and the resulting NJ tree is shown in Figure 5.3. The actin data suggested the existence of 5 different clades of isolates with well bootstrap values of 65%. Generally, actin data is useful for discrimination among these isolates. Actin was significantly better than both beta-tubulin and ITS at discriminating between these isolates.

The calmodulin gene was amplified by PCR using 2 primers cmd5 and cmd6 (Table 2.3). A problem was faced when we were amplifying it by PCR due to poor PCR yield and irregular and multiple peaks in the same position of the sequence chromatogram. DNA of these isolates was re-extracted and the calmodulin was re-amplified then sequenced. The calmodulin sequences were aligned and the resulting NJ tree is shown in Figure 5.4. Examination of the calmodulin data would suggest the existence of 5 different clades of isolates with good bootstrap values of 65%. Generally, actin data is useful in discriminating these isolates. Calmodulin was significantly better than both beta-tubulin and ITS at discriminating between these isolates.

Initially sequence alignment for each gene was conducted separately. Subsequently ITS, beta tubulin, actin and calmodulin sequences were combined, and a phylogenetic tree constructed. The combination of 4 gene datasets was most consistent with the existence of different clades of isolates with very poor bootstrap values of less than 50% except for 3 isolates (F/21295, F/21215 and soil 1) with 60%. Figure 5.5 shows a tree of a phylogenetic analysis produced by neighbor-joining of joined of ITS, partial β-tubulin, actin and calmodulin sequences. The tree was grouped the isolates into a minimum of 7 different clades. All isolates were identified as *A. fumigatus.* Two groups of clinical isolates grouped together, and these were sequential isolates from the same patient, introduced as 'similarity control' for the analysis. Appropriately the 2 sets grouped together, but were in distinct clades. The four clinical isolates were grouped with bootstrap values of 64%. But clades containing other isolates were not well supported by the bootstrap analysis suggesting different origins.

The clade comprising *Aspergillus* (soil 3) was distinct from *A. fumigates* complex. Nevertheless; this species was able to grow at 48^{o}C and was azole susceptible. Moreover, no alterations have been found in the *cyp51A* gene from this isolate. However, it appears to be a taxonomically discrete organism, possibly a new cryptic species, although this requires further investigation.

A. fumigatus clinical and environmental isolates have been grouped into different clades using a phylogenetic analysis produced by neighbor-joining of combined ITS, β-tubulin, calmodulin and actin sequences. This finding does not support the hypothesis that the origin of the resistant isolates could be the consequence of azole exposure in the environment but may support the hypothesis that this could be the consequence of prolonged azole therapy.

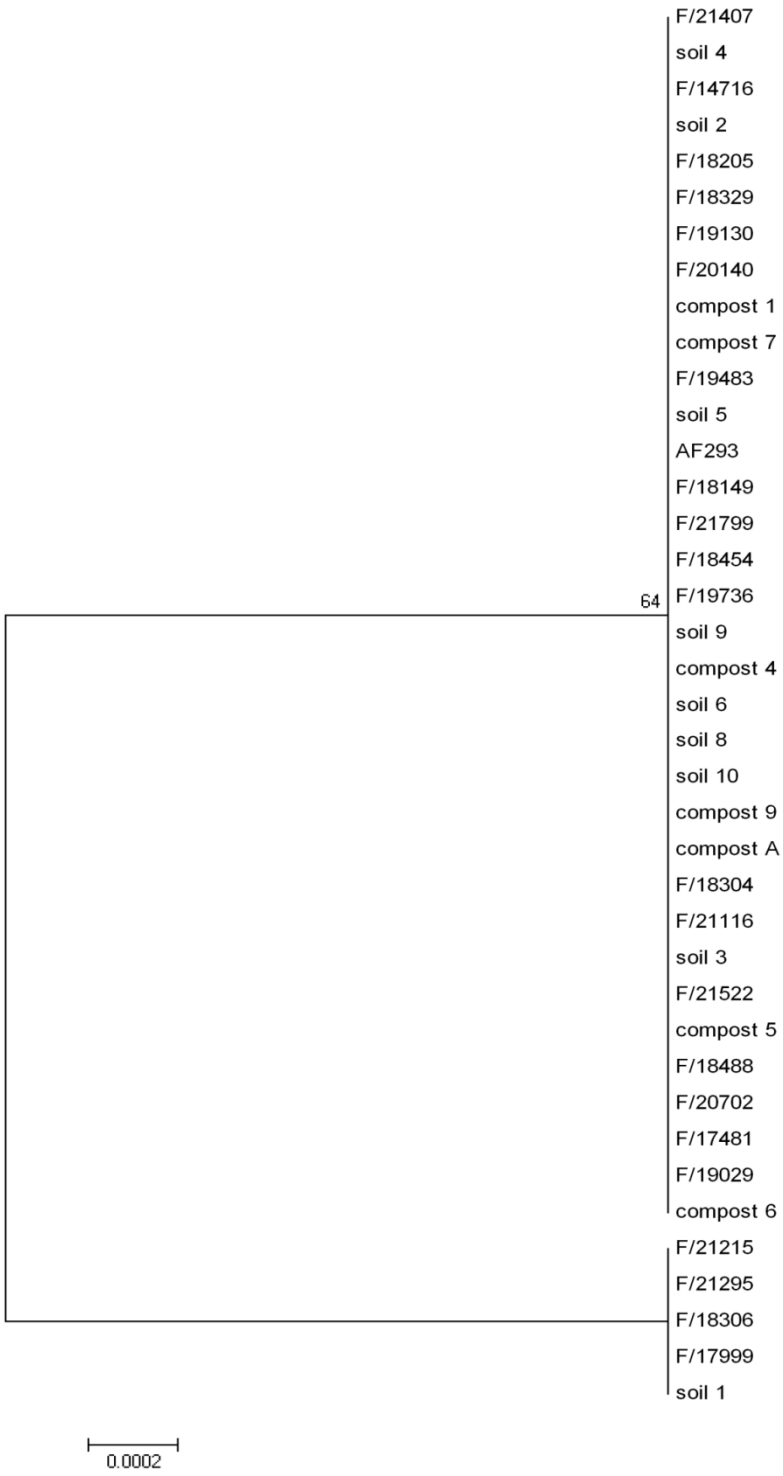

Figure 5.1 Phylogenetic tree obtained by phylogenetic analysis with 2,000 bootstrap replicates on the basis of the ITS sequences from 23 clinical and 16 environmental isolates included in the study

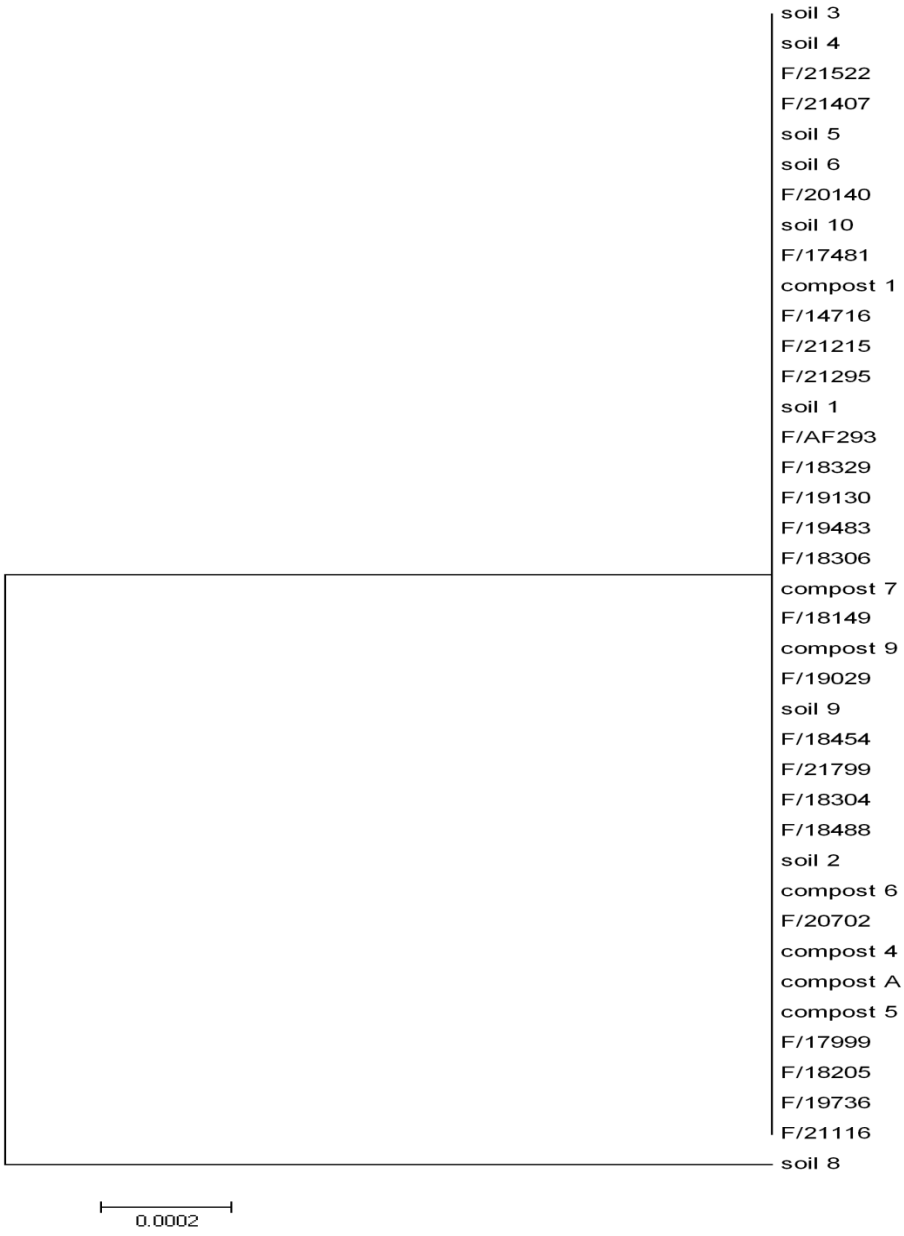

Figure 5.2 Phylogenetic tree obtained by phylogenetic analysis with 2,000 bootstrap replicates on the basis of the partial beta-tubulin sequences from 23 clinical and 16 environmental isolates included in the study

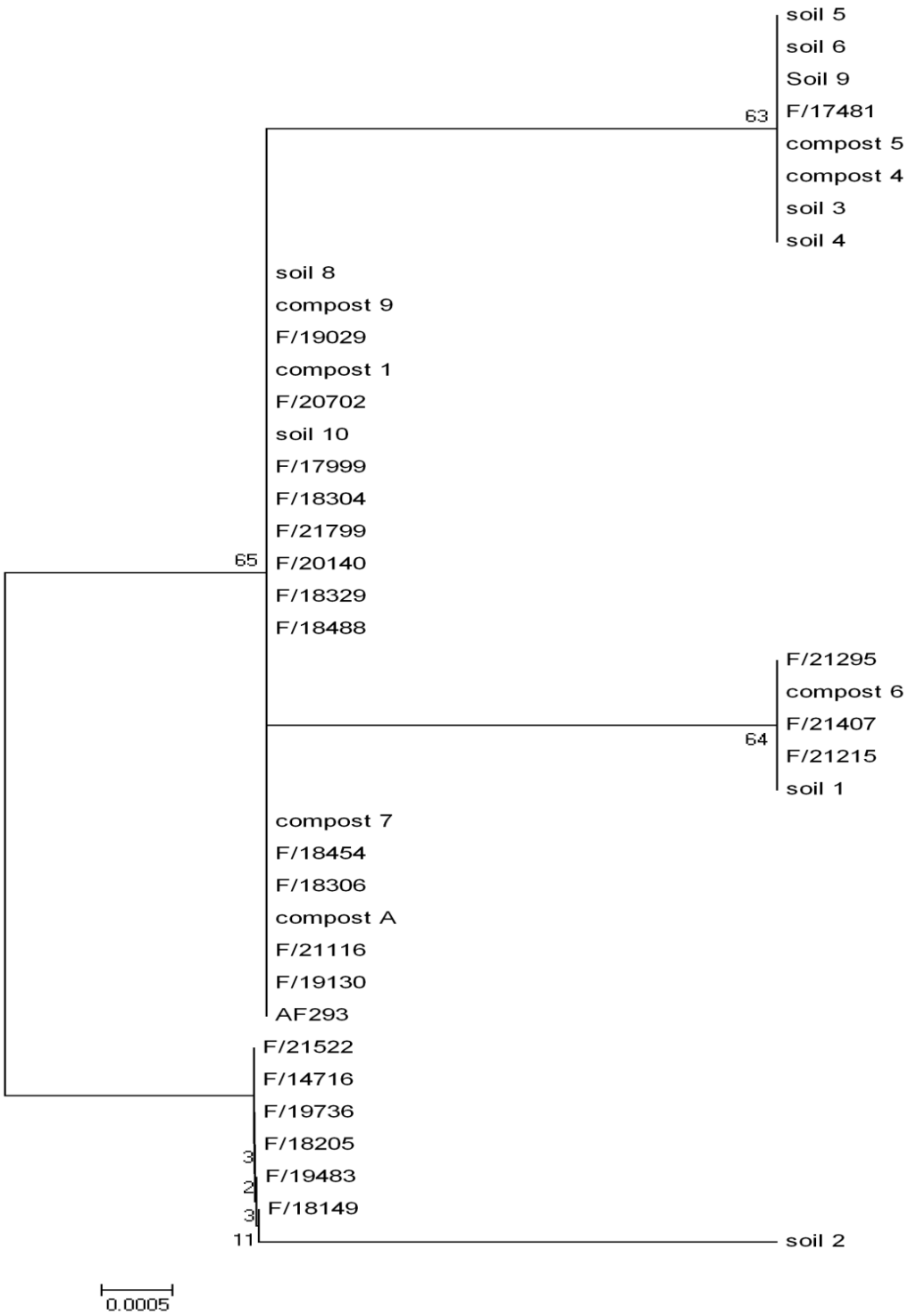

Fig ure 5.3 Phylogenetic tree obtained by phylogenetic analysis with 2,000 bootstrap replicates on the basis of the actin sequences from 23 clinical and 16 environmental isolates included in the study

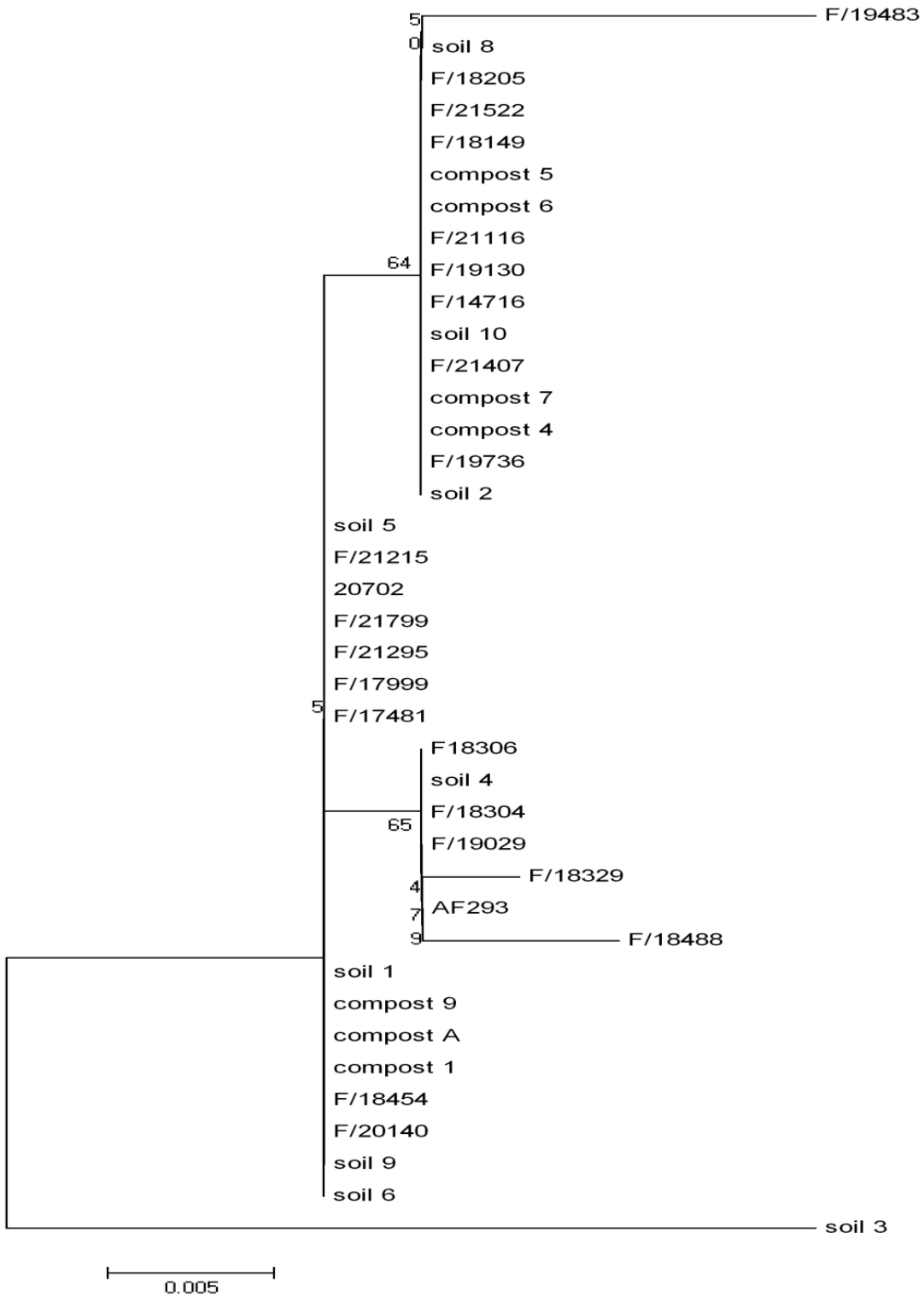

Figure 5.4 Phylogenetic tree obtained by phylogenetic analysis with 2,000 bootstrap replicates on the basis of the calmodulin sequences from 23 clinical and 16 environmental isolates included in the study

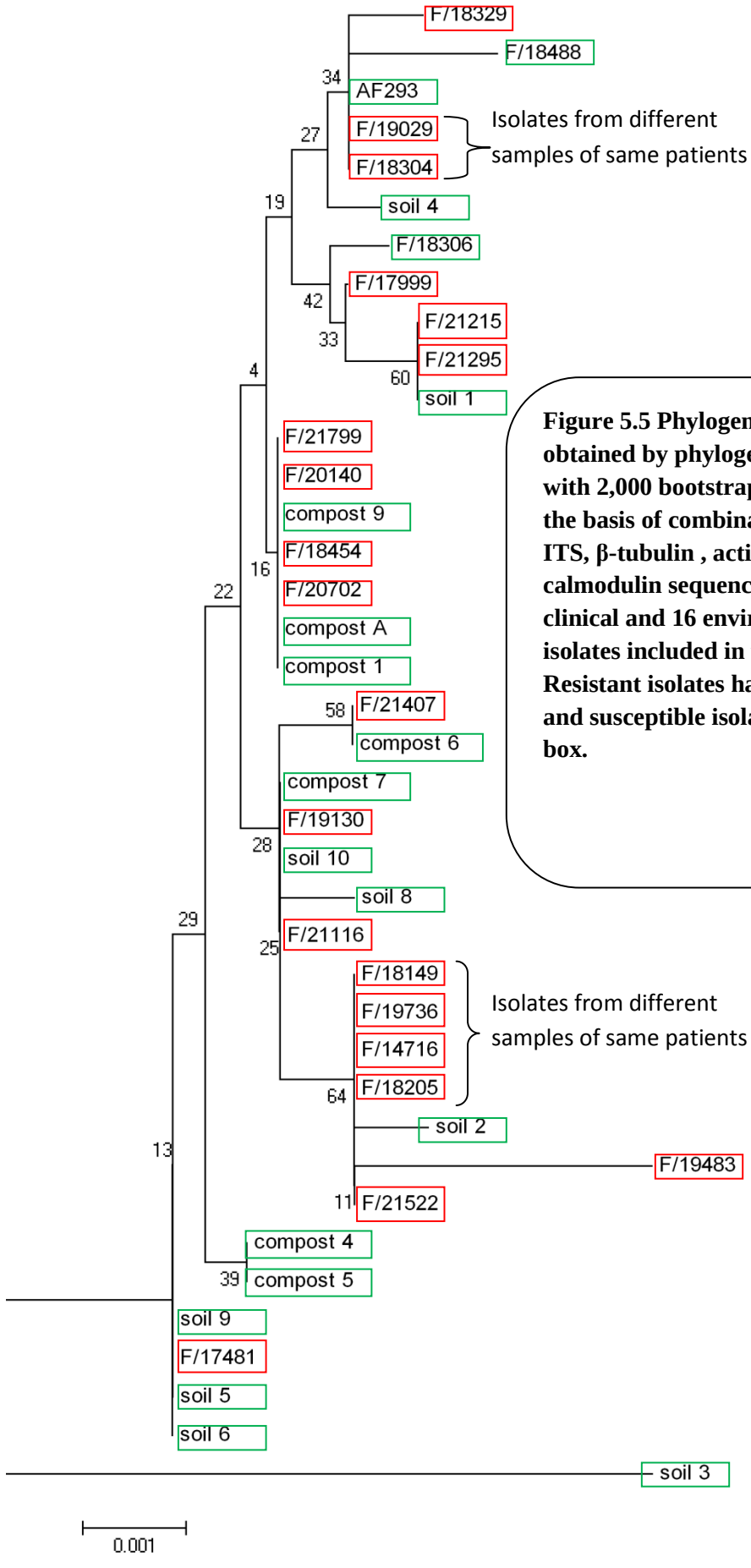

Figure 5.5 Phylogenetic tree obtained by phylogenetic analysis with 2,000 bootstrap replicats on the basis of combination of the ITS, β-tubulin , actin and calmodulin sequences from 23 clinical and 16 environmental isolates included in the study. Resistant isolates have a red box and susceptible isolates a green box.

5.3 Discussion

The 9 *A. fumigatus* environmental isolates from natural soil were azole susceptible with MIC ranges of 0.125-0.5 mg/l against ITR, 0.5-2 mg/l against VOR, and 0.03-0.06 mg/l against POS. Likewise the 7 *A. fumigatus* environmental isolates from compost were also susceptible with MIC ranges of 0.125-0.5 mg/l against ITR, 1-4 mg/l against VOR, and 0.125 mg/l against POS (Table 5.1). These results found that *A. fumigatus* environmental isolates were susceptible to ITR, VOR and POS. These results also suggest that environmental isolates of *Aspergillus* collected from natural soil are more susceptible to VOR than *A. fumigatus* environmental isolates that were collected from compost, although numbers are small.

The sequence analysis of the *cyp51A* revealed that no *cyp51A* mutations were found in *A. fumigatus* environmental isolates (Table 5.1) except one isolate with many mutations. The mutations found (F46Y, M172V, N248T, D255E, and E427K) do not confer azole resistance as they have been found in azole-susceptible strains (Rodriguez-Tudela et al. 2008).

5.3.1 Phylogenetic analysis

Macroscopic and microscopic morphology are not enough for *Aspergillus* identification to the species level. Misidentification was common when only morphological characteristics were used. Therefore multilocus sequence-based phylogenetic analyses have been used as a tool for phylogenetic analysis of species in this study.

Initially, *A. fumigatus* in this study were identified morphologically and confirmed by culturing them on SAB at 50°C, which excludes *A. lentulus* a closely related azole-resistant species (Alcazar-Fuoli et al. 2008;Yaguchi et al. 2007). The multilocus sequences were compared to GenBank records using BLAST (Blastn) to confirm that each isolate was *A. fumigatus*. High identity to *A. fumigatus* was found with all isolates.

Sequences were compared with those of *A. fumigatus* clinical isolates, all obtained from the Mycology Reference Centre, Manchester, UK (2008-2009). Sequence alignment for each gene was conducted separately. Subsequently ITS, beta tubulin, actin and calmodulin sequences were combined, and a phylogenetic tree constructed.

Several studies have shown that sequence-based identification using the ITS1 and ITS2 could be employed for species complex-level identification of *Aspergillus* (Hinrikson et al. 2005) and most *Mucorales* species (Schwarz et al. 2006) and for identification within some species complexes of *Fusarium* (Zhang et al. 2006;O'Donnell et al. 2008). In this study we evaluated the utility of the ITS1 and ITS2 sequences as targets for the identification and differentiation of our collection of environmental and clinical *A. fumigatius* isolates. High identity to *A. fumigatus* was found with all isolates when a systematic comparison of these sequences to those available in the GenBank database was conducted. In terms of differentiation, our dataset grouped into two clades using the ITS sequence (Figure 5.1) or the partial beta tubulin sequence region (Figure 5.2). Bootstrap values were all zero and branch lengths were short. ITS sequences did not help classify the species in this closely related group of organisms.

Confirming others' work, our finding suggests that *A. fumigatus* could not be differentiated by ITS alone (Hendolin et al. 2000;Hinrikson et al. 2005). Similarly partial beta - tubulin sequences provided insufficient resolution for our data perhaps due to the particularly close genetic relatedness of these isolates. Whereas beta – tubulin, actin and calmodulin have been used and recommended as good targets in differentiation (Balajee et al. 2005;Hong et al. 2005;Yaguchi et al. 2007;Samson and Varga 2009). The actin sequences were aligned and the resulting NJ tree is shown in Figure 5-3. The actin was significantly better than both beta - tubulin and ITS at discriminating between these isolates. The actin data suggested the existence of 5 different clades of isolates with bootstrap values of 65%. Generally, actin data is useful in discrimination within these isolates. The calmodulin sequences were aligned and the resulting NJ tree is shown in Figure 5.4. The calmodulin data suggested the existence of

5 different clades of isolates with bootstrap values of 65%. Therefore, ITS and beta - tubulin data was not useful in discrimination within these isolates. The calmodulin was found to be valuable as a molecular taxonomic target gene. It is significantly better than both beta-tubulin and ITS at discriminating between these isolates. Both actin and calmodulin grouped our dataset into 5 clades. The phylogenetic trees based on actin and calmodulin were largely in agreement. The calmodulin tree alone or actin tree alone was more supported than trees based on ITS or the partial beta- tubulin gene. Furthermore, we found calmodulin alone was better supported than the combination of the ITS and the partial beta beta - tubulin (data not shown). Therefore the data was useful in determining species in these isolates.

The ITS sequences were aligned and the resulting NJ tree is shown in Figure 5.1. With poor bootstrap values, the ITS region proved insufficient resolution between this group of closely related organisms. Therefore ITS data was not useful in determining species in these isolates.

The combination of sequences from 4 genes suggested the existence of different clades of isolates with very poor bootstrap values of less than 50% except 3 isolates (F/21295, F/21215 and soil 1) with 60%. The calmodulin or actin are significantly better than beta-tubulin and ITS at discriminating between these isolates. More subdivisions were possible using the combination of four loci.

A. fumigatus clinical and environmental have been grouped into different clades using a phylogenetic analysis produced by neighbor-joining of joined ITS, β-tubulin, calmodulin and actin sequences. In this study, calmodulin or actin sequences are the most promising loci for *A. fumigatus* identification, whereas partial beta tubulin or ITS sequencing is not enough for identification to the species level.

The clade comprising *Aspergillus* (soil 3) was distinct from *A. fumigatus* complex. Nevertheless; this species was able to grow at 48^{o}C and was azole susceptible.

Moreover, no alterations were found in the *cyp51A* gene. However, it is a discrete clade, possibly a new species, although this requires further investigation.

Many cryptic species are found in clinical and environmental isolates of the *A. fumigates* (complex). All environmental *A. fumigatus* complex isolates were ITR susceptible and no cross resistance was observed in this group of isolates. Our findings are in agreement with the previous report that azole-resistant isolates were never found in natural soil (Verweij et al. 2009b). The most likely explanation for this result is that natural soil, where *A. fumigatus* was isolated, was not exposed to azole compounds.

The susceptibility pattern of non- *A.fumigatus* may be different from that of *A. fumigatus.* The taxonomy of the species in this study does not suggest that any of the isolates were divergent and that there are no new sibling species in these isolates. Furthermore, the taxonomy emphasises the MIC distributions of the species. Molecular identification of Aspergilli is important because the efficacy of antifungal agents against different *Aspergillus* species that cause invasive infections varies. Therefore, identification of *Aspergillus* clinical isolates to species level may be important for the choice of appropriate antifungal therapy and epidemiological studies.

Chapter 6

Disruption of the transporter gene (*ABC11*), the major facilitator superfamily (*MFS56*) and hypothetical protein (*M85*) in *Aspergillus fumigatus* and their role in azole drug susceptibility

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6.1 Introduction

Aspergillus fumigatus is the most frequent causative agent of invasive aspergillosis in immunocompromised patients, chronic pulmonary aspergillosis and ABPA (Langfelder et al. 2002;Slaven et al. 2002). The triazoles (ITR, VOR and POS) are widely used to treat Aspergillus infection (Denning et al. 2011). Although *A. fumigatus* responds to these antifungals, some *A. fumigatus* strains have acquired resistance to antifungal agents, increasing the frequency of therapeutic failure of antifungals (Qiao et al. 2008;Verweij et al. 2009a). Thus, study of the mechanisms of antifungal resistance is important to understand and develop strategies to minimize and overcome resistance.

In resistant Aspergillus, the overexpression of efflux pumps contributes to reduce susceptibility by decreasing effective intracellular concentration of azoles (Del Sorbo et al. 2000;Langfelder et al. 2002). There are two classes of efflux transporters: ATP-binding cassettes (ABC) and major facilitator superfamily (MFS). ABC transporters are involved in the export or import of a wide variety of substrates.

A. fumigatus encodes more than 40 ABC transporters compared to 30 ABC transporters in *S. cerevisiae* (Mellado et al. 2007) and *A. fumigatus* has more than 100 major facilitator superfamily (MFS) proteins (Tekaia and Latge 2005). Recently genome sequencing of *A. fumigatus* has identified at least 327 genes that encode putative multidrug resistance efflux pumps, including 49 ABC type genes, and 278 genes that encode MFS proteins (Abad et al. 2010;Nierman et al. 2005b)

In *A. fumigatus*, four genes which encode ABC type transporters have been characterised: the *AfuMDR1, AfuMDR2 atrF* and *AfuMDR4* genes and a gene which encodes a protein of the MSF class, the *AfuMDR3* gene. These genes have been described and related to azole resistance in *A. fumigatus.* Other genes (abcA–E and mfsA–E) that encode for these types of transporter could be related to VOR resistance (Rementeria et al. 2005;Abad et al. 2010).

Two genes (*AfuMDR1* and *AfuMDR2*) predicted to encode ABC transporter proteins were identified in *A. fumigatus* (Tobin et al. 1997). Recently, over- expression of *atrF* gene was found to be correlated with *A. fumigatus* resistance to ITR (Slaven et al. 2002). Over - production of the *A. fumigatus* ABC transporter *AfuMDR1* in *S. cerevisiae* led to a significantly decreased sensitivity towards the antibiotic cilofungin (Tobin et al. 1997). Overe-xpression of one or both of two genes, *AfuMDR3* and *AfuMDR4*, efflux pump genes, of *A. fumigatus* are linked to high-level ITR resistance (Nascimento et al. 2003). The major facilitator superfamily (MFS) represents one of the two largest families of membrane transporters. *AfuMDR3* is the first fungal MFS transport gene identified which is linked to drug resistance (Nascimento et al. 2003).

The ABC and MFS transporters can also play a major role in antifungal resistance. As a result, these transporters are possible targets for antifungal drugs. Here in this chapter we describe the functional characterization and gene disruption *A. fumigatus* of new members of the ABC and MFS multidrug transporter gene family which have not yet been investigated; *ABC11*, *MFS56* and *M85*.

In vitro gene disruption or gene knock-out where the functional gene was replaced by a reporter gene such as the pyrithiamine (PT*)* resistance gene (ptrA). A specific single gene knock-out, which leaves other genes untouched, has been used in order to investigate whether or not the specific gene is involved in modulating susceptibility to azoles.

Knowledge of the subcellular localization of proteins within the cell of Aspergillus plays a significant role in characterizing the newly discovered protein and its corresponding gene and in the organization of the cell as a whole (Huh et al. 2003;Scott et al. 2005). Localization of a protein and its corresponding gene is established by visualization of GFP fusion proteins which have been tagged by fusion to green fluorescent protein (GFP) (Kumar et al. 2002). Green fluorescent protein (GFP), an intrinsically fluorescent molecule obtained from the jellyfish *Aequorea victoria*, is widely used to study protein-protein interactions, cell division, and gene expression in a variety of organisms in real time (Kumar et al. 2002;Kenri et al. 2004).

Reporters and epitope tags are fused to target genes (Kumar et al. 2002). Over - expressed protein products and reporter tags such as GFP increase in direct proportion to each other and vice versa, however weakly expressed protein may not yield sufficient protein to be visualized under fluorescence microscopy (Kumar et al. 2002). Few studies have characterized protein localization (Simpson et al. 2000;Kumar et al. 2002). Niedenthal et al. (1996) constructed GFP reporter fusions to three unknown open reading frames (ORFs) from *S. cerevisiae* Chromosome XIV and subsequently localized these chimeric GFP-fusion proteins by fluorescence microscopy (Kumar et al. 2002). 250 independent gene products were localized using GFP (Ding et al. 2000). GFP fluorescence does not require external cofactors; GFP signal can be monitored in living cells without disrupting cellular integrity in a simple eukaryotic cell (Huh et al. 2003).

6.2 Results

The DNA extraction methods; Mycxtra kit (2.2.3) and the method described in 2.2.3.1B, have been exploited in this study. The latter produced a sufficient quantity and quality of the extracted genomic DNA from *A. fumigatus* to perform PCR-based reactions and also to be used for other DNA manipulation techniques, such as DNA construction. However, amplification of a large fragment (>5 kb) such as *ABC11* gene using genomic DNA extracted by the Mycxtra kit (Myconostica Manchester, UK) failed to produce PCR product, although the yield of DNA was good enough. Nevertheless, DNA was re-extracted with a new kit of Mycxtra, and although PCR conditions (annealing and extending temperature) were changed, we did not succeed in obtaining PCR product.

Table 6.1 Amplification of novel gene flanking sequences from Af293 gDNA, the hph gene from pAN7-1, the PtrA gene from pPTRII and the GFP coding sequence from pFNO$_3$

DNA fragment	Gene	Length of the amplicon
Upstream flankging sequences	ABC11	1021 bp including 20 bp overlap
	MFS56	1020 bp including 20 bp overlap
	M85	1518 bp including 20 bp overlap
Downstream flankging sequences	ABC11	1035 bp including 20 bp overlap
	MFS56	1037 bp including 25 bp overlap
	M85	998 bp including 20 bp overlap
Hygromycin resistance gene sequences	hph	2828 bp including 20 bp overlap
GFP coding gene sequences	GFP	715 bp
Pyrithiamine resistant gene sequences	PtrA	2025bp

Several published fusion PCR protocols had previously been tested (Shevchuk et al. 2004) in our laboratory but they were irreproducible (Dr Marcin Fraczek, Manchester University, 2008-2011 personal communication). Primer design is a crucial step for fusion constriction. During this study, some attempts failed to create a gene knock-out fusion construct; for example, when we used primers designed to amplify the downstream flanking sequence, and one primer was complementary to the 5' end of the downstream flanking sequence and contained extensions of 25 bp identical to the 5' reverse sequence of the PtrA cassette. The other primer was complimentary to the 3' sequence of PtrA. However, two modified protocol techniques were used from Dr Marcin Fraczek (2010) and Dr Michael Bromley (2011), both of the University of Manchester, to provide the quantity and quality of fusion constructs required for transformation (2.2.9).

To analyse the importance of the 3 novel genes (*ABC11*, *MFS56* and *M85*) in the azole resistance mechanism in *A. fumigatus,* the corresponding genes were deleted from *A. fumigatus* strains A1160pyrG^{+} A 2-step fusion PCR (2.2.5.7) was performed for the 3 gene disruption constructs. 3 sets of primers were used for each gene (Table 2.5, 2.6 and 2.7). The upstream and downstream flanking regions of target genes from gDNA of Af293 and the pyrithiamine resistance gene (*ptrA*) from plasmid (pPTRII) were amplified (Figure 6.1, 6.2 and 6.3). A Phusion High Fidelity DNA polymerase was used to generate blunt-ended PCR products (2.2.5.7).

6.2.1 Disruption and PCR construction

To study the function of *ABC11* (AFUA-1G14330), *MFS56* (AFUA-1G05010) and *M85* (AFUA-5G07550) genes, disruption mutants were constructed using the methodology previously described (2.2.5.7).

For gene disruption, in order to splice 3 DNA fragments into a fusion PCR product, 6 specific primers were required to amplify these fragments. Four of these were used to amplify the upstream and downstream flanking sequences of the gene of interest to be

disrupted. The flanking gene fragments were first amplified separately by PCR using primers that produce ends overlapping the ptrA gene (Table 2.5, 2.6 and 2.7) and two specific primers (ptrA-F and -R) were designed to amplify the selective pyrithiamine-resistance gene (*PtrA*) from plasmid pPTRII (Takara, Japan). In addition, 2 nested primers were used to generate final constructs and check product specificity in the long PCR reaction used for cloning or transformation of the complete fusion product (Tables 2.5, 2.6 and 2.7 and Figures 6.1, 6.2 and 6.3). Similarly, for GFP fusion, in order to splice 3' promoter DNA fragments into a fusion PCR product, 6 specific primers were required to amplify these fragments. Two of them were used to amplify the upstream (promoter) of the interest gene (Table 2.5, 2.6 and 2.7), two of them were used to amplify GFP sequences, and two specific primers were designed to amplify the selective marker cassette (hph). In addition, 2 nested primers were used to generate the final constructs in the long PCR reaction used for cloning or transformation (Tables 2.5, 2.6 and 2.7 and Figures 6.7, 6.8 and 6.9).

Table 6.2 Expected fusion PCR amplified after the second step PCR for gene disruption

construct	Length of the amplicon (bp)				
	Forward & reverse primers	Nested primers (F & R)	X-F nest & PtrA-R	PtrA-F & PtrA-R	PtrA-F & X-R-nest
ABC11-ptrA-ABC11	4040	3947	3009	2025	2964
MFS56-PtrA-MFS56	4037	3956	2947	2025	3017
M85-PtrA-M85	4534	3832	2922	2025	3003

X, gene of intrest

Figure 6.1 Construction of gene replacement constructs using fusion PCR. The first PCR amplifies the upstream and downstream flanking region of ABC11 with 2 sets of primers. The second PCR amplifies the pyrithiamine (PtrA) gene which functions as a selectable resistant marker with primers (PtrA-F and PtrA-R). Fusion PCR is completed with the three amplified fragments and gene-specific primers

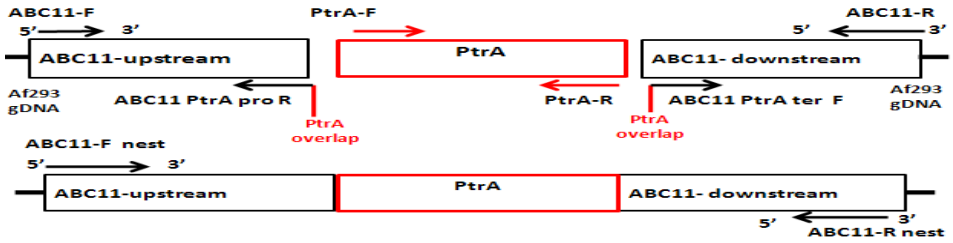


Figure 6.2 Construction of gene replacement constructs using fusion PCR. The first PCR amplifies the upstream and downstream flanking region of MFS56 with 2 sets of primers. The second PCR amplifies the pyrithiamine (PtrA) gene which functions as a selectable resistant marker with primers (PtrA-F and PtrA-R). Fusion PCR is completed with the three amplified fragments and gene-specific primers

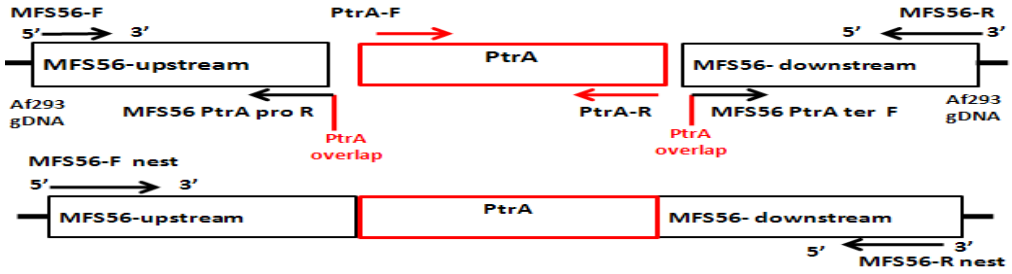
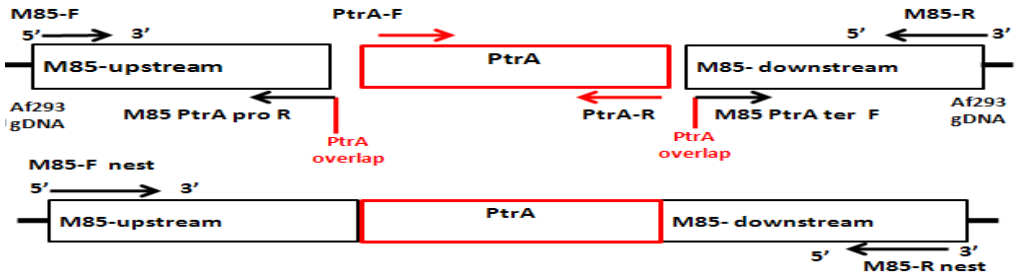


Figure 6.3 Construction of gene replacement constructs using fusion PCR. The first PCR amplifies the upstream and downstream flanking region of M85 with 2 sets of primers. The second PCR amplifies the pyrithiamine (PtrA) gene which functions as a selectable resistant marker with primers (PtrA-F and PtrA-R). Fusion PCR is completed with the three amplified fragments and gene-specific primers.



An outline of the fusion PCR for *ABC11* gene is described earlier (Table 2.5) (2.2.5.7) and Figure 6.1. Disruption constructs for the two other genes *MFS56* Figure 6.2 and *M85* Figure 6.3 are made in a similar manner but with different primers (Tables 2.6 and 2.7). . The sequences of three fragments were PCR amplified as previously described (2.2.5.6 and 2.2.5.7). For the first fragment of target gene (*ABC11*), ~1kb (1021bp) upstream flanking region (Table 6.1, Figure.6.1 and 6.4 A) was amplified with primers (ABC11F and ABCptrA-R). The second fragment, ~2kb (2025bp) *PtrA* cassette (Table 6.1, Figure.6.1 and 6.4 B) was amplified with specific primers (PtrA-F and PtrA-R). For the third fragment (Table 6.1, Figure.6.1 and 6.4 C), ~1kb (1035 bp) downstream flanking region was amplified using primers (ABCptrA- F and ABC11R) (Table 2.5).

A Phusion High Fidelity DNA polymerase was used in PCR for fragment amplification. Most fragments generated were a with single band high quantity PCR product. The three amplification fragments were purified using the QIAquick PCR purification kit (2.2.6). However, for the PCR products with more than one band, which was a non-specific band, a correct band and size was gel purified from an agarose slice using the QIAquick Gel Extraction Kit (Qiagen) (2.2.6).The three purified fragments from the first step PCR were analysed on a 1% agarose gel. Approximately 1µl of PCR product containing ~20 ng of each fragment was used in the second PCR step for PCR construction. To amplify and minimise any sequence changes during fusion PCR for a high molecular weight or a long construct, we used a high fidelity polymerase *Taq* DNA polymerase (Promega), AccuPrime Taq DNA Polymerase High Fidelity (Invitogen) and Phusion High Fidelity DNA Polymerase (Finnzymes).

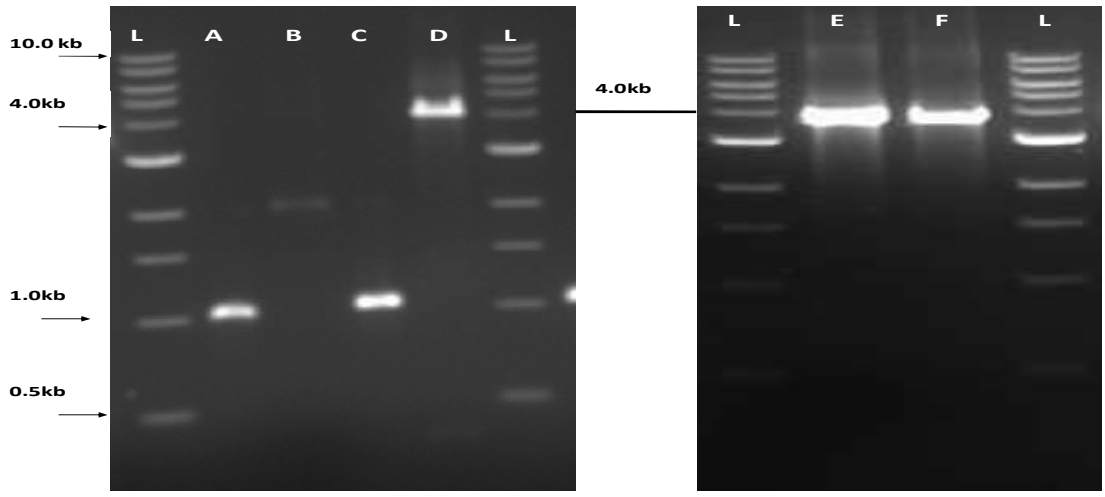


Figure 6.4 Agarose gel electrophoresis of 1st step fusion PCR. ~1kb upstream flanking region (A), ~2kb *PtrA* cassette (B), ~1kb downstream flanking region (C), approximately 4.0kb final construct bands (D, E and F) . 1kb DNA ladder (NEB) is presented in lane L.

We found AccuPrime Taq DNA Polymerase High Fidelity (Invitogen) was the most reliable and specific for long PCR products providing a good and specific yield of PCR product more reproducibly than others used in this study. Others used, mentioned above, produced a low quantity of specific target, did not amplify the desired products, or generated many non-specific low molecular weight fragments (data not shown). No fusion or non-specific fragments were generated in the second step PCR when the fragments of DNA included in the reaction were in low or high concentration, or owing to incorrect annealing temperatures. As a result we quantified the purified PCR products and used not more than 20ng for each fragment per reaction. Nested primers (Tables 2.5, 2.6 and 2.7) were used for the final step of fusion PCR to increase the specificity of the reaction. Furthermore, the primer concentration used in this step was important. 0.2-0.4 μM of each primer was found to be reliable and generated the expected size of constructs. The final fusion construct was cleaned with gel purification (Qiagen) before cloning and transformation. The expected bands (length) of three fragments, as well as the final constructs (Tables 6.1 and 6.2), are shown in Figures 6.4 and 6.5B. In order to determine whether the gene disruption construct had replaced the native gene in transformed strains genomic DNA isolated from mutant strains was amplified by PCR with a different set of a forward primer and a reverse primer located in a different region

of the new construct, and all PCR reactions resulted in the expected products To test whether or not the gene was not disrupted, a different primer set was used:ABC11-F and ABC11-R, ABC11-F nest and PtrA-R and PtrA-F and ABC11-R-nest for ABC11; MFS56-R nest and MFS56-F nest, MFS56-F nest and PtrA-R and PtrA-F and MFS56-R-nest for MFS56 and M85-R nest and M85-F nest, M85-F nest and PtrA-R and PtrA-F and M85-R-nest for M85. The expected PCR product size for untransformed genomic DNA was.6682 bp, 4010bp, and 2350 bp for *ABC11*, *MFS56* and *M85*, respectively (Figure 6.5A) and the expected PCR product size for strains which carried a precise gene replacement was 3947 bp, 3956 bp and 3832 bp.for *ABC11*, *MFS56* and *M85*, respectively (Figure 6.5B) confirmed by sequencing using nested primers (Appendix, A, B, and C).Strains which carried an insertion of the transforming DNA at a different site are expected to give a PCR product of ~2kb of each. A different size PCR product was obtained from the gDNAs extracted from some colonies after transformation. Therefore, PCR reactions confirmed that this gene was not disrupted by pyrithiamine, and may be the construct, ABC11-PtrA-ABC11, was transformed into another locus in the genomic DNA of this mutant (Figure 6.5A). Similarly, the *MFS56* and the *M85* was not disrupted. On the other hand, the PCR succeeded to amplify the construct from gDNAs extracted from some other mutants after transformation. However, the single PCR product and expected size confirmed that this *ABC11*gene was disrupted from genomic DNA of mutant (Figure 6.5B) and similarly for *MFS56* and *M85* (Figure 6.5B and 6.6).

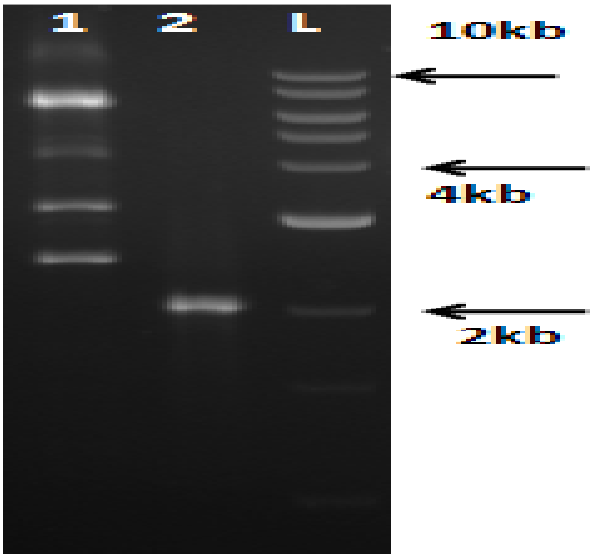


Figure 6.5A PCR screening of gene disruption transformed into A1160 *pyrg*$^{+}$ using nested primers (Tables 2.5) for ABC11

Lane 1-untransformed colony (~7kb)

Lane 2- untransformed colony (2kb)

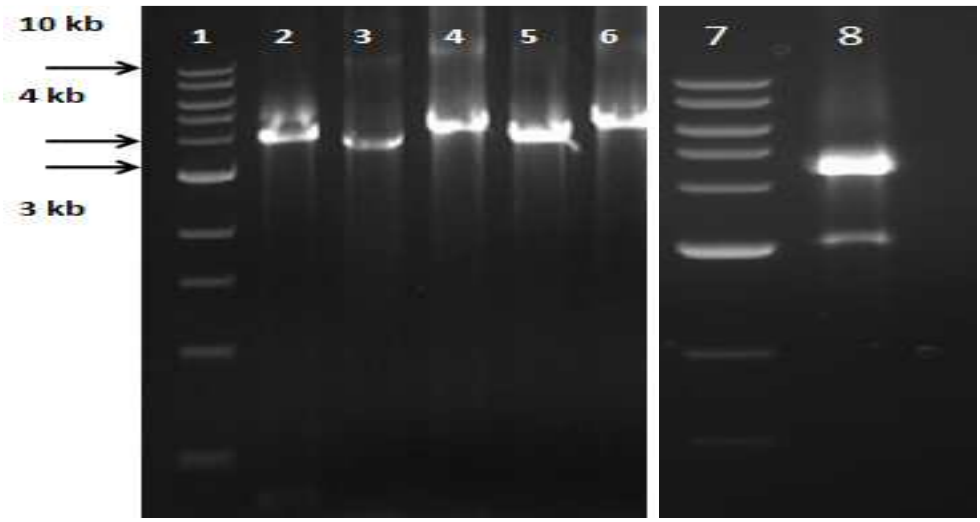


Figure 6.5B PCR screening of gene disruption and GFP fusion construct transformed into A1160 using nested primers (Tables 2.5, 2.6 and 2.7)

Lane 1-1 kb DNA ladder, Lane 2- ABC11-ptrA-ABC11 (4040 bp), Lane 3- M85-PtrA-M85 (3832 bp), Lane 4- M85-Pro-GFP-hph (4376 bp), Lane 5- MFS56-PtrA-MFS56 (3956 bp), Lane 6- MFS56-Pro-GFP-hph (4442 bp) and lane 8- MFS56-Pro-GFP-hph (4463 bp)

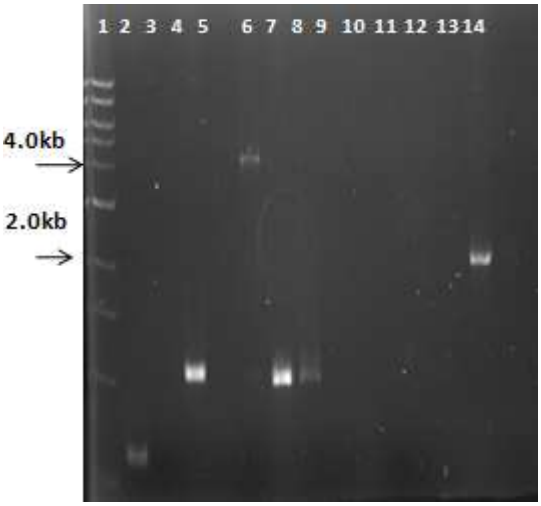


Figure 6.6

PCR screening of gene disruption construct transformed into A1160*pyrg*$^{+}$ using nested primers for MFS56 (Table 2.6)

Lane 6-PCR product (4kb) using nested primers

Lane 14- PCR product (2kb) using PtrA-F and PtrA-R

6.2.2 GFP – gene fusion PCR

The 3 fragments were separately amplified with 3 sets of primers (Tables 2.5, 2.6 and 2.7and Figures 6.7, 6.8 and 6.9); ~1kb upstream (promoter) flanking regions of target genes (Table 6.1): *ABC11* (AFUA-1G14330), *MFS56* (AFUA-1G05010) and *M85* (AFUA-5G07550) genes using Af293 as a source gDNA, were amplified with primers (Table, 2.5, 2.6 and 2.7), (715 bp) of the green fluorescent protein gene (GFP) from plasmid (pFNO3) was amplified using primers (Table 2.5, 2.6 and 2.7), and ~2kb (2828 bp) *PgpdA-hph-TtrpC* cassettes, including the hygromycin-B phosphotransfrase (hph) from plasmid (PAN7-1), was amplified using primers (Tables 2.5, 2.6 and 2.7), represented in Figures 6.7, 6.8 and 6.9. In the second step PCR a construct of 3 fragments was generated as previously described (2.2.5.6 and 2.2.5.7).The PCR conditions were similar to the gene disruption mentioned above (2.2.5.6 and 2.2.5.7). A Phusion High Fidelity DNA polymerase was used in PCR for fragment amplification. The three amplification fragments were purified using the QIAquick PCR purification kit. For the PCR products with more than one band, which was a non-specific band, a correct band and suspected size was gel purified froman agarose slice using the QIAquick Gel Extraction Kit (Qiagen) (2.2.6). A high concentration of specific products for constructs of three fragments was obtained (Figures 6.10, 6.11 and 6.12) using AccuPrime Taq DNA Polymerase (high fidelity) for assembling 3 fragments.

Table 6.3 Expected fusion PCR amplified after the second step PCR for GFP fusion

construct	Length of the amplicon (bp)				
	Forward& reverse primers	Nested primers (F & R)	X-F nest & GFP-R	GFP-F & GFP-R	GFP-F & hph R nest
ABC11-Pro-GFP-hph	4523	4463	1699	715	3479
MFS56-Pro-GFP-hph	4523	4442	1634	715	3479
M85-Pro-GFP-hph	5020	4376	1612	715	3479

Figure 6.7 Construction of GFP constructs using fusion PCR. The first PCR amplifies the upstream flanking region of ABC11 and the GFP with (GFP-F and GFP-R) primers. The second PCR amplifies the hygromycin-B (hph) gene which functions as a selectable resistant marker with primers (ABC11 GFPhph ter-Fand hph-R). Fusion PCR is completed with the three amplified fragments and gene-specific primers.

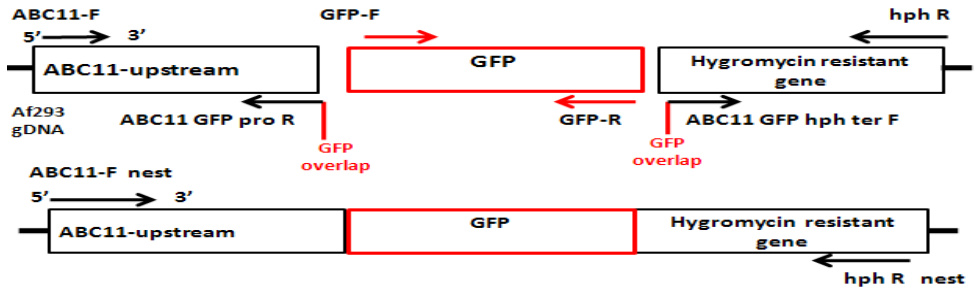


Figure 6.8 Construction of GFP constructs using fusion PCR. The first PCR amplifies the upstream flanking region of MFS56 and the GFP with (GFP-F GFP-R) primers. The second PCR amplifies the hygromycin-B (hph) gene which functions as a selectable resistant marker with primers (MFS56 GFPhph ter-F and hph-R). Fusion PCR is completed with the three amplified fragments and gene-specific primers.

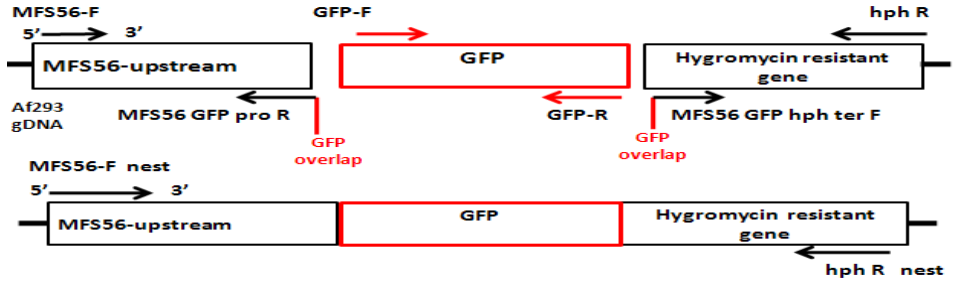
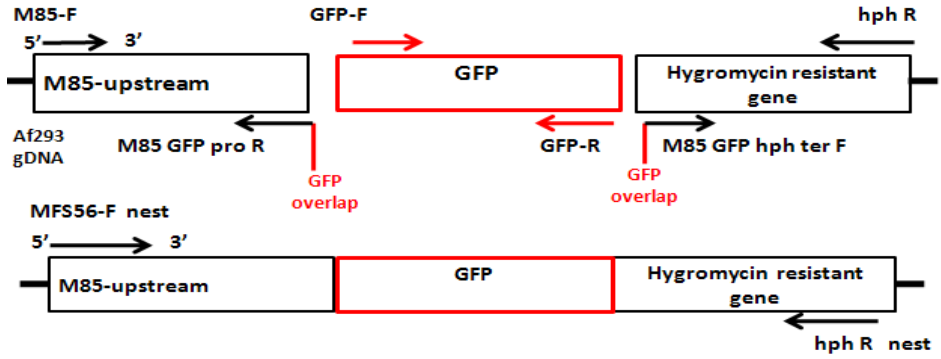


Figure 6.9 Construction of GFP constructs using fusion PCR. The first PCR amplifies the upstream flanking region of M85 and the GFP with (GFP-F GFP-R) primers. The second PCR amplifies the hygromycin-B (hph) gene which functions as a selectable resistant marker with primers (M85 GFPhph ter-F and hph-R). Fusion PCR is completed with the three amplified fragments and gene-specific primers.



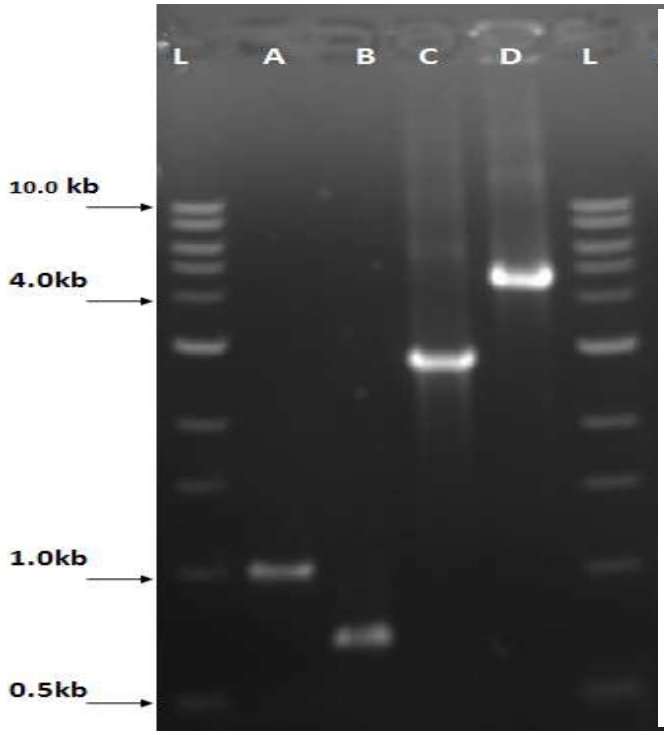


Figure 6.10

Agarose gel electrophoresis of 1st and 2nd step GFP fusion PCR for *ABC11*. ~1kb upstream flanking region of the gene (A), 715 bp *GFP* cassette (B), ~2.8kb reverse *PgpdA-hph-TtrpC* cassettes including hygromycin B phosphotransfrase (hph) (C), approximately 4.5 kb band (D) is the final construct consisting of fused upstream, *GFP* and *PgpdA-hph-TtrpC-* . 1kb DNA ladders (NEB) are presented in lane L

-

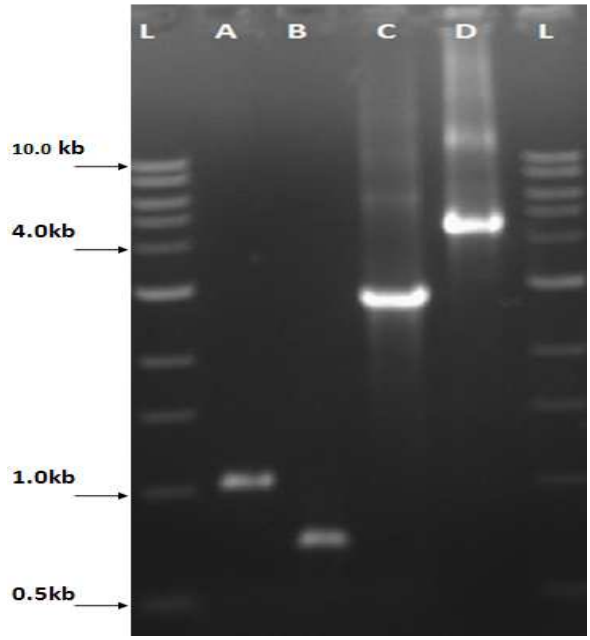


Figure 6.11

Agarose gel electrophoresis of 1st and 2nd step GFP fusion PCR for *MFS56*. ~1kb upstream flanking region the gene (A), 715 bp *GFP* cassette (B), ~2.8kb reverse *PgpdA-hph-TtrpC* cassettes including hygromycin B phosphotransfrase (hph) (C), approximately 4.5 kb band (D) showing the fusion product containing upstream, *GFP* and *PgpdA-hph-TtrpC-* regions. 1kb DNA ladders (NEB) are presented in lane L.

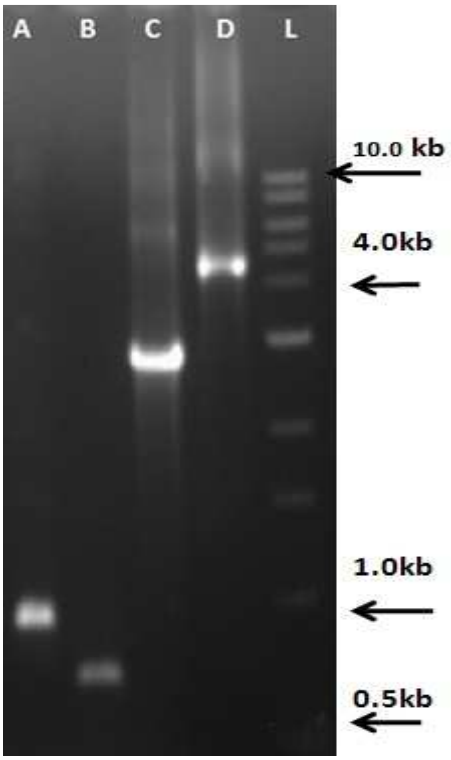


Figure 6.12

Agarose gel electrophoresis of 1st and 2nd step GFP fusion PCR for *M85.* ~1kb upstream flanking region the gene is presented (A), 715 pb *GFP* cassette (B), ~2.8kb reverse *PgpdA-hph-TtrpC* cassettes including hygromycin B phosphotransfrase (hph) (C), approximately 4.5 kb band (D) showing the fusion product containing upstream, *GFP* and *PgpdA-hph-TtrpC- regions*. 1kb DNA ladders (NEB) are presented in lane L.

6.2.3 Molecular cloning and DNA sequencing

The complete fusion constructs were gel purified as previously described (2.2.5.7 and 2.2.6) and cloned into the pGEM-T easy vector (Promega) (2.2.5.6 and 2.2.8) to obtain new plasmid, including a desired fusion.

For gene disruption, the correct construct of upstream and downstream flanking regions of target genes and pyrithiamine resistance gene (ptrA) cassettes was gel purified (2.2.5.7 and 2.2.6). Subsequently each purified PCR product of three genes was cloned into the pGEM-T easy vector. The correct size of constructs were confirmed by PCR amplification of the desired construct from a new plasmid of the PGEM-T vector using nested primers (Table 2.5, 2.6 and 2.7) and a different set of primers covered at least two fragments (Table 6.2). Constructs were sequenced to confirm correct splicing of PCR products (data not shown)

For GFP, the correct construct of promoter of target gene-GFP-reverse *PgpdA-hph-TtrpC* cassettes was gel purified. Subsequently each purified PCR product of three genes was cloned into the pGEM-T easy vector. To confirm correct cloning, PCR was carried out using nested primers (Table 2.5, 2.6 and 2.7) and a different set of primers covering at least two fragments (Table 6.3).The plasmids constructed for this study were stored at -80°C until required for transformation.

6.2.4 Transformation of *A. fumigatus*

Generation of *ABC11, MFS56* and *M85* deletion mutant strains and GFP mutant strains

To analyse the importance of the three unknown functional genes, the transporter gene (*ABC11*, *MFS56*) and the hypothetical protein (*M85*) in *A. fumigatus*, the corresponding genes were deleted independently in the *A. fumigatus* strains A1160 KU80pyrG^{+}. Transformation of *A. fumigatus* was carried out using protoplasts as described previously (2.2.9). We found that this protocol represents a highly efficient and reliable technique for protoplast production from *A. fumigatus*. The optimal conditions of growth for A1160 KU80pyrG^{+} strains were 14h in static cultures at 37°C. To harvest hyphae we used two methods: Pelleting the conidia by centrifugation, using a 50ml conical bottomed centrifuge tube or by filtration of hyphae with sterile Miracloth, which gave a high yield of protoplasts. The 4% Glucanex enzyme and 50 mM $CaCl_2$ + 0.6M KCl for 3h at 37°C in a shaker incubator were sufficient to produce >1x10^{7} protoplasts from 100 ml starting culture. Transformation was obtained when fresh protoplasts were used but not with protoplasts stored at 4°C for more than 24 h.

To characterise the unknown functional genes, a constructed fusion was generated, as described earlier (2.2.5.7). Two different marker genes were used; one coding for the pyrithiamine (*PtrA*) encoding gene of *A. oryzae*. This marker was used in the gene disruption experiment. The gene of interest has been replaced by a selectable marker. The other selection marker encoding for the hygromycin B phosphotransferase (hph)

gene, which is under the control of the strong gpdA promoter of *A. nidulans*, was used in the GFP – promoter fusion experiment.

Initially, plasmid pGEM-T easy including either constructs for gene disruption or GFP fusion were directly used for transformation using the first protocol (2.2.9), but we failed to obtain transformations. However, we successfully obtained transformed colonies when direct PCR products were used in the transformation. However, when transformation was repeated with the modified protocol (2.2.9) using either plasmid or PCR products transformed colonies were obtained.

In all transformation experiments a negative protoplast alone (no DNA) and a positive plasmid (pAN7-1) control were used. Transformations were based on selection with either hygromycin-B or pyrithiamine. Two different growth media were used in order to select mutant strains carrying either the target gene-*PtrA*–target construct (2.1.4.9.2) or gene-*GFP-PgpdA-hph-TrpC* constructs (2.1.4.9.1). Potential mutant strain knock-out ΔABC11, ΔMFS56, ΔM85 and A1160 *A. fumigatus* were cultivated on MM with 0.1 mg/l of pyrithiamine (2.1.4.9.2) (Kubodera et al. 2002), while target gene-*GFP-PgpdA-hph-TtrpC* fusion PCR was cultivated on YPS media containing 200 μg/ml of hygromycin-B (2.1.4.9.1). In order to prevent untransformed colonies from growing, several different antibiotic concentrations were tested. For gene disruption we used 0.2 mg/l of pyrithiamine in the medium (2.1.4.9.2) at pH 6.5. This concentration was not sufficient to stop the growth of untransformed protoplasts of A1160*pyrG*$^{+}$ even when we increased the concentration to 1 mg/l. As the possible transformants were still able to grow on these higher concentrations, we selected the fastest growing colonies and examined them by PCR using a set of primers (X-F nest and PtrA-R), (PtrA-F and PtrA-R) and (PtrA-F and X-R-nest)) designed to be diagnostic for gene disruption (Table 6.2). For the GFP experiment we used 200 μg/ml of hygromycin-B in the medium (yeast extract, peptone, and sucrose (YPS) pH 8.2). This concentration was sufficient to stop the growth of untransformed protoplasts of A1160*pyrG*$^{+}$.

Salt concentration and pH are crucial factors in the effectiveness of antibiotics. The concentration of 200 μg/ml of hygromycin prevented non-transformed colonies from growing. A few colonies that grew on selective media from each transformation experiment were selected for further analysis. To select the mutant strain *ΔABC11, ΔMFS56, ΔM85 A1160* of *A. fumigatus* strains, we cultivated each isolate on a Czapek-Dox (CD)-plate and MM (2.1.4.9.2) with pH 6.5 at 37^{o}C for 5 days using ranging from 1.0mg/L pyrithiamine in each media. The first visible growth was seen for transformed colonies after 72 h in both media.

The negative or non-transformed colonies which did not carry the markers were not grown in selective media. Transformants were streaked again at 37°C on MM agar plates including PT or YPS, including hygromycin for GFP mutants. Five drug resistant colonies of each transformant were then selected and sub-cultured on SAB for DNA extraction as previously described (2.2.3.1B) and for susceptibility test (2.2.2).

Genomic DNA from hygromycin-B resistant transformants, pyrithiamine resistant transformants and the parental strain were analysed by PCR and sequenced across the junctions using a different primers for gene disruption; (ABC11-F and ABC11-R), (ABC11-F nest and PtrA-R) and (PtrA-F and ABC11-R-nest) for ABC11 and (MFS56-R nest and MFS56-F nest), (MFS56-F nest and PtrA-R) and (PtrA-F and MFS56-R-nest) for MFS56., while for M85; (M85-R nest and M85-F nest), (M85-F nest and PtrA-R) and (PtrA-F and M85-R-nest) (Figures 6.1, 6.2, 6.3, 6.5B and 6.6) in order to investigate whether or not the genes of interest had been replaced by the pyrithiamine resistance cassette and to examine whether or not the target gene-*GFP-PgpdA-hph-TrpC* was integrated into the target gene loci in the chromosome of fungus. Nested primers (ABC11-R nest and ABC11-F nest), (MFS56-R nest and MFS56-F nest), and (M85-R nest and M85-F nest) were used to confirm that gene disruption was successfully transformed into gDNA. The length of the PCR product confirmed transformation in the correct locus for *ABC11* (Figure 6.4 and 6.5B) and similarly for *MFS56* and *M85* (Figure 6.5B and 6.6) GFP was confirmed in all selected transformed

colonies by Various primer sets (X-F nest and GFP-R) and (GFP-F and hph-R nest) and the transformation of gene fusion cassette into gDNA of A1160 was confirmed by nested primers (X-F nest and hph R nest) (Table 6.3 and Figure 6.5B).

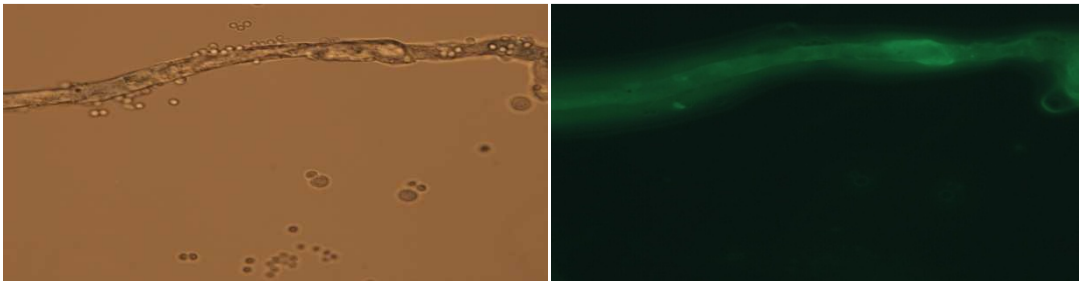
To test whether or not the gene was not disrupted, a different primers sets were used. A different size PCR product was obtained from the gDNAs extracted from some colonies after transformation. Therefore, PCR reactions confirmed that this gene was not disrupted by pyrithiamine, and may be the construct, ABC11-PtrA-ABC11, was transformed into another locus in the genomic DNA of this mutant. Similarly, the *MFS56* and the *M85* was not disrupted. On the other hand, the PCR succeeded to amplify the construct from gDNAs extracted from some other mutants after transformation. However, the single PCR product and expected size confirmed that this *ABC11*gene was disrupted from genomic DNA of mutant (Figure 6.4 and 6.5B) and similarly for *MFS56* and *M85* (Figure 6.5B and 6.6). The results obtained here demonstrate that this technique is clearly suitable for generating gene deletions in *A. fumigatus* strains.

6.2.5 In vivo expression of ABC11, MFS56 and M85 using GFP

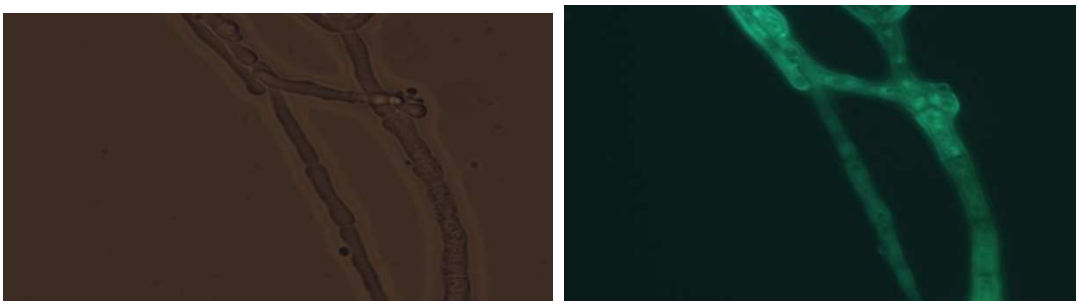
The gene-*GFP-PgpdA-hph-TrpC* mutants live-cell imaging

Different media (SAB broth and Vogel's MM) were used to grow gene-*GFP-PgpdA-hph-TrpC* mutants in order to express the target gene and GFP protein. The best expression of GFP protein was observed when a Vogel's MM medium with 0.25mg/l of itraconazole was used after 24h room temperature incubation. All 3 genes-GFP mutants were visualised by a fluorescent microscope. Different Expression was observed in the cell, as presented in Figure 6.13, distributed in the cytoplasm to the cell wall. Very low

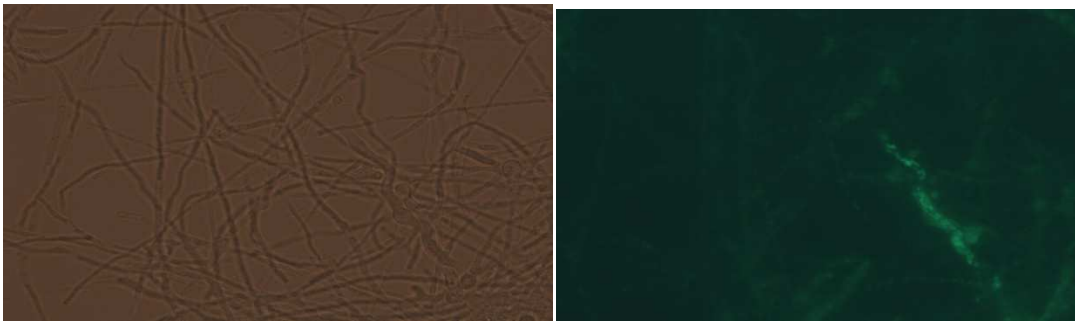
or no GFP expressions were observed during the first 6 hours of incubations (data not shown). Furthermore, no GFP were detected in the A1160 wild-type strain.



MFS56 GFP



AB11 GFP



M85 GFP

Figure 6.13 Live cell imageing of the target genes-GFP-mutants. Expression is observed for target genes.

6.2.6 Susceptibility testing

The individual MICs were determined using a EUCAST method with microdilution, using 96-well flat-bottomed microtitration plates as previously described in Table 2.9 (2.2.2) (Bueid et al. 2010). Mutants and parent strains were tested against itraconazole (ITR), voriconazole (VOR), and posaconazole (POS), using a hemacytometer for counting and adjusting *Aspergillus* spores to a final concentration of 5×10^4 CFU/ml. The microtitrations plates were used to load suspension and incubated at 37^oC for 48h. Experiments were repeated at least four times with each strain on four separate days. MICs were read by eye, with a no growth end point after 48 hours' incubation.

The mutant strains were analysed using minimal inhibitory concentration (MIC) assays for azole compounds (ITR, VOR and POS). The Δ*ABC11*, Δ*MFS56* and Δ*M85* knockout strains were more susceptible to azoles than the parent strains. All strains' individual MICs are shown in Table 6.4. The antifungal drug susceptibility profile of the mutants compared with the parental strain (A1160) showed that there were slight differences in susceptibility for the antifungal drugs tested. ITR MICs were two fold lower in *ABC11*-knockout strains, *MFS56*-knockout strains and *M85*-knockout strains, and four folds lower in *ABC11*-knockout strains, *MFS56*-knockout strains and *M85*-knockout strains than the parental WT strain (A1160) against voriconazole. The parental strain WT (A1160) is less susceptible (2 folds) than all three mutant strains against POS (Table 6.4).

Table 6.4 MICs of azoles antifungal for strains (A1160pyrG+) and their respective *ABC, MFS* and *M85* knock-out

Isolate	**ITR** [a] **MIC µg/ml**	**VOR** [b] **MIC µg/ml**	**POS** [c] **MIC µg/ml**
A1160pyrG$^+$	0.25	1	0.25
A1160pyrG$^+$ABC	0.125	0.25	0.06
A1160pyrG$^+$MFS	0.125	0.25	0.06
A1160pyrG$^+$M85	0.125	0.25	0.06

[a] Itraconazole, [b] voriconazole, [c] posaconazole

6.3 Discussion

Understanding of drug resistance mechanisms helps in understanding how resistance develops and may lead to invention of new drugs to prevent or delay the emergence of resistant fungal pathogens by interfering with the resistance mechanism. Few genes have been discovered to be involved in azole resistance in *A. fumigatus* such as *cyp51A, AfuMDR1, AfuMDR2,* and *AfuMDR3* (Nascimento et al. 2003). To find novel resistant genes other than those previously reported we employed a mutant strain of *A. fumigatus* A1160 (with deletions of *ABC11*, *MFS56*, and *M85*). Many strategies have been used for molecular characterizations of genes from *A. fumigatus*, such as mutagenesis (Firon et al. 2003). Disrupting genes at random by plasmid insertion has been achieved by inserting various DNA elements, such as transposons or plasmids, into the genome. The integration of transforming DNA mediated by the in vivo action of restriction enzymes (REMI) has been developed for *S. cerevisiae* and *Dictyostelium discoideum* (de Souza et al. 2000) Large-scale functional analysis and essential gene identification in *A. fumigatus* have proved more difficult (Hu et al. 2007).

Some methods are based on spontaneous gene mutations or creating constructs to examine the cellular localization of proteins encoded by these genes. Although mutagenesis has been proved for gene discovery and their functional study, identification of the gene of interest in *A. fumigatus* is required (Hu et al. 2007;Toews et al. 2004)

A strategy was used in this study for identifying these unknown genes and their products and to investigate whether or not they are important for azoles resistance. This was deleting the gene of interest by knock- out (gene disruptions) followed by comparing the drug sensitivity status of the mutant *A. fumigatus* strains. Although gene disruption methodologies have been adapted to *A. fumigatus*, they are limited owing to the organism's poor efficiency of homologous recombinatio (Hu et al. 2007;Toews et al. 2004). Thus we used a modified protocol (Dr Michael Bromley, Manchester University) in our laboratory, a 2-step PCR based method has been optimised for

reliable production of gene knock-out and gene fusion constructs that can be easily used to transform *A. fumigatus*. To identify these unknown genes and their products separately a specific gene knock-out was used.

The transporter *ABC11* (AFUA_1G14330) gene was chosen on the basis of homology to known *C. albicans* azole resistance gene, CDR1. Increased expression of CDR1 from *C. albicans* was found to confer resistance to several azole antifungal agents, including ITR (Sanglard et al. 1996). The two other genes *MFS56* (AFUA-1G05010) and *M85* (AFUA-5G07550) genes were also chosen as probable involvement in azole resistance from a previous insertional mutagenesis screen (Bowyer, personal communication).

6.3.1 Construction of PCR gene knock- out and GFP-gene fusion product

To characterize the role in drug resistance of the novel genes *ABC11, MFS56* and *M85* deletion mutants were generated by replacing the coding region of all three genes separately with the pyrithiamine (*PtrA*) encoding gene of *A. oryzae*. It was previously demonstrated to be useful as a dominant selectable marker for transformation of several species of filamentous fungi, including *A. oryzae* wild-type strain, *A. nidulans*, and *A. fumigatus* (Kubodera et al. 2002;Kubodera et al. 2000).

To test expression levels in vivo GFP was fused to the promoter of the three novel genes (*ABC11, MFS56* and *M85*).

Splicing two or more long fragments of DNA into one functional DNA molecule was restricted for short PCR (~3–4 kb) using overlap extension PCR protocol. Recently, existing protocols of overlap extension PCR create recombinant products up to 20kb long have been described, capable of fusing up to four fragments simultaneously

To observe the expression of the protein in the cell, we employed the GFP in the GFP experiment. The green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* (Shevchuk et al. 2004). 6 recombinant products were made. Homologous recombination was used in gene knockout experiments. This protocol accelerated the effort to knock out genes of interest in *A. fumigatus*.

Reverse primers were used to amplify the upstream region of the gene of interest (Tables 2.5, 2.6 and 2.7) and forward primers were used to amplify the downstream region of the gene of interest (Tables 2.5, 2.6 and 2.7), were required to contain 20 bp overlap tail at 5’ ends complementary to the end of the other fragment. Such a modification was crucial for successful fusion PCR. However, we failed to create some of the fusion constructs when we used primers with overlapping tails of 25 bp (data not shown), which may be due to a difference between Tm temperature of a set of primers.

Initially, the fragments of both constructs either for gene disruption or GFP fusion were amplified using a proofreading fidelity DNA polymerase that prevented A - tail formation at 3' ends of PCR amplified products. The creation of A - tails can cause mutation in the final fusion when a non-proofreading DNA polymerase is used.

Some factors may affect the final product: salt or ethanol in the buffers of the gel extraction and PCR purification kits, may inhibit polymerase activity and UV light used to visuialise PCR products on gels prior to gel purification may damage the DNA template. Therefore, an additional PE buffer wash was used to flush out the residual salt and ethanol in the PE buffer was removed by longer vacuum drying steps. Time of exposure of gels to UV light was minimised.

High quantity and quality of the final PCR fusion constructs were obtained when the Accuprime polymerase was used. The correct final fusion constructs were not always obtained when a conventional PCR Master Mix *Taq* DNA polymerase was used

Annealing temperature and rate of cooling from the DNA denaturation step to a lower annealing temperature play an important role in the primer annealing process in PCR. In this experiment, in the second PCR, change in temperature per second was limited to $0.1^{o}C$ to generate specific and correct fusion PCR molecules.

6.3.2 Cloning and transformation

The whole new modified plasmid and PCR product of all target construction, including the selective marker, have been used to transform the A1160 strain. The A1160 *Aspergillus* was used as the recipient strain for transformation. The KU80 *pyrG*$^{+}$ *A. fumigatus* strain (A1160) was used in this study in order to increase the frequency of heterologous gene integration (da Silva Ferreira et al. 2006) and selection of transformants was based on pyrithiamine**.** Therefore, mutant strains were capable of synthesizing all the thiamine compounds that the *A. fumigatus* could not produce in the presence of pyrithiamine. The replacement of the three genes was confirmed by PCR and sequencing of insertion site junctions.

Similarly, the A1160 used as the recipient strain for transformation and selection of transformants was based on hygromycin-B**.** Transformations were confirmed byPCR and sequencing of insertion site junctions**.**

The quality and quantity of protoplasts were important in transformation. The best yield was obtained from fungus grown in 20 ml Petri dish static cultures for 14 h at 37^{o}C in SAB broth medium containing 100 μg/ml ampicillin. Time, temperature and material used to treat and digest the cell wall of *Aspergillus* were crucial for quality of protoplasts. Despite using optimal conditions of time (14 h) and temperature (37 ^{o}C), good yields of protoplasts were infrequently obtained from hyphae when we used a rotary shaker for cultivation of *A. fumigatus,* but a high amount was obtained when a static culture was used. Assessment of cultures by microscopy suggested that cell lysis occurred when the time of incubation and temperature were increased. When fungus was grown for less than 14 h or more than 18 h we did not obtain good protoplast yields. The reason behind the latter may be that the fungal cells are older and have different cell wall structure, which affects efficiency of digestion. Adding polyethylene glycol (PEG6000) to protoplasts on ice affected transformation probably because ice made PEG precipitate and caused damage to the protoplasts. Therefore, we incubated mixtures with PEG at room temperature for 10 minutes before transferring protoplasts onto selective media

6.3.3 Genomic DNAs analysis

Genomic DNAs from pyrithiamine - resistant transformants and the parental strain were analysed by PCR and sequencing across the junctions using a different primers for gene disruption; (ABC11-F and ABC11-R), (ABC11-F nest and PtrA-R) and (PtrA-F and ABC11-R-nest) for ABC11 and (MFS56-R nest and MFS56-F nest), (MFS56-F nest and PtrA-R) and (PtrA-F and MFS56-R-nest) for MFS56., while for M85; (M85-R nest and M85-F nest), (M85-F nest and PtrA-R) and (PtrA-F and M85-R-nest).The PCR reaction confirmed the presence of the pyrithiamine gene in the chromosome of some of *ABC11, MFS56* and *M85* A1160 mutant isolates.

To examine whether or not the target gene-*GFP-PgpdA-hph-TrpC* was integrated into the target gene loci in the chromosome of fungus, nested primers (X-F nest and hph R nest) (Table 6.3 and Figure 6.5B) were used to confirm that gene *GFP-PgpdA-hph-TrpC* was successfully transformed into g DNA. The length of the PCR product confirmed transformation in the correct locus for *ABC11* and for *MFS56* and *M85* (Figure 6.5B). The PCR screening reaction for target gene-*GFP-PgpdA-hph-TrpC* mutants were performed using various primer sets (X-F nest and GFP-R) and (GFP-F and hph R-nest). GFP was confirmed in all selected transformed colonies by these primers, and the transformation of gene fusion cassette into gDNA of A1160 was confirmed by nested primers (Figure 6.5B). PCR confirmed the presence of the hygromycin-B gene in the gDNA of all 3 mutant strains. The KU80 pyrG+ *A. fumigatus* strain (A1160) was used as recipient for the gene knock-out and it is possible to test the products of these genes in mice.

6.3.4 Green Fluorescent Protein used as a reporter for protein expression in *A. fumigatus*

In vivo expression of the *ABC11, MFS56* and *M85* genes was performed by GFP tagging. The GFP expression was confirmed by fuorescent microscopy using a 480nm

excitation and a 504nm emission cut off filter on a Nikon Eclipse 80 microscope and the gene fusion was integrated into the correct locus of gDNA. This was confirmed by PCR with different sets of primers. (X-F nest and GFP R) and (GFP-F and hph R nest) (Table 6.3) All tested genes appeared to be expressed (Figure 6.13). The weak expression of these constructs has rendered localisation using fluorescence microscopy difficult. GFP expression was increased upon exposure to azoles although time constraints did not allow extensive study of these constructs. This indicates that these genes increase expression when *A. fumigatus* is exposed to azoles. These observations match those in Chapter 7 where induction of these genes by ITR is observed using RT-PCR. In resistant *Aspergillus*, the over-expression of efflux pumps contributes to the reduction of susceptibility by decreasing effective intracellular concentration of azoles (Langfelder et al. 2002;Del Sorbo et al. 2000). It might be possible to replace the weak promoter with a strong constitutive promoter such as beta tubulin or glucoamylase. The high level expression from such constructs would allow easier visualisation of the fused GFP.

6.3.5 Susceptibility

To investigate additional factors contributing to azole resistance, in vitro susceptibility of mutant and parent strains of *A. fumigatus* A1160 was examined (Table 6.4). The minimum inhibitory concentration (MIC) of azoles (ITR, VOR, POS) was measured in representative gene knock-out strains (Table 6.4). ABC and MFS transporters can play a major role in antifungal sensitivity and resistance (Del Sorbo et al. 2000). Decreased cytoplasmic drug accumulation in azole resistant *A. fumigatus* is based on increased efflux activity. ABC transporters have been described in several fungi and yeasts (Del Sorbo et al. 2000). The *A. fumigatus ABC11* gene, which encodes an ATP-binding cassette (ABC) transporter, is also differentially expressed in the presence of the antifungal itraconazole. In addition, *MFS56* and *M85* are highly induced in the presence of ITR. These genes protect cells against the effects of itraconazole.

These results clearly indicate that *ABC11, MFS56* are multidrug transporters involved in azole resistance. The *M85* gene also appears to play a role in azole resistance. Deletion

mutants of *ABC11, MFS56,* and *M85* in *A. fumigatus* display differential sensitivity to azole compounds. Two and four fold hyper-sensitivity was observed in *ABC11* mutant strains against ITR and VOR respectively. We also found that *ABC11 MFS56* and *M85 A. fumigatus* A1160 had cross-sensitivity to ITR, VOR and POS. Deletion strains of *ABC11, MFS56,* and *M85* displayed increased sensitivity to different classes of azole antifungal (ITR, VOR and POS)

We conclude that *A. fumigatus ABC11* and *MFS56* are outer-membrane pumps involved in efflux of azoles. In addition, MIC data for Δ*ABC11* Δ*MFS56* and Δ*M85* strains and over-expressing resistant strains (see Chapter 7) confirm that *ABC11, MFS56* and *M85* act to enhance the overall multidrug resistance of *A. fumigatus*. We demonstrated that the disruption of *ABC11, MFS56,* and *M85* increased the susceptibility of *A. fumigatus* to azoles.

Our findings suggest that the ABC and MFS transporter (ABC11, MFS56) and hypothetical protein (M85) multidrug factors are involved in the development of azole resistance in *A. fumigatus.* Despite the results obtained in this work, further studies are needed. Functional analysis of ΔABC11 ΔMFS56 and ΔM85 strains in vivo infection models to test the results obtained here are needed to confirm this indirect evidence. Additional studies could also include use of higher level expression GFP constructs fused to the coding region of these genes for protein localisation, use of alternative gene localisation markers such as RFP or combinations of gene disruption to determine whether the genes have an additive effect

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Chapter 7

Analysis of the expression of the *cyp51A*, *cyp51B*, *Afu MDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4*, *atrF*, *ABC11*, *MFS56*, and *M85*

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7.1 Introduction

Mutation in the target gene (cyp51A) is the most common mechanism of azole resistance in fungi. Two other mechanisms have been confirmed to some extent to play a role in azole resistance in clinical isolates of *A. fumigatus*; increased *cyp51A* copy number and efflux. Decreased intracellular level of azole accumulation due to overexpressed efflux pumps has been occasionally described as a cause of resistance in *A. fumigatus* (Nascimento et al. 2003;Manavathu et al. 1999;da Silva Ferreira et al. 2004;Chamilos and Kontoyiannis 2005;Qiao et al. 2008). Reduced intracellular concentration of ITR has been reported in one ITR resistant *A. fumigatus* clinical isolate (Denning et al. 1997b).

Sequence analysis of the genome of *A. fumigatus* has shown the presence of a large number of ABC and MFS transporters. The genome of *A. fumigatus* contains at least 49 ABC transporters and 278 major facilitator superfamily (MFS) genes (Nierman et al. 2005b;Chamilos and Kontoyiannis 2005;Qiao et al. 2008;Nierman et al. 2005a;Abad et al. 2010). Some of these genes have been investigated; The ABC transporters, *AfuMDR1, AfuMDR2, AfuMDR4* and *atrF* and major facilitator transporter gene, *AfuMDR3*, have been characterized in *A. fumigatus* (Nascimento et al. 2003;Slaven et al. 2002;Tobin et al. 1997). Although there are a number of reports which found over - expression of efflux pumps in azole resistant *A. fumigatus*, these were laboratory mutant strains of *A. fumigatus* not clinical isolates (Mellado et al. 2007). *AfuMDR1, AfuMDR2 AfuMDR3*, and *AfuMDR4* were found to be constitutively (basal) expressed in ITR resistant laboratory mutants. However, these genes had shown over - expression when the isolates have been exposed to ITR (Nascimento et al. 2003). Increased level of expression of the *atrF* gene in response to ITR was reported in one clinical strain (strain AF72) (Slaven et al. 2002). On the other hand a novel ABC transporter-encoding gene (*abcA*) of *A. fumigatus* was not found to influence susceptibility to triazoles or other antifungals either by expresssion or by gene knock out (Langfelder et al. 2002).

Another mechanism that may cause resistance in *A. fumigatus* is the over-expression of the target gene. Over-expression of the *cyp51A* gene was shown to confer ITR

resistance in *A. fumigatus* (Mellado et al. 2005). An increase in expression of the *cyp51A* gene was found in conjunction with a duplication of a 34 bp tandem repeat in the promoter region (Mellado et al. 2007). The increased target gene copy number led to resistance. One study has found that the increased target gene copy number led to an increase in mRNA level responsible for fluconazole resistance in *C. albicans* (Selmecki et al. 2006) and in *A. fumigatus* (Liu et al. 2004). Increased expression of the *cyp51A* gene in response to ITR was reported in one clinical isolate (Albarrag et al. 2011). A key step in the early stages of resistance development in *A. fumigatus* could be up-expression of genes of efflux pumps and related proteins. This increased efflux pump activity may allow for drug tolerance and/or resistance to relatively low intracellular concentrations of azoles, which may then allow development of stable resistance.

In this study three novel genes, *ABC11, MFS56* and *M85* as well as *cyp51A, cyp51B AfuMDR1, AfuMDR2 AfuMDR3,AfuMDR4* and *atrF* have been assessed using real-time PCR in azole susceptible and resistant isolates. In order to assess the contribution of transporters and other genes to resistance, particular attention was given to resistant isolates that did not carry a *cyp51*A mutation. Gene expression was tested after *in-vitro* exposure to ITR for 4 hours at 37 °C. Some resistant isolates have a mutation in the target *cyp51A* gene while the majority do not.

The transporter *ABC11* (AFUA_1G14330) gene was chosen on the basis of homology to known *C. albicans* azole resistance gene, CDR1. However, increased expression of CDR1 from *C. albicans* was found to confer resistance to several azole antifungal agents, including ITR (Sanglard et al. 1996). The two other genes *MFS56* (AFUA-1G05010) and *M85* (AFUA-5G07550) genes were also chosen as probable involvement in azole resistance from a previous insertional mutagenesis screen (Bowyer, personal communication).

7.2 Results

In this study, mechanisms of azole resistance were investigated for a collection of *A. fumigatus* clinical isolates; of these three ITR resistant isolates (ITR-R) had a known *cyp51A* mutation and 7 (ITR-R) carried no mutation. Additionally, 2 isolates resistant to only VOR as well as 2 ITR susceptible isolates were selected (Table 7.1). The level of expression of the novel transporters *ABC11, MFS56,* hypothetical protein *M85* and other genes *cyp51A, cyp51B, AfuMDR1, AfuMDR2, AfuMDR3, AfuMDR4, and atr-F* were assessed by real-time PCR for their expression in *A. fumigatus* after induction by ITR.

Relative quantification is performed based on the expression levels of the target genes versus a constitutively (basal) expressed housekeeping gene (beta-tubulin) that is not affected by different experimental factors (Fraczek et al. 2010). The levels of expression of the above genes' mRNA in azole-resistant clinical strains and azole-susceptible control strains were quantified by real-time PCR. In addition, parallel experiments were undertaken under controlled conditions to evaluate the effect of treatment with ITR on expression level of these genes (2.2.3.2 and 2.2.5.9) Total RNA samples were also used to estimate the relative basal amount of *cyp51A, cyp51B AfuMDR1, AfuMDR2 AfuMDR3, AfuMDR4, atrF, ABC11, MFS56* and *M85* expression. The expression levels of beta-tubulin gene were also measured as a control.

Two baffled conical flasks (40ml liquid medium (1% glucose with Vogel's salts) were inoculated with fresh *A. fumigatus* spores to a final concentration of $1x10^7$ spores/ml. ITR was added to one of two flasks in mid exponential phase at14-16hrs to a final concentration of 4.0 mg/l for resistant isolates with MIC >8mg/l. The second flasks were used as control. Sterile Miracloth (CalBoipchem, CA, USA) was used to harvest mycelia from all *A. fumigatus* isolates. Total RNA was extracted and quantified as previously described (2.2.3.2) then aliquoted into sterile tubes and stored immediately at -80°C till required.

Table 7.1 *A. fumigatus* (n: 14) clinical isolates used in gene expression experiments

Strain No.	Isolates	MIC (mg/l)			Mutation in *cyp51A*
		ITR	VOR	POS	
1	Af293	S	S	S	No mutation
2	F/15483	S	S	S	No mutation
3	F//17727	>8 [R]	8 [R]	>8 [R]	No mutation
4	F/17999	>8 [R]	8 [R]	0.5 [I]	No mutation
5	F/18085	>8 [R]	4 [R]	0.25 [S]	No mutation
6	F/18149	>8 [R]	>8 [R]	2 [R]	No mutation
7	F/20140	>8 [R]	2 [I]	0.5 [I]	No mutation
8	F/20451	>8 [R]	1 [S]	0.25 [S]	No mutation
9	F/19896	>8 [R]	1 [S]	0.125 [S]	No mutation
10	F/18304	>8 [R]	2 [I]	0.125 [S]	F46Y, M172V, N248T, D255E, E427K,
11	F/19980	>8 [R]	>8 [R]	0.5 [I]	H147Y ,G448S
12	F/20063	>8 [R]	1 [S]	0.5 [I]	A284T
13	F/18329	1 [S]	4 [R]	0.25 [S]	No mutation
14	F/18454	0.5 [S]	>8 [R]	0.06 [S]	No mutation

Primers (Table 2.8) were designed using Primer 3 software (Rozen and Skaletsky 2000), Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386 Source code available at http://fokker.wi.mit.edu/primer3/.

The primers selected were based on the following criteria: amplicon length between 100 and 210 bp, primer length 19 – 23 bp and GC content (35 - 65%). The primers were designed based on sequence deposited in Genbank (accession numbers: AF338659, AF338660, SFU62934, SFU62936, AF503774, AF503773, and AFU311940 for *cyp51A, cyp51B, AfuMDR1, AfuMDR2 AfuMDR3, AfuMDR4* and *atrF* respectively (Table 2.8) (Albarrag et al. 2011). For the three novel genes, the primers were designed

based on sequence deposited in CADRE (http://www.cadre-genomes.org.uk/index.html) with accession number AFUA_1G14330, AFUA_1G05010, and AFUA_5G07550 for *ABC11*, *MFS56* and *M85* respectively (Table 2.8) QRT- PCR was performed on total RNA using the Stratagene Brilliant II SYBR Green qRT-PCR kit in an Mx3005p real time PCR machine according to the manufacturers instructions.

The transcript levels of all genes mentioned above and the beta-tubulin comparator gene in all 14 treated and untreated *A. fumigatus* Table 7.1 were determined by quantitative real time PCR carried out in parallel with 3 independent replicate samples from each isolate for each gene as previously described (2.2.3.2 and 2.2.5.9) The qRT-PCR results were analysed by the $2^{-\Delta\Delta CT}$ method as described below (Schmittgen and Livak 2008) in an Excel spreadsheet. The C_T value of each independent gene for each isolate was normalised to corresponding β-tubulin values. The normalised treated sample values were calibrated to the values for untreated sample. No significant difference between the expression of β-tubulin in the different isolates and conditions was observed.

The data were analyzed using fold change = $2^{-\Delta\Delta CT}$

$2^{-\Delta\Delta CT}$ = ΔC_t of the sample (azole) -ΔC_t of the calibrator (non-azole).

e.g fold change = (C_T, target - C_T, beta tubulin) azole exposure - (C_T, target - C_T, beta tubulin) non-azole exposure.

Three independent replicate samples from each isolate for each gene were analysed. For each single run, the fold change of expression for each single gene was calculated as described above. Three novel genes, *ABC11*, *MFS56* and *M85* showed changes in expression levels in non-mutant ITR-R isolates related to an increase in ITR MIC values. Most (ITR-R) non-mutant isolates showed expression of almost all genes or induction of expression upon exposure to ITR and resulting alteration of antifungal susceptibility

The values presented in Table 7.3 are the differences in the levels of expression of 10 genes between the azole-resistant strains and azole-susceptible strains (Table 7.1) normalized to the levels of expression of the reference β-tubulin gene for each strain included in the study

7.2.1 Validation of real time PCR and specificity of primers

Initially, primers were designed based on criteria mentioned above. Each primer pair was validated by performing test qRT-PCR reactions and evaluating the resulting product with two quality control assays; a melt curve analysis with a single peak, and by agarose gel electrophoresis. Single fragments with correct expected sizes were produced for each PCR reaction (data not shown).

The primer design and the PCR reaction conditions (50°C for 60mins (for reverse transcription), 95°C for 10mins and 40 cycles of (95°C for 30s, 60°C for 1min, 72°C for 1min) were chosen (2.2.5.9) to provide optimal results. Initially, mRNA was converted into complementary DNA (cDNA) using reverse transcriptase then amplified by PCR and analysed by detection of fluorescence during the progress of the PCR then by agarose gel electrophoresis.

PCR controls include no-RNA and positive-RNA sample that had been previously shown to produce a product with a single melting curve peak. In addition, no - RT and no-primer controls were used in parallel in the RT-PCR. The housekeeping gene β-tubulin was validated and found to be stable and unaffected by treatment with ITR. The β-tubulin gene expression did not significantly change between treated and untreated, from samples that were taken after 4 hours of ITR exposure.

The optimal C_T detection threshold was chosen automatically by the Mx3005p software. It was used to calculate the C_T for all samples. Ethidium bromide in agarose gel electrophoresis was used to estimate quality and quantity of cDNA products of reverse transcriptase-PCR after each reaction but not for accurate quantitation.

7.2.2 Determination of relative expression level

The expression level of the previously described 7 genes as well as three novel genes (*cyp51A, cyp51B AfuMDR1, AfuMDR2 AfuMDR3, AfuMDR4, atrF, ABC11, MFS56* and *M85*) were analysed upon fungal growth *in-vitro* as described previously (2.2.3.2 and

2.2 .5.9) in two ITR susceptible isolates (AF293, F/15483) and in 12 ITR-R isolates, 3 of which have mutations in the *cyp51A* gene (Table 7.1).

ITR susceptible *A. fumigatus* isolates were used as a reference for expression in ITR–R isolates. Some isolates showed reduction in expression. If the first ΔC_t of the sample (azole) is greater than the second ΔC_t of the sample (non-azole) then the fold change ($2^{-\Delta\Delta CT}$) will be<1.

The results are presented in Table 7.3 and Figure 7.1 as a relative expression of all genes used in this study in response to ITR treatment. Table 7.4 and Figure 7.2 are presented as the log10 relative expression of these genes. The levels are initially calculated as a change in expression of the gene of interest relative to the internal control (β-tubulin) then levels in the treated (ITR) sample were compared with the untreated control.

Statistically significant differences were observed in the expression of *cyp51A*, *AfuMDR2*, and *AfuMDR4* genes with more than 2 - fold (Albarrag et al. 2011). In one study, significant differences were observed in the expression of *AfuMDR3* and *AfuMDR4* (from 1.3- to 3.1-fold) after exposure to ITR (Nascimento et al. 2003). In another study, significant expression of *atrF* (5-fold) was found in *A. fumigatus* grown with ITR (Slaven et al. 2002). In this study, thee pattern of the expression have been observed; a massive overexpression, overexpression and slight expression.

The basal level expression was investigated for all 10 genes to determine whether ITR stimuli induced or repressed expression. *cyp51A* was found to be constitutively (basal) expressed in ITR-R clinical isolates (Albarrag et al. 2011) and the overexpression of *cyp51A* gene responsible for broad-spectrum azole resistance in clinical isolates of *A. fumigatus* (Mellado, E., Alcazar-Fuoli, L., Garcia-Effron, G., Cuenca-Estrella, M., Rodriguez-Tudela, J.L., 2005b. Functional analysis of *Aspergillus fumigatus* C-5 sterol desaturases (*ERGA* and *ERGB*) genes in: 45th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Washington, DC, USA, Abstract # M-1590.). Constitutive (basal) expression of *cyp51* in the resistant strains *Penicillium digitatum* was about 100-fold higher than that in the sensitive strains (Hamamoto et al. 2000).

cyp51B plays a role in the growth rate and maintenance of membrane shape (Garcia-Effron, G., Mellado, E., Alcazar-Fuoli, L., Buitrago, M.J., Cuenca- Estrella, M., Rodriguez-Tudela, J.L., 2005. Role of *Aspergillus fumigatus* 14-alpha sterol demethylase (*cyp*51b) gene on cell growth and viability, antifungal susceptibility and sterol composition in 45th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Washington, DC, USA, Abstract # M-1591.). The expression of *cyp51B* increased (2 - fold) in clinical isolates of *A. fumigatus*, (Albarrag et al. 2011). In this study, the highest levels of constitutively (basal) expressed *cyp51B* mRNA were observed with the F/19980, F/18454 and F17727 isolates, resulting in 10.77-, 4.23- and 2.84- fold greater expression, respectively, than those of the susceptible strain, while no significant changes in *cyp51A* expression were observed in any isolates (Table 7.2). In contrast, no significant constitutively (basal) expressed of *AfuMDR1*, *AfuMDR2* *AfuMDR3*, *AfuMDR4* and *atrF* mRNA was observed for all isolates in this study (Table 7.2). Interestingly, Constitutive (basal) high level expression of the novel genes (*ABC11* and *M85*) was observed in some resistant isolates, low basal expression of *MFS56* mRNA was observed for all isolates (Table 7.2). The highest level of constitutive (basal) expression of *ABC11* was observed with the F/18304, F/20140 and F17727 isolates, resulting in 77.35-, 30.18- and 29.5-fold more copies of mRNA, respectively, than those of the susceptible strain. The highest level of constitutive (basal) expression of *M85* was observed with the F/19980 isolate, resulting in 34.70 fold more copies of mRNA than the susceptible strain.

To explore whether gene expression responded to the presence of the ITR, cells were treated with ITR at 2 µg/ml for susceptible strains or 4µg/ml resistant strains for 4 h at 37°C as described (2.2.3.2). The expression levels of *cyp51A* and *cyp51B* genes after exposure to ITR are shown in Table 7.3. The *cyp51B* gene in the susceptible strain (AF293) showed little induction (1.6-fold) by ITR, while the *cyp51A* gene was induced 2.88-fold (Table 7.3). Following exposure to ITR, over-expression of *cyp51A*, 1590.71- and 62.08- fold was observed in F/18304 and F/19980 isolates, respectively but no abnormal increase in gene expression was observed in F/19896 and F/20140. In the remaining resistant isolates, expression of *cyp51A* was slightly increased compared to AF293 after exposure to ITR (1.64-9.25-fold) (Table 7.3).

Table 7.2 The constitutively (basal) expression of 10 genes in azoles susceptible and resistant isolates. (NO, not observed)

Isolate	*cyp51A*	*cyp51B*	*MDR1*	*MDR2*	*MDR3*	*MDR4*	*Atr-F*	*ABC11*	*MFS56*	*M85*
AF293	0.22 ±0.033	1.06 ±0.29	0.035 ±0.016	0.014 ±0.0028	NO	NO	0.0008 ±0.00017	0.19 ±0.068	0.0005 ±0.0002	0.16 ±0.05
F/15483	0.39 ±0.18	0.98 ±0.41	0.29 ±0.089	0.21 ±0.08	0.0003 ±0.0001	NO	0.011 ±0.0007	5.9 ±0.10	0.016 ±0.002	6.77 ±1.51
F17727	0.23 ±0.22	2.84 ±4.90	0.59 ±0.69	0.039 ±0.031	NO	0.008 ±0.006	0.05 ±0.05	29.5 ±56.46	0.016 ±0.032	0.51 ±0.61
F/17999	0.38 ±0.39	1.2 ±0.42	0.088 ±0.026	0.028 ±0.0056	NO	0.011 ±0.0001	0.002 ±0.003	1.58 ±1.31	0.001 ±0.0008	0.64 ±0.054
F/18085	0.12 ±0.20	0.23 ±0.07	0.03 ±0.05	0.024 ±0.002	NO	NO	0.0001	1.19 ±0.44	0.001 ±0.002	0.045 ±0.1
F/18149	0.13 ±0.071	0.18 ±0.10	0.16 ±0.07	0.019 ±0.001	NO	NO	0.0012 ±0.0009	1.7 ±0.066	0.00071 ±0.0008	0.0193 ±0.0056
F/18304	0.00058 ±0.0005	1 ±0.990	0.122 ±0.085	0.0029 ±0.0029	NO	NO	0.035 ±0.0044	77.35 ±21.2	0.0002 ±0.0001	599.02 ±38.14
F/18329	0.005 ±0.0014	0.36 ±0.059	0.02 ±0.001	0.01 ±0.0002	NO	NO	0.00011	0.24 ±0.03	NO	0.0019 ±0.0002
F/18454	0.4 ±0.165	4.2 ±1.53	0.009 ±0.004	0.34 ±0.05	0.0012 ±0.0009	0.0003 ±0.0001	0.04 ±0.014	5.11 ±1.69	0.05 ±0.016	63.46 ±25.74
F/19896	NO	0.05568 ±0.014	0.0002 ±0.0001	0.027 ±0.0014	NO	NO	0.0015 ±0.0002	0.34 ±0.06	NO	0.002 ±0.0005
F/19980	0.51 ±0.20	10.77 ±2.085	0.27 ±0.062	0.37 ±0.12	0.046 ±0.0027	0.0007 ±0.0004	0.33 ±0.032	7.2 ±0.51	0.15 ±0.005	34.7 ±1.082
F/20063	0.16 ±0.13	0.644 ±0.47	0.153 ±0.105	0.046 ±0.027	NO	NO	0.0002 ±0.0001	0.34 ±0.30	0.0002 ±0.0002	0.003 ±0.0008
F/20140	0.0016 ±0.0014	0.33 ±0.024	0.27 ±0.085	0.1 ±0.002	NO	0.001 ±0.0002	0.009 ±0.003	30.18 ±5.15	0.002 ±0.0001	0.058 ±0.011
F/20451	0.098 ±0.188	0.558 ±0.879	0.0015 ±0.01	0.009 ±0.02	NO	0.0011 ±0.0009	0.0024 ±0.0016	4.53 ±3.61	0.0001 ±0.0001	0.16 ±0.113

Relative expressions of 10 genes in azoles susceptible and resistant isolates analysed by RT-PCR. The expression of each gene was normalized to β-tubulin. The data are the average of at least 3 independent determinations. ± represents the standard error of mean. The expression level of each gene was determined at 4 h at 37°C, prior mid exponential phase at14-16hrs

No resistant isolates showed significantly increased levels of *cyp51B* expression compared to the susceptible isolates except F/19896. The level of *cyp51B* expression increased 18.70- fold after exposure of the F/19896 isolate to ITR. In the remaining resistant isolates, expression of *cyp51B* was slightly incresed relative to expression in a susceptible isolate (F/15483) or decreased in F17727, F/17999, F/18085, F/18149, F/18304, F/19980 F/20063 (1.61-4.45-fold) (Table 7.3 and Figure 7.1). These results, showing that an increased level of expression of the *cyp51A* and *cyp51B* genes in clinical isolates following exposure to ITR, are in agreement with those observed by (Albarrag et al. 2011).

Increased expression of the *atrF* gene in response to ITR was reported in clinical strain AF72 (Slaven et al. 2002). In this study, expression levels of 20.11-, 3.82- and 2.20-fold greater than the reference AF293 strain were observed for *atrF* in F/17999, F/18149 and F/20140 respectively, However, there was a slight difference in expression of *atrF* gene in remaining resistant isolates relative to susceptible isolates (Table 7.3 and Figure 7.1).

In previous studies *AfuMDR1, AfuMDR2 AfuMDR3, AfuMDR4* genes had shown over - expression when the isolates have been exposed to ITR (Nascimento et al. 2003;da Silva Ferreira et al. 2004). In this study, The *Afumdr1*, *Afumdr2*, *Afumdr3* and *Afumdr4* genes were found to be induced 3.6-, 0.33-, 1.5- and 23-fold more, respectively, than in the reference strain. In contast, in one resistant mutant, the same genes were induced 16, 38-, 258- and 249- fold more, respectively, in the presence of ITR (da Silva Ferreira et al. 2004). In the current study, there was no significant difference in expression of the efflux pump transporters *AfuMDR1, AfuMDR2, AfuMDR3, AfuMDR4* in the majority of resistant isolates relative to susceptible isolates. One (F/18085) displayed a 220.14-fold increased level of expression of *AfuMDR1* relative to susceptible isolates, and with nearly twice the transcript levels for non-mutant resistant isolates (F/ 17999 , F/ 18149 and 18329) (Table 7.3 and Figure 7.1). Induction levels of 6.23-, 4.55-, 2.60- and 2.41-fold were observed for *AfuMDR2* in F/19980, F/18149, F/20063 and F/20063, F/17999, respectively, upon exposure to ITR, while increases of about 2.73-fold in only F/17999 for *AfuMDR3.*

Importantly, in the presence of ITR, significant overexpression of the three novel genes was observed especially *ABC11*. Overexpression levels of 31-.84.26.37-, 19.96-, 11.92- , 11.70-, 10.80-, 7.29- and 5.14-fold were observed for *ABC11* in F/20063, F/17999, F/18149, F/19896, F/18329, AF293, F/18085 and F/19980 respectively, while increases of 295.36-, 58.7-, 43.17-, 12.94-, 4.04-, 4.04- and 3.17-fold in the expression of *MFS56* were observed for F/17999, F/18085, F/20063, F/18149, F/19896, F/20140 and F/19980 isolates. While increases of about 410.47-, 91.26-, 4.31- and 3.69-fold in the expression of *M85* were observed for F/18085, F/20063, F/19896 and F/20140 (Table 7.3).

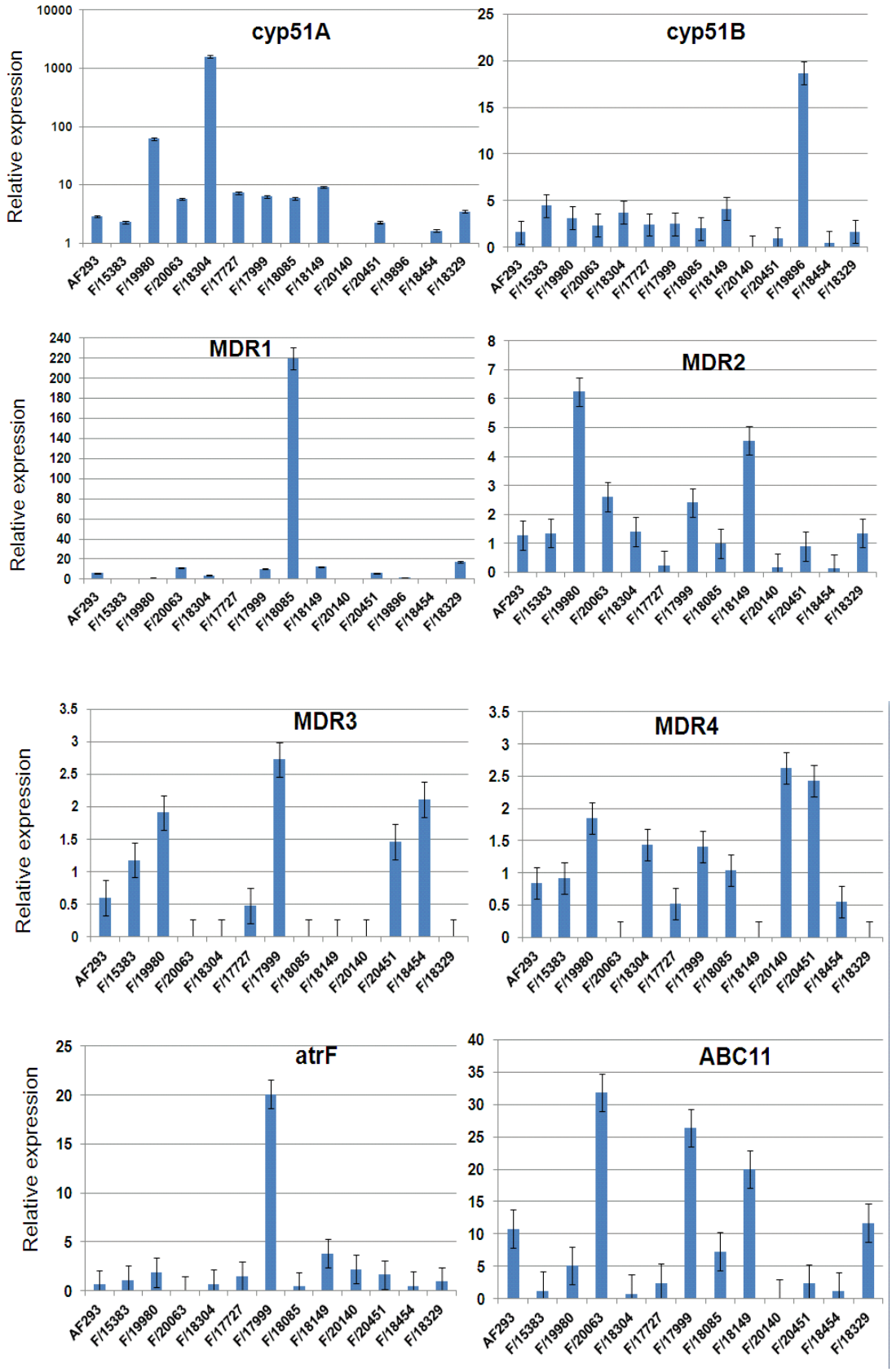
Following exposure to ITR, overexpression of the three novel genes *ABC11, MFS56* and *M85* was observed to some extent in all resistant isolates. The most prominent were those of F/20063, F/19896 and F/18085, which showed high-level expression of three genes. Three isolates, F/17999, F/18149 and F/19980, showed overexpression of two genes (*ABC11* and *MFS56*), 5 of these are without *cyp51A* mutation and one (F/20063) with point mutation (Table 7.3).

However, almost all the resistant isolates displayed significant increased levels of *cyp51A, ABC11, MFS56* and *M85* expression compared to the susceptible isolates.

Table 7.3 Relative expression of 10 genes in azoles susceptible and resistant isolates. (NO**,** not observed**)**

Isolate	***cyp51A***	***cyp51B***	***MDR 1***	***MDR 2***	***MDR 3***	***MDR 4***	***Atr-F***	***ABC11***	***MFS56***	***M85***
AF293	2.88 ±0.33	1.61 ±0.46	6.02 ±2.52	1.28 ±0.56	0.60 ±0.08	0.84 ±0.20	0.63 ±0.24	10.80 ±3.39	0.11 ±0.01	.05 ±0.01
F/15483	2.30 ±1.327	4.45 ±1.37	0.90 ±0.46	1.35 ±0.85	1.18 ±1.19	0.91 ±0.49	1.09 ±0.26	1.24 ±0.12	0.83 ±0.14	1.00 ±0.28
F17727	7.353 ±10.91	2.42 ±2.49	0.27 ±0.59	0.24 ±0.17	0.485 ±0.025	0.51 ±0.016	1.47 ±2.16	2.43 ±4.43	0.61 ±0.63	1.85 ±1.39
F/17999	6.37 ±2.46	2.48 ±1.12	10.35 ±5.89	2.41 ±0.78	2.73 ±1.16	1.40 ±0.68	20.11 ±21.64	26.37 ±16.62	295.36 ±581.86	1.86 ±1.12
F/18085	5.87 ±4.27	2.48 ±1.42	220.14 ±194.11	0.99 ±0.20	NO	1.04 ±0.05	0.43 ±0.12	7.29 ±6.84	58.71±129.30	410.47 ±915.40
F/18149	9.25 ±3.70	4.12 ±3.16	12.31 ±7.00	4.55 ±0.28	NO	NO	3.82 ±2.53	19.96 ±11.43	12.94 ±17.45	0.38 ±0.12
F/18304	1590.71 ±1452.49	3.74 ±2.13	3.87 ±3.04	1.40 ±1.20	NO	NO	0.66 ±0.19	0.78 ±0.16	0.57 ±0.44	0.09 ±0.01
F/18329	3.50 ±2.20	1.66 ±0.12	17.31 ±1.86	1.35 ±0.12	NO	NO	0.93 ±0.40	11.70 ±2.33	0.81 ±0.74	2.72 ±0.04
F/18454	1.64 ±1.46	0.49 ±0.10	1.22 ±0.18	0.12 ±0.06	2.11 ±2.81	0.55 ±0.15	0.45 ±0.19	1.17 ±0.14	0.38 ±0.10	0.38 ±0.13
F/19896	NO	18.70 ±24.82	1.36 ±0.56	1.86 ±0.30	NO	1.11 ±0.85	0.33± 0.10	11.92 ±7.73	4.04 ±1.63	4.31 ±1.31
F/19980	62.08 ±10.09	3.14 ±1.32	1.27 ±0.03	6.23 ±2.26	1.91 ±0.15	1.84 ±1.17	1.86 ±1.36	5.14 ±2.22	3.17 ±0.97	1.24 ±1.15
F/20063	5.75 ±4.65	2.37 ±2.09	11.63 ±4.56	2.60 ±1.34	NO	NO	NO	31.84 ±29.28	43.17 ±28.88	91.26 ±17.81
F/20140	NO	0.01 ±0.01	0.15 ±0.11	0.16 ±0.01	NO	2.63 ±2.13	2.20 ±1.84	0.09 ±0.01	4.04 ±0.65	3.69 ±1.64
F/20451	2.25 ±3.20	0.95 ±1.21	6.15 ±6.89	0.90 ±0.35	1.46 ±0.96	2.42 ±0.77	1.64 ±0.75	2.33 ±2.93	2.02 ±1.02	1.13 ±0.26

Relative expressions of 10 genes in azoles susceptible and resistant isolates analysed by RT-PCR. The expression of each gene was normalized to β-tubulin. The data are the average of at least 3 independent determinations. ± represents the standard error of mean. The expression level of each gene was determined at 4 h after the addition of ITR at 37°C, prior mid exponential phase at14-16hrs


cyp51A
cyp51B
MDR1
MDR2
MDR3
MDR4
atrF
ABC11
Relative expression
AF293
F/15383
F/19980
F/20063
F/18304
F/17727
F/17999
F/18085
F/18149
F/20140
F/20451
F/19896
F/18454
F/18329

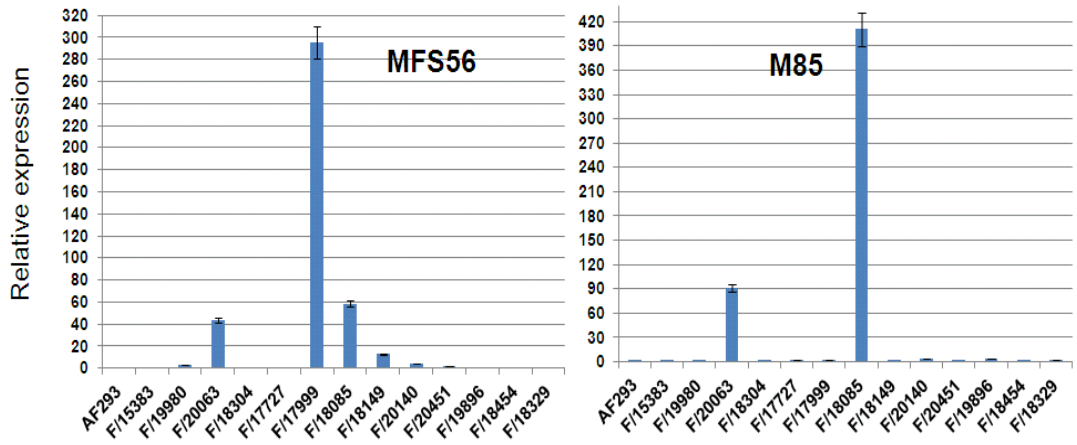


Figure 7.1 Relative expressions of 10 genes in azoles susceptible and resistant isolates analysed by RT-PCR. The expression of each gene was normalized to β-tubulin. The data are the average of at least 3 independent determinations. Error bars represents the standard error of mean. The expression level of each gene was determined at 4 h after the addition of ITR at 37°C, prior mid exponential phase at14-16hrs

Table 7.4 Log10 of relative expression of 10 genes in azole resistant and susceptible isolates

Isolate	*cyp51A*	*cyp51B*	*MDR1*	*MDR2*	*MDR3*	*MDR4*	*atr-F*	*ABC11*	*MFS56*	*M85*
AF293	0.46	0.20	0.78	0.10	-0.21	-0.07	-0.19	1.03	-0.94	-1.28
F/15483	0.36	0.64	-0.04	0.13	0.07	-0.03	0.04	0.09	-0.08	0.001
F17727	0.86	0.38	-0.55	-0.60	-0.31	-0.28	0.16	0.38	-0.21	0.26
F/17999	0.80	0.39	1.01	0.38	0.43	0.14	1.30	1.42	2.47	0.26
F/18085	0.76	0.30	2.34	-0.01	NO	0.02	-0.35	0.86	1.76	2.61
F/18149	0.96	0.61	1.09	0.65	NO	NO	0.58	1.30	1.11	-0.41
F/18304	3.20	0.57	0.58	0.14	NO	0.15	-0.17	-0.10	-0.23	-1.02
F/18329	0.54	0.22	1.23	0.13	NO	NO	-0.03	1.06	-0.09	0.43
F/18454	0.21	-0.30	0.086	-0.88	0.32	-0.25	-0.33	0.06	-0.41	-0.41
F/19896	NO	1.27	0.13	0.27	NO	0.04	-0.47	1.07	0.60	0.63
F/19980	1.79	0.49	0.10	0.79	0.28	0.26	0.27	0.71	0.50	0.09
F/20063	0.76	0.37	1.06	0.41	NO	NO	NO	1.50	1.63	1.96
F/20140	NO	-1.95	-0.82	-0.79	NO	0.42	0.34	-1.0	0.60	0.56
F/20451	0.35	-0.019	0.78	-0.04	0.16	0.38	0.21	0.36	0.30	0.05

NO, not observed

Log10 of relative expression of 10 genes in azoles susceptible and resistant isolates analysed by RT-PCR. The expression of each gene was normalized to β-tubulin. The data are the average of at least 3 independent determinations. ± represents the standard error of mean. The expression level of each gene was determined at 4 h after the addition of ITR at 37°C, prior mid exponential phase at14-16hrs

Generally, some genes were induced by ITR exposure while others were not.The *AfuMDR1*, *AfuMDR2*, *AfuMDR3*, *AfuMDR4*, and *M85* genes were repressed in the F/17727 isolate under ITR treatment. Genes *cyp51B*, *AfuMDR1*, *AfuMDR2* and *ABC11* were repressed in the F/20140 isolate under ITR treatment Figure 7.2. Interestingly, the *cyp51A* gene was induced during exposure to ITR for all isolates. c*yp51B* was repressed under ITR treatment in two isolates (F/20140 and F/18454), *AfuMDR1* was repressed during exposure to ITR in two isolates (F/17727 and F/20140), *AfuMDR2* was repressed under ITR treatment in 3 isolates (F/17727, F/20140 and F/18454), *AfuMDR3* was repressed under ITR treatment in two isolates (AF293 and F/17727), *AfuMDR4* was repressed during exposure to ITR in two isolates (F/17727 and F/18454), *atrF* was repressed under ITR treatment in several isolates (AF293, F/18304, F/18085, F/19896, and F/18454), *ABC11* was repressed in only one isolate (F/20140), *MFS56* was repressed in isolates with different susceptibility profiles AF293, F/15383, F/18304, F/17727, and F/18454 and finally *M85* was repressed in susceptible and resistant *A. fumigatus* isolates (AF293, F/18304, F/18149 and F/18454). However, repression of expression did not exceed 2-fold compared to the same gene expression in treated samples.

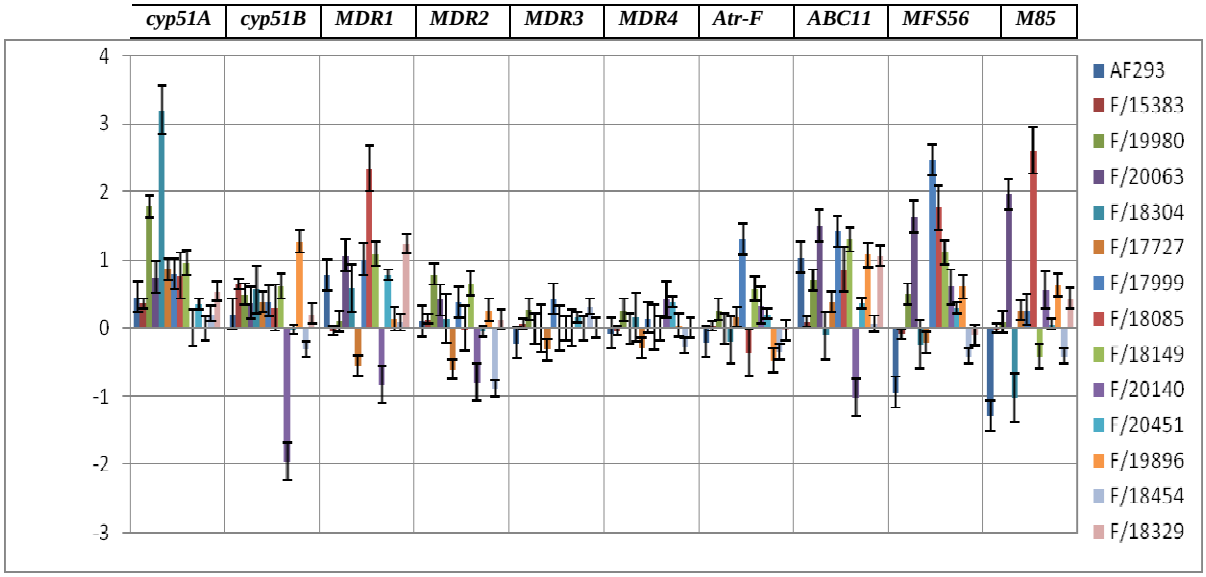


Figure 7.2 relative expressions of susceptible and resistant *A. fumigatus* isolates in response to ITR *in vitro*. The expression was analysed for untreated and treated isolates with ITR after 4 hours prior mid exponential phase at14-16hrs.

7.3 Discussion

Mutation in the target gene *cyp51A* is the most common mechanism of resistance in *A. fumigatus* clinical isolates because it leads to reduced binding of the drug (Howard et al. 2009;Howard et al. 2006;Mellado et al. 2004). But we recently found an increase in frequency of azole resistance without a corresponding mutation in the *cyp51A* gene (Bueid et al. 2010). Possible reasons for this could include over-expression of *cyp51A* or efflux pumps. Decreased intracellular level of azole due to over - expression of efflux pumps has been rarely described as a cause of resistance in some *A. fumigatus* isolates (Nascimento et al. 2003;Manavathu et al. 1999;da Silva Ferreira et al. 2004;Chamilos and Kontoyiannis 2005;Qiao et al. 2008). Reduced intracellular concentration of ITR has been reported in an ITR-R *A. fumigatus* clinical isolate (Denning et al. 1997b). *A. fumigatus MDR* (*AfuMDR*) pumps have been shown to be associated with increased resistance to ITR (Rajendran et al. 2011). Generally, overexpression of 14α-demethylase, the product of the *cyp51A* gene, associated with mutations in the target gene or with up-regulation of efflux pumps. However, over - expression of only *cyp51A* as the sole mechanism of resistance has not been described in resistant *A. fumigatus* clinical isolates and no particular pump has been identified to be responsible for efflux in *A. fumigatus* (Nascimento et al. 2003;Slaven et al. 2002;da Silva Ferreira et al. 2004;Mellado et al. 2007). However, overexpression of *cyp51A* was described as a sole of resistance mechanism in *Blumeriella jaapii* (Ma et al. 2006)

Quantitative expression analysis (real-time PCR) showed up to 62.08- and 1590.7-fold increase in the induction of expression of the *cyp51A* gene in the presence of ITR in (F/19980 and F/18304) respectively compared to that by the susceptible strain (AF293). This is massive increase in expression of *cyp51A* has not been reported previously in fungi, for example *in Penicillium italicum* (van den Brink et al. 1996) or in *A. fumigatus* (Mellado et al. 2007;Albarrag et al. 2011). It contrasts with other four other resistant isolates without mutations with nearly twice the transcript levels compared to susceptible isolates. Two factors may increase the *cyp51A* mRNA level; the first factor is the *cyp51A* gene copy number as described in *C. albicans* (Selmecki et al. 2006) and

C. glabrata (Marichal et al. 1997). A modification in the sequence of the *cyp51A* promoter is another possible factor behind the increased level of the *cyp51A.* The first factor was not investigated in this study. A duplication of a 34 bp tandem repeat in the promoter of *cyp51A* resulted in a 4 to 8 fold increase in expression (Mellado et al. 2007). Our isolates were sequenced to determine whether the tandem repeat was present but none was found in these isolates.

None of the resistant isolates displayed significant increased levels of *cyp51B* expression compared to the susceptible isolates except one resistant isolate (F/19896) that showed a 18-fold increase compared to the susceptible isolates (Table 7.3 and Figure 7.1). This over - expression of *cyp51B* has never been reported previously. *Cyp51B* plays a role in the growth rate and maintenance of membrane shape (Mellado et al. 2005;Garcia et al. 2005). *Cyp51A* and *cyp51B* are orthologues in *A. fumigatus* and have been shown to act in a compensatory manner in the ergosterol pathway (Warrilow et al. 2010). *cyp51B* displayed tight binding toward ITR and VOR whereas *cyp51A* did not exhibit tight binding with any of the azoles (Warrilow et al. 2010). Therefore, the over-expression of *cyp51B* associated with increased expression of 3 other transporters in one ITR-R isolate without a *cyp51A* mutation, might have some implications in azole resistance mechanisms.

In the remaining resistant isolates, *cyp51B* was slightly over-expressed, or under-expressed (1.61-4.45-fold) (Table 7.3 and Figure 7.1). Our results, showed that an increased level of expression of the *cyp51A* and *cyp51B* genes in clinical isolates following exposure to ITR, are in agreement with those observed by (Albarrag et al. 2011). Expression of *cyp51B* does not appears to be a common mechanism of resistance of 9 tested *A. fumigatus* clinical isolates as we found this in only one resistant isolate.

Although, 49 ABC transporter and 278 major facilitator superfamily (MFS) genes in the genome of *A. fumigatus* (Chamilos and Kontoyiannis 2005;Nierman et al. 2005a;Nierman et al. 2005b;Qiao et al. 2008;Abad et al. 2010), only a few studies have investigated their importance in azoles resistance. *AfuMDR1, AfuMDR2 AfuMDR3* and *AfuMDR4* were found to be constitutively (basal) overexpressed in ITR-R laboratory mutants (Nascimento et al. 2003). However, these genes had shown over - expression

when the isolates were exposed to ITR. Increased level of expression of the *atrF* gene in response to ITR was reported in one clinical strain (strain AF72) (Slaven et al. 2002). *A. fumigatus MDR* (*AfuMDR*) pumps have been described in several studies and have been shown to be associated with increased resistance to ITR (Rajendran et al. 2011). On the other hand, a ABC transporter-encoding gene (*abcA*) of *A. fumigatus* did not appear to play a role in susceptibility to azoles or other antifungals (Langfelder et al. 2002).

To address this deficiency in our understanding of resistance, we Investigated whether increased efflux pump expression, could account for resistance. The levels of expression and induction of the novel *ABC11*, *MFS56* and *M85* transporters as well as *cyp51A, cyp51B, AfuMDR1, AfuMDR2, AfuMDR3, AfuMDR4* and *atrF* mRNA in 12 azole-resistant clinical strains were compared with those in the *A. fumigatus* azole-susceptible control strains.

In previous studies statistically significant differences were observed in the expression of *cyp51A*, *AfuMDR2*, and *AfuMDR4* genes (Albarrag et al. 2011). In one study, significant differences were observed in the expression of *AfuMDR3* and *AfuMDR4* (from 1.3- to 3.1-fold) after exposure to ITR (Nascimento et al. 2003). In another study, significant expression of *atrF* (5-fold) was found in *A. fumigatus* grown with azole (Slaven et al. 2002). In this study, a three patterns of expression have been observed; a massive overexpression, overexpression and slight expression. In this study, there was no significant difference in expression or induction of efflux pump transporter *AfuMDR2 AfuMDR3, AfuMDR4* genes in resistant isolates relative to susceptible isolates. One isolate (F/18085) displayed a 220.14-fold increased level of *AfuMDR1* expression relative to susceptible isolates compared to other resistant isolates without mutations (F/ 17999, F/ 18149 and F/18329), (Table 7.3 and Figure 7.1). While for *AfuMDR3*, increases of about 2.73-fold in one ITR-R isolate without a *cyp51A* mutation (F/17999) were observed. In contrast, no significant difference in the expression of *AfuMDR4* was observed in all isolates (Nascimento et al. 2003;Albarrag et al. 2011). However the expression of *AfuMDR1* was associated with increased expression of 3

other transporters. Despite the prior work, it appears that *AfuMDR2 AfuMDR3, AfuMDR4* are unlikely to be involved in azole resistance.

A similar story was found with over-expression of the *atrF* gene product (AtrF) which was previously found to be over-induced in response to ITR. In one study, AF72 had approximately 5-fold higher levels of *atrF* mRNA than susceptible isolates AF10 and H06- 03 (Slaven et al. 2002). In this study, there was slight difference in expression of *atrF* gene in resistant isolates relative to susceptible isolates. However, in the ITR-R isolate (F/17999) without a *cyp51A* mutation studied here, significantly increased levels of *atrF* expression were fund compared to the susceptible isolates (Table 7.3 and figure 7.1). However, over-expression or over–induction of 2 novel transporters was also seen concurrently. Therefore overexpression of *atrF* is not solely responsible for azole efflux and resistance in *A. fumigatus.*

In this study, the transporter *ABC11* (AFUA_1G14330) gene was chosen on the basis of homology to known *Candidia. albicans* azole resistance gene, CDR1. This is not the published orthologue of CDR1 in *A. fumigatus* however the ABC11 gene is the second closest orthologue. Increased expression of CDR1 from *C. albicans* was found to confer resistance to several azole antifungal agents, including ITR (Sanglard et al. 1996).Over – expression or over - induction of *ABC11* as the sole mechanism of resistance has not been detected in any resistant *A. fumigatus* clinical isolate. However, increased expression of at least 2 genes was detected in several resistant *A. fumigatus* clinical isolates with no mutation in the *cyp51A.* The relative increases in the levels of *ABC11*expression obtained in the wild-type strain (AF293) was 10.80-fold, whereas the levels of *ABC11* transcription in isolates with and without a *cyp51A* mutation, ITR-R, F/20063, F/17999, F/18149, F/19896, F/18329, F/18085 and F/19980 were increased 31.84-, .26.37-, 19.96-, 11.92- , 11.70-, 7.29- and 5.14-fold respectively compared to the level of transcription in the wild-type (AF293) (Table 7.3 Figure 7.1).

Interestingly, Constitutive (basal) over - expression of *ABC11* and *M85* was observed in some resistant isolates, while no significant constitutively (basal) expressed of *MFS56* mRNA was observed for all isolates (Table 7.2). The highest level of basal constitutive over - expression of *ABC11* was observed with the F/18304, F/20140 and F17727

isolates, resulting in 77.35-, 30.18- and 29.5-fold more mRNA, respectively, than those of the susceptible strain. The highest level of constitutive (basal) over - expression of *M85* was observed with the F/19980 isolate, resulting in 34.70- fold mRNA than the susceptible strain. Constitutive (basal) overexpression of *ABC11* has been observed even in wild-type stain but less than those in resistant isolates. This finding agrees with the previous observations for *AfuMDR3* and *AfuMDR4* (Nascimento et al. 2003).

Major facilitator transporter gene (*AfuMDR3*) has been characterized in *A. fumigatus* (Nascimento et al. 2003;Slaven et al. 2002;Tobin et al. 1997) and has been found to be constitutively (basal) expressed in ITR-R laboratory mutants (Nascimento et al. 2003).

In this study, we observed low basal expression levels of the *MFS56* gene in the susceptible isolates AF293 and F/15483 and resistant isolates, F/17727, F/18304 and F/18454 at 0.0005- and 0.016-, 0.016-, 0.0002- and 0.05-fold respectively. The highest level of the *MFS56* expression induction was detected in (F/17999, F/18085, F/20063, F/18149, F/19896, F/20140 and F/19980) with about 295.36-, 58.7-, 43.17-, 12.94-, 4.04-, 4.04- and 3.17-fold increase respectively (Table 7.3 and Figure 7.1). However in all cases, *MFS56* is not the only transporter over - expressed and therefore may not be the sole factor responsible for resistance. Other transporter proteins other than *MFS56* whose expression is also increased could be responsible for resistance to ITR in these isolates.

Similarly, the M85 (AFUA 5G07550) was also chosen as probable involvement in azole resistance from a previous insertional mutagenesis screen (Bowyer personal communication). We also observed low basal expression levels of the *M85* gene in the two susceptible isolates and 3 ITR-R isolates. The highest level of the *M85* expression induction was detected in F/20063, F/18085 and F/19896 with 91.26-, 410.47- and 4.31-fold increase respectively (Table 7.3 and Figure 7.1). The *M85* is unlikely to be a sole factor responsible for resistance as other genes were also over - expressed. Other proteins rather than *M85* whose expression is also increased could be responsible for resistance to ITR in these isolates.

Interestingly, deletion of *ABC11*, *MFS56* and *M85* from a wild-type strain increased *A. fumigatus* susceptibility to azoles (chapter 6) and the increased expression of (*ABC11*, *MFS56* and *M85*) genes could account for resistance to ITR in *A. fumigatus* clinical isolate and this finding suggests that over - expression of *ABC11* is at least partially responsible for ITR resistance and could be considered a mechanism for the emergence of clinical resistance to azoles in *A. fumigatus*. However, high expression of *ABC11*, *MFS56* and *M85* was detected in all ITR-R *cyp51A*-mutant or *cyp51A*-non-mutant isolates compared to the susceptible isolates, suggesting that a combination of these genes could be responsible for resistance in the presence of ITR. Moreover, other transporter proteins other than *ABC11*, *MFS56* and *M85*, whose expression is also increased as part of this response, could be partly responsible for resistance to ITR in these isolates.

In this study, some ITR resistant strains showed either constitutive (basal) high-level expression or induction of *Afumdr3*, *Afumdr4*, and *AtrF* upon exposure to ITR. There was high agreement between our results (Table 7.4 and Figure 7.2) and the observations of Ferreira et. al (da Silva Ferreira et al. 2004). Over - expression of transporter gene (*CDR1*, *CDR2*, and *MDR1*) has been detected and shown to be a common mechanism of fluconazole resistance in *C. albicans* (Sanglard et al. 1997).

In this study, some of the ITR resistant mutants exhibited induced expression of some genes, when exposed to ITR. For example, high induction was observed for several genes in F/20063 F/180304. High inductions were observed for at least two of the novel genes (*ABC11*, *MFS56* and *M85*) together with up - regulation of *cyp51A* or c*yp51B* in 3 ITR-R without a *cyp51A* mutation. However, *AtrF* was induced in 4 ITR resistant non mutants isolates (Table 7.4 and Figure 7.2).

Induction of some efflux together with up - regulation of *cyp51A* or c*yp51B*, when *Aspergillus* is exposed to low levels of ITR during infection with inadequate azole therapy may allow reduced intracellular concentrations of azoles and selection of partially resistant strains which may then allow time for development of stable resistance (Howard et al. 2009;da Silva Ferreira et al. 2004;Howard and Arendrup 2011).

The response to VOR treatment was previously investigated in an isolate exposed to 0.5 mg/l VOR. 13 genes showed higher expression in the presence of VOR by real-time RT-PCR. *Afu*mdr1 (Afua_5g06070) was one of the most highly expressed genes (da Silva Ferreira et al. 2006). In this study, we exposed two VOR-R only isolates, without a *cyp51A* mutation to 2 µg/ml ITR, one of which (F/18329) exhibited an induction in the expression of 4 genes. Up-regulation was observed for *cyp51A*, *Afumdr1*, *ABC11* and *M85* at 3.50-, 17.31-, 11.70-, and 2.72- fold respectively. In contrast, F/18454 (VOR-R) did not exhibit a significant change in expression after 4 hours of ITR exposure. The possible explanation is that all efflux genes investigated here are not affected by ITR in this particular isolate.

In conclusion, reduced intracellular accumulation due to increased expression of efflux pumps have been described as a second molecular mechanism of resistance to azole drugs in ITR-R *A. fumigatus* strains. Many studies have revealed that drug efflux mediated by increased expression of ABC transporter is an important resistance mechanism in fungi. Increased expression of CDR1 *Candida albicans* was found to confer resistance to terbinafine, cycloheximide, and several azole antifungal agents, including ITR (Slaven et al. 2002). The major mechanism responsible for high-level azole resistance in clinical *Candida* isolates is over - expression of plasma membrane efflux pumps (Cannon et al. 2009). Increased levels of *ABC11* expression suggest a possible mechanism for azole resistance in ITR-R *A. fumigatus* strains. Mutant strain (ΔABC11) exhibited increased susceptibility to all azole (ITR, VOR, and POS) antifungal agents (chapter 6) and similarly increased levels of *ABC11* expression is observed in clinical resistant isolates, suggesting that *ABC11* transporter could be responsible for the azole resistant phenotype in some isolates. Similarly, there was a 2- to 400-fold increase in the level of *MFS56* or *M85* expression upon exposure to azoles which corresponded to a slight increase susceptibility for the respective gene knockouts (ΔMFS56 and ΔM85) (chapter 6) confirming that these genes may contribute to the azole resistant phenotype in ITR-R *A. fumigatus* strains. Over expression of *cyp51A* has been detected in both in mutant and non-mutant resistant isolates. This suggests that expression of *cyp51A* is not a sole resistance mechanism but may enhance undefined resistance mechanisms particularly in isolates do not carry mutation in *cyp51A*.

Disruption of *ABC11, MFS56* and *M85* in *A. fumigatus* had a small but detectable effect on azole susceptibility but following exposure to ITR, over - induction of novel genes *ABC11, MFS56* and *M85* was observed in all resistant isolates and high basal gene expression was observed for ABC 11 and M85 suggesting that they might be associated with azole resistance in clinical isolates. The over - expression and over – induction of these genes may cause ITR resistance through reduction of intracellular levels of azole. This finding suggest that overexpression of the novel genes are at least partially responsible for ITR resistance and could be considered mechanisms for the emergence of clinical resistance to azoles.

A further study is required to investigate the involvement of the novel efflux transporters *ABC11* and *MFS56* and hypothetical protein *M85* using mutant strains of ΔABC11, ΔMFS56 and ΔM85. Over expression of these genes would be interesting as well as gene knockout in the clinical isolates that show high expression.

Chapter 8

General discussion

8.1 General discussion

The increase in the prevalence of Aspergillus infections during the last two decades was accompanied by an increasing number of both immunosuppressed and immune compromised individuals. *Aspergillus fumigatus* is the most common aetiological agent causing invasive, chronic and allergic mould infections (Li et al. 2011;Denning and Perlin 2011). Triazole antifungal agents are the most effective and widely used in the management of Aspergillus diseases in agriculture (Verweij et al. 2009b) and in clinical settings (Denning and Perlin 2011).

Recently, acquired resistance to a number of antifungal components, especially triazole, has been predominately reported due to the widespread use of triazole antifungal agents (Nascimento et al. 2003;Howard et al. 2009;Qiao et al. 2008;Verweij et al. 2009b;Verweij et al. 2009a) or through exposure of isolates to azole fungicides in the environment (Van Der Linden et al. 2011). However, resistance to treatment with azole drugs is an emerging problem of considerable concern in human medicine.

No resistance was seen before the 1990s, perhaps because the filamentous fungi susceptibility method has changed more than once (Denning et al. 1997b). The global frequency of azole resistance is not clearly defined as results from many laboratories do not routinely test the susceptibility of their isolates of *Aspergillus* and they do not use a common standard method for susceptibility. Nevertheless laboratory techniques are being developed to identify and classify *Aspergillus* in clinical settings as well as to improve the susceptibility testing of filamentous fungi (EUCAST 2008;CLSI 2002)

Susceptibility

Initially, 64 *A. fumigatus* from the Mycology Reference Centre Manchester, UK (MRCM) culture collection isolates were re-identified by classical culture techniques and confirmed by culturing them on SAB at 50°C and ITS sequencing. The reason behind this was to exclude any closely related azole - resistant species. *A. calidoustus* for example, appears to be resistant to azoles, and *A. lentulus* and *Petromyces alliaceus*

have exhibited decreased susceptibility to anti-fungal drugs, especially the azoles and echinocandins and amphotericin B (Balajee et al. 2007a).

Although the susceptibility of 64 *A. fumigatus* MRCM culture collection isolates was determined in MRCM, we re-tested them for susceptibility against ITR, POS and VOR to confirm the MRCM results and to see whether there was susceptible profile change during storage. It was found that all MIC values for all *A. fumigatus* resistant isolates were consistent with clinical laboratory results with variation within ~a two-fold difference.

However, a modified EUCAST method which has been used in MRCM since 2001 was used. Standardization of *in-vitro* susceptibility testing methodology for *Aspergillus* gave precise and reproducible results in MRCM. EUCAST and mEUCAST methods are both broth dilution based and contain 2% glucose to reduce trailing end points, but differ with fungal inoculum size of 2.5 x 10^5cfu/ml VS of 2.5 x 10^4cfu/ml respectively. As a result of using comparable methods, detection of the change in frequency of resistance in *A. fumigatus* between 2001 and 2009 was possible. In addition, the results obtained here suggest the mEUCAST defined azole resistance may correlate with treatment failure in clinical settings, as *in-vitro* with treatment failure correlation has been observed and confirmed in animal models of fungal infection (Howard et al. 2009;Dannaoui et al. 1999;Denning et al. 1997a). Ongoing comparison of mEUCAST with EUCAST standard method shows equal or 1 dilution higher result with EUCAST. Using the EUCAST method would therefore lead to a slight increase in the frequency of reported resistance in *A. fumigatus* in Manchester.

In Manchester, UK, the frequency of ITR resistance was 5% from 2004 to 2007 (Howard et al. 2009) and the analysis has been updated in this study. In 2008, 23% were resistant to at least one azole while 31% were resistant in 2009. Using patients as the denominator rather than isolates (discounting additional isolates from patients with the same susceptibility pattern), the frequency of resistance remains high in the cases referred to in our laboratory during 2008-9 at 14% and 21%, respectively (see Table

3.1). Only 2 (10%) isolates from 2008 were VOR resistant. The remainder (16 in 2008 and 34 in 2009) were multi-azole resistant. Thus, during this period, 62 of 64 (97%) were ITR resistant, 2 of 64 (3%) were only VOR resistant and 50 of 64 (78%) cases were multi-azole resistant. Decreased susceptibility to VOR has already been described in clinical isolates and in laboratory mutants (Manavathu et al. 2001;Verweij et al. 2002), however, resistance to VOR only has not, to our knowledge, been reported in clinical isolates.

The risk of cross-resistance between triazoles in *A.fumigatus* was high (78%), which is of concern in human medicine with few choices of drug. thus new drug classes to treat aspergillosis are required. The reason behind the cross-resistance between triazoles in *A. fumigatus* may be because azoles inhibit the same target *cyp51A* gene.

Two possible reasons for the increasing overall rate of resistance of *A. fumigatus* found that compared to other centres, in the MRCM culture collection: a mEUCAST standard method has been used since 2001 and increasing specimen numbers received from patients failing therapy or problematic cases in MRCM, as a regional clinical diagnostic service. Improved MIC determinations have led to more reproducible and accurate detection of resistance, which has benefited the study of resistance mechanisms as well (Verweij et al. 2009a). However, the increased rate of azole resistance globally could be because ITR has been used for many years, for both therapy and prophylaxis. In contrast, other triazoles such as VOR and POS have recently been used with relatively low frequency compared to ITR.

Endpoint determination was problematic for some isolates; trailing growth of filamentous fungi remains a challenge because the endpoint is read visually rather than spectrophotmetically. However, as growth is usually in small micro-colonies, the optical endpoint reading is likely to be more sensitive than the spectrophotometric reading. We observed trailing growth during the MIC testing, which induced azole-resistant isolates, which may be due to increased expression of target gene or efflux pump. One study found that the main cause of this phenomenon in *C. albicans* is increased *ERG11* expression (Ribeiro and Paula 2007). However, a modification of methodology by

reduction in inoculum size reduces the appearance of trailing endpoints that allows a clearer MIC determination. Therefore, a recent study proposed breakpoints for *A. fumigatus* complex using the proposed EUCAST susceptibility testing methodology but is not yet ratified by the CLSI and EUCAST committees for *A. fumigatus* and the licensed azoles (Howard and Arendrup 2011). The breakpoints for ITR and VOR are <2 mg/l (susceptible), 2 mg/l (intermediate) and >2 mg/l (resistant); for POS, <0.25, 0.5 and >0.5 mg/l respectively. However, several conditions are required to establish the breakpoint including both intra- and interlaboratory reproducibility using isolates which are resistant to the agent(s) at a known level. Subsequent reports have confirmed resistance in *in vivo* models (Verweij et al. 2009a). Determining a breakpoint for resistance and using one standard MIC susceptibility test for fungi globally will enable data comparison.

Mechanisms of resistance

The mechanisms responsible for antifungal resistance are currently not clearly or distinctly defined. Azole resistance is usually caused by one or more of the following mechanisms: mutation in the target gene (cyp51A) or decreased intracellular concentration due to efflux or overexpression of target enzyme. In this study, we investigated alterations in *cyp51A* in all 64 azole resistant *A. fumigatus* clinical isolates. Although, all 16 *A. fumigatus* environmental isolates were susceptible to azoles, we also investigated alterations in *cyp51A*. Furthermore, the level of expression of the novel genes ABC transporters *ABC11*, *MFS56*, hypothetical protein *M85* and other genes *cyp51A, cyp51B, AfuMDR1, AfuMDR2, AfuMDR3, AfuMDR4, and atr-F* were assessed by real-time PCR for their expression in *A. fumigatus* after induction by ITR. In addition, we knocked out *ABC11, MFS56* and *M85* in a wild-type strain to see whether this had any effect on susceptibility.

In the isolates investigated here, the most remarkable trend is the increasing frequency of azole-resistant isolates without *cyp51A* mutations. Such isolates have been rarely reported elsewhere (Howard et al. 2009;Bellete et al. 2010). Prior to 2007 very few resistant isolates in MRCM had a wild-type *cyp51A* sequence. In 2008, of the 13 resistant isolates studied, 1 had a M220K mutation, 3 had the F46Y/ M172V/ E427K combination (which is probably not linked to resistance as it has seen in both

susceptible and resistant isolates (Howard et al. 2009;Rodriguez-Tudela et al. 2008), and the remaining 9 isolates had no *cyp51A* mutations. In 2009, 10 of 31 (32%) isolates tested had a wild-type *cyp51A* sequence (Figure 4.1). For patients, the frequency of mutations found in at least one isolate was 22% in 2008 and 58% in 2009 (Figure 4.1). Thus 43% of isolates and 54% of patients did not have a *cyp51A* mutation known to confer resistance (including two isolates that were VOR resistant only).

Interestingly, three patients had serial resistant isolates, some with *cyp51A* mutations, and others with wild-type sequences. Efflux-mediated resistance could be responsible, as it is a common mechanism in yeasts, although it has been mooted rarely in *Aspergillus* (Moore et al. 2000;Slaven et al. 2002). However, the level of expression of the *ABC11*, *MFS56*, *M85* and 7 genes including *cyp51A* and *cyp51B* were assessed in some azole resistant isolates. Following exposure to ITR, over - expression of most these genes was observed to some extent in all resistant isolates (Table 7.3).

Despite the influence of other mechanisms, cross-resistance patterns appeared to remain closely linked with the Cyp51A amino acid substitution. Isolates with G54R, P216L and G448S mutations are all associated with ITR and POS resistance, whilst remaining susceptible to VOR. Mutational hotspots confirmed to cause resistance have been characterized in the gene at codons 54 (Diaz-Guerra et al. 2003;Chen et al. 2005). 216 and 448 (Howard et al. 2009;Diaz-Guerra et al. 2003). We found isolates with five different amino acid substitutions at position M220, namely isoleucine (I), lysine (K), valine (V), arginine (R) and tryptophan (W), of which M220R and M220W have not been previously reported to our knowledge. However, all alterations at codon 220 are associated with ITR and POS resistance, but result in variable VOR MICs (typically raised). Mutations at codons 54 and 220 are reported with much greater frequency, and their link with azole resistance has been confirmed by transformation of the altered gene into wild-type strains, so these mutations have been correlated with high MIC, which leads to treatment failure either in animal models or in patients. These codons are known as hot spots (Howard and Arendrup 2011).

A novel finding is that two patients had one isolate each with a *cyp51A* (A284T) mutation (alanine to threonine substitution) conferring reduced susceptibility to ITR, VOR and POS. The novel alterations require further study to confirm their association with resistance by transformation into a wild-type strain. Two patients yielded an isolate each with F46Y/ M172V/ E427K mutations, one of which also had the mutations N248T and D255E. However, it is likely that these mutations are not associated with resistance, as they have been described previously in both susceptible and resistant isolates (Howard et al. 2009;Rodriguez-Tudela et al. 2008). However, in this study, we investigated both up-regulation of the *cyp51A* and effluxes in one ITR-R mutant isolate with these mutations and we observed a high level of constitutive (basal) overexpression of *ABC11*, while there was a massive over-expression of *cyp51A* following exposure to ITR. In contrast, variable expression of the remaining efflux genes studied here has been observed compared to that by the susceptible strain.

These results highlight the continuing increasing frequency and evolution of resistance mechanisms in *A. fumigatus*, in both azole-naive and azole-treated patients. The increasing rate of resistance is of concern. Furthermore the emergence of alternative mechanisms of resistance other than *cyp51A* mutations, including isolates resistant only to VOR with no target mutations detected, implies a quite distinct mechanism compared with previously reported resistant isolates. There appear to be differences in the geographical distribution of azole resistance in *A. fumigatus*, which cannot be explained by differences in methodology (as excellent concordance has been shown between CLSI and EUCAST methods). Since not all centres monitor the susceptibility of aspergilli to azoles the true incidence is unknown. Nonetheless, resistance has now been reported from many countries in Europe, China, Canada, India, Japan and the USA, as well as particularly high frequencies from the Netherlands and the north-west of the UK

Gene expression

In this study, the highest frequency of azole-resistant isolates was found without *cyp51A* mutations. This phenomenon has been seldom reported (Howard et al. 2009;Bellete et al. 2010).We also found a combination of mutation (46Y/ M172/ E427) in *cyp51A* known not to confer azole resistance. Therefore, azole resistant *A. fumigatus* isolates require additional investigation to determine their causative mechanism. However, up-regulation of the *cyp51A* gene has also been implicated in azole resistance (Mellado et al. 2007). Decreased intracellular level of azole accumulation due to over - expression of efflux pumps has occasionally been described as a cause of resistance in some *A. fumigatus* isolates (Nascimento et al. 2003;Manavathu et al. 1999;da Silva Ferreira et al. 2004;Chamilos and Kontoyiannis 2005;Qiao et al. 2008).For example, *A. fumigatus* pumps *AfuMDR* (Nascimento et al. 2003;Rajendran et al. 2011) and *atrF* (Slaven et al. 2002). Increased expression of CDR1 *Candida albicans* was found to confer resistance to terbinafine, cycloheximide, and several azole antifungal agents, including ITR (Slaven et al. 2002).

We observed a massive over-expression 1590.71 and 62.08 fold increase in the level of expression of the *cyp51A* gene in two isolates compared to that in the susceptible strain (AF293). We also observed nearly twice the transcript levels for non-mutant resistant isolates compared to susceptible isolates. However, up-regulation of the *cyp51A* gene in the susceptible isolates AF293 and F/15483 at 2.88 and 2.30 fold respectively have been observed but not detected in some resistant isolates (Table 7.3 and Figure 7.1). The reasons behind the increase in the *cyp51A* mRNA level may be: the copy number of *cyp51A* and a modification in the sequence of the *cyp51A* prompter. However, up-regulation of c*yp51A* alone has not been reported in clinical isolates, even in *C. albicans* to our knowledge (Ribeiro and Paula 2007). Over-expression of *cyp51A* is expected to increase MICs to all azoles and cross-resistance to azoles is also expected in *A. fumigatus* because the different azoles, inhibit the same target.

Expression of *cyp51A* and *cyp51B* has been observed in *A. fumigatus* (da Silva Ferreira et al. 2004), *Blumeriella jaapii* (Ma et al. 2006)*Candida glabrata* (Marichal et al. 1997) and *Venturia inaequalis* (Schnabel and Jones 2001) resulting from a chromosomal duplication and therefore an increase in copy number of the *cyp51A* gene. The *cyp51A*

gene copy number is described in *C. albicans* (Selmecki et al. 2006) and *C. glabrata* (Marichal et al. 1997). A modification in the sequence of the *cyp51A* promoter is another possible reason behind the increased level of *cyp51A* expression, which was not investigated in this study. A duplication of a 34 bp promoter tandem repeat of *cyp51A* results in an increase in expression (Mellado et al. 2007). Our isolates were sequenced for this tandem repeat but none was found in these isolates.

None of the resistant isolates displayed significantly increased levels of *cyp51B* expression compared to the susceptible isolates, except for one resistant isolate (Table 7.3 and Figure 7.1). Our results showed that an increased level of expression of the *cyp51A* and *cyp51B* genes in clinical isolates following exposure to ITR, are in agreement with those observed by (Albarrag et al. 2011). *Cyp51B* plays a role in the growth rate and maintenance of membrane shape (Garcia et al. 2005). However, *cyp51A* and cy*p51B* are orthologues in *A. fumigatus* and have been shown to act in a compensatory manner in the ergosterol pathway (Warrilow et al. 2010). Therefore, the high over-expression of *cyp51B* associated with increased expression of 3 other transporters in one ITR-R without a *cyp51A* mutation, might have some implications in azole resistance mechanisms.

In this study, the transporter *ABC11* (AFUA_1G14330) gene was chosen on the basis of homology to the known *C. albicans* azole resistance gene, CDR1. However, increased expression of CDR1 from *C. albicans* was found to confer resistance to several azole antifungal agents, including ITR (Sanglard et al. 1996). In this study, overexpression of a novel gene *ABC11* as the sole mechanism of resistance has not been detected in any resistant *A. fumigatus* clinical isolate. However, increased expression of at least 2 genes was detected in several resistant *A. fumigatus* clinical isolates with no mutation in the *cyp51A*. The relative increases in the levels of basal *ABC11* expression ranged from 5.14 to 31.84 in resistant isolates (see Table 7.3 Figure 7.1).

Interestingly, constitutive (basal) over-expression of *ABC11* and *M85* was observed in some resistant isolates, while no significant constitutive (basal) expression of *MFS56*

mRNA was observed for all isolates (Tble 7.2). However, constitutive (basal) over-expression *AfuMDR3* and *AfuMDR4* have been observed in mutant ITR-R isolates (Nascimento et al. 2003).

Over - expression of the *atrF* gene product (AtrF) in response to ITR was found to be correlated with ITR resistance (Slaven et al. 2002). In this study, a slight difference in expression of *atrF* gene in resistant isolates relative to susceptible isolates was observed. However, in the ITR-R isolate (F/17999) without a *cyp51A* mutation studied here, significantly increased levels of *atrF* expression were fund compared to the susceptible isolates (Table 7.3 and Figure 7.1). Expression of *atrF* does not appear to be a sole mechanism of resistance in resistant *A. fumigatus* clinical isolates.

The MFS56 (AFUA-1G05010) was chosen for its probable involvement in azole resistance from a previous insertional mutagenesis screen (Bowyer personal communication). In this study, we observed low basal expression levels of the *MFS56* gene in the susceptible and resistant isolates (Table 7.3 and Figure 7.1). The *MFS56* dose not appears to be a sole factor responsible for resistance. Other proteins as well as *MFS56* whose expression is also increased could be responsible for resistance to ITR in these isolates.

A similar story, the M85 (AFUA- 5G07550) was also chosen as probable involvement in azole resistance from a previous insertional mutagenesis screen We also observed low expression levels of the *M85* gene in the susceptible isolates AF293 and resistant isolates. (Table 7.3 and Figure 7.1) The *M85* is unlikely to be a sole factor responsible for resistance. Other proteins as well as *M85* whose expression is also increased could be responsible for resistance to ITR in these isolates.

Over-expression of the target gene or efflux pump can also contribute to resistance in *A. fumigatus,* which they can elevate the MIC to some extent, but not to the same extent as mutations that affect drug binding. Therefore, stable resistance is more likely to result from mutation rather than by up-regulation of *cyp51* or efflux. Increased expression of these genes allows the cells to persist within the host for a short period and subsequent

development of other more stable resistance mechanisms as reported with increased expression of *ERG11*in *C. albicans* (Ribeiro and Paula 2007).

In this study, various resistance mechanisms (up-regulation of c*yp51A* and expression of several efflux genes) were found in a number of clinical multi- azole resistant and pan-azole resistant isolates with wild-type *cyp51A*. However, up-regulation of c*yp51A* alone has not been observed in any resistant isolate.

Interestingly, deletion of *ABC11*, *MFS56* and *M85* from a wild-type strain increased *A. fumigatus* susceptibility to azoles and the increased expression of *ABC11*, *MFS56* and *M85* genes could account for resistance to ITR. These findings suggest that over-expression of *ABC11* is at least partially responsible for ITR resistance and could be considered a mechanism for the emergence of clinical resistance to azoles in *A. fumigatus*. However, over-expression or over-induction of *ABC11*, *MFS56* and *M85* was detected in almost all ITR-R isolates, either with or without *cyp51A* mutations compared to the susceptible isolates, suggesting that a combination of these genes could be responsible for resistance in the presence of ITR. However other proteins, whose expression is also increased as part of this response, could account for resistance to ITR in these isolates.

A. fumigatus has the capacity to form biofilms encased in a polymeric matrix, which is the most likely growth modality with a fungus ball (Loussert et al. 2010). The biofilm modality of *Aspergillus* growth may have a number of therapeutic implications for aspergillosis, including antifungal resistance (Bowyer et al. 2011). Expressions of efflux pumps have been observed in complex *A. fumigatus* biofilm populations, and this contributes to azole resistance (Rajendran et al. 2011). In addition to mutation in the target gene, efflux pump expression in biofilms is definitely other mechanisms that have yet to be characterised.

Taxonomy

Invasive aspergillosis acquired from the hospital environment has been reported with genetic relatedness between *A. fumigatus* isolates (Warris et al. 2003;Verweij et al.

2009a;Balajee et al. 2007a). The presence of sexual stage genes suggested that such a cryptic sexual stage exists in the human pathogen *A. fumigatus*. This may explain the multiple genotypes from the same geographical area and may explain the observed genetic variation.

Nowadays, a polyphasic taxonomy that combines morphological and molecular phylogenetic analyses is used to characterize *A. fumigatus* complex and analyse the genetic variation of strains or closely related species. This study and others demonstrated several cryptic species within the *Aspergillus* section *fumigati* which are indistinguishable on morphological characters alone. For this reason, clinical laboratories may report isolates of apparent *A. fumigatus* as *A. fumigatus* complex. In this study, we performed a polyphasic analysis of *A. fumigatus* clinical and environmental sources in order to examine the variability within the species and determine relatedness of the strains.

Initially, *A. fumigatus* in this study were identified morphologically and confirmed by culturing them on SAB at 50°C, which excludes *A. lentulus*, a closely related azole-resistant species (Yaguchi et al. 2007;Alcazar-Fuoli et al. 2008). The multilocus sequences were compared to GenBank records by BLAST.

The 9 *A. fumigatus* environmental isolates from natural soil were azole susceptible with MICs ranges of 0.125-0.5 mg/l against ITR, 0.5-2 mg/l against VOR, and 0.03-0.06 mg/l against POS. Likewise the 7 *A. fumigatus* environmental isolates from compost were also susceptible with MICs ranges of 0.125-0.5 mg/l against ITR, 1-4 mg/l against VOR, and 0.125 mg/l against POS (Table 5.1). These results found that *A. fumigatus* environmental isolates were susceptible to ITR, VOR and POS. These results also suggest that environmental isolates of *Aspergillus* collected from natural soil are more susceptible to VOR than *A. fumigatus* environmental isolates collected from compost, although numbers are small. Exposure of isolates to azole fungicides in the environment is the probably a predisposing factor for elevate MIC.

ITS sequences were helpful in identification of *Aspergillus* (Hinrikson et al. 2005), most *Mucorales* species (Schwarz et al. 2006), and within some species complexes of *Fusarium* (O'Donnell et al. 2008;Zhang et al. 2006). In this study, a high identity to *A.*

fumigatus was found with all isolates when conducting a systematic comparison of these sequences to those available in the GenBank database. In contrast, ITS sequences did not help classify the species in this closely related group of organisms (Figure 5.1) as other studies reported (Hinrikson et al. 2005;Hendolin et al. 2000). Similarly, the partial beta tubulin sequence region (Figure 5.2) provided insufficient resolution for our data perhaps due to the particularly close genetic relatedness of these isolates, whereas actin and calmodulin were found to be good molecular taxonomic targets in these isolates Figure 5.3 and Figure 5.4. Furthermore, we found calmodulin alone was more supported than the combination of the ITS and the partial beta-tubulin.

The clade comprising *Aspergillus* (soil 3) was distinct from *A. fumigatus* complex. This then, requires further investigation as it is possibly a new species. Future taxonomic works with this isolate and isolates with the same mutation combination is desirable. Molecular targets other than ITS and beta-tubulin may be required to provide more detail about evolutionary relationships.

Many cryptic species are found in clinical and environmental isolates of the *A. fumigatus* (complex). All environmental *A. fumigatus* complex isolates were ITR susceptible and no cross resistance in this dataset. Azole-resistant isolates were never found in natural soil (Verweij et al. 2009b), confirmed in this study; this may be because the natural soil, where *A fumigatus* was isolated was not exposed to azole compounds.

The complexity of the *Aspergillus* genus is a problematic issue in clinical laboratories. Morphological identification techniques and some target genes such as ITS, are not very helpful in classification of aspergillus. Therefore, alternative targets and polyphasic approaches may be important for identifying and classifying *A. fumigatus* strains accurately and quickly.

8.1.1 Conclusions

There has been a significant increase in *A. fumigatus* azole resistance in the UK and highly variable frequency worldwide as defined by *in-vitro* testing. Furthermore, the risk of cross-resistance between triazoles in *A. fumigatus* was high (78%), which is of concern in human medicine with few antifungal drug options. Thus new drug classes, particularly oral options, to treat aspergillosis are required. *In-vitro* and *in-vivo* correlation should be performed more widely particularly for strains with unknown resistance mechanisms. In recent years efforts have been made to standardize azole antifungal susceptibility testing. However, routine antifungal susceptibility testing is required to estimate the resistance incidence globally and enable data comparison.

Most remarkable is the increasing frequency of azole-resistant isolates without *cyp51A* mutations. Nevertheless, a variety of *cyp51A* mutations, both known and novel, were detected in azole resistant *A. fumigatus*. Evolution of resistance mechanisms in *A. fumigatus*, in both azole-naive and azole-treated patients, was detected; for example, a wild-type and *cyp51A* mutant isolates before and after resistance development were detected. Mutation position within the *cyp51A* gene was linked with resistance. Isolates with G54R, P216L and G448S mutations are all associated with ITR and POS resistance, whilst remaining susceptible to VOR. M220 modifications are associated with ITR and POS resistance, but result in variable VOR MICs. A combination of mutation (46Y/ M172/ E427) in *cyp51A* is known to occur frequently but not to confer azole resistance.

Efforts have been made to develop new molecular laboratory techniques to determine the resistance mechanisms. Advanced molecular techniques such as PCR can now be used to detect and identify *A. fumigatus* particularly those strains undetectable by culture, with a simultaneous evaluation of drug resistance (Denning and Perlin 2011). Furthermore, increased knowledge about drug resistance mechanisms may contribute to the development of new antifungal drugs, leading to more effective use of available antifungal agents and to controlling the development of resistance. The emergence of

alternative mechanisms of resistance other than *cyp51A* mutations implies a quite distinct mechanism compared with previously reported resistant isolates.

Molecular technologies have been used for sequencing of *Aspergillus* genomes. The complete sequence of Aspergillus helps identify new targets for antifungal drugs and enables investigative study of the basic biology of fungi. The information derived from the first complete genome sequence of a human pathogenic *A. fumigatus* will facilitate early diagnosis of IA.

Transformation-based approaches have already been used on fungi to study their mechanisms of resistance to antifungals. Therefore, deleting efflux transporters resulted in hypersensitivity of *A. fumigatus* to antifungals. Drug efflux mediated resistance appears to be another component of resistance in *A. fumigatus,* as overexpression of some efflux pumps has been correlated with antifungal resistance. Over-expression of *cyp51A* has been detected in both mutant and non-mutant resistant isolates. This suggests that expression of *cyp51A* may not be a sole resistance mechanism. There are increased levels of *ABC11* expression in many azole resistant isolates, suggesting that the *ABC11* transporter could be partially responsible for the azole resistant phenotype in some isolates. Deletion of this gene results in hypersensitivity to all azole (ITR, VOR, and POS) antifungal agents. Over-expression or over- – induction of the major facilitator gene *MFS56* is also associated with resistance, and deletion results in hypersensitivity to azole drugs. Similarly, hypothetical protein *M85* is also associated with resistance, and deletion results in hypersensitivity to azole drugs. This finding suggests that over-expression of the novel genes is at least partially responsible for ITR resistance and could be considered a mechanism for the emergence of clinical resistance to azoles. Gene expression analysis of Aspergillus and the response to antifungal exposure has extended our understanding of resistance mechanisms and evolution of resistance to antifungal agents, and identified genes associated with resistance.

In *A. fumigatus* few studies exist regarding ergosterol biosynthesis steps and the biochemistry of this pathway. More studies are required to characterise the genes involved in this pathway. Study and understanding of the ergosterol biosynthesis pathway in *A. fumigatus*, especially in resistant strains, may lead to alternative

mechanisms of antifungal drug resistance to enable the development of an effective agent.

Several laboratory methods have been used to differentiate between *Aspergillus* species, including phenotypic and genotypic. Morphological characteristics, ITS and beta-tubulin were not useful for classification in our data, for either environmental or clinical *Aspergillus*. Further study is required to determine the distinct species. However, the four gene sequences combination used in this study was found to better support the taxonomy in our data. Although azole-resistant *A. fumigatus* might be acquired from the environment, the taxonomy work on our environmental and clinical Aspergillus revealed no link between these isolates. However, aspergilloma or biofilms and long azole usage may be predisposing factors to the emergence of resistance, which is of concern in human medicine.

Chapter 9

References

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9.1 References

Abad, A., Fernandez-Molina, J. V., Bikandi, J., Ramirez, A., Margareto, J., Sendino, J., Hernando, F. L., Ponton, J., Garaizar, J. & Rementeria, A. (2010). What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis. *Rev Iberoam Micol,* 27(4)**,** 155-82.

Adam, O., Auperin, A., Wilquin, F., Bourhis, J. H., Gachot, B. & Chachaty, E. (2004). Treatment with piperacillin-tazobactam and false-positive Aspergillus galactomannan antigen test results for patients with hematological malignancies. *Clin Infect Dis,* 38(6)**,** 917-20.

Agarwal, R. (2009). Allergic bronchopulmonary aspergillosis. *Chest,* 135(3)**,** 805-26.

Albarrag, A. M., Anderson, M. J., Howard, S. J., Robson, G. D., Warn, P. A., Sanglard, D. & Denning, D. W. (2011). Interrogation of related clinical pan-azole-resistant Aspergillus fumigatus strains: G138C, Y431C, and G434C single nucleotide polymorphisms in cyp51A, upregulation of cyp51A, and integration and activation of transposon Atf1 in the cyp51A promoter. *Antimicrob Agents Chemother,* 55(11)**,** 5113-21.

Alcazar-Fuoli, L., Mellado, E., Alastruey-Izquierdo, A., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. (2008). Aspergillus section Fumigati: antifungal susceptibility patterns and sequence-based identification. *Antimicrob Agents Chemother,* 52(4)**,** 1244-51.

Alcazar-Fuoli, L., Mellado, E., Garcia-Effron, G., Buitrago, M. J., Lopez, J. F., Grimalt, J. O., Cuenca-Estrella, J. M. & Rodriguez-Tudela, J. L. (2006). Aspergillus fumigatus C-5 sterol desaturases Erg3A and Erg3B: role in sterol biosynthesis and antifungal drug susceptibility. *Antimicrob Agents Chemother,* 50(2)**,** 453-60.

Allende, M. C., Lee, J. W., Francis, P., Garrett, K., Dollenberg, H., Berenguer, J., Lyman, C. A., Pizzo, P. A. & Walsh, T. J. (1994). Dose-dependent antifungal activity and nephrotoxicity of amphotericin B colloidal dispersion in experimental pulmonary aspergillosis. *Antimicrob Agents Chemother,* 38(3)**,** 518-22.

Almeida, M. B., Bussamra, M. H. & Rodrigues, J. C. (2006). ABPA diagnosis in cystic fibrosis patients: the clinical utility of IgE specific to recombinant Aspergillus fumigatus allergens. *J Pediatr (Rio J),* 82(3)**,** 215-20.

Alvarez, C. A., Wiederhold, N. P., McConville, J. T., Peters, J. I., Najvar, L. K., Graybill, J. R., Coalson, J. J., Talbert, R. L., Burgess, D. S., Bocanegra, R., Johnston, K. P. & Williams, R. O., 3rd (2007). Aerosolized nanostructured itraconazole as prophylaxis against invasive pulmonary aspergillosis. *J Infect,* 55(1)**,** 68-74.

Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I. & Gottesman, M. M. (1999). Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol,* 39**,** 361-98.

Amitani, R., Taylor, G., Elezis, E. N., Llewellyn-Jones, C., Mitchell, J., Kuze, F., Cole, P. J. & Wilson, R. (1995). Purification and characterization of factors produced by Aspergillus fumigatus which affect human ciliated respiratory epithelium. *Infect Immun,* 63(9)**,** 3266-71.

Anaissie, E. J., Stratton, S. L., Dignani, M. C., Summerbell, R. C., Rex, J. H., Monson, T. P., Spencer, T., Kasai, M., Francesconi, A. & Walsh, T. J. (2002). Pathogenic Aspergillus species recovered from a hospital water system: a 3-year prospective study. *Clin Infect Dis,* 34(6)**,** 780-9.

Anderson, M. J., Gull, K. & Denning, D. W. (1996). Molecular typing by random amplification of polymorphic DNA and M13 southern hybridization of related paired isolates of Aspergillus fumigatus. *J Clin Microbiol,* 34(1)**,** 87-93.

Andrade, A. C., Del Sorbo, G., Van Nistelrooy, J. G. & Waard, M. A. (2000). The ABC transporter AtrB from Aspergillus nidulans mediates resistance to all major classes of fungicides and some natural toxic compounds. *Microbiology,* 146 (Pt 8)**,** 1987-97.

Aquino, V. R., Goldani, L. Z. & Pasqualotto, A. C. (2007). Update on the contribution of galactomannan for the diagnosis of invasive aspergillosis. *Mycopathologia,* 163(4)**,** 191-202.

Aravindhan, V., Sulochana, S., Narayanan, S., Paramasivam, C. N. & Narayanan, P. R. (2007). Identification & differentiation of Mycobacterium avium & M. intracellulare by PCR-RFLP assay using the groES gene. *Indian J Med Res,* 126(6)**,** 575-9.

Arendrup, M. C., Mavridou, E., Mortensen, K. L., Snelders, E., Frimodt-Moller, N., Khan, H., Melchers, W. J. & Verweij, P. E. (2010). Development of azole resistance in Aspergillus fumigatus during azole therapy associated with change in virulence. *PLoS One,* 5(4)**,** e10080.

Aufauvre-Brown, A., Cohen, J. & Holden, D. W. (1992). Use of randomly amplified polymorphic DNA markers to distinguish isolates of Aspergillus fumigatus. *J Clin Microbiol,* 30(11)**,** 2991-3.

Aviles, P., Falcoz, C., Guillen, M. J., San Roman, R., Gomez De Las Heras, F. & Gargallo-Viola, D. (2001). Correlation between in vitro and in vivo activities of GM 237354, a new sordarin derivative, against Candida albicans in an in vitro pharmacokinetic-pharmacodynamic model and influence of protein binding. *Antimicrob Agents Chemother,* 45(10)**,** 2746-54.

Bain, J. M., Tavanti, A., Davidson, A. D., Jacobsen, M. D., Shaw, D., Gow, N. A. & Odds, F. C. (2007). Multilocus sequence typing of the pathogenic fungus Aspergillus fumigatus. *J Clin Microbiol,* 45(5)**,** 1469-77.

Balajee, S. A., Gribskov, J. L., Hanley, E., Nickle, D. & Marr, K. A. (2005). Aspergillus lentulus sp. nov., a new sibling species of A. fumigatus. *Eukaryot Cell,* 4(3)**,** 625-32.

Balajee, S. A., Houbraken, J., Verweij, P. E., Hong, S. B., Yaghuchi, T., Varga, J. & Samson, R. A. (2007a). Aspergillus species identification in the clinical setting. *Stud Mycol,* 59**,** 39-46.

Balajee, S. A. & Marr, K. A. (2002). Conidial viability assay for rapid susceptibility testing of Aspergillus species. *J Clin Microbiol,* 40(8)**,** 2741-5.

Balajee, S. A., Nickle, D., Varga, J. & Marr, K. A. (2006). Molecular studies reveal frequent misidentification of Aspergillus fumigatus by morphotyping. *Eukaryot Cell,* 5(10)**,** 1705-12.

Balajee, S. A., Tay, S. T., Lasker, B. A., Hurst, S. F. & Rooney, A. P. (2007b). Characterization of a novel gene for strain typing reveals substructuring of Aspergillus fumigatus across North America. *Eukaryot Cell,* 6(8)**,** 1392-9.

Bart-Delabesse, E., Humbert, J. F., Delabesse, E. & Bretagne, S. (1998). Microsatellite markers for typing Aspergillus fumigatus isolates. *J Clin Microbiol,* 36(9)**,** 2413-8.

Barton, R. C., Hobson, R. P., Denton, M., Peckham, D., Brownlee, K., Conway, S. & Kerr, M. A. (2008). Serologic diagnosis of allergic bronchopulmonary aspergillosis in patients with cystic fibrosis through the detection of immunoglobulin G to Aspergillus fumigatus. *Diagn Microbiol Infect Dis,* 62(3)**,** 287-91.

Basich, J. E., Graves, T. S., Baz, M. N., Scanlon, G., Hoffmann, R. G., Patterson, R. & Fink, J. N. (1981). Allergic bronchopulmonary aspergillosis in corticosteroid-dependent asthmatics. *J Allergy Clin Immunol,* 68(2)**,** 98-102.

Baxter, C. G., Jones, A. M., Webb, K. & Denning, D. W. (2011). Homogenisation of cystic fibrosis sputum by sonication--an essential step for Aspergillus PCR. *J Microbiol Methods,* 85(1)**,** 75-81.

Bellete, B., Raberin, H., Morel, J., Flori, P., Hafid, J. & Manhsung, R. T. (2010). Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med Mycol,* 48(1)**,** 197-200.

Bernard, P. S. & Wittwer, C. T. (2000). Homogeneous amplification and variant detection by fluorescent hybridization probes. *Clin Chem,* 46(2)**,** 147-8.

Bertout, S., Renaud, F., Barton, R., Symoens, F., Burnod, J., Piens, M. A., Lebeau, B., Viviani, M. A., Chapuis, F., Bastide, J. M., Grillot, R. & Mallie, M. (2001). Genetic polymorphism of Aspergillus fumigatus in clinical samples from patients with invasive aspergillosis: investigation using multiple typing methods. *J Clin Microbiol,* 39(5)**,** 1731-7.

Blum, G., Perkhofer, S., Haas, H., Schrettl, M., Wurzner, R., Dierich, M. P. & Lass-Florl, C. (2008). Potential basis for amphotericin B resistance in Aspergillus terreus. *Antimicrob Agents Chemother,* 52(4)**,** 1553-5.

Bodey, G. P. & Vartivarian, S. (1989). Aspergillosis. *Eur J Clin Microbiol Infect Dis,* 8(5)**,** 413-37.

Bonnet, G., Tyagi, S., Libchaber, A. & Kramer, F. R. (1999). Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proc Natl Acad Sci U S A,* 96(11)**,** 6171-6.

Bowyer, P., Moore, C. B., Rautemaa, R., Denning, D. W. & Richardson, M. D. (2011). Azole antifungal resistance today: focus on Aspergillus. *Curr Infect Dis Rep,* 13(6)**,** 485-91.

Brown, G. D. (2011). Innate antifungal immunity: the key role of phagocytes. *Annu Rev Immunol,* 29**,** 1-21.

Bueid, A., Howard, S. J., Moore, C. B., Richardson, M. D., Harrison, E., Bowyer, P. & Denning, D. W. (2010). Azole antifungal resistance in Aspergillus fumigatus: 2008 and 2009. *J Antimicrob Chemother,* 65(10)**,** 2116-8.

Buffington, J., Reporter, R., Lasker, B. A., McNeil, M. M., Lanson, J. M., Ross, L. A., Mascola, L. & Jarvis, W. R. (1994). Investigation of an epidemic of invasive aspergillosis: utility of molecular typing with the use of random amplified polymorphic DNA probes. *Pediatr Infect Dis J,* 13(5)**,** 386-93.

Burnie, J. P., Coke, A. & Matthews, R. C. (1992). Restriction endonuclease analysis of Aspergillus fumigatus DNA. *J Clin Pathol,* 45(4)**,** 324-7.

Burnie, J. P., Matthews, R. C., Clark, I. & Milne, L. J. (1989). Immunoblot fingerprinting Aspergillus fumigatus. *J Immunol Methods,* 118(2)**,** 179-86.

Calderone, R. A. a. R. L. C. (2002). *Fungal pathogenesis : principles and clinical applications*.

Cannon, R. D., Lamping, E., Holmes, A. R., Niimi, K., Baret, P. V., Keniya, M. V., Tanabe, K., Niimi, M., Goffeau, A. & Monk, B. C. (2009). Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev,* 22(2)**,** 291-321, Table of Contents.

Carbone, I. a. L. K. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia,* 91(91)**,** 553–556

Cenci, E., Perito, S., Enssle, K. H., Mosci, P., Latge, J. P., Romani, L. & Bistoni, F. (1997). Th1 and Th2 cytokines in mice with invasive aspergillosis. *Infect Immun,* 65(2)**,** 564-70.

Chai, L. Y., Vonk, A. G., Kullberg, B. J. & Netea, M. G. (2011). Immune response to Aspergillus fumigatus in compromised hosts: from bedside to bench. *Future Microbiol,* 6(1)**,** 73-83.

Chamilos, G. & Kontoyiannis, D. P. (2005). Update on antifungal drug resistance mechanisms of Aspergillus fumigatus. *Drug Resist Updat,* 8(6)**,** 344-58.

Chazalet, V., Debeaupuis, J. P., Sarfati, J., Lortholary, J., Ribaud, P., Shah, P., Cornet, M., Vu Thien, H., Gluckman, E., Brucker, G. & Latge, J. P. (1998). Molecular typing of environmental and patient isolates of Aspergillus fumigatus from various hospital settings. *J Clin Microbiol,* 36(6)**,** 1494-500.

Chen, J., Li, H., Li, R., Bu, D. & Wan, Z. (2005). Mutations in the cyp51A gene and susceptibility to itraconazole in Aspergillus fumigatus serially isolated from a patient with lung aspergilloma. *J Antimicrob Chemother,* 55(1)**,** 31-7.

CLSI (2002). National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard-second edition. *Document M38- A2*.

da Silva Ferreira, M. E., Capellaro, J. L., dos Reis Marques, E., Malavazi, I., Perlin, D., Park, S., Anderson, J. B., Colombo, A. L., Arthington-Skaggs, B. A., Goldman, M. H. & Goldman, G. H. (2004). In vitro evolution of itraconazole resistance in Aspergillus fumigatus involves multiple mechanisms of resistance. *Antimicrob Agents Chemother,* 48(11)**,** 4405-13.

da Silva Ferreira, M. E., Kress, M. R., Savoldi, M., Goldman, M. H., Hartl, A., Heinekamp, T., Brakhage, A. A. & Goldman, G. H. (2006). The akuB(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in Aspergillus fumigatus. *Eukaryot Cell,* 5(1)**,** 207-11.

Dagenais, T. R. & Keller, N. P. (2009). Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis. *Clin Microbiol Rev,* 22(3)**,** 447-65.

Dannaoui, E., Borel, E., Monier, M. F., Piens, M. A., Picot, S. & Persat, F. (2001). Acquired itraconazole resistance in Aspergillus fumigatus. *J Antimicrob Chemother,* 47(3)**,** 333-40.

Dannaoui, E., Meletiadis, J., Tortorano, A. M., Symoens, F., Nolard, N., Viviani, M. A., Piens, M. A., Lebeau, B., Verweij, P. E. & Grillot, R. (2004). Susceptibility testing of sequential isolates of Aspergillus fumigatus recovered from treated patients. *J Med Microbiol,* 53(Pt 2)**,** 129-34.

Dannaoui, E., Persat, F., Monier, M. F., Borel, E., Piens, M. A. & Picot, S. (1999). In-vitro susceptibility of Aspergillus spp. isolates to amphotericin B and itraconazole. *J Antimicrob Chemother,* 44(4)**,** 553-5.

de Souza, C. C., Goldman, M. H. & Goldman, G. H. (2000). Tagging of genes involved in multidrug resistance in Aspergillus nidulans. *Mol Gen Genet,* 263(4)**,** 702-11.

de Valk, H. A., Meis, J. F., de Pauw, B. E., Donnelly, P. J. & Klaassen, C. H. (2007a). Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple Aspergillus fumigatus isolates from patients with invasive aspergillosis. *J Clin Microbiol,* 45(5)**,** 1415-9.

de Valk, H. A., Meis, J. F. & Klaassen, C. H. (2007b). Microsatellite based typing of Aspergillus fumigatus: strengths, pitfalls and solutions. *J Microbiol Methods,* 69(2)**,** 268-72.

Debeaupuis, J. P., Sarfati, J., Chazalet, V. & Latge, J. P. (1997). Genetic diversity among clinical and environmental isolates of Aspergillus fumigatus. *Infect Immun,* 65(8)**,** 3080-5.

Dee, T. H. (1975). Detection of Aspergillus fumigatus serum precipitins by counterimmunoelectrophoresis. *J Clin Microbiol,* 2(6)**,** 482-5.

Del Sorbo, G., Schoonbeek, H. & De Waard, M. A. (2000). Fungal transporters involved in efflux of natural toxic compounds and fungicides. *Fungal Genet Biol,* 30(1)**,** 1-15.

Denning, D. W. (1996). Therapeutic outcome in invasive aspergillosis. *Clin Infect Dis,* 23(3)**,** 608-15.

Denning, D. W. (1998). Invasive aspergillosis. *Clin Infect Dis,* 26(4)**,** 781-803; quiz 804-5.

Denning, D. W., Anderson, M. J., Turner, G., Latge, J. P. & Bennett, J. W. (2002a). Sequencing the Aspergillus fumigatus genome. *Lancet Infect Dis,* 2(4)**,** 251-3.

Denning, D. W., Clemons, K. V., Hanson, L. H. & Stevens, D. A. (1990). Restriction endonuclease analysis of total cellular DNA of Aspergillus fumigatus isolates of geographically and epidemiologically diverse origin. *J Infect Dis,* 162(5)**,** 1151-8.

Denning, D. W., Hanson, L. H., Perlman, A. M. & Stevens, D. A. (1992). In vitro susceptibility and synergy studies of Aspergillus species to conventional and new agents. *Diagn Microbiol Infect Dis,* 15(1)**,** 21-34.

Denning, D. W., Marinus, A., Cohen, J., Spence, D., Herbrecht, R., Pagano, L., Kibbler, C., Kcrmery, V., Offner, F., Cordonnier, C., Jehn, U., Ellis, M., Collette, L. & Sylvester, R. (1998). An EORTC multicentre prospective survey of invasive aspergillosis in haematological patients: diagnosis and therapeutic outcome. EORTC Invasive Fungal Infections Cooperative Group. *J Infect,* 37(2)**,** 173-80.

Denning, D. W., Park, S., Lass-Florl, C., Fraczek, M. G., Kirwan, M., Gore, R., Smith, J., Bueid, A., Moore, C. B., Bowyer, P. & Perlin, D. S. (2011). High-frequency triazole resistance found In nonculturable Aspergillus fumigatus from lungs of patients with chronic fungal disease. *Clin Infect Dis,* 52(9)**,** 1123-9.

Denning, D. W. & Perlin, D. S. (2011). Azole resistance in Aspergillus: a growing public health menace. *Future Microbiol,* 6(11)**,** 1229-32.

Denning, D. W., Radford, S. A., Oakley, K. L., Hall, L., Johnson, E. M. & Warnock, D. W. (1997a). Correlation between in-vitro susceptibility testing to itraconazole and in-vivo outcome of Aspergillus fumigatus infection. *J Antimicrob Chemother,* 40(3)**,** 401-14.

Denning, D. W., Ribaud, P., Milpied, N., Caillot, D., Herbrecht, R., Thiel, E., Haas, A., Ruhnke, M. & Lode, H. (2002b). Efficacy and safety of voriconazole in the treatment of acute invasive aspergillosis. *Clin Infect Dis,* 34(5)**,** 563-71.

Denning, D. W., Venkateswarlu, K., Oakley, K. L., Anderson, M. J., Manning, N. J., Stevens, D. A., Warnock, D. W. & Kelly, S. L. (1997b). Itraconazole resistance in Aspergillus fumigatus. *Antimicrob Agents Chemother,* 41(6)**,** 1364-8.

Diaz-Guerra, T. M., Mellado, E., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. (2003). A point mutation in the 14alpha-sterol demethylase gene cyp51A contributes to itraconazole resistance in Aspergillus fumigatus. *Antimicrob Agents Chemother,* 47(3)**,** 1120-4.

Dillon, H. K., Boling, D. K. & Miller, J. D. (2007). Comparison of detection methods for Aspergillus fumigatus in environmental air samples in an occupational environment. *J Occup Environ Hyg,* 4(7)**,** 509-13.

Ding, D. Q., Tomita, Y., Yamamoto, A., Chikashige, Y., Haraguchi, T. & Hiraoka, Y. (2000). Large-scale screening of intracellular protein localization in living fission yeast cells by the use of a GFP-fusion genomic DNA library. *Genes Cells,* 5(3)**,** 169-90.

Dixon, D. M., Polak, A. & Walsh, T. J. (1989). Fungus dose-dependent primary pulmonary aspergillosis in immunosuppressed mice. *Infect Immun,* 57(5)**,** 1452-6.

Eck, R. V. & Dayhoff, M. O. (1966). Evolution of the structure of ferredoxin based on living relics of primitive amino Acid sequences. *Science,* 152(3720)**,** 363-6.

Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B. & Nielsen, P. E. (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature,* 365(6446)**,** 566-8.

Escribano, I., Rodriguez, J. C., Llorca, B., Garcia-Pachon, E., Ruiz, M. & Royo, G. (2007). Importance of the efflux pump systems in the resistance of Mycobacterium tuberculosis to fluoroquinolones and linezolid. *Chemotherapy,* 53(6)**,** 397-401.

Espinel-Ingroff, A., Boyle, K. & Sheehan, D. J. (2001). In vitro antifungal activities of voriconazole and reference agents as determined by NCCLS methods: review of the literature. *Mycopathologia,* 150(3)**,** 101-15.

EUCAST (2008). Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee on Antimicrobial Susceptibility Testing EUCAST.EUCAST technical note on method for the determination of broth dilution minimum inhibitory

concentrations of antifungal agents for conidia-forming moulds. Clin Microbiol Infect 14: 982–984.
Evans, E. G. V. a. R., M. D. . (1989). *Medical mycology : a practical approach.*
.
Felsenstein, D., Carney, W. P., Iacoviello, V. R. & Hirsch, M. S. (1985). Phenotypic properties of atypical lymphocytes in cytomegalovirus-induced mononucleosis. *J Infect Dis,* 152(1)**,** 198-203.
Felton, T. W., Baxter, C., Moore, C. B., Roberts, S. A., Hope, W. W. & Denning, D. W. (2010). Efficacy and safety of posaconazole for chronic pulmonary aspergillosis. *Clin Infect Dis,* 51(12)**,** 1383-91.
Femenia, F., Huet, D., Lair-Fulleringer, S., Wagner, M. C., Sarfati, J., Shingarova, L., Guillot, J., Boireau, P., Chermette, R. & Berkova, N. (2009). Effects of conidia of various Aspergillus species on apoptosis of human pneumocytes and bronchial epithelial cells. *Mycopathologia,* 167(5)**,** 249-62.
Ferreira, M. E., Colombo, A. L., Paulsen, I., Ren, Q., Wortman, J., Huang, J., Goldman, M. H. & Goldman, G. H. (2005). The ergosterol biosynthesis pathway, transporter genes, and azole resistance in Aspergillus fumigatus. *Med Mycol,* 43 Suppl 1**,** S313-9.
Ferte, J. (2000). Analysis of the tangled relationships between P-glycoprotein-mediated multidrug resistance and the lipid phase of the cell membrane. *Eur J Biochem,* 267(2)**,** 277-94.
Firon, A., Villalba, F., Beffa, R. & D'Enfert, C. (2003). Identification of essential genes in the human fungal pathogen Aspergillus fumigatus by transposon mutagenesis. *Eukaryot Cell,* 2(2)**,** 247-55.
Fraczek, M. G., Rashid, R., Denson, M., Denning, D. W. & Bowyer, P. (2010). Aspergillus fumigatus allergen expression is coordinately regulated in response to hydrogen peroxide and cyclic AMP. *Clin Mol Allergy,* 8**,** 15.
Fryberg, M., Oehlschlager, A. C. & Unrau, A. M. (1973). Biosynthesis of ergosterol in yeast. Evidence for multiple pathways. *J Am Chem Soc,* 95(17)**,** 5747-57.
Galgiani, J. N. (1990). Susceptibility of Candida albicans and other yeasts to fluconazole: relation between in vitro and in vivo studies. *Rev Infect Dis,* 12 Suppl 3**,** S272-5.
Garcia, C. G., Garcia-Fernandez, M. A. & Sarnago Cebada, F. (2005). Aspergillus endocarditis. *Echocardiography,* 22(7)**,** 623-4.
Gehrt, A., Peter, J., Pizzo, P. A. & Walsh, T. J. (1995). Effect of increasing inoculum sizes of pathogenic filamentous fungi on MICs of antifungal agents by broth microdilution method. *J Clin Microbiol,* 33(5)**,** 1302-7.
Ghannoum, M. A. & Rice, L. B. (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev,* 12(4)**,** 501-17.
Gibson, U. E., Heid, C. A. & Williams, P. M. (1996). A novel method for real time quantitative RT-PCR. *Genome Res,* 6(10)**,** 995-1001.
Girardin, H., Latge, J. P., Srikantha, T., Morrow, B. & Soll, D. R. (1993). Development of DNA probes for fingerprinting Aspergillus fumigatus. *J Clin Microbiol,* 31(6)**,** 1547-54.
Glass, N. L. & Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol,* 61(4)**,** 1323-30.
Goel, G., Kumar, A., Puniya, A. K., Chen, W. & Singh, K. (2005). Molecular beacon: a multitask probe. *J Appl Microbiol,* 99(3)**,** 435-42.
Goodley, J. M., Clayton, Y. M. & Hay, R. J. (1994). Environmental sampling for aspergilli during building construction on a hospital site. *J Hosp Infect,* 26(1)**,** 27-35.

Gordon, M. A., Lapa, E. W. & Kane, J. (1977). Modified indirect fluorescent-antibody test for aspergillosis. *J Clin Microbiol,* 6(2)**,** 161-5.

Grau, S., Mateu-De, A. J., Alvarez-Lerma, F., Marin-Casino, M. & Torres-Rodriguez, J. M. (2006). Successful treatment of an Aspergillus fumigatus Infection with voriconazole via a nasogastric tube. *J Chemother,* 18(4)**,** 445-6.

Grazziutti, M., Przepiorka, D., Rex, J. H., Braunschweig, I., Vadhan-Raj, S. & Savary, C. A. (2001). Dendritic cell-mediated stimulation of the in vitro lymphocyte response to Aspergillus. *Bone Marrow Transplant,* 27(6)**,** 647-52.

Greer, N. D. (2003). Voriconazole: the newest triazole antifungal agent. *Proc (Bayl Univ Med Cent),* 16(2)**,** 241-8.

Gupta, P. R., Vyas, A., Meena, R. C., Sharma, S., Khayam, N., Subramanian, I. M., Kanoongo, D., Solanki, V. & Bansal, A. (2010). Clinical profile of pulmonary aspergilloma complicating residual tubercular cavitations in Northen Indian patients. *Lung India,* 27(4)**,** 209-11.

Hamamoto, H., Hasegawa, K., Nakaune, R., Lee, Y. J., Makizumi, Y., Akutsu, K. & Hibi, T. (2000). Tandem repeat of a transcriptional enhancer upstream of the sterol 14alpha-demethylase gene (CYP51) in Penicillium digitatum. *Appl Environ Microbiol,* 66(8)**,** 3421-6.

Hedayati, M. T., Pasqualotto, A. C., Warn, P. A., Bowyer, P. & Denning, D. W. (2007). Aspergillus flavus: human pathogen, allergen and mycotoxin producer. *Microbiology,* 153(Pt 6)**,** 1677-92.

Hendolin, P. H., Paulin, L., Koukila-Kahkola, P., Anttila, V. J., Malmberg, H., Richardson, M. & Ylikoski, J. (2000). Panfungal PCR and multiplex liquid hybridization for detection of fungi in tissue specimens. *J Clin Microbiol,* 38(11)**,** 4186-92.

Herbrecht, R. (2004). Voriconazole: therapeutic review of a new azole antifungal. *Expert Rev Anti Infect Ther,* 2(4)**,** 485-97.

Hernandez-Alles, S., Conejo, M., Pascual, A., Tomas, J. M., Benedi, V. J. & Martinez-Martinez, L. (2000). Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of Klebsiella pneumoniae. *J Antimicrob Chemother,* 46(2)**,** 273-7.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annu Rev Cell Biol,* 8**,** 67-113.

Hinrikson, H. P., Hurst, S. F., Lott, T. J., Warnock, D. W. & Morrison, C. J. (2005). Assessment of ribosomal large-subunit D1-D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important Aspergillus species. *J Clin Microbiol,* 43(5)**,** 2092-103.

Hong, S. B., Go, S. J., Shin, H. D., Frisvad, J. C. & Samson, R. A. (2005). Polyphasic taxonomy of Aspergillus fumigatus and related species. *Mycologia,* 97(6)**,** 1316-29.

Hong, S. B., Shin, H. D., Hong, J., Frisvad, J. C., Nielsen, P. V., Varga, J. & Samson, R. A. (2008). New taxa of Neosartorya and Aspergillus in Aspergillus section Fumigati. *Antonie Van Leeuwenhoek,* 93(1-2)**,** 87-98.

Hope, W. W., Walsh, T. J. & Denning, D. W. (2005a). The invasive and saprophytic syndromes due to Aspergillus spp. *Med Mycol,* 43 Suppl 1**,** S207-38.

Hope, W. W., Walsh, T. J. & Denning, D. W. (2005b). Laboratory diagnosis of invasive aspergillosis. *Lancet Infect Dis,* 5(10)**,** 609-22.

Howard, S. J. & Arendrup, M. C. (2011). Acquired antifungal drug resistance in Aspergillus fumigatus: epidemiology and detection. *Med Mycol,* 49 Suppl 1**,** S90-5.

Howard, S. J., Cerar, D., Anderson, M. J., Albarrag, A., Fisher, M. C., Pasqualotto, A. C., Laverdiere, M., Arendrup, M. C., Perlin, D. S. & Denning, D. W. (2009). Frequency and evolution of Azole resistance in Aspergillus fumigatus associated with treatment failure. *Emerg Infect Dis,* 15(7)**,** 1068-76.

Howard, S. J., Webster, I., Moore, C. B., Gardiner, R. E., Park, S., Perlin, D. S. & Denning, D. W. (2006). Multi-azole resistance in Aspergillus fumigatus. *Int J Antimicrob Agents,* 28(5)**,** 450-3.

Hu, W., Sillaots, S., Lemieux, S., Davison, J., Kauffman, S., Breton, A., Linteau, A., Xin, C., Bowman, J., Becker, J., Jiang, B. & Roemer, T. (2007). Essential gene identification and drug target prioritization in Aspergillus fumigatus. *PLoS Pathog,* 3(3)**,** e24.

Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S. & O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature,* 425(6959)**,** 686-91.

Hutchison, C. A., 3rd (2007). DNA sequencing: bench to bedside and beyond. *Nucleic Acids Res,* 35(18)**,** 6227-37.

Israel, H. L., Lenchner, G. S. & Atkinson, G. W. (1982). Sarcoidosis and aspergilloma. The role of surgery. *Chest,* 82(4)**,** 430-2.

Jensen, H. E. & Schonheyder, H. (1993). Experimental murine mycotic placentitis and abortion. A potent animal model (short communication). *J Exp Anim Sci,* 35(4)**,** 155-60.

Johnson, E. M., Oakley, K. L., Radford, S. A., Moore, C. B., Warn, P., Warnock, D. W. & Denning, D. W. (2000). Lack of correlation of in vitro amphotericin B susceptibility testing with outcome in a murine model of Aspergillus infection. *J Antimicrob Chemother,* 45(1)**,** 85-93.

Johnson, E. M., Szekely, A. & Warnock, D. W. (1998). In-vitro activity of voriconazole, itraconazole and amphotericin B against filamentous fungi. *J Antimicrob Chemother,* 42(6)**,** 741-5.

Joseph-Horne, T., Manning, N. J., Hollomon, D. & Kelly, S. L. (1995). Defective sterol delta 5(6) desaturase as a cause of azole resistance in Ustilago maydis. *FEMS Microbiol Lett,* 127(1-2)**,** 29-34.

Kahlmeter, G., Brown, D. F., Goldstein, F. W., MacGowan, A. P., Mouton, J. W., Odenholt, I., Rodloff, A., Soussy, C. J., Steinbakk, M., Soriano, F. & Stetsiouk, O. (2006). European Committee on Antimicrobial Susceptibility Testing (EUCAST) Technical Notes on antimicrobial susceptibility testing. *Clin Microbiol Infect,* 12(6)**,** 501-3.

Kedzierska, A., Kochan, P., Pietrzyk, A. & Kedzierska, J. (2007). Current status of fungal cell wall components in the immunodiagnostics of invasive fungal infections in humans: galactomannan, mannan and (1-->3)-beta-D-glucan antigens. *Eur J Clin Microbiol Infect Dis,* 26(11)**,** 755-66.

Kelly, S. L., Lamb, D. C., Corran, A. J., Baldwin, B. C. & Kelly, D. E. (1995). Mode of action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3 beta,6 alpha-diol. *Biochem Biophys Res Commun,* 207(3)**,** 910-5.

Kelly, S. L., Lamb, D. C., Kelly, D. E., Manning, N. J., Loeffler, J., Hebart, H., Schumacher, U. & Einsele, H. (1997). Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol delta5,6-desaturation. *FEBS Lett,* 400(1)**,** 80-2.

Kenri, T., Seto, S., Horino, A., Sasaki, Y., Sasaki, T. & Miyata, M. (2004). Use of fluorescent-protein tagging to determine the subcellular localization of mycoplasma pneumoniae proteins encoded by the cytadherence regulatory locus. *J Bacteriol,* 186(20)**,** 6944-55.

Khot, P. D., Ko, D. L., Hackman, R. C. & Fredricks, D. N. (2008). Development and optimization of quantitative PCR for the diagnosis of invasive aspergillosis with bronchoalveolar lavage fluid. *BMC Infect Dis,* 8**,** 73.

Kirkpatrick, W. R., McAtee, R. K., Fothergill, A. W., Rinaldi, M. G. & Patterson, T. F. (2000). Efficacy of voriconazole in a guinea pig model of disseminated invasive aspergillosis. *Antimicrob Agents Chemother,* 44(10)**,** 2865-8.

Kleinberg, M. (2006). What is the current and future status of conventional amphotericin B? *Int J Antimicrob Agents,* 27 Suppl 1**,** 12-6.
Knutsen, A. P., Bellone, C. & Kauffman, H. (2002). Immunopathogenesis of allergic bronchopulmonary aspergillosis in cystic fibrosis. *J Cyst Fibros,* 1(2)**,** 76-89.
Kontoyiannis, D. P. & Lewis, R. E. (2002). Antifungal drug resistance of pathogenic fungi. *Lancet,* 359(9312)**,** 1135-44.
Kostrikis, L. G., Tyagi, S., Mhlanga, M. M., Ho, D. D. & Kramer, F. R. (1998). Spectral genotyping of human alleles. *Science,* 279(5354)**,** 1228-9.
Kousha, M., Tadi, R. & Soubani, A. O. (2011). Pulmonary aspergillosis: a clinical review. *Eur Respir Rev,* 20(121)**,** 156-74.
Kozel, T. R. (1996). Activation of the complement system by pathogenic fungi. *Clin Microbiol Rev,* 9(1)**,** 34-46.
Kraemer, R., Delosea, N., Ballinari, P., Gallati, S. & Crameri, R. (2006). Effect of allergic bronchopulmonary aspergillosis on lung function in children with cystic fibrosis. *Am J Respir Crit Care Med,* 174(11)**,** 1211-20.
Kubodera, T., Yamashita, N. & Nishimura, A. (2000). Pyrithiamine resistance gene (ptrA) of Aspergillus oryzae: cloning, characterization and application as a dominant selectable marker for transformation. *Biosci Biotechnol Biochem,* 64(7)**,** 1416-21.
Kubodera, T., Yamashita, N. & Nishimura, A. (2002). Transformation of Aspergillus sp. and Trichoderma reesei using the pyrithiamine resistance gene (ptrA) of Aspergillus oryzae. *Biosci Biotechnol Biochem,* 66(2)**,** 404-6.
Kumar, A., Agarwal, S., Heyman, J. A., Matson, S., Heidtman, M., Piccirillo, S., Umansky, L., Drawid, A., Jansen, R., Liu, Y., Cheung, K. H., Miller, P., Gerstein, M., Roeder, G. S. & Snyder, M. (2002). Subcellular localization of the yeast proteome. *Genes Dev,* 16(6)**,** 707-19.
Kurup, V. P. & Kumar, A. (1991). Immunodiagnosis of aspergillosis. *Clin Microbiol Rev,* 4(4)**,** 439-56.
Lair-Fulleringer, S., Guillot, J., Desterke, C., Seguin, D., Warin, S., Bezille, A., Chermette, R. & Bretagne, S. (2003). Differentiation between isolates of Aspergillus fumigatus from breeding turkeys and their environment by genotyping with microsatellite markers. *J Clin Microbiol,* 41(4)**,** 1798-800.
Langfelder, K., Gattung, S. & Brakhage, A. A. (2002). A novel method used to delete a new Aspergillus fumigatus ABC transporter-encoding gene. *Curr Genet,* 41(4)**,** 268-74.
Lass-Florl C, C.-E. M., Denning DW, Rodriguez-Tudela J (2006). Antifungal susceptibility testing in Aspergillus spp. according to EUCAST methodology. *Medical Mycology,* 44**,** S319-S325.
Lass-Florl, C., Nagl, M., Speth, C., Ulmer, H., Dierich, M. P. & Wurzner, R. (2001). Studies of in vitro activities of voriconazole and itraconazole against Aspergillus hyphae using viability staining. *Antimicrob Agents Chemother,* 45(1)**,** 124-8.
Lass-Florl, C. & Perkhofer, S. (2008). In vitro susceptibility-testing in Aspergillus species. *Mycoses,* 51(5)**,** 437-46.
Latge, J. P. (1999). Aspergillus fumigatus and aspergillosis. *Clin Microbiol Rev,* 12(2)**,** 310-50.
Laufer, P., Fink, J. N., Bruns, W. T., Unger, G. F., Kalbfleisch, J. H., Greenberger, P. A. & Patterson, R. (1984). Allergic bronchopulmonary aspergillosis in cystic fibrosis. *J Allergy Clin Immunol,* 73(1 Pt 1)**,** 44-8.
Lay, M. J. & Wittwer, C. T. (1997). Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. *Clin Chem,* 43(12)**,** 2262-7.
Leenders, A., van Belkum, A., Janssen, S., de Marie, S., Kluytmans, J., Wielenga, J., Lowenberg, B. & Verbrugh, H. (1996). Molecular epidemiology of apparent outbreak of invasive aspergillosis in a hematology ward. *J Clin Microbiol,* 34(2)**,** 345-51.

Legendre, M., Pochet, N., Pak, T. & Verstrepen, K. J. (2007). Sequence-based estimation of minisatellite and microsatellite repeat variability. *Genome Res,* 17(12)**,** 1787-96.

Li, H., Barker, B. M., Grahl, N., Puttikamonkul, S., Bell, J. D., Craven, K. D. & Cramer, R. A., Jr. (2011). The small GTPase RacA mediates intracellular reactive oxygen species production, polarized growth, and virulence in the human fungal pathogen Aspergillus fumigatus. *Eukaryot Cell,* 10(2)**,** 174-86.

Li, R. K., Ciblak, M. A., Nordoff, N., Pasarell, L., Warnock, D. W. & McGinnis, M. R. (2000). In vitro activities of voriconazole, itraconazole, and amphotericin B against Blastomyces dermatitidis, Coccidioides immitis, and Histoplasma capsulatum. *Antimicrob Agents Chemother,* 44(6)**,** 1734-6.

Lin, D., Lehmann, P. F., Hamory, B. H., Padhye, A. A., Durry, E., Pinner, R. W. & Lasker, B. A. (1995). Comparison of three typing methods for clinical and environmental isolates of Aspergillus fumigatus. *J Clin Microbiol,* 33(6)**,** 1596-601.

Liu, W., May, G. S., Lionakis, M. S., Lewis, R. E. & Kontoyiannis, D. P. (2004). Extra copies of the Aspergillus fumigatus squalene epoxidase gene confer resistance to terbinafine: genetic approach to studying gene dose-dependent resistance to antifungals in A. fumigatus. *Antimicrob Agents Chemother,* 48(7)**,** 2490-6.

Liu, X. & Tan, W. (1999). A fiber-optic evanescent wave DNA biosensor based on novel molecular beacons. *Anal Chem,* 71(22)**,** 5054-9.

Lockhart, S. R., Frade, J. P., Etienne, K. A., Pfaller, M. A., Diekema, D. J. & Balajee, S. A. (2011). Azole resistance in Aspergillus fumigatus isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the cyp51A gene. *Antimicrob Agents Chemother,* 55(9)**,** 4465-8.

Loeffler, J. & Stevens, D. A. (2003). Antifungal drug resistance. *Clin Infect Dis,* 36(Suppl 1)**,** S31-41.

Loudon, K. W., Burnie, J. P., Coke, A. P. & Matthews, R. C. (1993). Application of polymerase chain reaction to fingerprinting Aspergillus fumigatus by random amplification of polymorphic DNA. *J Clin Microbiol,* 31(5)**,** 1117-21.

Loudon, K. W., Coke, A. P. & Burnie, J. P. (1995). "Pseudoclusters" and typing by random amplification of polymorphic DNA of Aspergillus fumigatus. *J Clin Pathol,* 48(2)**,** 183-4.

Loussert, C., Schmitt, C., Prevost, M. C., Balloy, V., Fadel, E., Philippe, B., Kauffmann-Lacroix, C., Latge, J. P. & Beauvais, A. (2010). In vivo biofilm composition of Aspergillus fumigatus. *Cell Microbiol,* 12(3)**,** 405-10.

Ma, Z., Proffer, T. J., Jacobs, J. L. & Sundin, G. W. (2006). Overexpression of the 14alpha-demethylase target gene (CYP51) mediates fungicide resistance in Blumeriella jaapii. *Appl Environ Microbiol,* 72(4)**,** 2581-5.

Mackay, I. M., Arden, K. E. & Nitsche, A. (2002). Real-time PCR in virology. *Nucleic Acids Res,* 30(6)**,** 1292-305.

Madan, T., Eggleton, P., Kishore, U., Strong, P., Aggrawal, S. S., Sarma, P. U. & Reid, K. B. (1997). Binding of pulmonary surfactant proteins A and D to Aspergillus fumigatus conidia enhances phagocytosis and killing by human neutrophils and alveolar macrophages. *Infect Immun,* 65(8)**,** 3171-9.

Maertens, J., Raad, I., Petrikkos, G., Boogaerts, M., Selleslag, D., Petersen, F. B., Sable, C. A., Kartsonis, N. A., Ngai, A., Taylor, A., Patterson, T. F., Denning, D. W. & Walsh, T. J. (2004). Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clin Infect Dis,* 39(11)**,** 1563-71.

Magee, B. B., D'Souza, T. M. & Magee, P. T. (1987). Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of Candida species. *J Bacteriol,* 169(4)**,** 1639-43.

Manavathu, E. K., Abraham, O. C. & Chandrasekar, P. H. (2001). Isolation and in vitro susceptibility to amphotericin B, itraconazole and posaconazole of voriconazole-resistant laboratory isolates of Aspergillus fumigatus. *Clin Microbiol Infect,* 7(3)**,** 130-7.

Manavathu, E. K., Cutright, J. L., Loebenberg, D. & Chandrasekar, P. H. (2000). A comparative study of the in vitro susceptibilities of clinical and laboratory-selected resistant isolates of Aspergillus spp. to amphotericin B, itraconazole, voriconazole and posaconazole (SCH 56592). *J Antimicrob Chemother,* 46(2)**,** 229-34.

Manavathu, E. K., Vazquez, J. A. & Chandrasekar, P. H. (1999). Reduced susceptibility in laboratory-selected mutants of Aspergillus fumigatus to itraconazole due to decreased intracellular accumulation of the antifungal agent. *Int J Antimicrob Agents,* 12(3)**,** 213-9.

Mann, P. A., Parmegiani, R. M., Wei, S. Q., Mendrick, C. A., Li, X., Loebenberg, D., DiDomenico, B., Hare, R. S., Walker, S. S. & McNicholas, P. M. (2003). Mutations in Aspergillus fumigatus resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase. *Antimicrob Agents Chemother,* 47(2)**,** 577-81.

Marichal, P., Vanden Bossche, H., Odds, F. C., Nobels, G., Warnock, D. W., Timmerman, V., Van Broeckhoven, C., Fay, S. & Mose-Larsen, P. (1997). Molecular biological characterization of an azole-resistant Candida glabrata isolate. *Antimicrob Agents Chemother,* 41(10)**,** 2229-37.

Matsuda, H., Kohno, S., Maesaki, S., Yamada, H., Koga, H., Tamura, M., Kuraishi, H. & Sugiyama, J. (1992). Application of ubiquinone systems and electrophoretic comparison of enzymes to identification of clinical isolates of Aspergillus fumigatus and several other species of Aspergillus. *J Clin Microbiol,* 30(8)**,** 1999-2005.

Matsuo, T. (1998). In situ visualization of messenger RNA for basic fibroblast growth factor in living cells. *Biochim Biophys Acta,* 1379(2)**,** 178-84.

Maxam, A. M. & Gilbert, W. (1977). A new method for sequencing DNA. *Proc Natl Acad Sci U S A,* 74(2)**,** 560-4.

Meletiadis, J., Mouton, J. W., Meis, J. F., Bouman, B. A. & Verweij, P. E. (2002). Comparison of the Etest and the sensititre colorimetric methods with the NCCLS proposed standard for antifungal susceptibility testing of Aspergillus species. *J Clin Microbiol,* 40(8)**,** 2876-85.

Mellado, E., Alcazar-Fuoli, L., García-Effrón, G., Alastruey-Izquierdo, A., Cuenca-Estrella, M. & Rodríguez-Tudela, J. L. (2006). New resistance mechanisms to azole drugs in Aspergillus fumigatus and emergence of antifungal drugs-resistant A. fumigatus atypical strains. *Medical Mycology,* 44**,** S367-S371.

Mellado, E., Diaz-Guerra, T. M., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. (2001). Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in Aspergillus fumigatus and other Aspergillus species. *J Clin Microbiol,* 39(7)**,** 2431-8.

Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. (2004). Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of Aspergillus fumigatus are responsible for resistance in vitro to azole antifungal drugs. *Antimicrob Agents Chemother,* 48(7)**,** 2747-50.

Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Melchers, W. J., Verweij, P. E., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. (2007). A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrob Agents Chemother,* 51(6)**,** 1897-904.

Mellado, E., Garcia-Effron, G., Buitrago, M. J., Alcazar-Fuoli, L., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. (2005). Targeted gene disruption of the 14-alpha sterol demethylase (cyp51A) in Aspergillus fumigatus and its role in azole drug susceptibility. *Antimicrob Agents Chemother,* 49(6)**,** 2536-8.

Mengoli, C., Cruciani, M., Barnes, R. A., Loeffler, J. & Donnelly, J. P. (2009). Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis. *Lancet Infect Dis,* 9(2)**,** 89-96.

Mondon, P., Brenier, M. P., Symoens, F., Rodriguez, E., Coursange, E., Chaib, F., Lebeau, B., Piens, M. A., Tortorano, A. M., Mallie, M., Chapuis, F., Carlotti, A., Villard, J., Viviani, M. A., Nolard, N., Bastide, J. M., Ambroise-Thomas, P. & Grillot, R. (1997). Molecular typing of Aspergillus fumigatus strains by sequence-specific DNA primer (SSDP) analysis. *FEMS Immunol Med Microbiol,* 17(2)**,** 95-102.

Moore, C. B., Sayers, N., Mosquera, J., Slaven, J. & Denning, D. W. (2000). Antifungal drug resistance in Aspergillus. *J Infect,* 41(3)**,** 203-20.

Morschhauser, J. (2010). Regulation of multidrug resistance in pathogenic fungi. *Fungal Genet Biol,* 47(2)**,** 94-106.

Mosquera, J. & Denning, D. W. (2002). Azole cross-resistance in Aspergillus fumigatus. *Antimicrob Agents Chemother,* 46(2)**,** 556-7.

Mosquera, J., Warn, P. A., Morrissey, J., Moore, C. B., Gil-Lamaignere, C. & Denning, D. W. (2001). Susceptibility testing of Aspergillus flavus: inoculum dependence with itraconazole and lack of correlation between susceptibility to amphotericin B in vitro and outcome in vivo. *Antimicrob Agents Chemother,* 45(5)**,** 1456-62.

Mouas, H., Lutsar, I., Dupont, B., Fain, O., Herbrecht, R., Lescure, F. X. & Lortholary, O. (2005). Voriconazole for invasive bone aspergillosis: a worldwide experience of 20 cases. *Clin Infect Dis,* 40(8)**,** 1141-7.

Mueller, U. G. & Wolfenbarger, L. L. (1999). AFLP genotyping and fingerprinting. *Trends Ecol Evol,* 14(10)**,** 389-394.

Mullins, J., Hutcheson, P. S. & Slavin, R. G. (1984). Aspergillus fumigatus spore concentration in outside air: Cardiff and St Louis compared. *Clin Allergy,* 14(4)**,** 351-4.

Munn, A. L., Heese-Peck, A., Stevenson, B. J., Pichler, H. & Riezman, H. (1999). Specific sterols required for the internalization step of endocytosis in yeast. *Mol Biol Cell,* 10(11)**,** 3943-57.

Nascimento, A. M., Goldman, G. H., Park, S., Marras, S. A., Delmas, G., Oza, U., Lolans, K., Dudley, M. N., Mann, P. A. & Perlin, D. S. (2003). Multiple resistance mechanisms among Aspergillus fumigatus mutants with high-level resistance to itraconazole. *Antimicrob Agents Chemother,* 47(5)**,** 1719-26.

Nei, M., and S. Kumar (2000). *Molecular Evolution and Phylogenetics*. Oxford and New York: Oxford University Press

Nierman, W. C., May, G., Kim, H. S., Anderson, M. J., Chen, D. & Denning, D. W. (2005a). What the Aspergillus genomes have told us. *Med Mycol,* 43 Suppl 1**,** S3-5.

Nierman, W. C., Pain, A., Anderson, M. J., Wortman, J. R., Kim, H. S., Arroyo, J., Berriman, M., Abe, K., Archer, D. B., Bermejo, C., Bennett, J., Bowyer, P., Chen, D., Collins, M., Coulsen, R., Davies, R., Dyer, P. S., Farman, M., Fedorova, N., Feldblyum, T. V., Fischer, R., Fosker, N., Fraser, A., Garcia, J. L., Garcia, M. J., Goble, A., Goldman, G. H., Gomi, K., Griffith-Jones, S., Gwilliam, R., Haas, B., Haas, H., Harris, D., Horiuchi, H., Huang, J., Humphray, S., Jimenez, J., Keller, N., Khouri, H., Kitamoto, K., Kobayashi, T., Konzack, S., Kulkarni, R., Kumagai, T., Lafon, A., Latge, J. P., Li, W., Lord, A., Lu, C., Majoros, W. H., May, G. S., Miller, B. L., Mohamoud, Y., Molina, M., Monod, M., Mouyna, I., Mulligan, S., Murphy, L., O'Neil, S., Paulsen, I., Penalva, M. A., Pertea, M., Price, C., Pritchard, B. L., Quail, M. A., Rabbinowitsch, E., Rawlins, N., Rajandream, M. A.,

Reichard, U., Renauld, H., Robson, G. D., Rodriguez de Cordoba, S., Rodriguez-Pena, J. M., Ronning, C. M., Rutter, S., Salzberg, S. L., Sanchez, M., Sanchez-Ferrero, J. C., Saunders, D., Seeger, K., Squares, R., Squares, S., Takeuchi, M., Tekaia, F., Turner, G., Vazquez de Aldana, C. R., Weidman, J., White, O., Woodward, J., Yu, J. H., Fraser, C., Galagan, J. E., Asai, K., Machida, M., Hall, N., Barrell, B. & Denning, D. W. (2005b). Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. *Nature,* 438(7071)**,** 1151-6.

O'Donnell, K., Sutton, D. A., Fothergill, A., McCarthy, D., Rinaldi, M. G., Brandt, M. E., Zhang, N. & Geiser, D. M. (2008). Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the Fusarium solani species complex. *J Clin Microbiol,* 46(8)**,** 2477-90.

O'Gorman, C. M., Fuller, H. T. & Dyer, P. S. (2009). Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. *Nature,* 457(7228)**,** 471-4.

Oakley, K. L., Moore, C. B. & Denning, D. W. (1998). In-vitro activity of voriconazole against Aspergillus spp. and comparison with itraconazole and amphotericin B. *J Antimicrob Chemother,* 42(1)**,** 91-4.

Ortiz, E., Estrada, G. & Lizardi, P. M. (1998). PNA molecular beacons for rapid detection of PCR amplicons. *Mol Cell Probes,* 12(4)**,** 219-26.

Osherov, N., Kontoyiannis, D. P., Romans, A. & May, G. S. (2001). Resistance to itraconazole in Aspergillus nidulans and Aspergillus fumigatus is conferred by extra copies of the A. nidulans P-450 14alpha-demethylase gene, pdmA. *J Antimicrob Chemother,* 48(1)**,** 75-81.

Ostrosky-Zeichner, L. (2004). Novel approaches to antifungal prophylaxis. *Expert Opin Investig Drugs,* 13(6)**,** 665-72.

Pao, S. S., Paulsen, I. T. & Saier, M. H., Jr. (1998). Major facilitator superfamily. *Microbiol Mol Biol Rev,* 62(1)**,** 1-34.

Paphitou, N. I., Ostrosky-Zeichner, L., Paetznick, V. L., Rodriguez, J. R., Chen, E. & Rex, J. H. (2002). In vitro activities of investigational triazoles against Fusarium species: effects of inoculum size and incubation time on broth microdilution susceptibility test results. *Antimicrob Agents Chemother,* 46(10)**,** 3298-300.

Patel, R. & Paya, C. V. (1997). Infections in solid-organ transplant recipients. *Clin Microbiol Rev,* 10(1)**,** 86-124.

Paterson, P. J., Seaton, S., Prentice, H. G. & Kibbler, C. C. (2003). Treatment failure in invasive aspergillosis: susceptibility of deep tissue isolates following treatment with amphotericin B. *J Antimicrob Chemother,* 52(5)**,** 873-6.

Patterson, T. F., Fothergill, A. W. & Rinaldi, M. G. (1993). Efficacy of itraconazole solution in a rabbit model of invasive aspergillosis. *Antimicrob Agents Chemother,* 37(11)**,** 2307-10.

Patterson, T. F., Miniter, P., Ryan, J. L. & Andriole, V. T. (1988). Effect of immunosuppression and amphotericin B on Aspergillus antigenemia in an experimental model. *J Infect Dis,* 158(2)**,** 415-22.

Petrikkos, G. & Skiada, A. (2007). Recent advances in antifungal chemotherapy. *Int J Antimicrob Agents,* 30(2)**,** 108-17.

Petti, C. A., Polage, C. R. & Schreckenberger, P. (2005). The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. *J Clin Microbiol,* 43(12)**,** 6123-5.

Pfaller, M. A., Messer, S. A., Boyken, L., Rice, C., Tendolkar, S., Hollis, R. J. & Diekema, D. J. (2008). In vitro survey of triazole cross-resistance among more than 700 clinical isolates of Aspergillus species. *J Clin Microbiol,* 46(8)**,** 2568-72.

Pfaller, M. A., Messer, S. A., Hollis, R. J. & Jones, R. N. (2002). Antifungal activities of posaconazole, ravuconazole, and voriconazole compared to those of itraconazole and

amphotericin B against 239 clinical isolates of Aspergillus spp. and other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. *Antimicrob Agents Chemother,* 46(4)**,** 1032-7.

Pfeiffer, C. D., Fine, J. P. & Safdar, N. (2006). Diagnosis of invasive aspergillosis using a galactomannan assay: a meta-analysis. *Clin Infect Dis,* 42(10)**,** 1417-27.

Piatek, A. S., Tyagi, S., Pol, A. C., Telenti, A., Miller, L. P., Kramer, F. R. & Alland, D. (1998). Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis. *Nat Biotechnol,* 16(4)**,** 359-63.

Powderly, W. G., Kobayashi, G. S., Herzig, G. P. & Medoff, G. (1988). Amphotericin B-resistant yeast infection in severely immunocompromised patients. *Am J Med,* 84(5)**,** 826-32.

Prasad, R., Panwar, S. L. & Smriti (2002). Drug resistance in yeasts--an emerging scenario. *Adv Microb Physiol,* 46**,** 155-201.

Promper, C., Schneider, R. & Weiss, H. (1993). The role of the proton-pumping and alternative respiratory chain NADH:ubiquinone oxidoreductases in overflow catabolism of Aspergillus niger. *Eur J Biochem,* 216(1)**,** 223-30.

Punt, P. J., Oliver, R. P., Dingemanse, M. A., Pouwels, P. H. & van den Hondel, C. A. (1987). Transformation of Aspergillus based on the hygromycin B resistance marker from Escherichia coli. *Gene,* 56(1)**,** 117-24.

Qiao, J., Liu, W. & Li, R. (2008). Antifungal resistance mechanisms of Aspergillus. *Nihon Ishinkin Gakkai Zasshi,* 49(3)**,** 157-63.

Raclasky, V., Trtkova, J., Ruskova, L., Buchta, V., Bolehovska, R., Vackova, M. & Hamal, P. (2006). Primer R108 performs best in the RAPD strain typing of three Aspergillus species frequently isolated from patients. *Folia Microbiol (Praha),* 51(2)**,** 136-40.

Rajendran, R., Mowat, E., McCulloch, E., Lappin, D. F., Jones, B., Lang, S., Majithiya, J. B., Warn, P., Williams, C. & Ramage, G. (2011). Azole resistance of Aspergillus fumigatus biofilms is partly associated with efflux pump activity. *Antimicrob Agents Chemother,* 55(5)**,** 2092-7.

Rementeria, A., Lopez-Molina, N., Ludwig, A., Vivanco, A. B., Bikandi, J., Ponton, J. & Garaizar, J. (2005). Genes and molecules involved in Aspergillus fumigatus virulence. *Rev Iberoam Micol,* 22(1)**,** 1-23.

Rex, J. H., Pfaller, M. A., Walsh, T. J., Chaturvedi, V., Espinel-Ingroff, A., Ghannoum, M. A., Gosey, L. L., Odds, F. C., Rinaldi, M. G., Sheehan, D. J. & Warnock, D. W. (2001). Antifungal susceptibility testing: practical aspects and current challenges. *Clin Microbiol Rev,* 14(4)**,** 643-58, table of contents.

Ribeiro, M. A. & Paula, C. R. (2007). Up-regulation of ERG11 gene among fluconazole-resistant Candida albicans generated in vitro: is there any clinical implication? *Diagn Microbiol Infect Dis,* 57(1)**,** 71-5.

Richard, J. L., Dvorak, T. J. & Ross, P. F. (1996). Natural occurrence of gliotoxin in turkeys infected with Aspergillus fumigatus, Fresenius. *Mycopathologia,* 134(3)**,** 167-70.

Richardson, M. D. (2005). Changing patterns and trends in systemic fungal infections. *J Antimicrob Chemother,* 56 Suppl 1**,** i5-i11.

Richardson, M. D., Stubbins, J. M. & Warnock, D. W. (1982). Rapid enzyme-linked immunosorbent assay (ELISA) for Aspergillus fumigatus antibodies. *J Clin Pathol,* 35(10)**,** 1134-7.

Rinyu, E., Varga, J. & Ferenczy, L. (1995). Phenotypic and genotypic analysis of variability in Aspergillus fumigatus. *J Clin Microbiol,* 33(10)**,** 2567-75.

Rippon, J. W. (1982). *Medical mycology : the pathogenic fungi and the pathogenic actinomycetes*. Philadelphia London, Saunders.

Rodriguez-Tudela, J. L., Alcazar-Fuoli, L., Mellado, E., Alastruey-Izquierdo, A., Monzon, A. & Cuenca-Estrella, M. (2008). Epidemiological cutoffs and cross-resistance to azole drugs in Aspergillus fumigatus. *Antimicrob Agents Chemother,* 52(7)**,** 2468-72.

Ronning, C. M., Fedorova, N. D., Bowyer, P., Coulson, R., Goldman, G., Kim, H. S., Turner, G., Wortman, J. R., Yu, J., Anderson, M. J., Denning, D. W. & Nierman, W. C. (2005). Genomics of Aspergillus fumigatus. *Rev Iberoam Micol,* 22(4)**,** 223-8.

Rosenberg, M., Patterson, R., Mintzer, R., Cooper, B. J., Roberts, M. & Harris, K. E. (1977). Clinical and immunologic criteria for the diagnosis of allergic bronchopulmonary aspergillosis. *Ann Intern Med,* 86(4)**,** 405-14.

Ross, C. F. (1951). A case of pulmonary aspergillosis. *J Pathol Bacteriol,* 63(3)**,** 409-16.

Rozen, S. & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol,* 132**,** 365-86.

Ruan, B., Lai, P. S., Yeh, C. W., Wilson, W. K., Pang, J., Xu, R., Matsuda, S. P. & Schroepfer, G. J., Jr. (2002). Alternative pathways of sterol synthesis in yeast. Use of C(27) sterol tracers to study aberrant double-bond migrations and evaluate their relative importance. *Steroids,* 67(13-14)**,** 1109-19.

Sabatelli, F., Patel, R., Mann, P. A., Mendrick, C. A., Norris, C. C., Hare, R., Loebenberg, D., Black, T. A. & McNicholas, P. M. (2006). In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. *Antimicrob Agents Chemother,* 50(6)**,** 2009-15.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol,* 4(4)**,** 406-25.

Samson, R. A. & Varga, J. (2009). What is a species in Aspergillus? *Med Mycol,* 47 Suppl 1**,** S13-20.

Sanger, F. & Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol,* 94(3)**,** 441-8.

Sanglard, D., Coste, A. & Ferrari, S. (2009). Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation. *FEMS Yeast Res,* 9(7)**,** 1029-50.

Sanglard, D., Ischer, F., Monod, M. & Bille, J. (1996). Susceptibilities of Candida albicans multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob Agents Chemother,* 40(10)**,** 2300-5.

Sanglard, D., Ischer, F., Monod, M. & Bille, J. (1997). Cloning of Candida albicans genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. *Microbiology,* 143 (Pt 2)**,** 405-16.

SAST (2007). Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds.

Schaffner, A. (1989). Experimental basis for the clinical epidemiology of fungal infections. A review. *Mycoses,* 32(10)**,** 499-515.

Schaffner, A., Douglas, H., Braude, A. I. & Davis, C. E. (1983). Killing of Aspergillus spores depends on the anatomical source of the macrophage. *Infect Immun,* 42(3)**,** 1109-15.

Schmittgen, T. D. & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc,* 3(6)**,** 1101-8.

Schnabel, G. & Jones, A. L. (2001). The 14alpha-Demethylasse(CYP51A1) Gene is Overexpressed in Venturia inaequalis Strains Resistant to Myclobutanil. *Phytopathology,* 91(1)**,** 102-10.

Schwarz, P., Bretagne, S., Gantier, J. C., Garcia-Hermoso, D., Lortholary, O., Dromer, F. & Dannaoui, E. (2006). Molecular identification of zygomycetes from culture and experimentally infected tissues. *J Clin Microbiol,* 44(2)**,** 340-9.

Scott, M. S., Calafell, S. J., Thomas, D. Y. & Hallett, M. T. (2005). Refining protein subcellular localization. *PLoS Comput Biol,* 1(6)**,** e66.

Selmecki, A., Forche, A. & Berman, J. (2006). Aneuploidy and isochromosome formation in drug-resistant Candida albicans. *Science,* 313(5785)**,** 367-70.

Shao, P. L., Huang, L. M. & Hsueh, P. R. (2007). Recent advances and challenges in the treatment of invasive fungal infections. *Int J Antimicrob Agents,* 30(6)**,** 487-95.

Shevchuk, N. A., Bryksin, A. V., Nusinovich, Y. A., Cabello, F. C., Sutherland, M. & Ladisch, S. (2004). Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. *Nucleic Acids Res,* 32(2)**,** e19.

Simpson, D. A., Feeney, S., Boyle, C. & Stitt, A. W. (2000). Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. *Mol Vis,* 6**,** 178-83.

Slaven, J. W., Anderson, M. J., Sanglard, D., Dixon, G. K., Bille, J., Roberts, I. S. & Denning, D. W. (2002). Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. *Fungal Genet Biol,* 36(3)**,** 199-206.

SNEATH, P. H. A. a. R. R. S. (1973). Numerical taxonomy. W. H. Freeman and Company, San Francisco. SOURDIS, J., and M. NEI. 1988. Relative efficiencies of the maximum parsimony and distance-matrix methods in ob- taining the correct phylogenetic tree. Mol. Biol. Evol. 5: 298-311. SWOFFORD, D.

Snelders, E., van der Lee, H. A., Kuijpers, J., Rijs, A. J., Varga, J., Samson, R. A., Mellado, E., Donders, A. R., Melchers, W. J. & Verweij, P. E. (2008). Emergence of azole resistance in Aspergillus fumigatus and spread of a single resistance mechanism. *PLoS Med,* 5(11)**,** e219.

Spiess, B., Buchheidt, D., Baust, C., Skladny, H., Seifarth, W., Zeilfelder, U., Leib-Mosch, C., Morz, H. & Hehlmann, R. (2003). Development of a LightCycler PCR assay for detection and quantification of Aspergillus fumigatus DNA in clinical samples from neutropenic patients. *J Clin Microbiol,* 41(5)**,** 1811-8.

Steinbach, W. J. & Stevens, D. A. (2003). Review of newer antifungal and immunomodulatory strategies for invasive aspergillosis. *Clin Infect Dis,* 37 Suppl 3**,** S157-87.

Sterling, T. R. & Merz, W. G. (1998). Resistance to amphotericin B: emerging clinical and microbiological patterns. *Drug Resist Updat,* 1(3)**,** 161-5.

Stiefel, M., Reiss, T., Staege, M. S., Rengelshausen, J., Burhenne, J., Wawer, A. & Foell, J. L. (2007). Successful treatment with voriconazole of Aspergillus brain abscess in a boy with medulloblastoma. *Pediatr Blood Cancer,* 49(2)**,** 203-7.

Takacs, T., Jeney, C., Kovacs, L., Mozes, J., Benczik, M. & Sebe, A. (2008). Molecular beacon-based real-time PCR method for detection of 15 high-risk and 5 low-risk HPV types. *J Virol Methods,* 149(1)**,** 153-62.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol,* 24(8)**,** 1596-9.

Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci U S A,* 101(30)**,** 11030-5.

Tan, W., Fang, X., Li, J. & Liu, X. (2000). Molecular beacons: a novel DNA probe for nucleic acid and protein studies. *Chemistry,* 6(7)**,** 1107-11.

Tekaia, F. & Latge, J. P. (2005). Aspergillus fumigatus: saprophyte or pathogen? *Curr Opin Microbiol,* 8(4)**,** 385-92.

Thelwell, N., Millington, S., Solinas, A., Booth, J. & Brown, T. (2000). Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res,* 28(19)**,** 3752-61.

Tilden, E. B., Hatton, E. H., Freeman, S., Williamson, W. M. & Koenig, V. L. (1961). Preparation and properties of the endotoxins of Aspergillus fumigatus and Aspergillus flavus. *Mycopathologia,* 14**,** 325-46.

Tobin, M. B., Peery, R. B. & Skatrud, P. L. (1997). Genes encoding multiple drug resistance-like proteins in Aspergillus fumigatus and Aspergillus flavus. *Gene,* 200(1-2)**,** 11-23.

Toews, M. W., Warmbold, J., Konzack, S., Rischitor, P., Veith, D., Vienken, K., Vinuesa, C., Wei, H. & Fischer, R. (2004). Establishment of mRFP1 as a fluorescent marker in Aspergillus nidulans and construction of expression vectors for high-throughput protein tagging using recombination in vitro (GATEWAY). *Curr Genet,* 45(6)**,** 383-9.

Tomee, J. F., Kauffman, H. F., Klimp, A. H., de Monchy, J. G., Koeter, G. H. & Dubois, A. E. (1994). Immunologic significance of a collagen-derived culture filtrate containing proteolytic activity in Aspergillus-related diseases. *J Allergy Clin Immunol,* 93(4)**,** 768-78.

Traunmuller, F., Popovic, M., Konz, K. H., Smolle-Juttner, F. M. & Joukhadar, C. (2011). Efficacy and safety of current drug therapies for invasive aspergillosis. *Pharmacology,* 88(3-4)**,** 213-24.

Tuon, F. F. (2007). A systematic literature review on the diagnosis of invasive aspergillosis using polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples. *Rev Iberoam Micol,* 24(2)**,** 89-94.

Tyagi, S., Bratu, D. P. & Kramer, F. R. (1998). Multicolor molecular beacons for allele discrimination. *Nat Biotechnol,* 16(1)**,** 49-53.

Tyagi, S. & Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol,* 14(3)**,** 303-8.

van Belkum, A., Melchers, W. J., Ijsseldijk, C., Nohlmans, L., Verbrugh, H. & Meis, J. F. (1997). Outbreak of amoxicillin-resistant Haemophilus influenzae type b: variable number of tandem repeats as novel molecular markers. *J Clin Microbiol,* 35(6)**,** 1517-20.

van Belkum, A., Quint, W. G., de Pauw, B. E., Melchers, W. J. & Meis, J. F. (1993). Typing of Aspergillus species and Aspergillus fumigatus isolates by interrepeat polymerase chain reaction. *J Clin Microbiol,* 31(9)**,** 2502-5.

van Belkum, A., Scherer, S., van Alphen, L. & Verbrugh, H. (1998). Short-sequence DNA repeats in prokaryotic genomes. *Microbiol Mol Biol Rev,* 62(2)**,** 275-93.

Van Cutsem, J., Van Gerven, F., Van de Ven, M. A., Borgers, M. & Janssen, P. A. (1984). Itraconazole, a new triazole that is orally active in aspergillosis. *Antimicrob Agents Chemother,* 26(4)**,** 527-34.

van den Brink, H. J., van Nistelrooy, H. J., de Waard, M. A., van den Hondel, C. A. & van Gorcom, R. F. (1996). Increased resistance to 14 alpha-demethylase inhibitors (DMIs) in Aspergillus niger by coexpression of the Penicillium italicum eburicol 14 alpha-demethylase (cyp51) and the A. niger cytochrome P450 reductase (cprA) genes. *J Biotechnol,* 49(1-3)**,** 13-8.

van den Hazel, H. B., Pichler, H., do Valle Matta, M. A., Leitner, E., Goffeau, A. & Daum, G. (1999). PDR16 and PDR17, two homologous genes of Saccharomyces cerevisiae, affect lipid biosynthesis and resistance to multiple drugs. *J Biol Chem,* 274(4)**,** 1934-41.

Van Der Linden, J. W., Warris, A. & Verweij, P. E. (2011). Aspergillus species intrinsically resistant to antifungal agents. *Med Mycol,* 49 Suppl 1**,** S82-9.

Vassiloyanakopoulos, A., Falagas, M. E., Allamani, M. & Michalopoulos, A. (2006). Aspergillus fumigatus tricuspid native valve endocarditis in a non-intravenous drug user. *J Med Microbiol,* 55(Pt 5)**,** 635-8.

Velez, J. D., Allendoerfer, R., Luther, M., Rinaldi, M. G. & Graybill, J. R. (1993). Correlation of in vitro azole susceptibility with in vivo response in a murine model of cryptococcal meningitis. *J Infect Dis,* 168(2)**,** 508-10.

Verweij, P. E., Howard, S. J., Melchers, W. J. & Denning, D. W. (2009a). Azole-resistance in Aspergillus: proposed nomenclature and breakpoints. *Drug Resist Updat,* 12(6)**,** 141-7.

Verweij, P. E., Meis, J. F., Sarfati, J., Hoogkamp-Korstanje, J. A., Latge, J. P. & Melchers, W. J. (1996). Genotypic characterization of sequential Aspergillus fumigatus isolates from patients with cystic fibrosis. *J Clin Microbiol,* 34(10)**,** 2595-7.

Verweij, P. E., Oakley, K. L., Morrissey, J., Morrissey, G. & Denning, D. W. (1998). Efficacy of LY303366 against amphotericin B-susceptible and -resistant Aspergillus fumigatus in a murine model of invasive aspergillosis. *Antimicrob Agents Chemother,* 42(4)**,** 873-8.

Verweij, P. E., Snelders, E., Kema, G. H., Mellado, E. & Melchers, W. J. (2009b). Azole resistance in Aspergillus fumigatus: a side-effect of environmental fungicide use? *Lancet Infect Dis,* 9(12)**,** 789-95.

Verweij, P. E., Te Dorsthorst, D. T., Rijs, A. J., De Vries-Hospers, H. G. & Meis, J. F. (2002). Nationwide survey of in vitro activities of itraconazole and voriconazole against clinical Aspergillus fumigatus isolates cultured between 1945 and 1998. *J Clin Microbiol,* 40(7)**,** 2648-50.

von Eiff, M., Roos, N., Schulten, R., Hesse, M., Zuhlsdorf, M. & van de Loo, J. (1995). Pulmonary aspergillosis: early diagnosis improves survival. *Respiration,* 62(6)**,** 341-7.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & et al. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res,* 23(21)**,** 4407-14.

Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J,* 1(8)**,** 945-51.

Walsh, T. J., Anaissie, E. J., Denning, D. W., Herbrecht, R., Kontoyiannis, D. P., Marr, K. A., Morrison, V. A., Segal, B. H., Steinbach, W. J., Stevens, D. A., van Burik, J. A., Wingard, J. R. & Patterson, T. F. (2008). Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clin Infect Dis,* 46(3)**,** 327-60.

Wang, L., Yokoyama, K., Miyaji, M. & Nishimura, K. (2000). Mitochondrial cytochrome b gene analysis of Aspergillus fumigatus and related species. *J Clin Microbiol,* 38(4)**,** 1352-8.

Wanger, A., Mills, K., Nelson, P. W. & Rex, J. H. (1995). Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B-resistant Candida isolates. *Antimicrob Agents Chemother,* 39(11)**,** 2520-2.

Warrilow, A. G., Melo, N., Martel, C. M., Parker, J. E., Nes, W. D., Kelly, S. L. & Kelly, D. E. (2010). Expression, purification, and characterization of Aspergillus fumigatus sterol 14-alpha demethylase (CYP51) isoenzymes A and B. *Antimicrob Agents Chemother,* 54(10)**,** 4225-34.

Warris, A., Klaassen, C. H., Meis, J. F., De Ruiter, M. T., De Valk, H. A., Abrahamsen, T. G., Gaustad, P. & Verweij, P. E. (2003). Molecular epidemiology of Aspergillus fumigatus isolates recovered from water, air, and patients shows two clusters of genetically distinct strains. *J Clin Microbiol,* 41(9)**,** 4101-6.

Weinberger, M., Elattar, I., Marshall, D., Steinberg, S. M., Redner, R. L., Young, N. S. & Pizzo, P. A. (1992). Patterns of infection in patients with aplastic anemia and the emergence of Aspergillus as a major cause of death. *Medicine (Baltimore),* 71(1)**,** 24-43.

Weiner, M. H. & Coats-Stephen, M. (1979). Immunodiagnosis of systemic aspergillosis. I. Antigenemia detected by radioimmunoassay in experimental infection. *J Lab Clin Med,* 93(1)**,** 111-9.

Whitcombe, D., Theaker, J., Guy, S. P., Brown, T. & Little, S. (1999). Detection of PCR products using self-probing amplicons and fluorescence. *Nat Biotechnol,* 17(8)**,** 804-7.

Wills, J. W., Troutman, W. B. & Riggsby, W. S. (1985). Circular mitochondrial genome of Candida albicans contains a large inverted duplication. *J Bacteriol,* 164(1)**,** 7-13.

Wong, M. L. & Medrano, J. F. (2005). Real-time PCR for mRNA quantitation. *Biotechniques,* 39(1)**,** 75-85.

Woodcock, A. A., Steel, N., Moore, C. B., Howard, S. J., Custovic, A. & Denning, D. W. (2006). Fungal contamination of bedding. *Allergy,* 61(1)**,** 140-2.

Xi, C., Balberg, M., Boppart, S. A. & Raskin, L. (2003). Use of DNA and peptide nucleic acid molecular beacons for detection and quantification of rRNA in solution and in whole cells. *Appl Environ Microbiol,* 69(9)**,** 5673-8.

Xiao, L., Madison, V., Chau, A. S., Loebenberg, D., Palermo, R. E. & McNicholas, P. M. (2004). Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from Aspergillus fumigatus and Candida albicans provide insights into posaconazole binding. *Antimicrob Agents Chemother,* 48(2)**,** 568-74.

Yaguchi, T., Horie, Y., Tanaka, R., Matsuzawa, T., Ito, J. & Nishimura, K. (2007). Molecular phylogenetics of multiple genes on Aspergillus section Fumigati isolated from clinical specimens in Japan. *Nihon Ishinkin Gakkai Zasshi,* 48(1)**,** 37-46.

Zander, D. S. (2005). Allergic bronchopulmonary aspergillosis: an overview. *Arch Pathol Lab Med,* 129(7)**,** 924-8.

Zhanel, G. G., Karlowsky, J. A., Harding, G. A., Balko, T. V., Zelenitsky, S. A., Friesen, M., Kabani, A., Turik, M. & Hoban, D. J. (1997). In vitro activity of a new semisynthetic echinocandin, LY-303366, against systemic isolates of Candida species, Cryptococcus neoformans, Blastomyces dermatitidis, and Aspergillus species. *Antimicrob Agents Chemother,* 41(4)**,** 863-5.

Zhang, N., O'Donnell, K., Sutton, D. A., Nalim, F. A., Summerbell, R. C., Padhye, A. A. & Geiser, D. M. (2006). Members of the Fusarium solani species complex that cause infections in both humans and plants are common in the environment. *J Clin Microbiol,* 44(6)**,** 2186-90.

Zmeili, O. S. & Soubani, A. O. (2007). Pulmonary aspergillosis: a clinical update. *QJM,* 100(6)**,** 317-34.

10.0 Appendices

Appendix, A

ABC11PtrA ABC 11

CAGGACCTTCTTGAGTTGCTGTCATGTTGTATACGGTGTAACTGTTCTCTATACCTTCGACGGTGTGGTCTATTTTAG
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CTCGACATGAACTCGGCCGAGGATGCCATCGTCAAGAACACCCGCGAGGTTACTAAGGGCTTGATAATCGGC
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ACCCGAATGGGTTCAGTGCATGAACCGGATTTGTCTTACGGTCTTTGACGATAGGGGAATGATGATTATGTGA
TAGTTCTGAGATTTGAATGAACTCGTTAGCTCGTAATCCACATGCATATGTAAATGGCTGTGTCCCGTATGTAA
CGGTGGGGCATTCTAGAATAATTATGTGTAACAAGAAAGACAGTATAATACAAACAAAGATGCAAGAGCGGCT
CATCGTCACCCCATGATAGAAGGGTTCGACGTGACAGCCTGACCTCCTTCCCTTCTAGCTCCTTCGTGGATATTTAA
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ACTTCGTGTCACGGCTTGCTCGTGTGTACACGCTTGGTACATGCACATACATATACACTTTTGCTCTCCTTTGCTTCTTT
GGATATACCTTACCGATTTTGAATTATCCTCTCGTCTTCTGGGTCAGATTCTTAGATTTTAGCATACTGCGGACATTTC
CAGCGGTGACACAATATAGACACATTATGTCATATCATCTAGTTTATAAAAAACAAAAATCTTTTGAAGTATGCTCTG
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GCATGCAGCCCTGCAGCTGGATCTACCATGTGAACGCCTCTTCGACAAAATCGACCACCGCGCCCTTCCCGATGTTAT
TTCGCGCCGCGGCGCGGACCTCATCTGCAAAGGCGCCCGGCCCACACACGGAGACGATGGTTGCGCCGACGCGTTTG
GGGATGACTTCACTCAGGACGACGTCGGGGCGGCAGCGGCCCGGGAACATCTGGACCGTGGCGCTGGGGGAGACGA
TCTCCCGG**CTGCTCTTGGGTTTGGAGAC**

Appendix, B

MFS56PtrAMFS56

AGTGTTGCCGAAATCGAGGGCAGTGCCTCCGCCGCCGCGCTCGCCGAGCTCATGAACAACCCAGAAGCAGGGTGATA
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Appendix, C

M85PtrAM85

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