Genetic and virulence variation of the population of environmental and clinical isolates of the pathogenic *Aspergillus fumigatus*

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Abbreviations

Acquired immune deficiency (AIDS)

Allergic aspergillosis sinusitis (AAS)

Allergic bronchopulmonary aspergillosis (ABPA).

Alveolar macrophages (AMs)

Arbitary primed PCR (AP-PCR)

Chronic obstructive pulmonary disease (COPD)

Chronic pulmonary aspergillosis (CPA)

Colony forming unit (CFU)

Colony radial growth rate (Kr)

Complementary DNA (cDNA)

Cell Blood count (CBC)

Deoxyribonucleic acid (DNA) Diethylpyrocarbonate (DEPC)

Ethylenediaminetetra-acitic acid disodium salt (EDTA)

Genomic deoxyribonucleic acid (gDNA)

Gliotoxin (GT) Glycosylphosphatidylinositol (GPI)

Invasive pulmonary aspergillosis (IPA)

Luria-Bertani medium (LB)

Microsatellite Length polymorphism (MLP)

Microsatellite Polymorphism (MSP)

Phosphatidylcholine (PC)

Phospholipase A (PLA)

Phospholipase B (PLB)

Phospholipase C (PLC)

Phospholipase D (PLD)

Phospholipases (PL)

Polyethylene Glycol (PEG)

Polymerase chain reaction (PCR)

Polymorphic Microsatellite Markers (PPM)

polymorphonuclear neotrophils (PMNs)

Potato Dextrose Agar (PDA)

Quantitative –Polymerase chain reaction (Q-PCR)

Random amplified polymorphic DNA (RAPD)

Restriction fragment length polymorphism (RFLP)

Ribonucleic acid (RNA)

Ribosomal DNA (rDNA)

Sequence type (ST)

Sequence-specific DNA primer (SSDP)

Severe asthma with fungal sensitization (SAFS)

Short Tandem Repeats (STRs)

Single Sequence Repeats (SSR)

Spearman's Rank Order Coefficient (SROC)

Standard error of mean (SEM)

Tris acetic EDTA buffer (TAE)

Abstract

Aspergillus fumigatus has long been a focus of research, as it is the cause of the majority of Aspergillus infections. *A. fumigatus* is widely distributed in the environment and mainly distributed in air as conidia and is the main source of lung infection. *A. fumigatus airborne* counts were determined monthly during two years from the outside air environment at the University of Manchester campus and compared to total fungal airborne counts. Total fungal airborne counts were strongly seasonally associated with peak counts occurring during the summer months reaching 1,100-1400 CFU $m^{-3}$and were correlated positively with mean temperature (R^2=0.697). In contrast, *Aspergillus fumigatus* counts were not seasonally associated and gave persistent low levels of between 3-20 CFU $m^{-3}$and were not correlated with mean temperature. A random selection of Manchester environmental isolates collected over one year along with clinical patient isolates and environmental isolates from the air from Dublin were analysed for genetic diversity using two combined RAPD primers. RAPD analysis revealed that the Manchester environmental isolates represented a genetically diverse population while the clinical isolates were less diverse and formed three major clusters. The Dublin isolates were the least diverse, probably due to their isolation at a single time point. When the pathogenicity of clinical and Dublin isolates were compared with a random selection of Manchester isolates in a wax moth model, as a group, clinical isolates were significantly more pathogenic than environmental isolates. Moreover, when relative pathogenicity of individual isolates was compared, clinical isolates were the most pathogenic, Dublin isolates the least pathogenic and Manchester isolates showed a range of pathogenicities suggesting that selection for the most pathogenic isolates from the environment occurs during patient infection. When the expression of secreted phospholipases *in vitro* during wax moth larvae of a range of isolates displaying varying degrees of pathogenicity was compared, two phospholipase C genes, *Af*plcA and *Af*plcC were strongly correlated with pathogenicity. *Af*plcC was by far the most highly expressed, however a Δ*Af*plcC knockout strain did not show attenuated virulence compared to the wild type in wax moth larvae.

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 any other university or other institute of learning

Copyright statement

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CHAPTER 1

1.1. General Introduction

The genus Aspergillus is composed of over 250 species that have a broad distribution in the environment and are amongst one of the most commonly isolated organisms found in soil, foods, decaying vegetation, organic remains and in the air (Klich, 2006, Bennett, 2009). Although most species are widely distributed, only a small number cause a direct threat to human health. For example, *A .parasiticus* and *A. flavus* can contaminate food stuffs with mycotoxins, principally afaltoxins (Rustom, 1997), while *A. fumigatus*, and to a lesser degree, *A. terreus* and *A. flavus* can directly cause opportunistic diseases in humans and other animals (Aspergillosis) with *A. fumigatus* responsible for >90% of cases (Stevens et al., 2000, Latgé, 2003, Dagenais and Keller, 2009).

Several species of Aspergillus have high biotechnological value. For example, *A. niger* is used to produce citric acid as well as a range of industrially important enzymes such as glucoamylase and *A. oryzae* is important in many far Eastern food fermentations such as Soy sauce production (Archer, 2000). In addition, *A. nidulans*, which has a well-known sexual stage, has for decades been used as a model eukaryotic organism to study classical genetics and more recently gene function (Nierman et al., 2005).

1.2. History

Aspergillus was first recognized in 1729, by Italian priest and biologist Pier Antonio Micheli (1729). Viewing the fungus under the microscope he found that it was the same shape as the aspergillum (holy water sprinkler), and named it accordingly. In 1926, (Thom and Church) classified the genus and provided clear guidelines for identification of Aspergillus. In 1965, (Raper and Fennell) added to this work by including several new species in their comprehensive text on the genus. They classified Aspergillus into subgenera and groups, but this classification was replaced in 1985 by Gams, who further classified the groups into sections. (Gams et al., 1985). Currently there are approximately 250 named species and this number may grow with the increasing application of DNA based phylogeny rather than on relying on morphological markers alone (Geiser, 2009).

The first case of infection caused by Aspergillus was reported in 1845 in Edinburgh while the first reported invasive case was in a 3-year-old child in 1890 and proved fatal. However, it was not until 1953 that the pathologist Rankin first described invasive aspergillosis in a patient who had developed aplastic anaemia as a consequence of chloramphenicol toxicity (Denning et al., 2002a). In the last thirty years, the number of cases of disease caused by *A. fumigatus* has increased dramatically due to a rise in the number of immunocompromised patients due to the advent of AIDS, drug treatments affecting immunity and a rise in solid organ transplantation amongst others (Latge, 1999, Dagenais and Keller, 2009).

1.3. The Clinical Spectrum of Aspergillosis

Aspergillosis is the term used to describe infection caused by members of the genus Aspergillus that largely affect immunocompromised individuals and is usually opportunistic. The majority of cases of aspergillosis are caused by *A. fumigatus* with *A. flavus* considered the second most important pathogen, while *A. niger*, *A. terreus* and *A. nidulans* are less frequent (Dagenais and Keller, 2009) Aspergillosis is classified according to a clinical spectrum (Rinaldi, 1983, Denning et al., 1990), and varies between fatal and mild infections, and allergic syndromes in atopic individuals. After inhalation of the spores the infection travels via the respiratory tract, causing pulmonary infection and possible dissemination to other parts of the body according to the immunological status of the patient. Infection can also enter through open wounds due to trauma or surgical interventions, mainly to the kidney, liver and bones (Nampoory et al., 1996, Singh et al., 1997, Steinfeld et al., 1997).

1.3.1. Allergic Aspergillosis

Allergic aspergillosis includes extrinsic allergic alveolitis, severe asthma with fungal sensitization (SAFS) and allergic bronchopulmonary aspergillosis (ABPA). ABPA usually affects patients suffering from inherited genetic disease such as cystic fibrosis and these individuals are targets for various organisms that cause lung infections due to inability to clear mucous from the lungs (Zirbes and Milla, 2008). Allergic aspergillosis evolves from acute corticosteroid-responsive asthma and progresses towards fibrotic end-stage lung disease (Patterson et al., 1982). Usually the complication of allergic aspergillosis is bronchiectasis, with recurrent pulmonary infiltrates. The main diagnostic criteria of allergic aspergillosis are elevated blood level with IgE antibodies, which can be detected by precipitation test, and blood eosinophilia (Tomee et al., 1995).

The common side effects of allergic aspergillosis include fever, cough, malaise, weight loss, wheezing and lung ailments (Stevens, 2007). Patients recover gradually if treated properly with antifungal medications. Allergic aspergillosis can be successfully treated with corticosteroid and intraconazole (Stevens et al., 2000), but the prolonged use of corticosteroids can lead to various side effects.

1.3.1.1. Allergic Bronchopulmonary Aspergillosis (ABPA)

ABPA is commonly seen in patients with a prolonged history of asthma (McAdams et al., 1995) and is associated with the presence of plugs of inspissated mucus containing Aspergillus and eosinophils (Glimp and Bayer, 1983). The fungi enter the airway lumen with the subsequent outcome of production of a constant supply of antigen. An allergic reaction with immunoglobin E and immunoglobin G occurs. The inflammatory cells and the immune complexes settle in the bronchus producing necrosis and eosinophils. This ultimately damages the bronchial wall (McAdams et al., 1995). The common symptoms of the disease are malaise, fever, wheezing, cough with sputum and chest pain. Radiological examination of the patient can help trace the disease (Binder et al., 1982, Thompson et al., 1995).

1.3.2. Pulmonary Aspergillosis

Pulmonary aspergillosis is a hypersensitivity reaction to Aspergillus antigens, which affects patients suffering from asthma (Rubin, 2004). CT scan is normally used to identify the disease. Pulmonary aspergillosis is further divided into five groups:

1.3.2.1. Colonizing Aspergillosis (Aspergilloma)

This Aspergillus infection appears as a mass of entangled fungal hyphae combined (fungal ball) with cellular debris along with the mucus in the pulmonary cavity. There is no tissue invasion in this type of aspergillosis (Gefter, 1992, Aquino et al., 1994). It is usually caused due to sarcoidosis or tuberculosis and may be associated with AIDS, pulmonary sequestration-like thickening of the cavity wall and adjacent pleura, or bronchogenic cyst (Logan and Müller, 1996). The common symptoms of the infection are poor lung function and haemoptysis and variable fever (Glimp and Bayer, 1983). Chest x-rays will show a coin-like mass in the lung, over which there will be a radiolucent.

1.3.2.2. Semi-invasive Aspergillosis (Chronic Necrotizing Aspergillosis)

Chronic necrotizing aspergillosis is associated with the presence of necrotic tissue and granulomatous inflammation with associated factors such as diabetes mellitus, alcoholism, ageing, chronic debilitating illness, COPD (chronic obstructive pulmonary disease) and prolonged corticosteroid therapy (Franquet et al., 2000). Clinical symptoms of the disease include fever, cough with sputum and other constitutional symptoms. Almost 15% of patients are reported to have haemoptysis (Gefter et al., 1981). X-rays and CT scans can help to evaluate the exact condition of the patient (Brown et al., 1994).

1.3.2.3. Airway-invasive Aspergillosis

In this disease, the Aspergillus is present deep towards the airway basement membrane (Logan et al., 1994). It is seen particularly in AIDS and neutropenic patients (Brown et al., 1998, Won et al., 1998). There is a thickening of the bronchial and tracheal wall with acute tracheobronchitis, bronchiolitis and bronchopneumonia.

1.3.2.4. Angioinvasive Aspergillosis

Angioinvasive aspergillosis is normally caused due to severe neutropenia in the immunosuppressed patient. Often however, high-dose steroids inhibit the functioning of the neutrophils, so that despite a normal neutrophil count, the individual suffers from severe functional neutropenia (Curtis et al., 1979). The clinical diagnosis of this ailment is quite difficult and therefore it has a high mortality rate (Gefter et al., 1985).

1.3.2.5. Chronic Pulmonary Aspergillosis (CPA)

Chronic pulmonary aspergillosis (CPA) is a common form of aspergillosis, characterized by slow progression of the underlying chronic cavitary lung infection over months and years (Grahame-Clarke et al., 1994). It may be due to chronic obstructive lung disease (COPD), lung irradiation, lung resection or lung infarction. Prior tuberculosis and cystic fibrosis may also result in CPA. The symptoms of CPA are similar to those of IPA, except that in CPA the progression of the disease takes place slowly rather than severely.

The sub acute or semi-invasive pulmonary aspergillosis form of CPA is known as chronic necrotizing pulmonary aspergillosis (CNPA) (Binder et al., 1982); (Yousem, 1997, Soubani and Chandrasekar, 2002, Denning, 2003). CNPA patients may complain of mild immune dysfunction due to the use of corticosteroids or the presence of diabetes. It is very difficult to differentiate CPA from other chronic lung problems like upper lobe lung cancer, chronic cavitary lung infections, epidemic lung infection (*Cocidioides immitis* and *Histoplasma capsulatum*), atypical mycobacterial infection and *Mycobacterium tuberculosis* (Denning, 2003).

1.3.3. Invasive Pulmonary Aspergillosis

Invasive pulmonary aspergillosis (IPA) is a fatal disease seen in immunocompromised patients (Shiraishi et al., 2006, Agarwal, 2009). A patient may suffer from IPA as a result of an immunosuppressive therapy and organ transplantation. Symptoms include cough, fever, joint pain, shortness of breath, chest pain, respiratory problems such as crackles due to plural rub, and weight loss (Goldman and Ausiello, 2007). There is a malignant neoplastic disorder and generally a productive cough and haemoptysis up to bleeding that can become dangerous. The various diagnostic tests that may be carried out to confirm the presence of the fungus are chest x-ray bronchoscopy, blood test, CT scan of the chest, sputum culture and fungal stains.

IPA is a critical infection and is difficult to cure. However, antifungal medications such as amphotericin B are recommended to control the infection and problems of immunity (Blot et al., 2002). In some cases amphotericin B and a combination of caspofungin and voriconazole is being used to treat patients suffering from disseminated aspergillosis involving the brain, lungs and thyroid gland as well as acute lymphoblastic leukaemia, however the disease is often fatal (Zwitserloot et al., 2008).

1.3.4. Aspergillosis of the Paranasal Sinuses

Aspergillosis of the nose and the paranasal sinuses are divided into four types, allergic, invasive, non-invasive and fulminant (Rowe-Jones, 1993). Invasive aspergillosis of the nose and the paranasal sinuses are most prevalent among patients with leukaemia and granulocytopenia (Landoy et al., 2006). It is usually caused by *A. fumigatus* and *A. flavus*. Early detection of the disease through culture and histologic examination of biopsy material can help to control the infection. The treatment involves a regimen of amphotericin B along with surgical debridement for eradicating the infection. Otherwise, invasive aspergillosis of the nose and paranasal sinuses has a high mortality rate**.**

Previous studies have confirmed that semi-invasive aspergillosis of the paranasal sinuses is accompanied by bone destruction and erosion, but without any fungal tissue invasion (Romelt and Newman, 1982, Von Haacke, 1982, Oyarzabal et al., 2000) . The bone destruction may sometimes extend towards the orbit or into the anterior cranial fossa, especially in an immunosuppressed patient. Fortunately, the course of treatment, involving debridement of the infected region and oral itraconazole, has been successful in fighting against the disease without any recurrence for at least five years. The non-invasive disease may sometimes develop into invasive disease with the passage of time (Sarati and Lucent, 1988). Almost 28% of patients suffering from allergic aspergillosis sinusitis (AAS). are expected to itraconazole are recommended together (Herbrecht et al., 2002). However, voriconazole and caspofungin are being tested and have revealed some superior results (Taccone et al., 2003). Surgical excision of the infected lung section is often the ultimate remedy (Matt et al., 2003). Early diagnosis of the disease helps to initiate an early treatment regimen.

1.3.5. Disseminated Aspergillosis

Disseminated aspergillosis is a fatal infection which progresses quickly and normally influences immunosuppressed patients with previous history of corticosteroid treatment, cytotoxic chemotherapy or haematological malignancy, neutropenic patients, bone marrow and lung transplant patients (Prescott et al., 1992). About 25% of patients with IPA develop disseminated infection through haematogenous distribution, and of these, gastrointestinal involvement is reported in almost 45% of patients (Prescott et al., 1992). The gastrointestinal invasion may lead to complications such as bowel obstruction and angioinvasion with secondary bowel ischemia, perforation and necrosis (Young et al., 1970, Kinder and Jourdan, 1985, Watts and Chandler, 1997). However, these patients are rarely diagnosed with bowel involvement except during autopsy, as symptoms such as GI bleeding, abdominal pain and GI obstruction are generally concealed by the infected demonstration of the disease (Kinder and Jourdan, 1985).

Infection is primarily from the lungs and later after invasion of the lung tissue leads to the distribution of the fungus into the bloodstream, GI tract and other vital organs. The patient may then present with diseases such as vascular occlusion, haemorrhage or infarction (Marterre et al., 1992). undergo bone transplantation development or bone erosion at the infected site (Hartwick and Batrakis, 1991). Diagnosis is made on the basis of the behaviour of the disease, clinical status, and Cell Blood count (CBC) report, radiological findings and surgical conclusion (Rowe- Jones, 1993). CT scan of the infected region can clearly indicate the extent of fungal involvement (De Carpentier et al., 1994).

1.4. Treatment of Aspergillosis

Fungal infections are quite difficult to treat. Most are caused by Aspergillus species during critical care in patients with leukaemia and HIV/AIDS. Around 10 to 25% of all the leukaemia patients suffer from invasive infection caused by *A. fumigatus* (Nierman et al., 2005). An increase in the number of immunosuppressed patients has marked an increase in severe and fatal IA, which is the most common mould infection today.

There are three main classes of antifungal drugs. Firstly, polyenes, comprising amphotericin B, amphotericin B deoxycholate and its lipid formulations, and nystatin (including liposomal nystatin). However, these therapies are limited by the occurrence of considerable toxicity, mainly resulting in renal failure. The triazole group; itraconazole, the recently introduced voriconazole and posaconazole are often prescribed as they have considerably reduced side effects. Voriconazole has been found to be superior when compared with the polyenes and is the drug of first choice, as flucytosine is used in combination with other antifungal drugs (Herbrecht et al., 2002). Other drugs include the echinocandins such as caspofungin, licensed for the treatment of invasive aspergillosis, the recently introduced micafungin and the allylamines such as terbinafine, terbinafine would not be used to treat aspergillosis due to poor systemic drug exposure (Kontoyiannis and Bodey, 2002, Odds et al., 2003, Groll and Kolve, 2004, Kontoyiannis, 2012).

1.4.1 Azoles

A class of five-membered nitrogen heterocyclic ring compounds that contain at least one other non-carbon atom, sulphur, oxygen or nitrogen is known as an azole (Eicher and Hauptmann, 2003). The effectiveness of azoles in the treatment of Aspergillus disease was established in 1984 (Heeres et al., 1984, Van Cutsem et al., 1984). The first oral medication for invasive aspergillosis was itraconazole, followed by voriconazole, which emerged in 1996

as an antifungal with anti-Aspergillus properties (George et al., 1996, Oakley et al., 1998) and became the first-line therapy for invasive aspergillosis (Herbrecht et al., 2002, Denning et al., 2002b). More recently, posaconazole has come into existence, while ravuconazole and BAL8557 are in the process of development.

Triazole and imidazole are synthetic antifungal drugs that help in inhibiting the cytochrome P450 114a-demethylase enzyme (Lamb et al., 1995). This enzyme is essential because it is involved in synthesis of the fungal cell membrane, conversion of lanosterol into ergosterol and blocking steroid synthesis.

1.4.1.2 Azoles Mode of actions

All the azoles act as ergosterol (cell membrane) synthesis inhibitors and aid in drug-ergosterol interaction (Schutze et al., 1994). They act by inhibiting the lanosterol 14α-demethylase, which is the gene product of ERG11 (cyp51 in Aspergillus). This gene helps in the biosynthetic manufacturing of ergosterol from lanosterol in the fungi. If this enzyme remains inactive it is an indication that 14α sterols are not demethylated, and C14 sterols accumulate and enter into the plasma membrane of the fungus, causing growth arrest in *Candida albicans* and *Saccharomyces cerevisiae* (Kelly et al., 1995, Kelly et al., 1997). It also increases the sensitivity to reactive oxygen species (Shimokawa and Nakayama, 1992). Voriconazole acts primarily by inhibition of cytochrome P450 14a- demethylase (P45014DM), an enzyme involved in sterol biosynthesis leading from lanosterol to ergosterol (Odds et al., 1986, Patterson, 1999). The inhibition of cytochrome P450 14a-demethylase depends upon the dose of the medication. The liver metabolizes Voriconazole and the enzymes involved in the metabolism process include CYP 2C9, CYP2C19 and CYP 3A4.

The mode of action of posaconazole is similar to that of all the other azole antifungal agents (Refer to Mode of Action of voriconazole). However, posaconazole is a more potent inhibitor of sterol C14 demethylation as compared to itraconazole (Munayyer et al., 1996). It

is more effective than other triazoles such as itraconazole and fluconazole in the prevention of invasive fungal infections, but the side effects are more severe than for other triazoles (Cornely et al., 2007). The common side effects of PCZ when taken in small doses, alone or with other drugs, include headaches, nausea, lethargy and dryness of the mouth (Courtney et al., 2004, Krieter et al., 2004, Courtney et al., 2005, Sansone-Parsons et al., 2007).

1.4.2 Amphotericin B

Amphotericin B is a polyene antibiotic used for the treatment of life- threatening aspergillosis (Schiller and Fung, 2007, Arnold et al., 2010) but has to be administered intravnously. Patients with serious infections are given the maximum tolerated dose of atericin (Schiffman and Klein, 1977). Amphotericin B is administered intravenously via slow IV infusion of 0.1 mg/ml concentration over approximately 2 to 6 hours, while keing the ptient under close observation. Faster administration may lead to arrhythmias, and the total daily dose should not exceed 1.5 mg/kg, as that can lead to cardio-respiratory arrest in the patient.

Amphotericin B damages the cell membrane by binding to the fungal sterol ergoterol preferentially compared to human cholesterol forming ions channels and leakage. (Conner et al., 1971, Woods et al., 1974). Some common effects of amphotericin B are chills, drness of mouth, nausea, vomiting, drowsiness, muscle ache, fever, seizure, dark urine and clay-coloured stool, jaundice, diarrhoea, weight loss, headache, skin infection, infsion-related toxicity, renal failure, low potassium level, low magnesium level and loss of appetite (Sheehan et al., 1999).

1.4.3 Itraconazole

Itraconazole has been used successfully to treat allergic bronchopulmonary aspergillosis (Stevens et al., 2000). It bears a slight resemblance to the liver cytochromal P450 enzyme, which reduces the risk of interaction with other drugs (Van Cauteren et al., 1987, Saag and Dismukes, 1988). The drug is not preferred if the patient is allergic to other similar drugs like fluconazole or ketoconazole, if they suffer or have suffered from cgetive heart failure (De Gans et al., 1989, Heykants et al., 1987). For women if they are pregnant or plan to become pregnant, Itraconazole is circulated with the breast milk, hence should also be avoided by nursing mothers. Before beginning on the regimen of itraconazole, the physician should make sure that the patient does not suffer from any kidney, liver or heart diseases, nor does he have a history of stroke, breathing disorder or cystic fibrosis.

Itraconazole is given both intravenously and orally. The dose for IV itraconazole administration is 200 mg twice a day for two days and thereafter 200 mg/day. Itraconazole capsules are generally taken on an empty stomach at least an hour prior to or after a meal. The dose prescribed for oral intake is 100 to 400 mg daily (Degreef and DDoncker, 1994, Dupont et al., 1996, Haria et al., 1996), and treatment generally continues for about 1 to 6 months. The medication is very effective and has shown minimal side effects. Rsearch on 43 people treated with itraconazole revealed that 93% of them did not have any signs of recurrence for a year post-operatively (Yagi et al., 1999). Amphotericin B or IV amphotericin in lipid base is prescribed in life threatening cases, where the infection has spread widely, and in fulminant fungal infection (Rowe-Jones, 1993, Luna et al., 2000). The mode of action of itraconazole is similar to that of other azoles (Gilbert, 2006). Due to its capacity to restrain cytochrome P450 3A4, the drug is used carefully with regard to its interaction with other drugs, the drug is eliminated by means liver mtabolism. Multiple doses of itraconazole lead to saturation of some hepatic metabolism pathways. Common side effects are similar to those of fluconazole,

and include hypokalemia, oedema, headaches, altered mental states and high blood pressure (Denning et al., 1989). Hepatotoxicity is rarely noticed with it, and the drug occasionally causes some mild side effects (Sheehan et al., 1999).

1.4.4 Voriconazole

In 2002 the FDA approved voriconazole for the treatment of invasive aspergillosis, *Scedosporium apiospermum* and Fusarium species. It can fight against a variety of microorganisms, such as Aspergillus species (Clancy and Nguyen, 1998) *Scedosporium aiospernum*, *Fusarium* sp, *Cryptococcus neoformans* and *Candida* sp, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Penicillium marneffei* and *Coccidioides immitis* (Rinaldi, 1983, Arikan et al., 1999). The enhanced activity of the medication against fluconazole-resistant *Candida krusei*, *Candida guilliermondii* and *Candida glabrata* is extraordinarily effective (Nguyen and Yu, 1998, Lozano- Chiu et al., 1999). Voriconazole can be taken both orally and intravenously. During clinical trials, favourable responses were received with doses of 200 mg, given orally twice a day; and doses of 3 to 6 mg/kg, administered intravenously every 12 hours (Sheehan et al., 1999).

The most common side effects of voriconazole include vision defects, among them photophobia, blurred vision, and changes in colour vision persisting for the initial few days of treatment and then normalizing (Sheehan et al., 1999). Normally only 30% of patients experience this vision disorder for longer than 30 minutes after initiation of the therapy. It is mainly due to the blockage of receptor de-excitation during voriconazole therapy. Other side effects include skin rashes and elevated liver enzymes.

1.4.4. Posaconazole

Posaconazole is a triazole antifungal drug (Rachwalski et al., 2008) particularly recommended for treating oral and throat yeast infections as it slows down the fungi growth and treats the infection that otherwise could not be treated with other medications. Those with weaker capability to fight against the fungal infection are prescribed posaconazole. It is particularly used to treat invasive infections caused by Aspergillus (Walsh et al., 2007). and Candida species (Li et al., 2004). It is taken orally, usually three times a day with a full meal or liquid nutritional supplement. The duration of the treatment depends upon the general health of the patient and the type and intensity of the infection. Before commencement of the treatment the physician should be fully informed about any allergic reaction towards the drugs and any diseases the patient might be suffering, such as kidney, liver or heart disorder, low levels of potassium, magnesium or calcium in blood, or any eating disorder. The doctor should also be informed in case the patient develops severe vomiting or diarrhoea with the commencement of the treatment.

Oral doses of 800 mg/day of posaconazole divided into 2 to 4 doses are recommended for aspergillosis in immunosuppressive patients intolerant of antifungal therapy, depending upon the condition of the patient (Ullmann et al., 2006, Vazquez et al., 2006, Ullmann et al., 2007, Cornely et al., 2007). The gastrointestinal absorption of the drug is enhanced when taken with food or a nutritional supplement, regardless of gastric pH (Courtney et al., 2003, Courtney et al., 2004). It is metabolized in the liver and almost three-fourths are excreted in the faeces.

1.4.5 Echinocandin class

Echinocandin class of antifungal agents and has the potential to be either additive or synergistic with polyenes and azoles. The inhibition of the fungal cell wall synthesis by inhibiting the enzyme β(1,3)-D-Glucan synthase as it is important in cell wall assembly (Onishi, et. al. 2000, Stanley, et. al., 2003) such as lipopeptide, pneumocandines, echinocandins and glycolipid papulacandins acidic terpenoids, caspofungin, micafungin and anidulafungin, the papulocandins. Disruption of the fungal cell wall results in osmotic stress, lysis, and death of the microorganism. In contrast, cell wall of yeasts includes. polymers of glucose, mannose, and *N*-acetylglucosamine, forming the polysaccharides, glucan, mannan, and chitin, respectively, Glucan is a microfibril composed of 3 helically entwined linear polymers of glucose. Glucan is the major component of the fungal cell wall, accounting for 30%–60% of the cell wall of *Saccharomyces* and of *Candida.* The level of oral absorption of the echinocandins and pneumocandins is low (Stanley, et. al., 2003). the level of oral absorption of the echinocandins and pneumocandins is low thus, we have sought new chemical structures with the mode of action of lipopeptide antifungal agents but with the potential for oral absorption.

Caspofungin it may prove to be useful in empirical therapy for suspected invasive fungal infections In vitro studies and some animal studies almost always indicate an absence of antagonism when caspofungin is combined with azole or polyene antifungal agents. Caspofungin has an excellent safety profile. In vitro studies have demonstrated a 5–10-fold increase in the inhibitory activity of micafungin, compared with that of caspofungin, against *C. albicans, C. glabrata, C. tropicalis,* and *C. dubliniensis* (Stanley, et. al., 2003). The lack of antagonism when micafungin is combined with other antifungal drugs is encouraging; however, clinical trials are critical to determine whether combination therapy (e.g., micafungin plus voriconazole) is superior to monotherapy (e.g., voriconazole) for invasive aspergillosis (Chandrasekar and Sobel, 2006).

1.5. Pathogenicity (virulence) factors of *Aspergillus fumigates*

A. fumigatus causes infection in immunocompromised patients, and is normally unable to develop disease in immunocompetent patients. However, while *A. fumigatus* spores represent only ca. 0.3% of all Aspergillus species spores present in the air, they account for 90% of cases (Rementeria et al., 2005). A number of factors assist in the progression of the infection in the immunocompromised host; these include the virulence of the organism with capacity to establish the infection, and delays in diagnosis and treatment, which reduce the chance of successful treatment (Paterson et al., 2003).

The recent whole genome sequencing of *A. fumigatus* has deepened our knowledge of the organism and has revealed for example that there is a hidden sexual stage as well as revealing previously unknown secondary metabolite pathways (O´Gorman et al., 2009).

1.5.1. Conidia

A.fumigatus is a highly sporulating organism that is ubiquitous in nature with the spores commonly found in the air and general environment. It has been estimated that on average, a person will inhale several hundred spores and owing to the small size of the conidia, which range between 2 and 3µm in diameter, they can reach the lower part of the lung (alveoli) (Latge, 1999). In immunocompetent patients the innate-immune system will eliminate the spores from the body by mucociliary clearance and phagocytic cells (Behnsen et al., 2007). The conidia not destroyed by alveolar macrophages (AMs) and those escaping are killed by polymorphonuclear neotrophils (PMNs) even after germination and hyphae production (Behnsen et al., 2007). Immunocompromised people on the other hand are at risk of developing pulmonary or invasive Aspergillosis (Rhodes, 2006).

The process of pathogenicity of *A. fumigatus* requires adhesion to the host. The next step is germination, then invasion by hyphae of the lung tissues and dissemination by the blood vessels. Although *A. fumigatus* spores do not predominate in the air, their capability to cause infection to humans is high, perhaps due to the ability of the conidia to bind to extracellular matrix proteins, such as fibronectin, laminin, type IV collagen, complement, albumin, Ig's and surface proteins (Latge, 1999, Wasylnka and Moore, 2000).

The hydrophobic nature of the conidia is due to the presence in the outer surface of protrusions, termed rodlets, which are absent in the hyphae structure (Ibrahim-Granet and D'Enfert, 1997, Aimanianda et al., 2009). The binding process is non-specific as the hydrophobic rodlets bind to hydrophobic pockets present in albumin, collagen, laminin and fibrinogen (Thau et al., 1994). However, changes in expression or deletion of the genes do not explain differences in pathogenicity (Hohl and Feldmesser, 2007). Virulence levels are unaffected, most probably because these mutant strains retain their ability to bind laminin and fibrinogen in a specific manner (Thau et al., 1994). The protein encoded by this gene, RodAp, has a low molecular weight (16KDa), is moderately hydrophobic and is rich in cysteine, and appears to protect the conidia from destruction by alveolar macrophages.

Sialic acid residues, which are present on the conidial surface, have been suggested to have a different function in binding to fibronectin and initiating the aspergilloma or invasive disease. It is also suggested that the virulence of *A. fumigatus* relies upon the adhesion of the conidia to the host cells, mainly to the extracellular matrix (ECM)- fibronectin, laminin, IV collagen, intact lung cell basal lamina *in vivo* (Wasylnka and Moore, 2000).

1.5.2. The Cell Wall

The main composition of the cell wall is polysaccharide, composed of α-(1, 3) glucan, β-(1, 3), glucan β-(1, 6) branches; linear β-(1, 3), β-(1, 4) glucan, chitin, and galactomannan (Gastebois et al., 2009). The polysaccharide has attached to it nine glycosylphosphatidylinositol (GPI)-like proteins. A study by (Li et al., 2007) has shown that the disruption of Af*pig*-a gene inhibits the synthesis of the GPI- linked proteins, resulting in the production of hypovirulant strains, along with a slow growth rate. However, the disruption of the GPI-like protein Ecm33p causes the conidia to germinate rapidly and is more virulent with the conidia more resistant to phagocytosis by alveolar macrophages.

The cell wall pigment melanin has suggested being an important factor in the virulence of *A. fumigatus*, as well as *Cryptococcus neoformans* and *Wangiella dermatitidis* (Dixon et al., 1987). The functions of this pigment include protection against UV light and against high temperature. Moreover, it diminishes the phagocytosis process, and increases fungal resistance to reactive oxygen species and to cell lysis (Brakhage and Liebmann, 2005). These pigments give a greyish-green colour to the conidia, due to accumulation of 1,8-Dihydroxynaphthalene-melanin (Youngchim et al., 2004, Brakhage and Liebmann, 2005). Whereas in hyphae the colour is diminished, melanin gives the spore ability to resist environmental. Conditions for a long time (Rementeria et al., 2005). Observations by (Tsai et al., 1998) have revealed that the pathogenicity of the non-pigmented, white conidia due to loss of polyketide biosynthesis is reduced compared to the wild type (Rementeria et al., 2005).

A number of enzymatic reactions are involved in the synthesis of melanin, and these enzymes are encoded in six genes: pksP/alb1, ayg1, arp1, arp2, abr1 and abr2 representing a polyketide synthsase, a scytalone dehydrogenase and hydroxynaphalene reductase respectively (Tsai et al., 1999). The deficiency of ΔpksP, which encodes the polyketide synthstase, results in production of non- pigmented conidia, which are more prone to phagocytosis, and produce a greater amount of reactive oxygen species by neutrophils if compared to the wild type (Tsai et al., 1998, Jahn et al., 2002) The loss of pksP enhances exposure of β-(1,3) glucan (polysaccharaides) to the innate immune system (Kessler et al., 2002).

1.5.3. Thermotolerance and growth rate

The filamentous fungi comprise a network (mycelia) of hyphae interwoven together. The hyphae are tube-like structures, filled with scaffolds and organelles. The mycelia derive their development from germination of spores. The first step in the formation of a new fungal colony is the germination of spores to form a germ tube; this step is critical for the fungi to grow and to establish their development at elevated temperatures.

The growth rate of filamentous fungi may be measured using different methods. The colony radial growth rate measurement method has been found to be reproducible and not affected by variation in the number or spores (inoculums size).

This method can be performed using solid media, and the diameter of the colony can be measured at time intervals. The inoculation process uses either a spore suspension or a mycelia plug. To calculate the colony radial growth the colony diameters are measured with time, and then plotted on a graph (Withers et al., 1995, Van de Vondervoort et al., 2004) with the slope of line measuring linear colony expansion.

The growth rate can also be measured by absorbance of fungal biomass, using turbidometric measurement (Granade et al., 1985). This method has recently been modified by (Meletiadis et al., 2001), the main change introduced was the inclusion of a chromogenic metabolic indicator MTT and uses of microtitre plates to make continuous measurements of changes in the optical density of the fungal growth over time; it is also used for susceptibility testing of filamentous fungi. There is a correlation between the growth rate and the virulence of the organism with organisms with a higher growth rate tending to be more virulent. The growth rate of *A. fumigatus* is assumed to be an important factor influencing the pathogenesis, but this requires further investigation (Paisley et al., 2005).

A. fumigatus has the distinguishing feature of being thermotolerant, with ability to cause infection to humans and animals (birds) and adapted to temperatures up to 70°C. (Nierman et al., 2005, Araujo and Rodrigues, 2004) found a strong relationship between germination rate and frequency as opportunistic pathogens. *A. fumigatus* germinated at 37°C and 42°C had a higher percentage of germination of between 50-60% compared to less than 40% for *A. flavus* and *A. niger* (Araujo and Rodrigues, 2004). It is not known what genes are responsible for thermotolerance in *A. fumigatus* (Hohl and Feldmesser, 2007) although (Nierman et al., 2005) found that there were 323 genes differentially expressed at 48°C compared to 37°C.

1.5.4. Toxins and Secondary Metabolites

The main distinctive feature of filamentous fungi is the production of natural products or secondary metabolites and mycotoxins. The production of many secondary metabolites are thought to play a role in the virulence of *A. fumigatus* and include fumagillin, gliotoxin, fumitremorgin, verruculogen, fumigaclavine, helvolic acid and sphingofungins (Latge, 1999). The genes encoding these secondary metabolites in *A. fumigatus* are arranged in 26 clusters whereas in *A. oryzae* and *A. nidulans* only 13 clusters are present (Nierman et al., 2005).

Mycotoxins are one of the virulence factors that bypass the immune system, especially in immunocompromised patients, and may play an important role in the invasion of human immunity (Kamei and Watanabe, 2005). The most abundant mycotoxins are gliotoxin (hydrophobic metabolites), which belong to the class of epipolythiodioxopoperazines compounds, characterized by the presence of a quinoid moiety and disulfide bridge across a piperazine ring (Latge, 1999, Waring and Beaver, 1996). This compound is detected in patient serum with invasive aspergillosis, and might occasionally be detected in patients without IA (Lewis et al., 2005). *A. fumigatus* strains that do not produce a gliotoxin are less virulent than producing strains (Tao et al., 2006). Gliotoxin inhibits the NADPH oxidase activity responsible for the neutrophils oxidative burst (Tsunawaki et al., 2004). However, virulence tests in mice have shown that deletion of the gene *gli*P or *gli*Z which block gliotoxin production do not affect virulence suggesting that gliotoxin might not be important for virulence in every host (Hohl and Feldmesser, 2007). However, Sugui et al., (2007) found that gliotoxin is an important factor for virulence, which is mainly dependent on the status of the immunosuppressive regimen. This correlates with the finding by (Orciuolo et al., 2007) that methylprednisolone and the production of gliotoxin from *A. fumigatus* leads to inflammatory reaction, along with impairment of T cell function and development of invasive aspergillosis, and consequently an increase in morbidity.

1.5.5. Proteases

Secreted proteolytic activity has been intensively investigated, and the presence of different secreted proteases has been demonstrated on the surface of fungal elements colonizing mucosa and penetrating tissues during disseminated infections.

Proteases catalyse the proteolytic cleavage of peptide bonds (CO-NH) of proteins (Monod et al., 2002). The classification and nomenclature of all proteases, together with further information about them, can be found in the MEROPS database accessible at http://www.merops.ac.uk/merops/merops.htm. The proteases are initially classified following their mode of action and their active sites. Aspartic, cysteine, metallo, serine and threonine proteases are recognized as the major classes of proteases, as well as proteases with unknown catalytic mechanism. The proteases can be either endoproteases (or endopeptidases) or exoproteases (or exopeptidases). The endoproteases cleave peptide bonds internally within a polypeptide. The exoproteases cleave peptide bonds only at the N- or the C terminus of polypeptide chains.

Extracellular proteases are secreted by many microorganisms and by fungi from their mycelia, and *A. fumigatus* secretes a range of proteases and other extracellular hydrolases such as lipases, phosphatases and glycosyl hydrolases that are able to degrade macromolecules which are used for growth (Robson et al., 2005).

A number of studies have suggested that the extracellular proteases play a major role in the pathogenicity of the *A. fumigatus*, along with other endoproteases (including alkaline protease, metalloprotease and aspartic protease), which have also been identified (Latge, 2001). Proteases target the lung tissue, allowing *A. fumigatus* to invade the tissue causing cellular damage that facilitates fungal adhesion (Tomee et al., 1995). Proteases also help the spread of allergens through epithelial cells and the production of inflammatory chemokines and cytokines (Knutsen et al., 2004, Pylkkanen et al., 2004).

There is no evidence to date that loss of specific protease genes impacts on *A. fumigatus* virulence. Single *alp*, *pep* or *mep* mutants and a double *alp pep* mutant were not shown to have attenuated virulence in mice (Monod et al., 1993, Monod et al., 2002, Jaton-Ogay K. et al., 1994, Reichard et al., 1997) however as the activities of several proteases are redundant, loss of one protease activity may be compensated by others and therefore a role for secreted proteases cannot be ruled out (Latge, 1999).

1.5.6. Phospholipases

Phospholipases are a heterogeneous group of enzymes that hydrolyse phospholipids into fatty acids and other lipopholic substances (Rementeria et al. 2005). There are two major categories, the acyl hydrolases and the phosphodiesterases, and four major classes termed A, B, C and D. The acyl hydrolases consist of phospholipases A_1 (PLA_1) and A_2 (PLA_2), which cleave the SN-1 and SN-2 acyl chains respectively. However, phospholipase B cleaves both bonds have been shown to have transacylase activity since they are able to convert lysophospholipids and free fatty acids into phospholipids. Phospholipase C enzymes are phosphodiesterases that cleave the glycerophosphate bond, while phospholipase D enzymes remove the base group of phospholipids (Köhler et al., 2006).

In *Cryptococcus neoformans* and *Candida albicans*, PLB is the only secreted extracellular phospholipase and deletions have been shown to reduce virulence in both organisms (Leidich et al., 1998, Ghannoum, 2000, Cox et al., 2001, Theiss et al., 2006). Moreover, inhibitors of PLB have been shown to reduce the levels of *C.neoformans* in the brains of infected mice (Ganendren et al., 2004).

In contrast, genome analysis of *A. fumigatus* has revealed that it contains one putative secreted PLA, two PLB's, three PLC's and secretion of extracellular PLA, PLB and PLC activities have been demonstrated (Birch et al., 1996, Birch et al., 2004, Köhler et al., 2006, Robson, 2009). The genes encoding the two extracellular PLB's have been shown to be upregulated in the presence of phospholipid (Shen et al., 2004) but to date, there has been no research into the role of phospholipases in the pathogenicity of *A. fumigatus*. Extracellular phospholipases are thought to potentially be important in pathogenicity because infection is generally through the lung and the small size of the *A. fumigatus* conidia mean it will come into contact with the surfactant coating the alveoli which is composed of ca. 90% phospholipid (Morgenroth, 1988, Veldhuizen et al., 1998) Thus germination and growth will occur in a highly phospholipid rich environment.

1.7 Molecular Typing of Aspergillus fumigatus

For the epidemiological study of any infectious disease it is essential to differentiate between isolates of the same species. In order to understand the mode of transmission, spread of the organism, and virulence, typing of an organism is mandatory. Usually, verification of epidemiological relationships is determined by species identification and morphological typing. However, *A. fumigatus* is highly diverse in nature and therefore requires sensitive techniques. There is no gold standard typing method for *A. fumigatus* with several methods in current use, each possessing both advantages and disadvantages (Spreadbury et al., 1990, Denning et al., 1990, Aufauvre-Brown et al., 1992, Vos et al., 1995, Balajee et al., 2008, Lasker, 2002, Thierry et al., 2010). Several studies have compared the different molecular typing methods and concluded that using combinations of more than one typing method provides the best discrimination, because it gives more emphasis to the genetic information of the fungus.

1.7.1 Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD), also termed Arbitary primed PCR (AP-PCR), is a relatively recent technique demonstrated by (Welsh and McClelland, 1990), and is the most widely used method for typing *A. fumigatus*. This technique is based on the PCR reaction, but in RAPD the fragments amplified are random, and are generated using primers of arbitrary sequence. The single primer is normally between 8 and 20 base pairs (bP), and the PCR reaction is carried out at low annealing temperature (Welsh and McClelland, 1990). The primer will anneal to the DNA template at different sites and PCR will generate a number of arbitrary fragments, usually between 100 (bp) and 4 kb. Following amplification, the fragments are separated by agarose gel electrophoresis, stained by ethidium bromide and visualized under UV light, along with a specific molecular weight ladder. The results will be different bands with different fragment sizes, enabling unrelated isolates to be differentiated.

The advantages of this method are that the procedure is rapid, inexpensive, can be applied to a large number of isolates and requires no previous sequence information (Bertout et al., 2001). Recent studies (Loudon et al., 1994, Mondon et al., 1997, Menotti et al., 2005) have found that RAPD has a high discriminatory power, is reproducible and requires only a small amount of DNA. On the other hand, as RAPD is a PCR-enzymatic reaction it can be affected by a number of factors including concentration of DNA, concentration of PCR components, and PCR conditions. The method can therefore be laboratory dependant, and so requires specific designed protocols in order to be reproducible (Savelkoul et al., 1999, de Valk et al., 2007). The absence of such conditions can lead to mismatched pairing, lack of reproducibility and difficulty in interpretation of results (Bart-Delabesse et al., 2001, Lasker, 2002).

A considerable amount of literature has been published on RAPD. This method was used by Aufauvre-Brown et al. (1992) to test nine different isolates of *A. fumigatus* with 44 different primers (10 base primers). Primer 108 is the most frequently used for RAPD studies (Lasker, 2002). Mondon et al. (1997) studied 108 primers, and found that while they had a high discriminatory power, reproducibility was low. More recently, Menotti et al. (2005) tested 21 environmental isolates and 26 clinical isolates and reported 34 distinct profiles, indicating a high level of diversity in the *A. fumigatus* strain in the clinical and environmental population.

1.7.1 Sequence-specific DNA primer (SSDP)

Preliminary work using sequence specific DNA primers (SSDP) was undertaken by Mondon et al. (1997). SSDP is a PCR-based typing method, developed from RAPD. Based on the RAPD profiles, a discriminating band(s) between isolates is sequenced and used for the design of one or more specific primers. PCR with this primer will show either the presence or absence of discriminating fragments. The advantage of SSDP is that it requires higher

stringency in the PCR compared to RAPD and has a high discriminatory power and level of reproducibility.

1.7.2 Restriction fragment length polymorphism without hybridization (RFLP)

In restriction fragment length polymorphism (RFLP) analysis, genomic DNA is digested by restriction enzyme(s), resulting in a profile composed of different lengths of restricted fragments (Denning et al., 1990, Burnie et al., 1992). Restriction endonucleases generally recognize 6 bp sequences. Restricted fragments are separated by gel electrophoresis in agarose and visualized under UV light after staining with ethidium bromide. According to sequence differences in the DNA of the isolate, different numbers and sizes of fragment are developed, with fragments reaching up to 10,000 bp. This technique shows the polymorphism between the species, and different banding patterns may differentiate different isolates. (Girardin et al., 1993) found that *Eco*RI-digested DNA did not show high discriminatory power between *A. fumigatus* isolate.

1.7.3 Restriction fragment length polymorphism with hybridization (RFLP)

RFLP with hybridisation is an extension of the RFLP technique. Following digestion and separation of DNA fragments, electrophoresis DNA is transferred onto a nylon membrane supported by southern blotting, and hybridized with either *Xba*I, *Xho*I, or *Sal*I**,** a labelled DNA probe. A number of different probes have been used: a heterologous probe (Spreadbury et al., 1990) was used specifically for the non-transcribed region of the rRNA gene; retrosposon -like element repetitive DNA probes include *Afut*1 (Neuveglise et al., 1996, Batista et al., 2008), *Afut*2 and *Afut*4A and M13 Phage (Anderson et al., 1996). Hybridization patterns obtained with *Afut*I enabled higher reproducibility (Chazalet et al., 1998, Debeaupuis et al., 1997).

The main disadvantages of RFLP are that it requires a large amount of highly purified DNA, it is time consuming and labour intensive, and the probe is designed only for a specific species (Lin et al., 1995, Lasker, 2002). The use of radioactive labelled probe is another disadvantage. Furthermore, the reproducibility of this technique depends on the band intensity, and any variations in the process may affect the final result (de Valk et al., 2007, Savelkoul et al., 1999, Bart-Delabesse et al., 2001). Like any other fingerprinting band-based technique RFLP is complex, and dependence on the band intensity makes interpretation more difficult (Savelkoul et al., 1999, Bart-Delabesse et al., 2001).

1.7.4 Amplified fragment length polymorphism (AFLP)

AFLP was first described in by Vos et al., (1995). There are two restriction enzymes, one a rare cutter and the other a common cutter, selected to cut the adapter of double stranded DNA which is ligated to the end of the restriction fragments using PCR after designing suitable primers for each step. Alternatively, fluorescent or radioactive labelled primers, or polyacrylamide gel electrophoresis, may be used (Mueller and Wolfenbarger, 1999). This method is similar to RAPD in that there is no need to know any specific DNA sequence. Although reported to be highly reproducible and to have a high degree of discriminatory power compared to other methods, only a few studies have employed AFLP for molecular typing of *A. fumigatus* (de Valk et al., 2007).

1.7.5 Microsatellites or short tandem repeats (MSTR)

Microsatellite Length polymorphism (MLP) is also called single sequence repeats (SSR), or Short Tandem Repeats (STRs) (de Valk et al., 2005). It is referred to as Microsatellite Polymorphism (MSP) (Bertout et al., 2001), or Polymorphic Microsatellite Markers (PPM) (Lasker, 2002). STR is a relatively recent procedure, used since the mid 1990s, for forensic purposes. The single sequence repeats are known for the high level of polymorphism present in the nuclear and organellar DNA, short segments of DNA that have a repeated sequence of adjacent units of 1-6 pb in length, such as CACACACA (van Belkum et al., 1999). This repeated length is found in the genomic region (coding region) and in the non-coding intron regions (Li et al., 2002). These repetitive sequences are found randomly distributed in the genomes of most eukaryotic gens and prokaryotic genomes (Li et al., 2002) including fungi (Bretagns et al., 1997).

The MLP is performed using PCR with the choice of primers being dependant on the flanking sequences. The labelled fluorescent primers can be used at several microsatellite loci, and these amplified markers are separated by denaturing gel electrophoresis, stable-based polyacrylamide gel electrophoresis or capillary- based polyacrylamide-derived gel. The PCR products can be separated and determined according to their size and sequence (Pasqualotto and Denning, 2007). The number of repeats in each marker can be deduced from the size of the fragments. All repeat numbers of the analyzed markers form a genotype for each individual isolate. This technique was first used by (Bart-Delabesse et al., 1998) for typing *A. fumigatus*. Four different dineucleotides markers were used to discriminate among the unrelated isolates, and a discriminatory power of 0.994 was obtained. The polymorphism is measured by changes in the number of repeats. This method shows high discrimanatory power at 0.994 (Bart-Delabesse et al., 1998).

More recently, a new panel of nine short tandem repeats, called STR*Af* (STR of *A. fumigatus*) have been described. This technique uses three different STRS, (three dinucleotide loci, three trinucleotide). A discriminatory power of 0.9994 was obtained. The STR*Af* technique is applied for typing clinical, environmental and outbreak setting (de Valk et al., 2007, Lasker, 2002, Bart-Delabesse et al., 1998, de Valk et al., 2005, Bain et al., 2007, Balajee et al., 2008).

The use of MLP offers a number of technical advantages over many other fingerprinting techniques. These include high reproducibility, because of the high stringency condition in PCR reaction, as well as the automated sizing of the fragments. This method also allows large numbers of isolates to run in a short time period, along with different multicolour multiplex (markers), and the results are exchangeable between laboratories (de Valk et al., 2005). Even where there is variation between experiments (different enzymes, number of thermocycles and annealing temperature), the method does not lead to false typing results (de Valk, 2007a). The discriminatory power of this technique can identify the existence of a mixture of several isolates in one sample

Since the microsatellite technique depends on the fragment electrophoretic mobility to the corresponding number of repeats, attempts have been made to standardize discrepancy power in relation to human forensics by constructing a reference fragment, and a specific allelic ladder which can be developed for each locus. Unfortunately, the mobility of the fragment relies on many factors, including the denaturing compound, the exact base composition, the sequence of the fragment, temperature, the different fluorescent labels, and the type of matrix (de Valk et al., 2007, Pasqualotto and Denning, 2007). A calibration method for electrophoreses machine has been found by generating a reference of sizing values (determined by sequencing) for a range of alleles that can be calibrated. Finally, by using the SERV, and on the basis of the nucleotide sequencing, the electrophoresis machine calculates which microsatellite loci are changeable (Klaassen, 2009).

1.7.6 Multilocus sequence typing (MLST)

MLST is a method to type multiple loci. This technique is used to characterize isolates of bacterial species using the DNA sequencing of internal fragments of multiple (usually seven) loci. Approximately 400-500 bp regions are analyzed using an automated sequencing machine, then compared within the species. Distinct alleles - according to the differences in the sequence, are assigned for each isolate among the species. The sequence type (ST) will define the differences for each isolate at distinct alleles. Nucleotide differences between strains can be checked at a variable number of genes (generally seven) depending on the degree of discrimination desired. By comparing the STs region between isolates, the degree of similarity between isolates can be determined.

Bain et al. (2007) first reported the use of an MLST panel for sub-typing *A. fumigatus*. They analyzed 100 isolates, both clinical and environmental, and found that the developed MLST system had low discriminatory power at 0.93.

Aims and Objectives

Aspergillus fumigatus is an important and significant opportunistic pathogen of humans, birds, reptiles and other organisms with a worldwide distribution in the environment and is associated with decaying plant material and vegetation. Its ability to infect the host appears to be multifactorial; relying on a number of factors with no single pathogenicity factor(s) and appears to be genetically diverse in the environment.

In this study, outdoor, airborne levels of *A. fumigatus* were monitored monthly over a two year period to measure spore concentration and to determine if these levels fluctuated between seasons (Chapter 3).

To investigate whether this population was genetically diverse, a random selection of strains isolated from during the first twelve months was subjected to RAPD analysis using two RAPD primers and the combined data subjected to cluster analysis. To investigate whether clinical isolates showed any significant variation from this population, this analysis was repeated alongside clinical isolates from infected patients. To determine if the pathogenicity potential of the environmental population was variable, a number of randomly selected isolates were used to infect wax moth larvae and pathogenicity determined on the basis of larval survival over seven days and compared to clinical isolates (Chapter 4).

Secreted phospholipases have been suggested to potentially play an important role in pathogenicity as infection largely occurs via inhalation of spores and the lung surface contains a high concentration of phospholipid. Therefore, the expression of secreted phospholipase genes during infection of wax moth larvae was monitored by real time PCR by comparing expression relative to the housekeeping gene actin. One highly expressed phospholipase C gene, *Af*plcC, was replaced with the hygromycin selectable marker gene and the pathogenicity of the *Af*plcC knockout strain in wax moth larvae compared to the wild type strain (Chapter 5).

The work presented in this thesis was carried out entirely by the author (Fadwa Alshareef).

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CHAPTER 2

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Solutions and buffers

Solutions and buffers were prepared using double distilled water (ddH2O). The pH was measured at room temperature and when necessary was adjusted using 1M NaOH or HCl. Solutions and buffers referred to as sterile were autoclaved for 15 min at 121^{O}C, 15 psi. All commercially available media were prepared according to manufacturer's instructions.

2.1.2. Primers

All primers used in this research are shown below and were stored at -20°C until required. All primers were purchased from Eurofins, (UK) except primers used for real- time PCR of phospholipase gene expression which were purchased from Life Technologies (formally Invitrogen, UK). Primers were designed using primer3 software (Rozen and Skaletsky, 2000). The primers for the B-tubulin (Staab et al, 2009), the ITS primers were used as indicated by (White et al, 1990) and for the RAPD Primers from

2.1.2.1. Internal Transcribed Spacer Region (ITS) primers

ITS-1	5'-TCCGTAGGTGAACCTGCGG-3'
ITS-4	5'-TCCTCCGCTTATTGATATGC-3'

2.1.2.2. β-tubulin primers

β-tubI	5'-AATTGGTGCCGCTTTCTCGG -3'
βtubII	5'-AGTTGTCGGGACGGAATAG-3'

2.1.2.3. APD primers

R108	5'-AGTGCACACC-3'
C90	5`-GGGGGTTAGG-3'
R151	5`-GCTGTAGTGT-3'
C08	5`-AGGATGTCGAA-3'

2.1.2.4. Primers used for construction of phospholipase gene knockouts

	Primer sequences for PLB1
1	5'-CATAGCTGCGTCTCCACTGC-3'
2	3'-GCGCCCACTCCACATCTCCACTCGATTTGGGTTGTATTATGCCGCC TCAC-5'
3	5’-GTGAGGCGGCATAATACAACCCAAATCGAGTGGAGATGTGGAGT GGGCGC-3’
4	3’-TTTTCGAATAATTCCAAAATGAAACCTATAGAATCATCCTTATTCG TTGA-5’
5	5’-TCAACGAATAAGGATGATTCTATAGGTTTCATTTTGGAATTATTCG AAAA-
6	3’-TCCCAGTTCGTCGTCCGTAG-5’
1N	5`-GATCTGTGGTCCTCGGCTCA-3`
6N	3’-CGACTAACCACAAAGGCGCA-5’

	Primer sequences for PLB3
1	5’-GTCGGGTTGATGAGCGTGAG-3`
2	3`-GCGCCCACTCCACATCTCCACTCGAATCGGTGCCGTTGGATGTCT TCCAG-5`
3	5`-CTGGAAGACATCCAACGGCACCGATTCGAGTGGAGATGTGGAGT GGGCGC-3`
4	3`-ATCAAACAAAGTCTACTGCCATCTGCTATAGAATCATCCTTATTC GTTGA-5`
5	5`-TCAACGAATAAGGATGATTCTATAGCAGATGGCAGTAGACTTTGT TTGAT-3`
6	3`-TTCTGAAGCGGTGGTGCTTG-5`
1N	5`-CGTCGGTGATTCGGATGGTG-3`
6N	3`-CCATCTGGACCCGGCAAAAG-5`

	Primer sequences for PLCA
1	5`-AGCTTCTGGAGCACGTTCCT-3`
2	3`-GCGCCCACTCCACATCTCCACTCGATTTGACAGTGGTTCTGTA ACCCTTT-5`
3	5`-GCGCCCACTCCACATCTCCACTCGATTTGACAGTGGTTCTGTA ACCCTTT-3`
4	3`-AACATCTATCAGGATCTGACTACATCTATAGAATCATCCTTAT TCGTTGA-5`
5	5`-TCAACGAATAAGGATGATTCTATAGATGTAGTCAGATCCTGA TAGATGTT-3`

6	3`-TCGTCATACCTCCGACCAGC-5
1N	5`-GGCAATGCCCAGTCAACGAT-3`
6N	3`-TCGTCATACCTCCGACCAGC-5`

	Primer sequences for PLCB
1	5`-GGACCAATTCCAAACCCAGACG-3`
2	3`-GCGCCCACTCCACATCTCCACTCGATCTGCAGAGATGAAGCAG AGAAGAC-5`
3	5`-GTCTTCTCTGCTTCATCTCTGCAGATCGAGTGGAGATGTGGAGT GGGCGC-3`
4	3`-CCATGTCCCATATTTAGTTCCATGTCTATAGAATCATCCTTATT CGTTGA-5`
5	5`-TCAACGAATAAGGATGATTCTATAGACATGGAACTAAATATGG GACATGG-5`
6	3-TTCAGTGTCTTGCATGTCGTCG-3`
1N	5`-TGCATAATTTCCCCGCCTGC-3`
6N	3`-CACAGTCAAGCCAGGAGCAC-5`

	Primer sequences for PLCC
1	5`-GACAGTTGGAATTGCGGCCT-3`
2	3`-GCGCCCACTCCACATCTCCACTCGAGATGATGGACGCAGCTCT  TACCCAT-5`

3	5`-ATGGGTAAGAGCTGCGTCCATCATCTCGAGTGGAGATGTGGAG  TGGGCGC-3`
4	3`-TTTCTGAGCAGGTTTTGCATGAAATCTATAGAATCATCCTTATT  CGTTGA-5`
5	5`-TCAACGAATAAGGATGATTCTATAGATTTCATGCAAAACCTGC  TCAGAAA-3`
6	3`-ACCAAGCTGAGGAGCGAAGT-5`
1N	5`-CAAGCCAAGGAACGCCATGA-3`
6N	3`-CTGAGGGTGAGATGGAGCGT-5`

2.1.2.4 Real-time PCR primers

	Sequence from (5` to 3`)
PLB1	5’-TGTTGGCTTTGTCATGGGTA-3’
	3’-GATGTCGGTGAAGACGGATT-5’
PLB2	5’-CAGGCATTTCTGCAGATGAA-3’
	3’-GGCTCGCATAGCGATAGAAC-5’
PLB3	5’-CCAATGGAGCTGGAGCTATC-3’
	3’-TGCAGTAGACCACCGAGATG-5’
PLCA	5’-CGGCGAGGTCATCAACTACT-3’
	3’-AAGTGCCGGACGTCAGATAG-5’
PLCB	5’-ACAGGTCGAGCAGAAGGGTA-3’
	3’-GGGGTAAAGGGCTCAAAGTC5’-
PLCC	5’-CGGACGCTCTCTTCTTCAAC-3’
	3’-ATGGAGTTGGTGTCGAGGTC-5’
ACTIN	5’-TGCTCCTCCTGAGCGTAAAT-3’
	3’-ACATCTGCTGGAAGGTGGAC-5’

2.2 Organisms

2.2.1 *A. fumigatus* strains

Sequenced strains Af293 and Af1163 were kindly supplied by Dr Paul Bowyer (Faculty of Health and Medical Sciences, University of Manchester, UK). Clinical isolates were kindly supplied by Dr Caroline Moore and were obtained from the Wythenshaw hospital culture collection (University Hospital of South Manchester,

Wythenshaw, Manchester, UK). Environmental Dublin *A. fumigatus* isolates were kindly supplied by Prof. Hubert Fuller (University College Dublin, Ireland). These isolates were obtained from the air at the Belling field campus, University College Dublin on the 19th November 2005 and were putatively identified as *A. fumigatus* based on microscopic observations but had not been confirmed by sequencing.

2.2.2 E. coli strains

Escherichia coli (α-select Chemically Competent Cells) strains were routinely maintained in LB-broth supplemented with 100 µg ml^{-1} ampicillin (prepared as 10 mg ml^{-1} filtered sterilized stock solution stored at -20^{O}C). Strains were stored on LB agar plates at 4°C for up to one month or at -80°C in LB broth containing 50% (v/v) glycerol.

2.2.3 Wax moth larvae

Wax moth larvae (*Galleria mellonela*) were obtained from Live Food Direct Ltd (Sheffield UK). Larvae (ca. 0.3-0.4 g in body weight) were supplied in boxes in wood shavings and after visual inspection and removal of dead larvae, were stored in the dark at 4°C for up to ten days.

2.3 Methods

2.3.1 Growth and maintenance of *A. fumigatus*

2.3.1.1 Manipulation of spores of A. fumigatus

Precautions were taken when working with *A. fumigatus* spores to prevent cross contamination between samples and escape into the laboratory by carrying out all manipulations in a Class II microbiological safety cabinet. The work surfaces of the cabinet were cleaned thoroughly before and after use with 10% (v/v) TriGene (Medichem International, UK) followed by 70% (v/v) Industrial Methylated Spirits.

2.3.1.2 General cultivation of A. fumigatus *isolates*

Aspergillus fumigatus isolates were grown in 500 ml vented tissue culture flasks containing 50 ml of PDA agar (ForMedium, UK). Flasks were streaked with spores and incubated at 37°C for a minimum of 7 days or until confluent growth was obtained.

2.3.1.3 Preparation of spore suspensions

Following a minimum of 7 days growth at 37°C, spore suspensions were prepared by adding 10-20 ml of sterile DW 0.05% (v/v) Tween 20, tightening the lid and shaking vigorously to dislodge the spores. The spore suspensions were separated from mycelial fragments and conidiophores by filtering through a double layer of sterile lens tissue (Whatman, UK). If required, spore suspensions were then centrifuged at 3000 rpm for 15 min at room temperature, the supernatant removed and spores re-suspended in 2 ml of sterile 0.05% (v/v) Tween 20. Spore suspensions were stored at 4^0C for up to 3 months.

2.3.1.4 Storage of A. fumigatus *isolates*

For long-term storage, 900 µl of spore suspension was mixed with 900 µl of sterile glycerol in triplicate sterile CryoTubes (Nunc, Thermo Fisher Scientific, UK) and stored at -80°C. For short term storage, tissue culture flasks with confluent sporulating mycelium or spore suspensions were stored at 4°C.

2.3.1.5 Measurement of spore count and viability

The viability of the spores was assessed using the colony count method. For each suspension, the spore concentration was determined by counting using an improved, Haemocytometer Chamber (Improved Neubauer, Hawksley, UK). Spore suspensions were then adjusted to 108 spores per ml, and serially diluted tenfold to 102 spores per ml in sterile 0.05% (v/v) Tween 20. 100 µl of 10^3 and 10^2 spores per ml dilutions were spread evenly over three replicate PDA plates with a sterile spreader and incubated at 37°C for 48 h.

2.3.1.6 Growth of *A. fumigatus* in liquid culture

Spore suspensions were used to inoculate either 50 ml of PDB (Oxoid Limited, Hampshire, UK) in a 250 ml Erlenmeyer flask or 5-10 ml of PDB in a conical tube (Corning, UK), to a final concentration of 10^8 spores per ml. Cultures were grown at 37°C for maximum three days in a G25 orbital incubator (New Brunswick Scientific Inc., Edison New Jersey, USA) at 250 rpm.

2.3.1.6 Colony radial growth rate (Kr)

Colony radial growth rate (Kr) measurements were performed as described by Robson et al (1995). Petri dishes containing 20 ml of medium were inoculated centrally by dipping the tip of a sterile cocktail stick into the spore suspension and gently piercing the centre of the plate. Plates were incubated at 37°C and readings taken twice a day by measuring the

diameter of each colony in (average of perpendicular and horizontal measurement). The colony radial growth rate was determined by plotting colony radius against time and calculating the slope of the regression line.

2.3.2 Determination of total fungal viable and *A. fumigatus* viable airborne spore concentrations

Airborne total viable fungal counts were determined at ca. monthly intervals over a two year period (March 2009 to February 2011 inclusive) outside the Michael Smith building on the University of Manchester campus (GPS latitude 53°27′53.7834″, longitude 2°13′41.5884″) using a Super 10/180-SAS Isolator DuoSAS 360 (Cherwell Laboratories, UK). The lid was sterilized by autoclaving or wiping with 70% (v/v) industrial methylated spirits and the air was sprayed with 70% (v/v) industrial methylated spirits while the air flow was running for 30 s prior to air sampling. Air was aspirated through a 219-hole sieve head plate onto a PDA plate (90 mm diameter) supplemented with 0.05% (w/v) chloramphenicol (Sigma-Aldrich, UK) to suppress bacterial growth. The samples were collected at ca.1.5 m above the ground between 10:00 am and 12:00 noon when no rain fall was occurring. A total volume of 500 L of air was sampled in standard mode six times in succession. Three plates were incubated at 45°C and three at 25°C. Plates were inspected for growth after three days and the number of colonies visible to the naked eye counted and numbers corrected using the manufactures positive-hole conversion (Cherwell laboratories, UK). The total concentration of airborne viable fungal spores per cubic meter of air was calculated from the total fungal counts at 25°C and the total concentration of airborne viable putative *A. fumigatus* spores was calculated from the counts at 45°C. Putative *A. fumigatus* colonies were sub-cultured using a sterile toothpick onto PDA in tissue culture flasks and universal tubes. Only one main morphological type was ever recovered during the two year sampling period at 45°C and was provisionally identified as *A. fumigatus* based on the re-definition of Aspergillus section

Fumigati as proposed by (Hong et al., 2005) by macroscopic and microscopic characteristics. In total, 220 putative *A. fumigatus* isolates were collected over the two year period. Randomly selected isolates were further characterised by ITS rDNA sequencing (White et al, 1990) and by PCR-RFLP of a β-tubulun (*benA*) amplicon (Staab et al, 2009).

2.3.3 Virulence determination in wax moth larvae

Wax moth larvae (WML) (Live Food Direct Ltd. Sheffield, UK) were stored in wood shavings in the dark at 4°C for up to 10 days before use. WML larvae in groups of 30 were inoculated with 10 µl (this is what I mint of spore suspensions) of an *A. fumigatus* spore suspension into the haemocoel through the last right or left proleg, using a Hamilton 1 ml gastight syringe (Fisher Scientific, UK) with a 10 µl repeating dispenser attachment (Jaytee Biosciences Ltd, UK). Spore concentrations of $1X10^{8,}$ to $1X10^{3}$ per ml were used to infect larvae andincubated at 37°C in the dark in Petri dishes (10 larvae per plate) and the mortality monitored daily for 7 days. Mortality was assessed by lack of movement in response to stimulation and by discoloration (melanisation) of the cuticle. The LD90 (lowest spore concentration that kills 90% of WML) for each isolate was determined from a plot of percentage mortality against time since inoculation.

2.3.3.1 Statistical analysis

2.3.3.1.1 Man-Whitney U test (Stat Direct)

The survival of the WML was analysed using the Mann Whitney U test (Stats Direct) with a P value of >0.05 consider significant and was used to compare differences in survival rate of larvae when inoculated with different strains.

2.3.3.1.2 Kaplan-Meier test

A nonparametric estimate of the mean survival time for WML was obtained as the area under the Kaplan-Meier estimate of the survival curve. Kaplan meier also known as the product limit estimator, an estimator for estimating the survival_function from lifetime data. In medical research, it is often used to measure the fraction of patients living for a certain amount of time after treatment. And a score for the rank order of day 7 (0) score (when the outcome is unknown) and a code given as (1) when the time it must be recorded whether or not the case has reached the endpoint (is censors).

2.3.4 General DNA manipulation and analysis

2.3.4.1. Fungal genomic DNA extraction

Fungal genomic DNA was extracted from mycelium grown for 16-18 h in Vogel's medium in liquid culture at 37°C on a rotary shaker (New Brunswick, UK) at 250 rpm. Mycelium was harvested by vacuum filtration through Whatman 54 filter paper, washed with distilled water, transferred to a 15 ml polypropylene tube and flash frozen in liquid nitrogen. Mycelium was either immediately used for DNA extraction or stored at -80°C until required. Frozen mycelium was ground in liquid nitrogen to a powder in a mortar and pestle pre-cooled to -20°C or freeze-dried by lyophilisation for two days and genomic DNA was extracted with chloroform/isoamyl alcohol or with a DNeasy Plant minikit (Qiagen, UK).

2.3.4.1.1 Chloroform: Isoamyl alcohol extraction

Ground mycelium was added to 50 ml Falcon tubes (on ice) to the 10 ml mark and an equal volume of pre-heated DNA extraction buffer (0.7 M NaCl, 1 M $Na_2(SO_3)$, 0.1 M Tris-HCl (pH 7.5), 0.05 M EDTA, 1% (w/v) SDS, 65°C) was added and mixed thoroughly with a pipette tip and incubated at 65°C for 30 min to terminate nuclease activity and induce protein denaturation (James et al., 2000). After incubation, 10 ml of chloroform: isoamyl alcohol

(24:1) was added, vortexed vigorously and placed on ice for 30 min. Following centrifugation at 5000 rpm for 30 min at 4°C, the top aqueous layer was transferred to a new sterile tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added, vortexed and placed on ice for 15 min. Following centrifugation at 5000 rpm for 15 min, the supernatant was transferred to a new 50 ml falcon tube and an equal volume of isopropanol added, mixed by inversion, left for 15 min at room temperature to precipitate the nucleic acids, and centrifuged for 10 min at 5000 rpm at 4°C. After removing the supernatant, the DNA pellet was left to air dry, re-suspended in 2 ml of DEPC water and incubated at 37°C for 15 min. 1 ml of 7.5 M NH_4 acetate was added, mixed by inversion and placed on ice for 1 h. The suspension was then centrifuged at 5000 rpm for 30 min, the supernatant removed to a new tube and 1.6 ml of isopropanol added. The tube was mixed by inversion and left at room temperature for 10 min to allow the DNA to precipitate, centrifuged at 5000 rpm for 15 min and the supernatant removed. The DNA pellet was washed with 0.5 -1 ml 70% (v/v) ethanol and centrifuged at high speed for 10 min. The supernatant was removed and the pellet air dried. The DNA pellets were then re-suspended in 0.5-1 ml DEPC water or TE buffer (pH 7.5) and left overnight at 4°C Aliquots were frozen and stored at -80°C until required.

2.3.4.1.2 DNeasy Plant Mini Kit

The DNeasy® Plant Mini Kit (Qiagen Ltd, UK) was used according to the manufacturer's instructions by following the protocol for purification of total DNA from plant tissue. Approximately 0.3-0.4 ml of ground mycelium was added to a 1.5 ml eppendorf tube and vortexed vigorously with 800 µl buffer API containing 4 µl of RNase a stock solution (100 mg ml^{-1}) and incubated for 30 min at 65°C with occasional mixing. After the addition of 260 µl buffer AP2, the tube was incubated on ice for 15 min before pipetting the lysate onto a QIA shredder minispin column and centrifuged for 2 min at 14,000 rpm. The collected supernatant was mixed with 1.5 volumes of buffer AP3 containing ethanol, transferred onto a

DNeasy mini spin column and centrifuged for 1 min at 8,000 rpm and the supernatant discarded. Following the addition of 700 µl of Buffer AW, the spin column was centrifuged for 1 min at 8000 rpm and the supernatant discarded Following the addition of a further 700 µl of Buffer AW, the column was centrifuged at 14,000 rpm for 2 min and the supernatant discarded. After the addition of 50 µl buffer AE, the column was incubated for 5 min at room temperature, centrifuged for 1 min at 8000rpm and the eluate collected. Aliquots were frozen and stored at -80°C until required.

2.3.4.2 Bacterial plasmid extraction

For rapid screening of recombinant transformants, plasmid DNA was prepared from *E. coli* cultures using a QIAprep Spin Miniprep kit (Qiagen, UK) according to manufacturer's instructions. 2 ml of LB broth containing 4 µl ampicillin (100 µg ml^{-1} ampicillin) in falcon tubesDiscarded were inoculated and incubated for 6-8 h at 37°C on a rotary shaker at 225 rpm, and subcultured into 3 ml of LB broth containing 6 µl ampicillin (100µg ml^{-1} ampicillin) and incubated at 37°C for 18 h on a rotary shaker at 225 rpm. Cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C. Plasmid DNA was extracted and purified using a QIAGEN Plasmid Mini Kit according to the manufacturer's instructions. Following centrifugation, the bacterial pellet was resuspended in 300 µl of PI buffer containing RNase A and LyseBlue reagent and transferred to a micro centrifuge tube. Following the addition of 300 µl of P2 buffer, cells were mixed by inverting the tube and incubated at room temperature for 5 min. 300 µl of chilled buffer P3 was added, mixed immediately and incubated on ice for 5 min and centrifuged at 13000 rpm for 5 min. The supernatant was carefully removed, and applied to a QIAGEtip 20 column that had 1 ml Buffer QBT added and allowed to enter the column by gravity. The column was washed with 2 ml of buffer QC and the DNA eluted with 0.8 ml buffer QF. The DNA was precipitated by adding 0.7 volumes (0.56 ml per 0.8 ml of elusion volume) of room-temperature isopropanol

to the eluted DNA, gently mixed and centrifuged at 13000 rpm for 30 min. The supernatant was carefully removed from the DNA pellets and discarded and the pellet washed with 1 ml of 70% (v/v) ethanol and centrifuged for 10 min. After carefully decanting the supernatant, the DNA pellet was air dried for 5-10 min and redissolved in 30 µl TE buffer (10 mM Tris-HCL, pH 7.5, 0.1 mM EDTA). Aliquots were frozen and stored at - 80°C until required.

2.3.4.3 Quantification of DNA and RNA

A NanoDrop 1000 Spectrophotometer (Thermo Scientific, UK) was used to measure DNA and RNA concentrations in samples. The measurement program was initialized with 1 µl of DEPC water loaded onto the pedestal and the machine set to zero. This was repeated a second time before the addition of 1 µl of the sample and the sample absorbance measured at 280 and 260 nm. The 260/280 nm ratio for all samples was between 1.8-2.0.

2.3.4.4 Gel electrophoresis

Agarose gels (0.8 to 1.0 % (v/v) were prepared and run in Tris-acetate EDTA buffer (TE pH 8.0,[0.04 M Tris, 0.001 M EDTA, 65% (v/v) glacial acetic acid]. Agarose (Melford Laboratories, UK) was dissolved by heating in TAE buffer and allowed to cool to Ca. 50°C before pouring into the gel mould. After setting at room temperature, the gel was covered with TAE buffer. Gel loading buffer (0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, 30% (v/v) glycerol, 5 mM EDTA, 10 mM Tris-HCl, pH 7) was added to DNA samples before loading onto the gel (usually 2 µl of loading buffer in 10 µl of DNA sample). Gels were run for Ca. 1 h in TAE buffer at a constant voltage of 70V. Gels were stained in a bath of TAE buffer containing ethidium bromide (10 mg ml^{-1}, Sigma) for ca. 20 min and visualized and photographed under UV light using UVItec transilluminator and imaging system (UVtec Ltd. Cambridge, UK) to detect each DNA amplicon and its size. Hyperladder I, IV or V was used on every gel to enable the size and concentration of products to be estimated. The ladder used depended on the size of the products expected.

2.3.5 Polymerase chain reaction (PCR)

Special precautions were taken to minimize the potential for contamination of the polymerase chain reaction **(**PCR). All PCR components were handled with a separate dedicated set of micro-pipettes using pre-sterilized aerosol-resistant tips. Negative controls, containing the same concentration of components as the other tubes but without template DNA, were also included to check for contamination. To further minimize risk, highly purified water (DEPC standard) was used in all steps. A positive control using genomic DNA from *Af*293 was included to verify the integrity of all the components of the PCR.

Standard PCR amplification was carried out using separate *Taq* polymerase, buffer, dinucleotides and $MgCl_2$ if required (Bioline, UK). A premix of these reagents was normally prepared before addition of primers in 50 µl reaction volumes followed by addition of target DNA. Amplification was carried out using either a Thermal iCycler (Bio-Rad, UK) or Applied-Biosystems thermal cycler (Applied Biosystems, UK). After amplification a 5 µl of PCR product was analysed by gel electrophoresis.

2.3.5.1 ITS sequencing

The internal transcribed spacer (ITS) region is the most extensively sequenced DNA region in fungi. 18S-ITS1 and ITS4-28S primers amplify the highly variable ITS1 and ITS4 sequences surrounding the 5.8S-coding sequence and is situated between the small subunit-coding sequence (SSU) and the large subunit-coding sequence (LSU) of the ribosomal operon (White et al., 1990). The ITS region has been used extensively for phylogenetic analysis, as it shows variation between different species but with minor variations within strains of the same species.

PCR was performed using 50 µl of reaction mixture containing ~100 ng of genomic DNA (20 ng μl^{-1}). The final concentration of components in the PCR reaction was 1x PCR

buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8 and 0.1% stabilizer), 1.5 mM $MgCl_2$, 0.02 mM of ITS-1 and ITS-4 primers (Eurofins, UK), 0.2 mM of dNTPs (Bioline, UK) and 0.5 U of *Taq* DNA polymerase (Bioline, UK). The PCR was carried out under the following conditions: Denaturation at 95°C for 1 min, followed by annealing at 55°C for 1 min for 40 cycles and finally extension at 72°C for 1 min.

2.3.5.2 Ben A PCR restriction fragment polymorphism

The ITS region is known to be inadequate for discriminating between closely related species within the Aspergillus section Fumigati such as *A. fumigatus*, *A. lentulus*, and *Neosartorya fischerii*. However, sufficient variation in the intragenic regions between these species has been shown in the β-tubulin encoding gene *benA* enabling discrimination of these closely related strains (Staab et al, 2009) and was used to further verify strain identity (Balajee et al., 2007).

PCR was performed using 50 µl of reaction mixture containing 100 ng of genomic DNA (20 ng μl^{-1}). The final concentration of components in the PCR reaction was 1 x PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8 and 0.1% stabilizer), 1.5 mM $MgCl_2$, 0.2 pM of each primer, βtubI and βtubII (Eurofins, UK) , 0.2 mM of dNTPs and 0.5 U of *Taq* DNA polymerase. The PCR was carried out under the following conditions: initial denaturation at 94°C for 2 minutes followed by three cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s. The reactions were terminated with a final incubation at 72°C for 5 min, following the last cycle (Staab et al., 2009).

10 µl of the *benA* PCR amplicon was added to 1 x NE buffer 1 (New England Biolabs, UK), 100 µg/ml BSA (New England Biolabs, Ipswich, United Kingdom) and 1.0 unit of BccI enzyme (New England Biolabs, Ipswich, UK) and incubated at 37°C for 1 h. Banding patterns were examined after resolving on an agarose gel.

2.3.5.3 Random amplification of polymorphic DNA (RAPD)

A total of 106 isolates were analyzed using RAPD as described by Dyer *et al.* (1993). The primers used for random amplification of polymorphic DNA (RAPD) typing were R108, C90, R151 and C08 (Eurofins, UK). Fingerprinting of *A. fumigatus* proceeded as follows. The reaction mixture contained the amplification buffer (160 mM $(NH4)2SO4$, 67 mM Tris-HCl, pH 8.8, 0.1% stabilizer), 3 mM $MgC12$, 200 µM of each dNTP, 20 pmol primer, 0.5-1 U *Taq* Polymerase (Bioline, UK) and ~50 ng genomic DNA template and the volume made up to 50 µl with DEPC water. The reaction conditions were standardized to ensure optimal reproducibility of RAPD profiles in subsequent investigations. DNA from isolate *Af*293 was included on every gel to enable normalisation of band positions between gels. PCR was performed using an iCycler (Bio-Rad, UK) under the following conditions: Denaturation at 93^{O}C for 2 min, 40 cycles at 93°C for 30 s (ramp rate 0.5^{O}C/s), 40 s annealing at 40°C and ramp rate 0.5^{O}C/s for 30 s followed by extension step at 72°C : 1 min 20s (ramp rate 0.3^{O}C/s) and a final extension of 5 min at 72°C. The amplified products (25 µl) were run on 1.5% (w/v) agarose gels in TAE buffer at 50 V for up 3 h. Gels were stained with ethidium bromide (10 mg/ml, Sigma) and PCR products were visualized and photographed under UV using a UVItec transilluminator and imaging system (UVtec Ltd. Cambridge, UK). Band sizes were determined by comparing their position against hyperlader VI (Bioline, UK). Only clear and reproducible RAPD PCR bands were included in the analysis and were scored as either absent (0) or present (1). The data from all primers were pooled for each isolate and a pair's similarity matrix was calculated with Jaccard's coefficient with the program FREETREE v. 0.9.1.50. A bootstrapped dendrogram with 1000 replications was produced with NJ plot.

2.3.6. Purification of PCR products

2.3.6.1 Purification from PCR

When required, PCR products were purified using a QIAquick PCR purification kit, following the micro-centrifuge protocol, according to the manufacturer's instructions (Qiagen Ltd, Crawley, United Kingdom). All centrifugation steps were performed at 13,000 rpm. Five volumes of PBI buffer (after addition of pH indicator) was added to one volume of PCR product and vortexed. The mixture was applied to a QIAquick column centrifuged for 30-60 s at 13000 rpm, and the supernatant discarded. 750 µl of PE buffer was applied to the column, centrifuged for 30-60 s and the supernatant discarded. After a second centrifugation for 1 min, any further supernatant was discarded and the DNA eluted by applying 50 µl of EB buffer and the supernatant collected following centrifugation at 13000 rpm for 1 min.

2.3.6.2. Purification from agarose gels

PCR products were separated on a 1% (w/v) UltraPure™ agarose gel (Invitrogen) and visualized under low-strength UV light. A clean scalpel was used to cut the band to be purified, and placed in a clean eppendorf tube and extracted using a QIAquick Gel Extraction Kit (Qiagen Ltd., Crawley, United Kingdom) according to the manufacturer's instructions. Three volumes of DNA-Binding buffer (QG) were added to the excised band and heated at 50°C for 10 min to dissolve the agarose. After gently mixing, an equal volume of isopropanol was added and applied to a QIAquick spin column and centrifuged at 13,000 rpm for 1 min. After discarding the supernatant, 0.5 ml of QC buffer was added, the column centrifuged for one min at 13,000 rpm, and 0.75ml of 70% (v/v) ethanol added. After centrifugation at 13000 rpm for 1 min, 50 µl of elution buffer EB (10 mM Tris-HCL, pH 8.5) was added, the column centrifuged for 1 min at 13000 rpm and the supernatant collected.

2.3.7. Cloning procedures in bacteria

2.3.7.1. Preparation of competent cells

The α-Selected Chemically Competent *E. coli* cells (Bioline, UK), contain a lacZ marker that provides α-complementation of the β-galactosidase gene for blue/white colour screening enabling transformed cells containing the DNA insert (white colonies) to be distinguished from untransformed cells (blue colonies) following induction with IPTG in the presence of X-Gal. The cells were stored at -80°C until required and thawed on ice with gentle swirling according to the manufacturer's instructions.

2.3.7.2 Cloning of PCR amplicons

DNA fragments generated by PCR were cloned into the pGEM-T vector (Promega Inc., Madison, Wisconsin, USA). This vector has a single 3´-thymidine residue at the insertion site of the vector to facilitate cloning of *Taq* DNa polymerase amplified DNA fragments, which have a single deocyadenosine added, independent of the template, to the 3´ ends of the PCR products. This vector shares other features such as multiple cloning sites with a range of restriction enzyme recognition sites, *LacZ* gene for blue-white selection and an ampicillin gene for antibiotic selection. 1µl of appropriate vector (50 ng), 15 µl of 10X ligation buffer, 15 µl of purified PCR products approximately 50-200 ng of target DNA, 1 µl of T4 DNA ligase (1 unit/µl) ligation reaction were incubated at 4°C over night.

2.3.7.3 Transformation of competent cells

Frozen competent cells were thawed, gently mixed to ensure all cells were in suspension, and 50 µl of mixed with 15 µl of ligation reaction in 12 ml Falcon tubes and incubated on ice for 30 min. Cells were heat shocked using water bath at 42°C for approximately ~30-45 s (without shaking) and placed immediately on ice for 2 min. Cells were diluted to a final volume of 1 ml by the addition of 935 ml of SOC media (Super Optimal Broth with Catabolite Repression) containing 2% (w/v) Tryptone, 0.5% (w/v) yeast extracts,0.4% (w/v) glucose, 10 mM NaCl, 10 mM $MgCl_2$, 2.5 mM KCl and 10 mM $MgSO_4$. Cells were incubated at 37°C for 1 h at 2000 rpm on a rotary shaker to allow expression of the antibiotic resistance gene product before the antibiotic was added.

2.3.7.4 Blue /white selection

Transformed cells were identified by blue/white selection. Transformation of the vector into competent cells disrupts the lac operon and after induction with IPTG in the presence of the substrate X-Gal, remain white while untransformed cells that retain a functioning lac operon turn blue. 40 µl of X-Gal/IPTG stock solution (400 µg ml^{-1} in formamide) was gently spread over the surface of an LB plate containing 100 µg ml^{-1} ampicillin and air dried for 10 mins. 100 µl of transformed cells were then spread gently across the surface of the plate and allowed to dry for 5 min before incubating at 37°C for 24 h. Plates were transferred to 4°C for 3 h to enable colour development and white colonies selected with sterile toothpicks for further analysis. The transformation effeciensy was (4.76667e-3 ng of DNA).

2.3.8. DNA sequencing

The sequencing mixtures were composed of forward and reverse primers and sequence reactions were performed in a volume of 10 µl, containing 20 ng of purified PCR product and 4 pmol of either forward or reverse primer for amplification. DNA was sequenced by the dideoxy termination method at the University of Manchester sequencing facility and the sequences were edited using Finish software and Finch TV trace viewer software (Geospiza, Inc.) DNA sequences were used as queries using BLASTn algorithm searches against the NCBI database. Sequences were also used as queries for the BLAST pairwise alignment algorithm to draw distance trees, enabling isolates to be identified more accurately.

2.3.9. Amplification of sequence upstream and downstream of phospholipase genes and amplification of the hygromycin marker

For homologous recombination and replacement of the target phospholipase gene with the selectable marker for hygromycin resistance, three overlapping amplicons consisting of the gene sequence for the hygromycin selective marker and upstream and downstream sequence of the targeted phospholipase gene were generated by PCR and the final construct generated by overlapping PCR using nested primers. The final construct consisted of the hygromycin resistance gene flanked by homologous sequence upstream and downstream of the targeted phospholipase gene enabling targeted gene replacement by homologous recombination following fungal transformation.

The DNA of *Af*1163 strain was used to prepare upstream and downstream amplicons of the phospholipase gene while the middle part of the cassette was formed from the marker pAN7.1. The primers used to amplify sequence upstream of the *plc C* gene were generated using primers 1 & 2, primers 3 & 4 were used to generate the marker gene hygromycin and primers 5 & 6 used to amplify sequence upstream of the *plc C* gene. Conventional PCR reaction performed using the reaction mixture contained the amplification buffer 5X Phusion™ polymerase with HF Buffer (Finnzymes, New England BioLabs), 200 µM of each

dNTP, 0.5 µM of each primer, 0.2 µl Phusion™ polymerase and ~30 ng of genomic DNA of the *Af*plcC gene and 1µl of DNA of the Marker (pAN7.1) in a volume mixture of 20 µl. The following parameters were used for PCR using the Applied biosystem: 30 s of denaturation at 98°C, 35 cycles of 15 s at 98°C, 30 s annealing at 52°C and 40 s extension at 72°C with a final extension of 40 s at 72°C. The whole amplified products were run on UltraPure™ agarose 0.8 % (w/v) gels in TE buffer at 80 V for 30 min. Gels were stained with ethidium bromide (10 mg/ml, Sigma) and visualized using a UVItec transilluminator and imaging system (UVtec Ltd. Cambridge, UK). A surgical blade was used to excise the relevant band(s) followed by gel extraction using QIAquick® Gel Extraction according to the manufacturer's instructions.

2.3.9.1 Generation of the cassette by overlapping PCR

Approximately 0.15-0.2 µl of purified product from each of the three previous PCR purified reactions were used to generate the construct. 5X Phusion™ polymerase HF Buffer, 200 µM of each dNTP, 0.5µM of each of the nested primers (1N and 6N), and 0.02 U/µl Phusion™ polymerase (Finnzymes, New England, BioLabs® Inc.UK) in a volume mixture of 20 µl. Conditions were as follows: 40 sec of denaturation at 98°C, 35 cycles of 10 s at 98°C, 30 s annealing at 68°C and 40 s extension at 72°C with a final extension of 40 s at 72°C. The PCR routine used to amplify the construct from three templates involved multiple different cycles and ramp rates.

Following resolution on an agarose gel of 0.8%, at 80 V, PCR products were stained with ethidium bromide visualised under a UV transluminator and the appropriate sized amplicon removed using a surgical blade followed by gel extraction using QIAquick® Gel Extraction kit according to the manufacturer's instructions.

2.3.9.2 Poly-A tail

Phusion enzyme generates PCR products with blunt ends and addition of thymidine at the 5' and 3' ends was required to enable ligation into the cloning vector. To achieve this, 30 µl of eluted purified PCR products were added to buffer (3.55 ul) (160 mM $(NH4)2SO4$, 67 mM Tris-HCl, pH 8.8), (1.06 µl) 1X MgC12, (0.6 µl)1X dNTP,(0.355µl)1X Taq Polymerase (Bioline, UK) for a stock solusion of 50X, and The PCR routine run iiCycler (Bio-Rad, UK) 20 min for 72°C. Detection of specific amplicon products using UV transluminator and surgical blades where cut followed by gel extraction using a QIAquick® Gel Extraction kit.

2.3.10. Phospholipase knockouts in *A. fumigatus*

2.3.10.1 PCR of the inserted cassette from plasmid using nested primers

The PCR reaction of 50 µl was used containing 10 µl, 5X phusion HF buffer 10 mM of DNTPS, and 0.5 µM of each primer (1N and 6N), phusion DNA polymerase 0.5 µl and the plasmid approximately 0.5 µl and varied according to concentration. The reaction conditions were as follows, step one: initialization step, 98°C for 45 s, followed by 35 cycles (98°C for 15 s, annealing 55°C for 30 s and 72°C for 20 s) followed by extension for 72°C for 3 min. The amplicon was separated in a 0.8% (w/v) agarose gel and the required band purified as described in section (2.6.1) and used for transformation.

2.3.10.2 Linearised plasmid

Purified plasmid was linearised using the restriction enzyme BcuI (SpeI) (Fermentas, Life Sciences). 4 µl of 10X Tango buffer and 3 µl of enzyme was added to 30 µl of DNA and made up to a final volume of 40 µl with DEPEC water, vortexed briefly and incubated overnight at 37°C. 20 µl was used for fungal transformation.

2.3.11 Fungal transformation

2.3.11.1 Preparation of A. fumigatus protoplasts

Eight Petri dishes containing 25 ml of SDB media were inoculated with 60 µl of a $1X10^7$ spore per ml suspension and incubated at 37°C or until there is a confluent growth on the surface of the plate. After checking for confluent growth, mycelium was filtered through a sterile funnel lined with a double layer of sterile lens tissue (Whatman) and excess media removed by gently squeezing with a sterile plastic spreader. Mycelium was resuspended in 15 ml of protoplasting buffer (0.6 M KCl and 50 mM $CaCl_2$) containing 3% (w/v) Glucanex (NovoNordisk, Denmark) in a Corning tube at 30^o C with gentle shaking for ca. 3 h until protoplast formation was observed under a light microscope. Protoplasts were filtered through a sterile funnel lined with a double layer of lens tissue (Whatman), and the filtrate containing the protoplasts centrifuged at 3000 rpm for 15 min at 4°C. The protoplast pellet was gently re-suspended in 1 ml of ice cold protoplasting buffer and 100 µl of protoplasts used per transformation.

2.3.11.2 Transformation

15 µl (~2 µg) of the extracted plasmid, linearised plasmid or PCR amplified cassette was added to 100 µl of protoplasts gently mixed and incubated on ice for 20 min. 1 ml of 60% (v/v) PEG (600) (Sigma, UK) at room temperature was slowly added and incubated at room temperature for 10 min to catalyse protoplast fusion and DNA uptake.

Malt extract agar was autoclaved at 121°C for 10 min, allowed to cool to ca.55°C, before adding of 50x diluted stock solution of a volume approximately 2ml/100ml solution, filter sterilized hygromycin B (Melford Laboratories Ltd) added to a final concentration of 200 µg/ml. 200 µl of protoplasts were gently mixed with 20 ml of medium, poured into a Petri dish and allowed to solidify. Plates were incubated at 37°C until growth was detected. If no growth was detected after 7 days the transformation was regarded as negative. Colonies were picked and transferred onto SAB agar containing Hygromycin B (200 µg/ml) using sterile toothpicks.

2.3.11.3 Checking for homologous recombination

After checking purity of growth, DNA was extracted from putative transformants as described in section (2.5.2.1**)** and screened by PCR to determine if the construct had integrated into the fungal genome through homologous recombination. The screening PCR reaction contained 0.5 µl of each appropriate fusion primer, with 1X Phusion™ polymerase Master Mix in HF buffer in a 25 µl final volume mix. Tubes were heated for 45 s at 98°C to activate the polymerase, followed by 35 cycles of denaturation at 98°C for 15 s, annealing at 55°C for 30 s and elongation of 72°C for 20 s per expected Kb of predicted product size. The reactions terminated at 72°C held for 3 min. Products were run on a 1% (w/v) agarose gel and visualized after ethidium bromide staining under UV light.

2.3.12 Quantitation of relative gene expression of phospholipases by real- time PCR

To avoid RNA degradation or contamination with genomic DNA, certain precautions were taken. RNA samples were kept on ice during processing or stored at - 80°C. All RNA manipulations and PCR amplifications were performed in UV- resistance hood equipped with UV lamp (UVP, California, USA) The UV light was turned on for a minimum of 15 min prior to manipulating RNA to destroy any DNA material present within the working area

2.3.12.1 RNA extraction

The mycelia growth was harvested after overnight incubation on orbital shaking for 18 h at 37°C by vacuum filtration and mycelium flash frozen immediately in liquid nitrogen. For wax moth larvae, individual larvae were flash frozen in liquid nitrogen and ground to a powder with a mortar and pestle in liquid nitrogen prior to RNA extraction.

Approximately ~50 mg of ground sample was transferred to an eppendorf tube and RNA extracted using a Qiagen RNeasy Plant mini kit (Qiagen, UK) according to the manufacturer's instructions. 6 µl of β-mercaptoethanol was added to 600 µl of buffer RLT before adding to the ground sample, vortexing and incubated for 2 min at 56^{O}C. The whole lysate was transferred to a QIA shredder spin column, centrifuged for 2 min at 13,000 rpm and the supernatant collected. 500µl of ethanol was added, gently mixed by inversion, transferred onto an RNeasy spin column and centrifuged at 13,000 rpm for 1 min and the supernatant discarded. 700 µl buffer RW 1 was applied to the column, centrifuged at 13,000 rpm) for 1 min and the supernatant discarded. 500 µl of RPE buffer was then added to the column, centrifuged at 13,000 rpm for 1 min and the supernatant discarded. Finally, RNA was eluted from the column by adding 50 µl RNase-free water directly the spin column and centrifuged at 13,000 rpm for 1 min. Then RNA concentration and purity was determined using a Nano Drop 1000 spectrophotometer (Thermo Scientific, UK) and stored in aliquots at -80°C until required.

2.3.12.2 cDNA synthesis

Removing genomic DNA is critical for accurate qRT-PCR, analysis. Any contaminating genomic DNA was removed according to the QUantiTect Reverse Transcription the protocol (Qiagen, UK). 2 µl of gDNA Wipeout buffer and 14 µl of RNase-free water were added to ca. 0.8 -1 µg of RNA in a 0.5 ml PCR tube and the sample incubated for 2 min at 42° C before placing on ice. 1 µl of Quantiscript Reverse Transcriptase, 4 µl of 5X Quantiscript RT Buffer, 1 µl of RT primer mix and 6 µl of master mix were added to the sample and incubated for 15 min at 42° C followed by 3 min incubation at 95°C to inactivate Quantiscript Reverse transcriptase. The sample was then diluted four fold by the addition of 60 µl RNase-free water.

2.3.12.3 Quantitative real time-PCR

QRT-PCR was performed in triplicate for each sample using SYBR-Green to detect PCR products in 96 well plates according to the manufacturer's instructions (BioRad, UK). Controls in which DEPC water replaced the sample and where no reverse transcriptase was added were included to check there was no contamination with genomic DNA.

2 µl of diluted cDNA was added to 18 µl of Master Mix (New England Biolabs, UK) which contained thermopol buffer (10x) for a volume of reaction 1X (2 µl), 0.4 µl dNTPs (10 mM, Bioline, UK), 0.6 µl of MgCl2 (Bioline, UK), 0.75 µl Sober Green (1/2000 dilution, Sigma-Aldrich, UK), 0.2 µl Thermostable Taq DNA polymerase (New England BioLabs., UK and 0.06 µl of forward and reverse primers (50 µM). The PCR reaction was performed using a Chromo4 machine (BioRad, UK) according to the following protocol: step one initial denaturation at 94°C for 10 min for 1 cycle, step two: denaturation at 94°C for 30 s, annealing 60°C for 30 s, and extension for 72°C for 1 min for 35 cycles, step three: final extension 72°C for 5 min for one cycle only. Melting curves for each reaction were conducted at 60-90°C. Data from the QRT-PCR was analysed using Chromo4 software (BioRad, UK).

2.3.12.4 Relative gene experssion

In order to quantify the expression of phospholipase genes in vivo in wax moth larvae, the Ct value for each phospholipase genes was compared to the Ct value for the housekeeping gene actin. The average Ct value for the phospholipase (CtPL) was deducted from the average Ct value for actin (CtAct) (mean at least three replicates) to give the value ΔCt, and converted to a fold change using 2ΔCt.

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Chapter 3

Title: Prevalence, persistence and phenotypic variation of *Aspergillus fumigatus* in the outdoor environment in Manchester (UK) over a two year period.

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3.1. Abstract

Aspergillus fumigatus is considered by many to be the world's most harmful mould. It is an opportunistic fungal pathogen which causes Invasive Aspergillosis (IA) in immunosuppressed patients, is widely distributed in the environment, and air is the main source for the transporting the conidia and is the main source of pulmonary lung infection. In this study, air sampling of total viable fungal spores and viable *A. fumigatus* spores was conducted monthly over a two year period (2009 and 2010) close to Manchester city centre, UK. Total viable airborne fungal counts varied seasonally, peaking in thesummer and autumn in both years reaching peak levels of ca. 1,100 to 1,400 CFU m^3 and was positively correlated to mean temperature (R^2=0.697). By contrast, *A. fumigatus* viable airborne counts were not seasonally associated with persistent low levels of between 3 and 20 CFU m^3 and was not correlated with mean temperature (R^2=0.018). A total of 220 isolates of *A. fumigatus* were recovered and ITS and partial sequencing of the β-tubulin gene of 34 randomly selected isolates confirmed them as *A. fumigatus*. When the colony radial growth rates were determined, the highest rates were observed on potato dextrose agar, followed by Vogel's medium supplemented with phosphatidylcholine and the lowest on Vogel's medium alone. When compared to clinical isolates, clinical isolates had a significantly higher colony radial growth rate on potato dextrose agar compared to environmental isolates.

3.2. Introduction

A .fumigatus is an opportunistic pathogen widely distributed in the environment and is associated with decomposing plant material and is ubiquitous worldwide (Tekaia and Latge, 2005, Pringle et al., 2005, O´Gorman, 2011). Due to the small size of it's asexual conidia, spores are readily transmissible in the air and have a low settling rate compared to other Aspergillus species (Gregory, 1973). The air is considered the main medium for the transport of conidia, which are involved in pulmonary infections in immunocompromised individuals, leading to a spectrum of diseases ranging from to pulmonary infection to systemic fungal infection (O'Gorman and Fuller, 2008,) and although not the predominant fungal species in the airborne mycoflora (O'Gorman and Fuller, 2008, Griffin et al., 2003), it has been estimated that individuals may inhale several hundred conidia a day (Hospenthal et al., 1998).

Total airborne fungal counts are generally much higher in the summer and autumn months and influenced by temperature and humidity with *Aspergillus*, *Penicillium*, *Alternaria* and *Cladosporium* sp usually the predominant fungal species in the airborne mycoflora with peak concentrations typically in the range 10^2 to 10^4 CFU m^{-3} (Frey and Durie, 1960, Beaumont et al., 1985, Shelton et al., 2002, Kasprzyk, 2008, Oliveira et al., 2009, Gonçalves et al., 2010 , Grinn-Gofroń, 2011, Sabariego et al., 2012). Few studies have specifically measured *A. fumigatus* airborne spore counts, with those that have giving conflicting reports as to seasonal effects on spore concentration. Some studies have reported an increase in the autumn and winter months, possibly due to an abundance of decaying plant material due to autumn leaf fall (O'Gorman and Fuller, 2008, Guinea et al., 2006) whereas in other studies a lack of seasonal variation in airborne conidial numbers has also been reported (Infante et al., 1995, O'Gorman and Fuller, 2008). With the exception of locations close to composting facilities, where concentrations of up to ca. $1x10^5$ CFU m^{-3} have been reported (Fischer et al., 1998, Deacon et al., 2009), or close to building demolition work which are associated with elevated counts (Srinivasan et al., 2002, Oren et al., 2001, Arnow et al., 1991) airborne counts have so far generally been reported to be in the range 0

to 70 CFU m^{-3} (O´Gorman, 2011). In indoor environments, conidial numbers are generally similar or lower than outside unless buildings suffer from damp conditions although fungal spores are associated with dust particles which carry microbes, including fungal spores (Rintala, et al., 2012) and potential reservoirs such as air conditioning systems, carpets and the presence of pets (Dharmage et al., 1999, Anaissie et al., 2001, Kemp et al., 2002., Cho et al., 2006, Dassonville et al., 2008, Hamada and Abe, 2010). Monitoring and controlling *A. fumigatus* airborne counts are critical in hospital environments as the presence of *A. fumigatus* has been linked to nosocomial aspergillosis outbreaks (Streifel and Lauer, 1983, Bouza et al., 2002, Hansen et al.,2008). However, to date the minimal airborne concentration of *A. fumigatus* spores necessary to cause infection in patients with significant immunodeficiency remains unknown. Although concentrations below 1 CFU m^{-3} have been shown to be sufficient to cause outbreaks in immunocompromised patients (Arnow et al., 1991, Sherertz et al., 1987), the level of *A. fumigatus* considered to present a risk for immunocompromised patients is >10 CFU m^{3} (Denning, 1996). Previous studies monitoring airborne *A. fumigatus* counts have relied heavily on morphological features for presumptive identification with few studies using rDNA for verification. *A. fumigatus* belongs to the section *Fumigati* which was recently reclassified to contain 28 species (Samson et al., 2006). With several highly related strains being difficult to separate based on morphological parameters alone (Balajee et al., 2005, Alcazar-Fuoli et al., 2008, Serrano et al., 2011) and leaving open the possibility that other related species may form a significant proportion of the population previously thought to have been *A. fumigatus*. The aim of this present study was to monitor the total airborne viable fungal count and the airborne putative *A. fumigatus* count over a two year period in the air at an inner city site in Manchester UK to add further information on the variation in monthly levels of airborne *A. fumigatus* conidia. In addition, after presumptive identification of *A. fumigatus* strains by morphological examination, randomly selected strains were further characterised based on ITS sequencing and restriction digest analysis of a *benA* amplicon to further verify the identity of the isolates.

3.3. Materials and methods

3.3.1. Determination of total fungal viable and *A. fumigatus* viable airborne spore concentrations

Airborne total viable fungal counts were determined at ca. monthly intervals over a two year period (March 2009 to February 2011 inclusive) outside the Michael Smith building on the University of Manchester campus (GPS latitude 53°27′53.7834″, longitude 2°13′41.5884″) using a Super 10/180-SAS Isolator DuoSAS 360 (Cherwell Laboratories, UK). The lid was sterilized by autoclaving or wiping with 70% (v/v) industrial methylated spirits and the air was sprayed with 70% (v/v) industrial methylated spirits while the air flow was running for 30 s prior to air sampling. Air was aspirated through a 219-hole sieve head plate onto a PDA plate (90 mm diameter) supplemented with 0.05% (w/v) chloramphenicol to suppress bacterial growth. The samples were collected at ca.1.5 m above the ground between 10:00 am and 12:00 noon when no rain fall was occurring. A total volume of 500 L of air was sampled in standard mode six times in succession. Three plates were incubated at 45°C and three at 25°C.

Plates were inspected for growth after three days and the number of colonies visible to the naked eye counted and numbers corrected using the manufactures positive-hole conversion (Cherwell laboratories, UK). The total concentration of airborne viable fungal spores per cubic meter of air was calculated from the total fungal counts at 25°C and the total concentration of airborne viable putative *A. fumigatus* spores was calculated from the counts at 45°C. Putative *A. fumigatus* colonies were sub-cultured using a sterile toothpick onto PDA in tissue culture flasks and universal tubes. Only one main morphological type was ever recovered during the two year sampling period at 45°C and all isolates were provisionally identified as *A .fumigatus* based on the re- definition of Aspergillus section *Fumigati* as proposed by Hong et al. (2008) by macroscopic and microscopic characteristics. In total, 220 putative *A. fumigatus* isolates were collected over the two year period and spore suspensions stored in 50% (v/v) glycerol at -80°C.

3.4. Results

3.4.1. Monthly variation in total culturable and *A. fumigatus* culturable airborne fungal spores during 2009-2010

Air samples were taken at monthly intervals in the city of Manchester, next to the Michael Smith Building, University of Manchester (GPS latitude 53°27′53.7834″, longitude 2°13′41.5884″) over a two year sampling period from March 2009 to February 2011 inclusive. The total culturable fungal airborne counts ranged from 6 to 1333 CFU m^{-3} over the two year sampling period. Total culturable airborne spores showed a strong seasonal variation in both years, with the highest total counts occurring during August and September in 2009 and July to August in 2010. In both years, counts fell sharply in November and remained low during the winter before increasing rapidly in May (Fig 3.1a). The mean total concentrations were significantly higher in the summer season (June to August) and autumn season (September to November) compared to the spring season (March to May) and winter season (December to February) ($P<0.05$, Figure 3.2a). With the exception of the summer season, there was no significant difference ($P<0.05$) between comparable seasons over the two years (Fig 3.2a). When the mean monthly temperature and mean total culturable airborne fungal count were examined over the two year period, a significant ($P<0.05$) positive correlation ($R^2=0.697$) was found such that higher airborne counts were associated with higher temperatures (Fig 3.3).

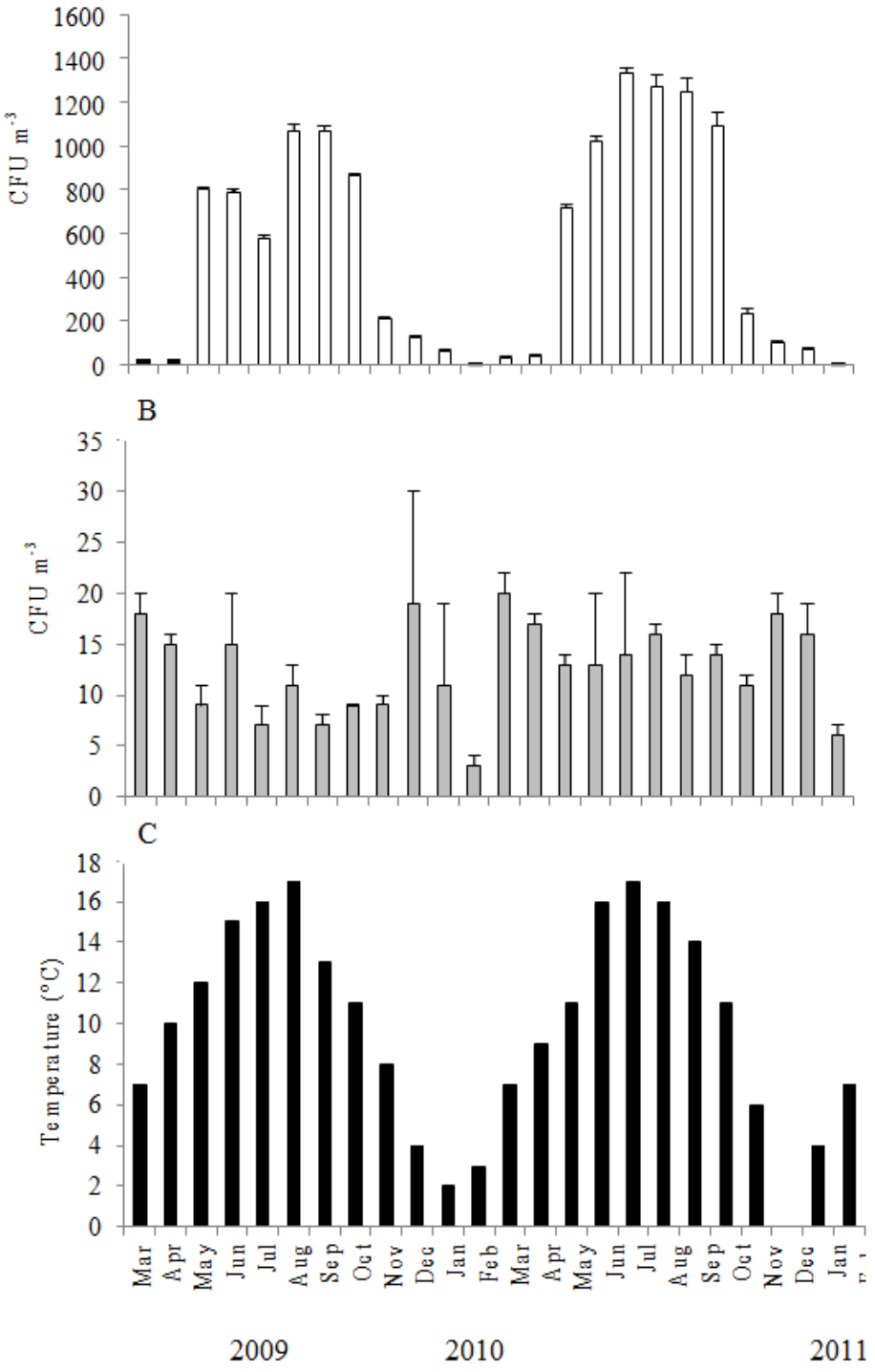


Figure 3.1. Monthly variation in: a) the total viable airborne fungal count (CFU m^{-3}) determined on PDA media at 25°C; b) the putative A. fumigatus airborne count (CFU m^{-3}) determined on PDA at 45°C and c) mean temperature. Air samples were collected near the Michael Smith Building, University of Manchester, UK between 2009 and 2011. Results represent the means of three replicates ± standard error of the mean. Monthly mean temperatures for Manchester were obtained from the NationalClimate Data Centre, UK. +/- (SEM, *standard error*).

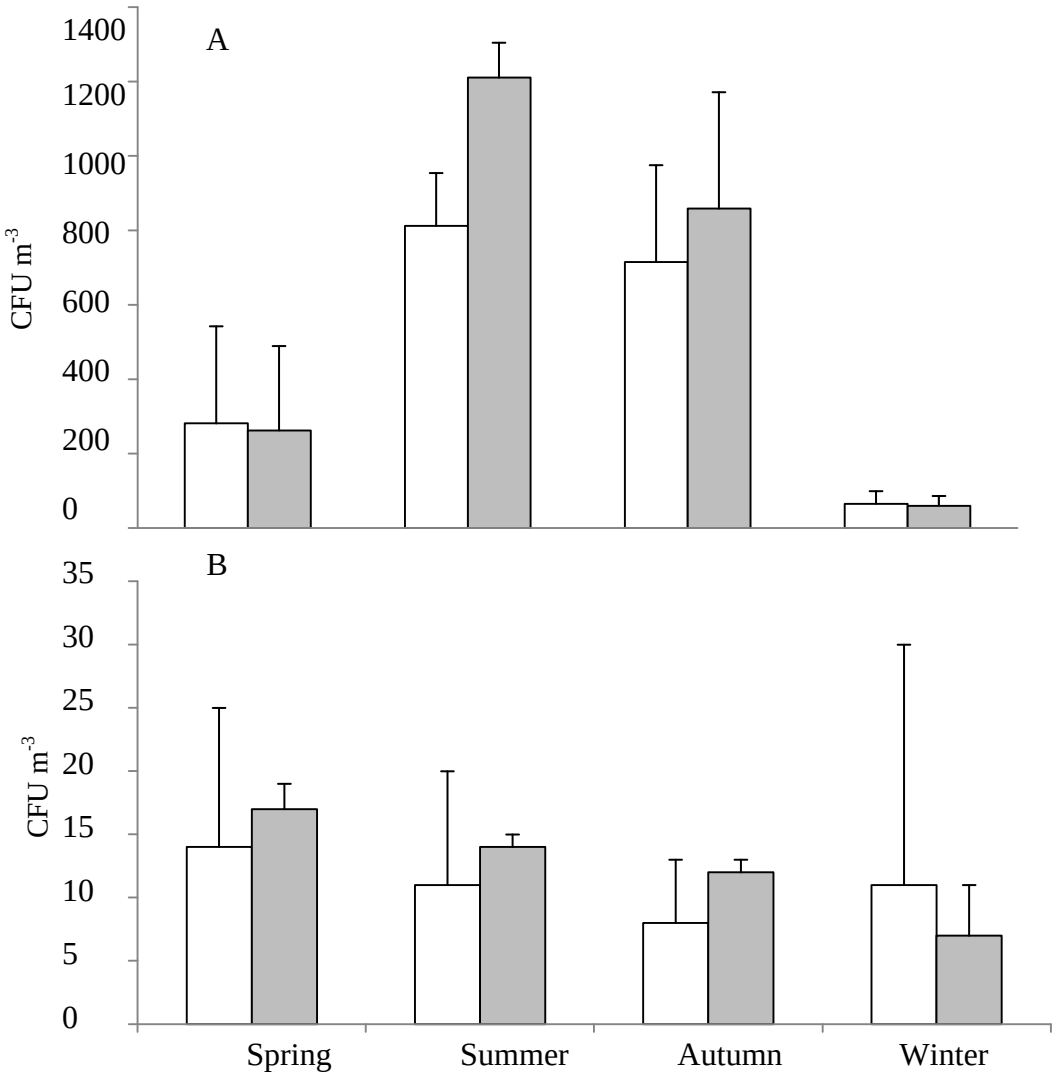


Figure 3.2. Seasonal variation in: A) the total viable airborne fungal count (CFU m^{-3}) determined on PDA media at 25°C; B) the putative A. fumigatus airborne count (CFU m^{-3}) determined on PDA at 45°C. Air samples were collected near the Michael Smith Building, University of Manchester, UK between 2009-2010 and results represent the mens – SEM ±.

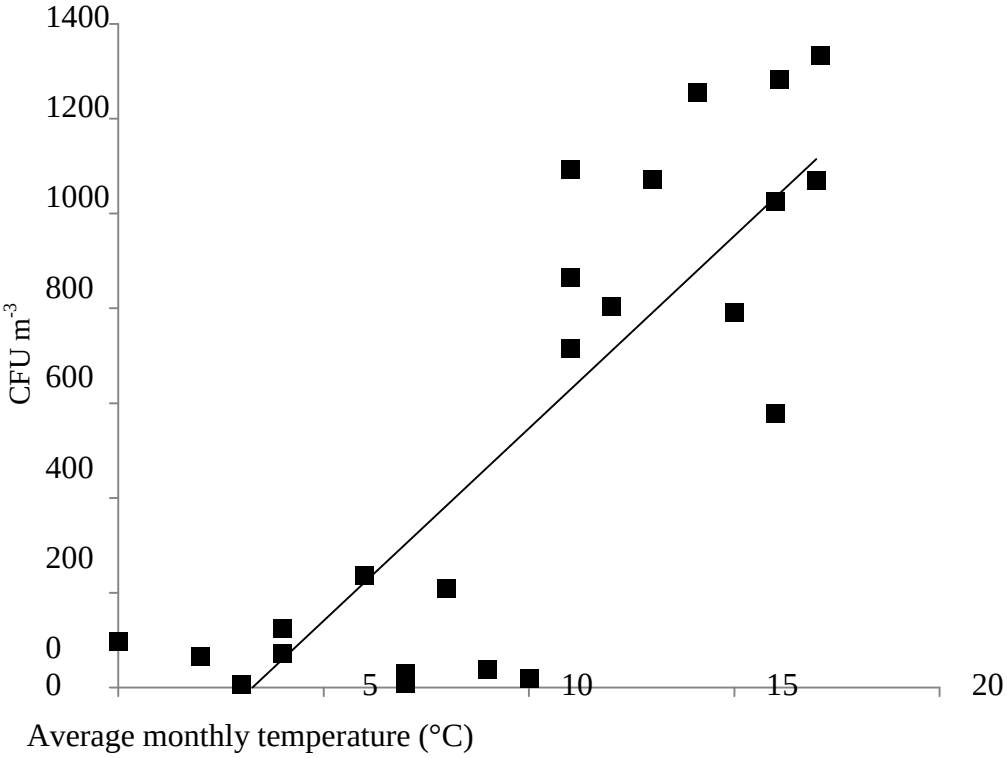


Figure 3.3. Correlation between total fungal airborne count and average monthly temperature. Air samples were collected near the Michael Smith Building, University of Manchester, UK between 2009 and 2011. Monthly mean temperatures for Manchester were obtained from the National Climate Data Centre, UK.

Year	Month	Total count (CFU m^{-3})	*A. fumigatus* count (CFU m^{-3})	Percentage *A. fumigatus*
2009	March	19±2	18±2	94.7
	April	20±3	15±1	75.0
	May	803±11	9±2	1.1
	June	791±10	15±5	1.9
	July	577±14	7±2	1.2
	August	1069±28	11±2	1.0
	September	1072±18	7±1	0.7
	October	864±8	9±0	1.0
	November	209±8	9±1	4.3
	December	124±5	19±11	15.3
2010	January	65±7	11±8	16.9
	February	6±1	3±1	50.0
	March	31±5	20±2	64.5
	April	39±5	17±1	43.6
	May	716±20	13±1	1.8
	June	1025±18	13±7	1.3
	July	1333±24	14±8	1.1
	August	1273±54	16±1	1.3
	September	1245±69	12±2	1.0
	October	1092±63	14±1	1.3
	November	237±20	11±1	4.6
	December	97±9	18±2	18.6
2011	January	73±3	16±3	21.9
	February	8±1	6±1	75.0

+/- (Standard Error)

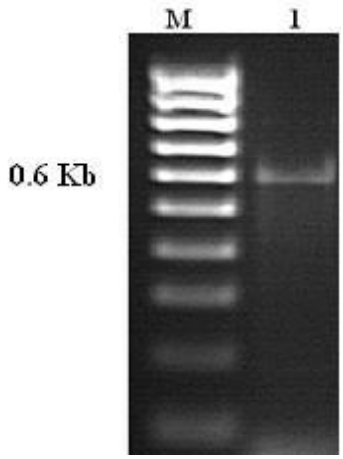
Table.3.1 A. fumigatus airborne count as a percentage of the total airborne fungal count between 2009 and 2011. In Jan-April % of Aspergillus fumigatus is much higer and propabley significant higher. Air samples were collected near the Michael Smith Building, University of Manchester, UK.

By contrast, the putative *A. fumigatus* airborne counts fluctuated between 2 and 20 CFU m^{-3} throughout the two year period (Fig 3.1b) and there was no significant ($P>0.05$) seasonal variation (Fig 3.2b) and no significant correlation ($R^2=0.018$) with temperature (data not shown). Consequently, *A. fumigatus* airborne spores as a percentage of the total fungal airborne spores varied between 80 – 95% in the winter season to less than 1% in the summer and autumn seasons in both years (Table 3.1).

3.4.2. *A. fumigatus* identification

A total of 303 putative *A. fumigatus* strains were isolated over the two year study and were initially identified as *A. fumigatus* based on morphological characteristics and growth at 45°C. 34 strains were randomly selected and subjected to ITS sequencing and included clinical, Manchester environmental Deleted strains. Most isolates showed no differences in the sequence while 7 showed between 1 and 8 single nucleotide polymorphisms (results not shown); however, as *taq* polymerase and not proof reading polymerase was used, this may be due to the infidelity of the polymerase. Nonetheless, sequences were congruent with *A. fumigatus*. It has been shown recently that ITS sequencing alone is insufficient to discriminate between the closely related species *A. fumigatus, Aspergillus lentulus* and *Neosartorya fischerii* within the section Fumigati but that these strains could be discriminate by comparing variable regions within the *benA* gene encoding β-tubulin by restriction digest PCR amplification (Balajee et al., 2007). PCR *benA* amplicons of all 34 strains gave a single product of the predicted size which after digestion with BccI resolved as three distinct bands of 0.25, 0.15 and 0.1 Kb (Figure 3.4.). The number and size of these fragments were in agreement with those published for *A. fumigatus* (Staab et al., 2009).

A



B

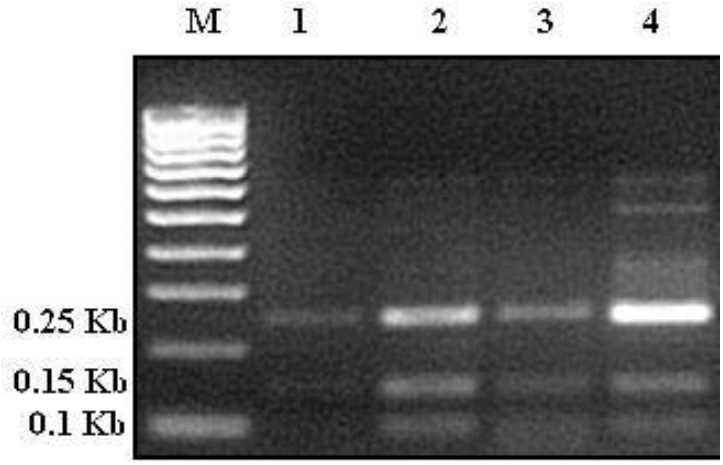


Figure 3.4. (A) Representative single 0.6 Kb PCR product generated from the benA gene using BtI and BtII primers. Lane M = Hyperladder IV (Bioline, UK), Lane 1= example of the PCR product generated from an A. fumigatus isolate. (B) Representative banding pattern from four A. fumigatus isolates after restriction digestion of the 0.6 Kb benA amplicon with the restriction enzyme BccI. Three fragments generated were ca. 0.25Kb, 0.15Kb, and 0.1Kb. M = Hyperladder IV (Bioline, UK), Lane 1-4 = four different A. fumigatus isolates.

3.4.3. Colony radial growth rate of environmental and clinical isolates.

The colony radial growth rate (Kr) of all isolates was determined on PDA and for a sub set of three for 25 isolates determined on Vogel's modified medium containing glucose or containing glucose supplemented with phosphatidylcholine (PC). Phosphatidylcholine has previously been shown to stimulate the colony radial growth rate of *A. fumigatus* (Dr GD Robson, unpublished). The colony radial growth rates for the Manchester environmental isolates, clinical isolates and Dublin environmental isolates are shown in Fig 3.5, 3.6 and 3.7 respectively. In all cases, the colony radial growth rates of all isolates were tested by ANOVA (SPSS V.19), significantly ($P<0.05$) higher on PDA and Vogel's medium containing PC compared to Vogel's medium alone. The colony radial growth rates of Manchester environmental isolates varied on PDA from ca. 426 to 545 μmh^{-1}. The Dublin environmental isolates ranged from ca. 452 to 496 μmh^{-1} while the clinical isolates all had colony radial growth rates in excess of 489 and up to 565 μmh^{-1}. A greater range of colony radial growth rates were observed on both Vogel's medium alone and on Vogel's medium supplemented with PC. For the Manchester environmental isolates, the colony radial growth rates ranges from ca. 235 to 397 μmh^{-1} on Vogel's medium alone and from ca. 404 to 550 μmh^{-1} on Vogel's medium supplemented with PC. The colony radial growth rates of the Dublin environmental isolates ranged from ca. 299 to 382 μmh^{-1} on Vogel's medium alone and from ca. 428 to 454 μmh^{-1} on Vogel's medium supplemented with PC. The colony radial growth rates of the clinical isolates ranged from ca. 260 to 397 μmh^{-1} on Vogel's medium alone and from ca. 378 to 500 μmh^{-1} on Vogel's medium supplemented with PC.

When the mean colony radial growth rates for the Manchester environmental, Dublin environmental and clinical isolates were compared for each medium, there was no significant difference between the groups with the exception of PDA where the mean colony radial growth rate of the clinical isolates was significantly ($P<0.05$) higher (513) μmh^{-1} compared to 465 and 481 for the Manchester and Dublin environmental strains respectively) (Table 3.2).

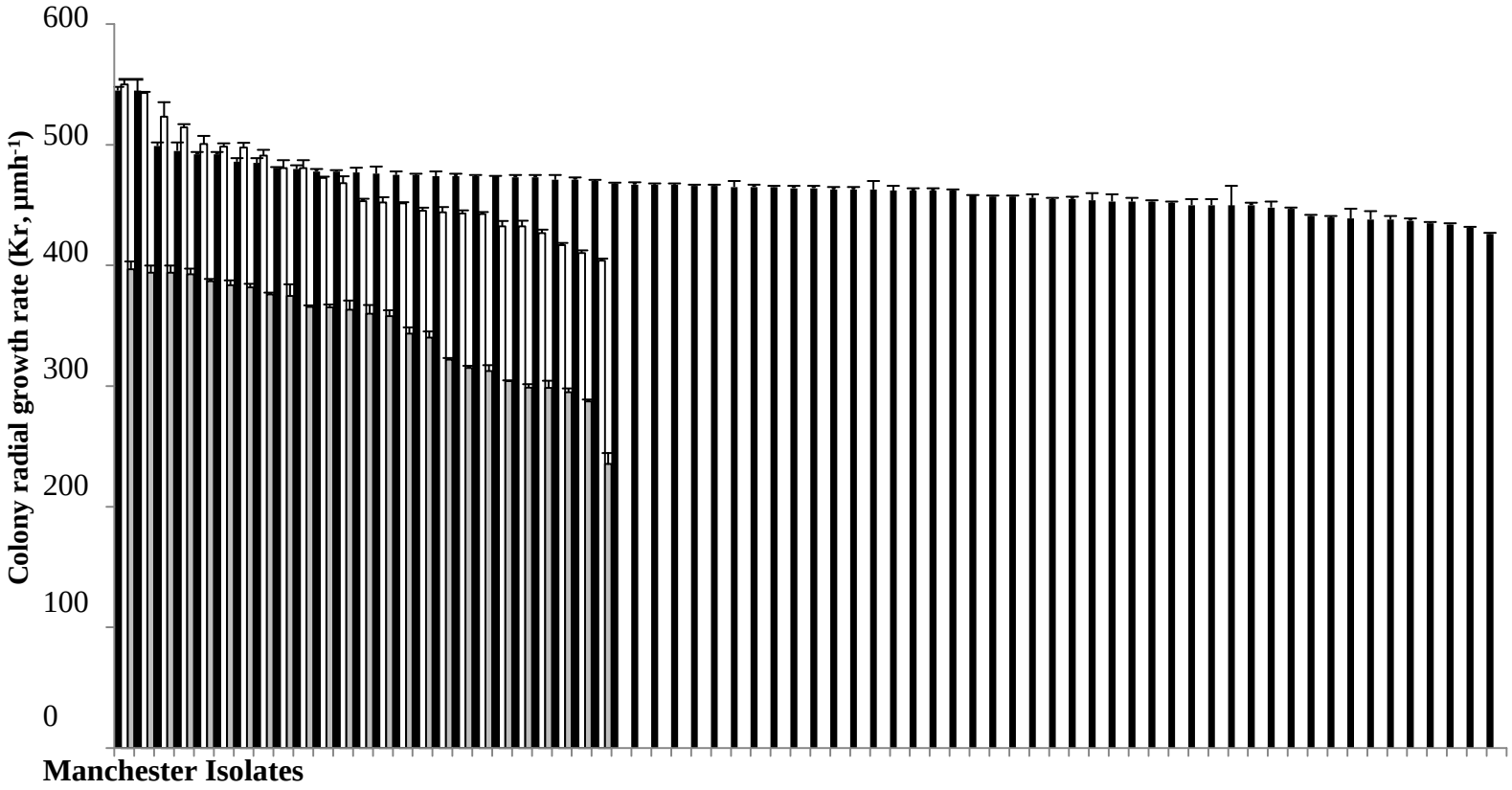


Figure 3.5. Colony radial growth rate (Kr) of Manchester environmental isolates of A. fumigatus on PDA (all isolates,■) and on Vogel's medium containing glucose with (□) or without (■) phosphatidylcholine (25 isolates) at 37^{O}C. Individual results represent the means of 4 replicates ± SEM.

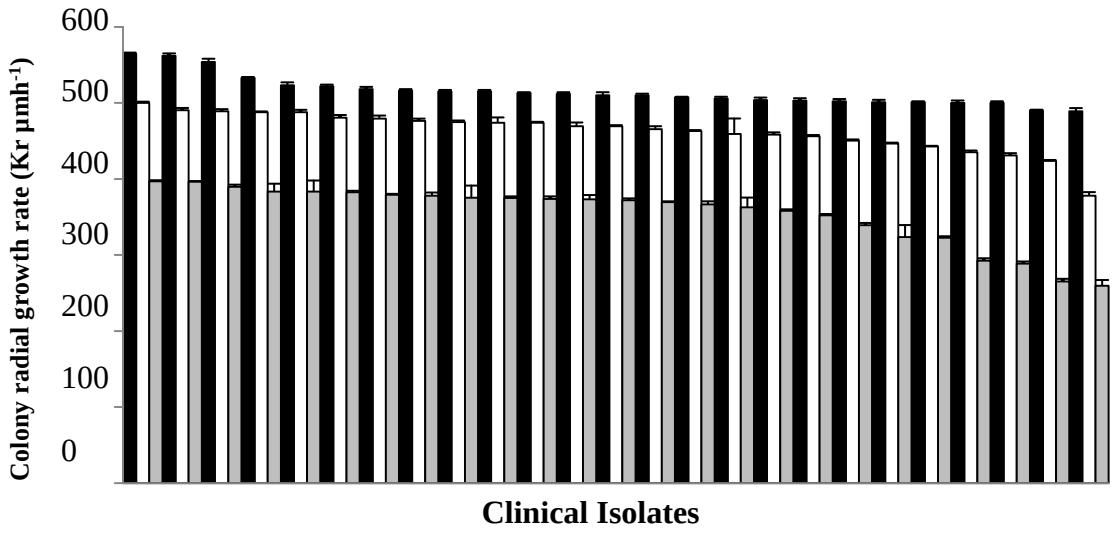


Figure 3.6. Colony radial growth rate (Kr) of clinical isolates of A. fumigatus on PDA · (■)*and on Vogel's medium containing glucose with* (□) *or without* (■)*phosphatidylcholine at 37^{O}C. Individual results represent the means of 4 replicates ± SEM.*

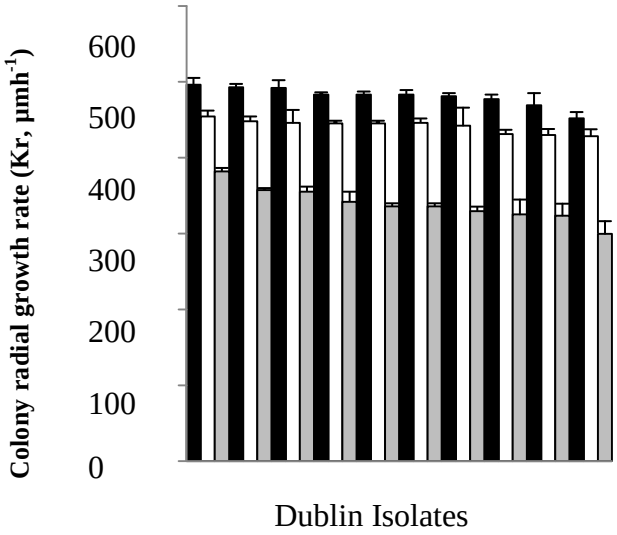


Figure 3.7. Colony radial growth rate (Kr) of Dublin environmental isolates of A. fumigatus on PDA (■) *and on Vogel's medium containing glucose with* (□) *or without* (■) *phosphatidylcholine at 37^{O}C. Individual results represent the means of 4 replicates ± SEM.*

Media	Manchester Environmental N=25	Dublin environmental N=25	Clinical N=25
Vogel's –PC Glucose	346±9 μmh^{-1}	339±8 μmh^{-1}	354±8 μmh^{-1}
Vogel's +PC	467±8 μmh^{-1}	441±3 μmh^{-1}	462±5 μmh^{-1}
PDA	465±3 μmh^{-1}	481 ±4 μmh^{-11}	513±4 μmh^{-1}

Table 3.2. Mean colony radial growth rate (Kr) of Manchester environmental, Dublin environmental and clinical isolates of A. fumigatus on PDA and Vogel's medium in the presence and absence of phosphatidylcholine (PC). Results are group means ± SEM.

3.5. Discussion

This study provides information on the level of cultrable *A. fumigatus* airborne spores from the outdoor environment in Manchester in comparison to the total culturable airborne fungal spores over a two year period. Determining the airborne spore level over a prolonged period of time is important as some authors have suggested that levels of the opportunistic pathogen of >10 CFU m^{-3} may pose a significant risk of pulmonary infection in immunocompromised individuals (Denning, 1996) and levels >1 CFU m^{-3} were associated with a fourfold increase the levels of nosocomial invasive aspergillosis (Arnow et al., 1991). Despite the importance of *A. fumigatus* as an opportunistic pathogen and its ubiquitous occurrence in the environment, there have been relatively few studies on the annual levels of airborne *fumigatus* spores in outdoor environments (O´Gorman, 2011). More studies are needed to gain a more comprehensive view of their frequency and seasonal variation in outdoor environments.

A total of 220 *A. fumigatus* isolates were recovered in this study and putatively identified on the basis of morphological characteristics and growth at 45°C. Sequencing of the ITS1 and ITS4 region of the rDNA of a number of randomly selected isolates indicated they belonged to the section *Fumigati*. Recently, it has become apparent that a number of isolates phenotypically highly similar to *A. fumigatus* are in reality, closely related species within the section *Fumigati* and that ITS sequencing alone is insufficient to resolve them (Balajee et al., 2005, Balajee et al., 2006, Hong et al., 2008, Staab et al., 2009). However, it has been shown that there is interspecies variability at intronic regions in the sequence of the gene encoding β-

tubulin (*benA*) sufficient to resolve three of the major species within the section Fumigati, namely *A. fumigatus*, *A. lentulus* and *N. udagawae* (Staab et al., 2009). Using a PCR-restriction fragment polymorphism of the benA amplicon of the strains subjected to ITS sequencing, these strains were confirmed as isolates of *A. fumigatus* (Fig 3.4). Differentiating *A. fumigatus* from closely related species in the section *Fumigati* is important clinically as the related species have been shown to exhibit variable levels of azole resistance (Balajee et al., 2005). In addition, it also confirms that at least the majority of airborne spores thought to be *A. fumigatus* based on morphology in this study are *A. fumigatus*.

The annual variation in the total airborne fungal count in Manchester varied widely between >10 and 1,333 CFU m3 and was strongly seasonally associated with higher concentrations in summer and autumn and lower concentrations in winter and spring over the two year period (Figs 3.1, 3.2). Such peaks in the total airborne fungal counts over the summer and autumn periods has been observed in many studies in temperate countries where warmer summer weather and autumnal leaf fall are thought to play important factors in increasing fungal growth, sporulation and subsequently airborne spore numbers (Larscn, 1981, Shelton et al., 2002, Millington and Corden, 2005, Guinea et al., 2006, O'Gorman and Fuller, 2008, Fang et al., 2008, Gonçalves et al., 2010 , Grinn-Gofroń, 2011). In our study, this seasonal variation in total counts was also strongly positively associated with the average monthly temperature (Fig 3.3) but not with humidity or rainfall (results not shown). While some studies have found a correlation with other meteorological factors, including humidity, rainfall and wind speed, others have found little or no correlation (Li and Kendrick, 1995, Kasprzyk, 2008, Oliveira et al., 2009) .While many studies have determined that various genera such as Alternaria, Cladosporium, Aspergillus and Penicillium are largely responsible for these seasonal fluctuations with the dominant genera varying

depending on geographical location (Mediavilla et al., 1997), few studies have focused on specific species and in particular *A. fumigatus* (O´Gorman, 2011). In those studies that have been conducted, the majority report a small rise in airborne *A. fumigatus* counts during the winter. In a study of the outdoor air in Cambridge, UK, Hudson (1973) reported elevated *A. fumigatus* airborne counts during the winter months (ca. 6 to 14 CFU m-3) and the highest levels of airborne *A. fumigatus* spores were also reported to be present in the winter by Mullins et al (1976) in Cardiff, UK. A later study in Cardiff which compared airborne spore counts for *A. fumigatus* with those obtained in St Louis, USA also confirmed higher counts in the winter months (Mullins et al., 1984). A study in Spain, which compared airborne *A. fumigatus* counts from several locations across the province of Madrid, including both urban and rural sites, found peak counts in the autumn (Guinea et al., 2006). By contrast, a study at Michigan, USA indicated a weak seasonal trend peaking slightly in the summer (Solomon et al., 1978). While a study at four sites in Dublin, Ireland, showed no seasonal trend at all (O'Gorman and Fuller, 2008). In this present study, no seasonal variation was observed in the airborne counts of *A. fumigatus* over the two year period with numbers falling within the range 3 to 20 CFU m^{-3} (Fig 3.1, Table 3.1). The persistence of low levels of *A. fumigatus* throughout the year is consistent with many other studies where *A. fumigatus* has been specifically monitored. An early study in Cambridge, UK, reported levels of 0.13 to 13 conidia m-3 while in a year-long study in Cardiff, UK and St Louise, USA, average spore concentrations were found to be 11.3 and 13.5 m^{-3} respectively (Hudson, 1973, Mullins et al., 1984). In a study in Michigan, USA, mean *A. fumigatus* airborne counts were 6.25 CFU m^{-3} (Mullins et al., 1984) while Shelton et al (2002) reported a median *A. fumigatus* count of 20 $CFUm^{-3}$ and a study in Austria recorded a mean count of 20.3 CFU m^{-3} (Blum et al., 2008). In a study at various sites around Dublin, Ireland, normal counts were found to be <10 CFU m^{-3} with occasional counts of up to 400 CFU m^{-3} which was attributed to occasional capture of a conidiophores (O'Gorman and Fuller, 2008). The only exceptions to this low

level are where studies have examined counts close to composting facilities, where airborne counts was 10^4 CFU m^{-3} have been reported as *A. fumigatus* is thermophilic and high levels of growth are associated with the composting process (Fischer et al., 1998, Tekaia and Latge, 2005, Deacon et al., 2009). In urban areas, the lack of seasonality and persistence throughout the year independent of temperature may perhaps suggest that the source of the spores may be from buildings and ventilation systems rather than from decomposing vegetation and therefore not affected by seasonal fluctuations in temperature. Many studies have found a causative link between local building renovation and demolition work and an increase in the number of airborne *A. fumigatus* fungal spores (Blum et al., 2008, Chazalet et al., 1998). This present study falls broadly in line with the majority of other previous studies that suggest *A. fumigatus* is present at low but persistent levels in the outdoor environment despite differences in geographical location, sampling methods and culture media. To examine any variation in phenotypic characteristics within the Manchester environmental isolates and between these isolates and environmental isolates from Dublin and clinical isolates, colony radial growth rates (Kr) were determined on three different media. Colony radial growth rates of the all the isolates showed a wide variation on all media but every isolate was consistently and significantly ($P<0.05$) higher of PDA and on Vogel's medium supplemented with phosphatidylcholine (PC). When the mean colony radial growth rate of Manchester environmental, Dublin environmental and clinical environmental isolates were compared, there was no difference on Vogel's medium or Vogel's medium supplemented PC. In contrast, The effect of PC is potentially highly significant as the surfactant coating the lung surface is made up of ca. 90% phospholipid of which PC is the most abundant (Morgenroth, 1988, Bernhard et al., 2001). However, the group mean of the colony radial growth rate on PDA media of the clinical isolates was significantly higher than the group means for the Manchester and Dublin environmental isolates. This increase in the hyphal extension rate is not apparently due to a change in the specific growth rate (Robson, unpublished data) but due to a change in morphology with higher hyphal extension rates being achieved with a reduction

in branching frequency. In the lung in immunocompetent hosts, conidia are rapidly removed by phagocytosis (Latgé, 2001) before they achieve a minimum germ tube length to escape this immune response (Waldorf, 1989). Hence, a faster extension rate in response to PC may be opprtunistic response in enabling germinating conidia to achieve a germ tube length big enough to evade phagocytosis more quickly. A morphologically similar response has been reported previously for the cereal plant pathogen *Fusarium graminearum* and the related non-pathogenic Quorn mycoprotein fungus *F. venenatum* in response to choline and the related compound betaine (WiIebe et al., 1989, Robson et al., 1995). Both choline and betaine levels peak during anther emergence in wheat which are primarily the infection sites of *F. graminearum* and has been postulated to aid in the infection process by stimulating rapid extension across the host (Strange and Smith, 1971, 1974, 1978). Moreover, mutants in impaired in choline transport in *F. venenatum* were no longer responsive to choline suggesting uptake was essential and an internal site of action (Robson et al., 1995) and a similar loss of response to PC has been shown in choline transport mutants in *A. fumigatus* (Robson, unpublished). This strongly suggests that choline or phosphorylcholine liberated from PC as the result of extracellular phospholipase activity (Birch et al., 1996, Robson, 2008). is likely to be the active compound eliciting the observed morphological effect. The morphological response of *A. fumigatus* to PC, correlation between pathogenicity and the extent of the morphological effect and its possible role in lung infection all warrant further investigation.

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3.6. Reference

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Chapter 4:

Title: Genetic and virulence variation in an environmental population of the opportunistic pathogen *Aspergillus fumigatus*

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4.1. Abstract

Environmental populations of the opportunistic pathogen *Aspergillus fumigatus* have been shown to be genotypically diverse and to contain a range of isolates with varying pathogenic potential. In this study, we combined two RAPD primers to investigate the genetic diversity of environmental isolates from Manchester collected monthly over one year alongside Dublin environmental isolates and clinical isolates from patients. RAPD analysis revealed a diverse genotype but with three major clinical isolate clusters. When the pathogenicity of clinical and Dublin isolates were compared with a random selection of Manchester isolates in a wax moth model, as a group, clinical isolates were significantly more pathogenic than environmental isolates. Moreover, when relative pathogenicity of individual isolates was compared, clinical isolates were the most pathogenic, Dublin isolates the least pathogenic and Manchester isolates showed a range of pathogenicities. Overall, this suggests that the environmental population is genetically diverse displaying a range of pathogenicities and that the most pathogenic strains from the environment are selected during patient infection.

4.2. Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen that can cause Aspergillosis in immunocompromised patients, such as those with neutropenia, or on immunosuppressive therapy following solid organ transplantation (Abad et al., 2010, Ben-Ami et al., 2010, Dagenais and Keller, 2009, Latge, 1999, Paris and Latge., 2001, Rementeria et al., 2005). The principle route of infection is through the inhalation of airborne spores where due to their small spore size, they are able to reach the alveoli (Dagenais and Keller, 2009, Ibrahim-Granet et al., 2003, Latge, 1999, Philippe et al., 2003). While other Aspergilli can cause opportunistic infections (such as *A. terreus* and *A. flavus*), *A. fumigatus* accounts for around 90% of all cases despite airborne spore numbers only accounting for less than 1% of all Aspergillus spores (Rementeria et al., 2005). A number of studies have shown a high degree of genetic variation within the *A. fumigatus* population and while some studies have indicated potential geographical subgroups, comparisons between clinical isolates and environmental isolates have largely been unable to distinguish these populations (Araujo et al., 2010, Aufauvre-Brown et al., 1992, Balajee et al., 2008, Chazalet et al., 1998, Denning et al., 1990, Duarte-Escalante et al., 2009, Leenders et al., 1999, Menotti et al., 2005). Moreover, epidemiological studies have largely failed to match genotypes in infected patients with genotypes found in hospitals, in part due to the high level of genetic diversity in the environmental populations (Araujo et al., 2010, Bart-Delabesse et al., 1999, Chazalet et al., 1998, Guinea et al., 2011, Leenders et al., 1999, de Valk et al., 2007) and similar findings have been reported in avian populations (Arné et al., 2011 , Lair-Fulleringer et al., 2003, Olias a et al., 2011). While genotyping of isolates from some individual patients has revealed infection with multiple isolates, most patien appear to be colonised by only one genotype despite exposure to a genetically diverse population (Alvarez-Perez et al., 2009, Bart-Delabesse et al., 1999, Chazalet et al., 1998, Guinea et al., 2011, Menotti et al., 2005, Tang et al., 1994, de Valk et al., 2007). This has led to the suggestion that the lung environment

selects for a strain or strains from the environment that are the most adapted and therefore pathogenic (Cimon et al., 2001; , Guinea et al., 2011, de Valk et al., 2007).

In this study we investigated the genetic variation in airborne environmental *A. fumigatus* strains isolated over a 12 month period from the outdoor environment in Manchester, UK, Dublin environmental strains isolated from the outdoor environment at one sample time and clinical isolates using two RAPD primers. In addition, we also compared the relative pathogenicity of environmental and clinical isolates in a wax moth larvae model and report that clinical isolates were at the most pathogenic end of the spectrum while environmental isolates were found throughout the spectrum from high to low suggesting clinical isolates represent the most pathogenic strains from the environment.

4.3. Materials and Methods

4.3.2. Virulence determination in wax moth larvae

Wax moth larvae (WML) (Live Food Direct Ltd. Sheffield, UK) were stored in wood shavings in the dark at 4°C for up to 10 days before use. WML larvae in groups of 30 were inoculated with 10 µl of an *A. fumigatus* spore suspension -Appendix I- into the haemocoel through the last right or left proleg, using a Hamilton 1 ml gastight syringe (Fisher Scientific, UK) with a 10 µl repeating dispenser attachment (Jaytee Biosciences Ltd, UK). Spore concentrations of $1X10^{8,}$ to $1X10^{3}$ per ml were used to infect larvae and incubated at 37°C in the dark in Petri dishes (10 larvae per plate) and the mortality monitored daily for 7 days. Mortality was assessed by lack of movement in response to stimulation and by discoloration (melanisation) of the cuticle. The LD90 (lowest spore concentration that kills 90% of WML) for each isolate was determined from a plot of percentage mortality against time since inoculation.

The survival of the WML was analysed using the Mann Whitney U test (Stats Direct) with a P value of >0.5 consider significant and was used to compare differences in survival rate of larvae when inoculated with different strains. A nonparametric estimate of the mean survival time for WML was obtained as the area under the Kaplan-Meier estimate of the survival curve.

4.3.3. DNA extraction

Ground mycelium was added to 50 ml Falcon tubes (on ice) to the 10 ml mark and an equal volume of pre-heated DNA extraction buffer (0.7 M NaCl, 1 M $Na_2(SO_3)$, 0.1 M Tris-HCl (pH 7.5), 0.05 M EDTA, 1% (w/v) SDS, 65°C) was added

and mixed thoroughly with a pipette tip and incubated at 65°C for 30 min to terminate nuclease activity and induce protein denaturation (James et al., 2000). A NanoDrop 1000 Spectrophotometer (Thermo Scientific, UK) was used to measure DNA concentrations in samples.

4.3.3. Random amplification of polymorphic DNA (RAPD)

A total of 106 isolates were analyzed using RAPD as described by Dyer et al. (1993). The primers used for random amplification of polymorphic DNA **(**RAPD) typing were R108 (5'-AGTGCACACC-3'), C90 (5`-GGGGGTTAGG-3'), R151 (5`-GCTGTAGTGT-3') and C08 (5`-AGGATGTCGAA-3') (Eurofins, UK). The samples were run on agarose gels (2.5 % (v/v) in Tris-acetate EDTA buffer (TE pH 8.0,[0.04 M Tris, 0.001 M EDTA, 65% (v/v) glacial acetic acid], agarose (Melford Laboratories, UK). Only primers R108 and C08 were used to compare isolates and RAPD bands included in the analysis were scored as either absent (0) or present (1). The data from all primers were pooled for each isolate and a pair's similarity matrix was calculated with Jaccard's coefficient with the program FREETREE v. 0.9.1.50. A bootstrapped dendrogram with 1000 replications was produced with NJ plot.

4.4 Results

4.4.1. RAPD fingerprinting of *A. fumigatus* isolates

Four primers were initially used for RAPD fingerprinting, (R108, C80, and R151 and C90) with seven *A. fumigatus* isolates (Fig 4.1) and repeated at least two times to evaluate reproducibility. The four primers produced a range of RAPD bands, which ranged from 0.4 Kb to 6 Kb. All four primers gave several strong bands that were reproducible along with many fainter bands. Of the four primers tested, R108 and RC80 gave the most reproducible results in a pilot study and were selected (R108 and RC80) for fingerprinting the remaining isolates and had a discriminatory power of 0.992 and 0.868 respectively against all isolates (Bikandi et al., 2004). Only the strongest and reproducible bands were included in the analysis and fainter and variable bands were excluded. To increase discrimination, the presence or absence of bands from both primers were pooled prior to analysis. As the number of isolates exceeded the maximum number of wells on a gel, *Af*293 was run on every gel as an internal control.

RAPD analysis of the Manchester and Dublin environmental isolates showed a high genetic diversity in the population (Fig 4.2). With few exceptions, isolates collected at the same time points could be distinguished from each other indicating multiple sources of airborne spores. In addition, comparison of Manchester isolates collected monthly for 12 months indicated that the source of airborne spores varied at each time point. When environmental isolates were analysed along with clinical isolates, clinical isolates displayed a degree of clustering (Fig 4.3). Twenty of the twenty four clinical isolates formed three clusters accounting for twelve, five and four isolates though with the exception of isolates 22178 and 22636 and isolates 17871 and 16258, they could be distinguished from each other.

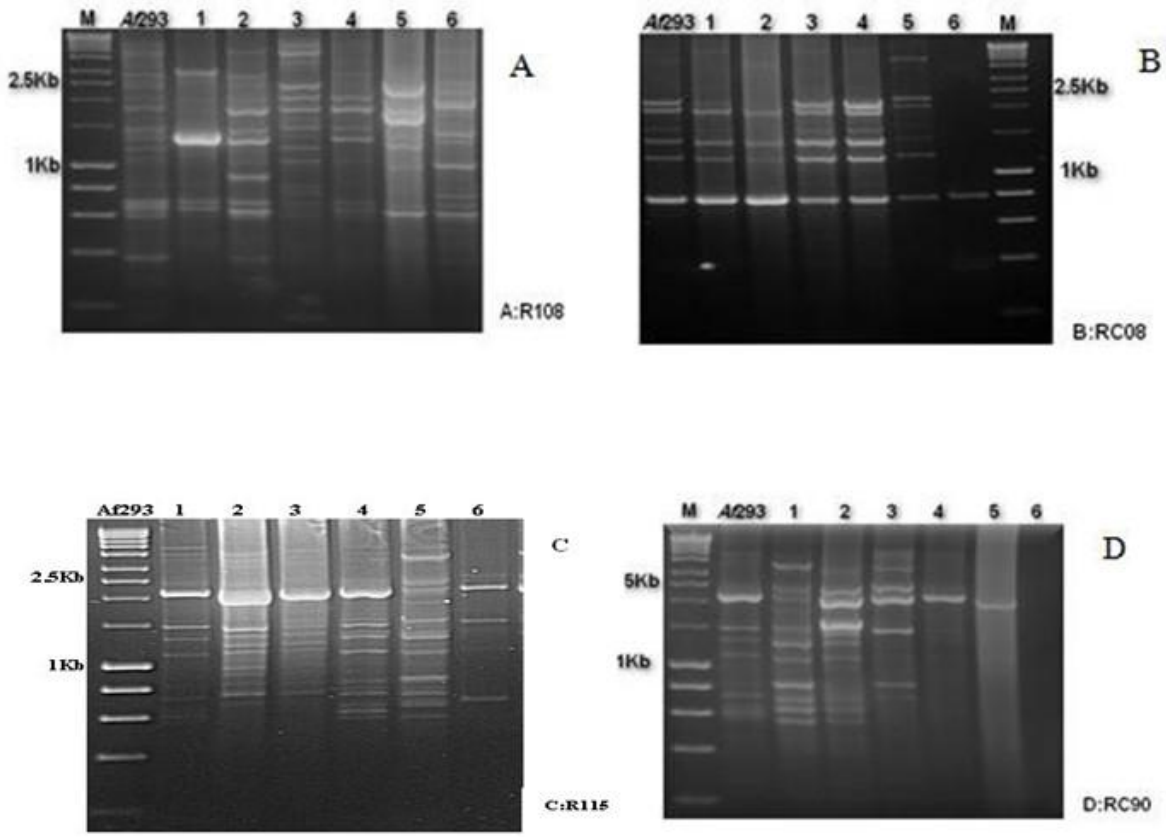

Figure 4.1. Random amplification of polymorphic DNA (RAPD) of Aspergillus fumigatus *isolates obtained from Manchester isolates (1&2), Dublin (2&3) and Clinical isolates (4&5) Hyperlader I (Bioline) (M), A: Primer R108; B: RC08. C:R151;D:RC90. The samples runs on a 2% agarose gels stained with ethidium bromide, along with Hyperlader I as a molecular Marker (M).*

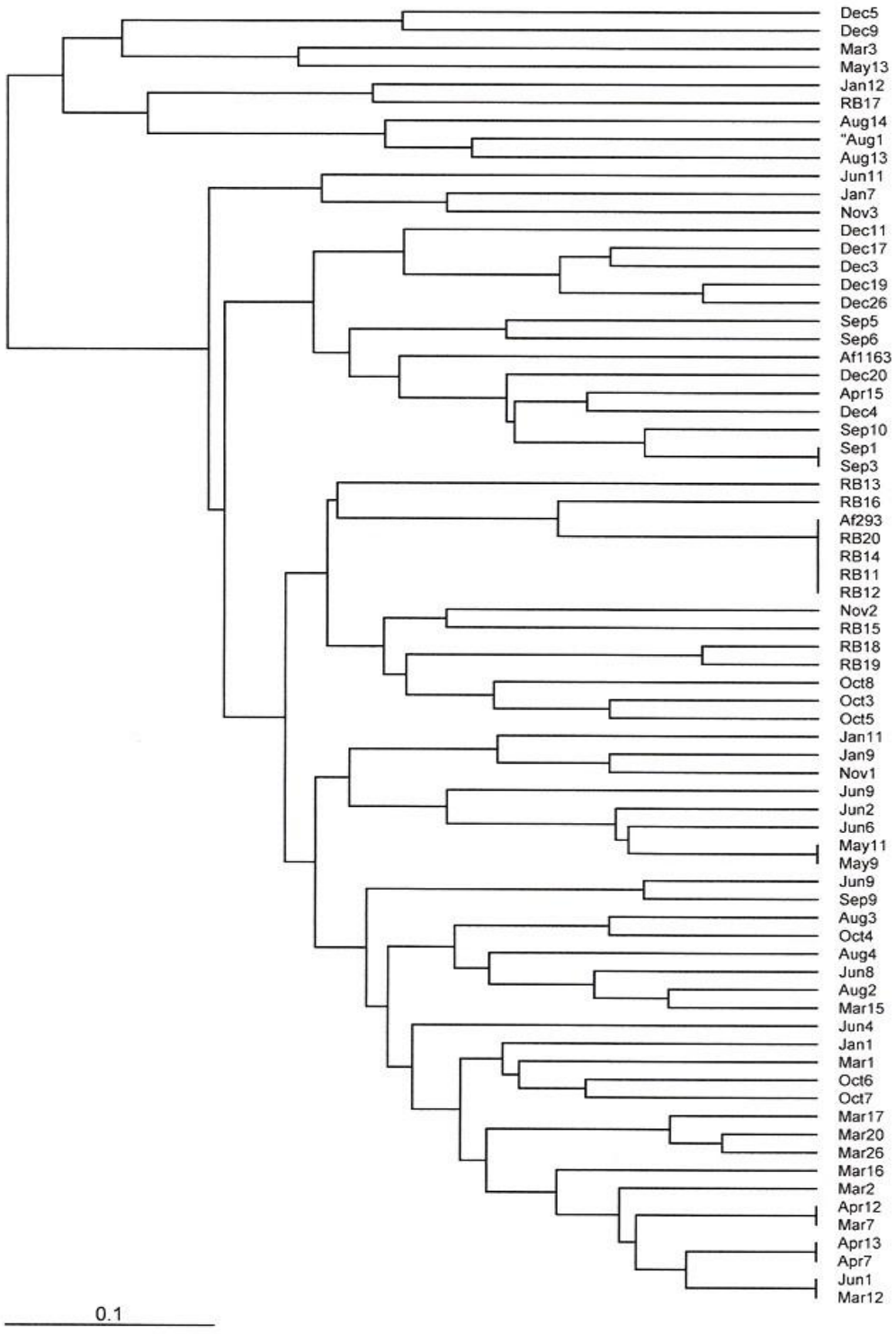

Figure 4.2. Dendrogram analysis of RAPD fingerprinting of A. fumigatus *environmental isolates. Manchester airborne isolates (March-2009-February-2010, month of isolation indicated by code and number), Dublin airborne isolates (RB).*
Bootstrap was 1000.

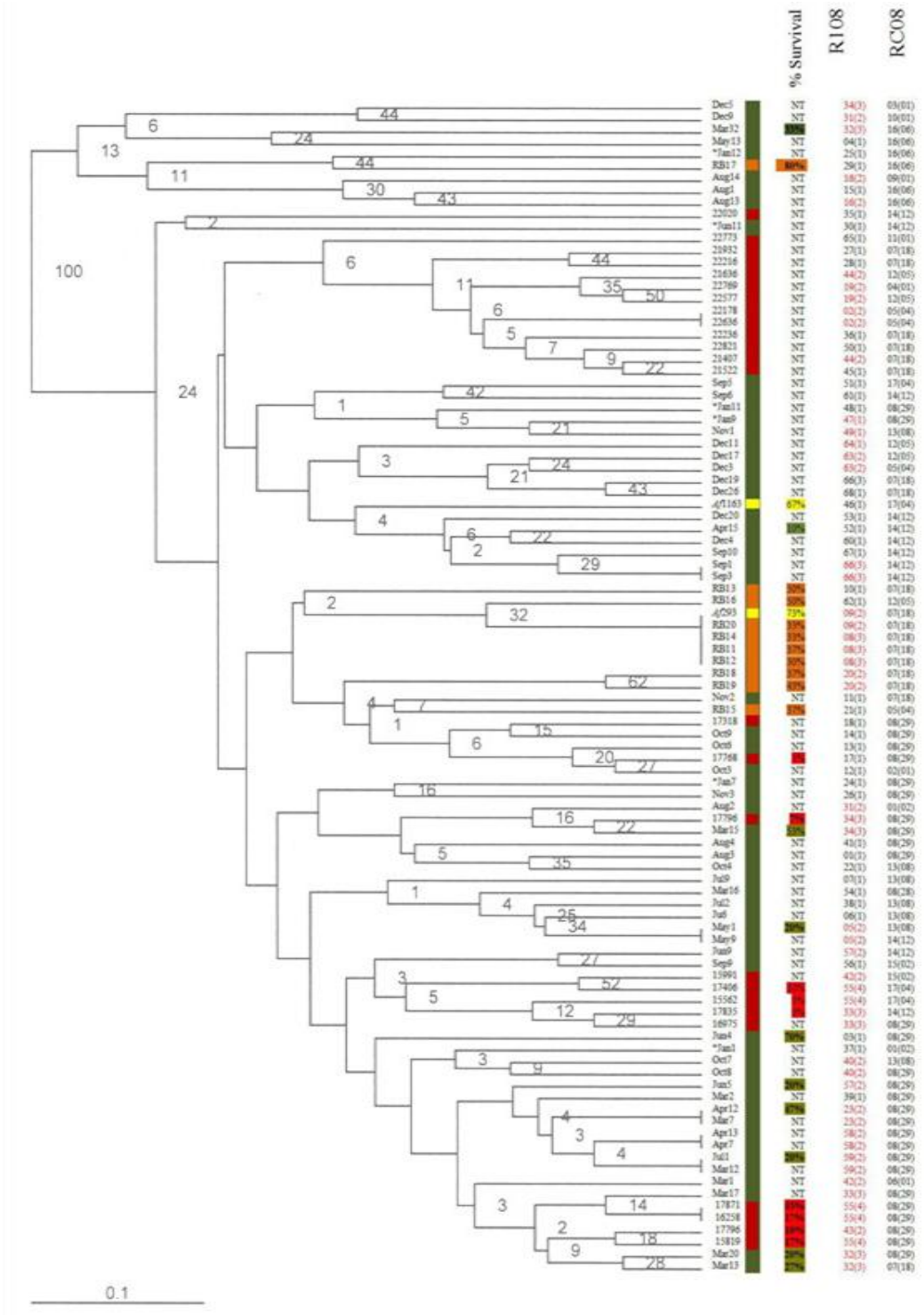

Figure 4.3. Dendrogram analysis of RAPD fingerprinting of A. fumigatus *isolates (March-2009-February-2010); Manchester airborne isolates (month of isolation indicated by code and number), Clinical isolates (code number), and Dublin airborne isolates (RB). The banding patterns and the type number for the primer R108 and C08 are shown separate columns. The % of survival rate of tested isolates were determined in a Wax moth larvae model (NT = not tested), (*isolates from 2009- 2010).* ■ Manchester isolates, ■ Dublin isolates, ■ clinical isolates, ■ sequenced strains.

4.4.2. Virulence testing in wax moth larvae (*Galleria menollela*)

The virulence of ten Manchester environmental isolates, ten Dublin environmental isolates, ten clinical isolates and sequenced strains *Af*293 and *Af*1163 were determined in wax moth larvae inoculated with $1X10^3$ to $1X10^6$ spores per larva by monitoring survival over seven days (Figs 4.4, 4.5, 4.6). Initial spore inoculums size had a large effect on wax moth larvae survival in all strains with all strains showing the greatest survival after seven days with $1X10^3$ spores and lowest survival with $1X10^6$ spores. The greatest discrimination in survival between the different strains was seen at an inoculums level of $1X10^5$ spores per larva. In order to compare the relative pathogenicity of the Manchester, Dublin and sequenced strains, survival over seven days at each spore inoculum for each group was combined and subjected to a Mann-Whitney (Fig 4.7) and Kaplan-Meier (Fig 4.8) analyses. The clinical isolates were more significantly more pathogenic than the Dublin and Manchester isolates ($p<0.0001$) at the four different doses. Moreover, the Manchester isolates were significantly more pathogenic than the Dublin isolates (10^6 and 10^5, $p<0.0001$; 10^4, $p= 0.0135$; 10^3, $p = 0.0043$). The greatest variation in pathogenicity was found amongst the Manchester isolates. When the percentage survival after four days for each strain at an inoculums of $1X10^5$ spores per larva were compared (cobined data), the clinical isolates clustered together as the most pathogenic, the Dublin isolated the least pathogenic and the Manchester isolates showed a broad spectrum in pathogenicity (Figs 4.7, 4.8).

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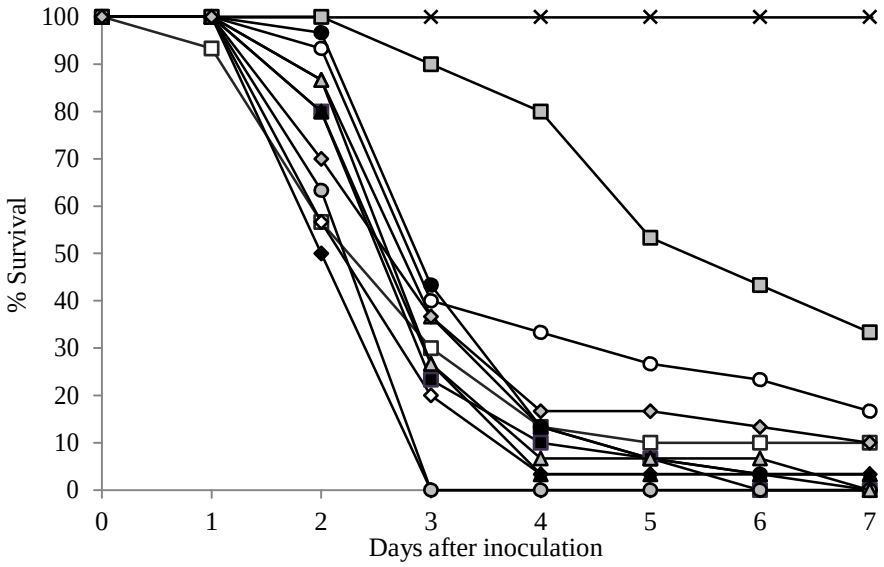
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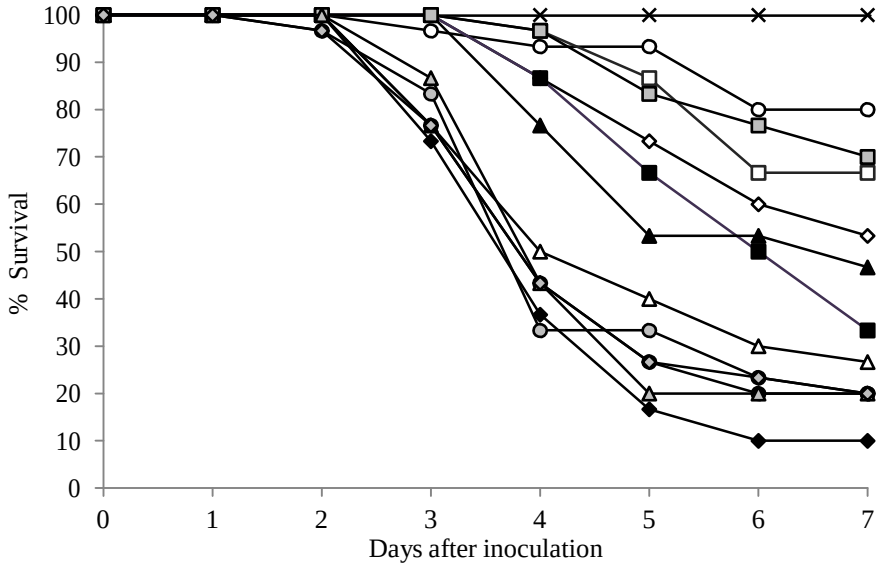
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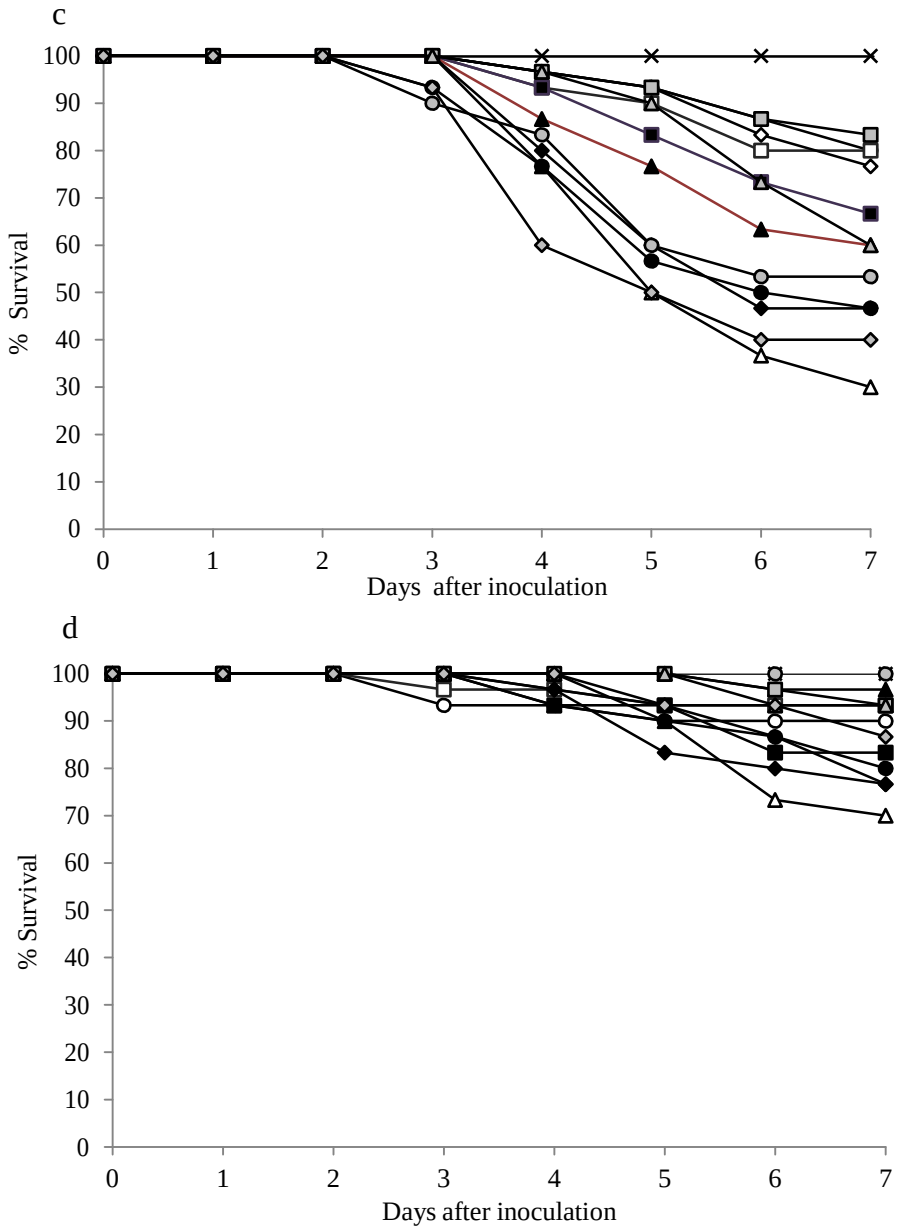

Figure 4.4. Influence of spore inoculums concentration on the survival of wax moth larvae. 30 wax moth larvae were inoculated with Manchester environmental isolates of A. fumigatus *to give (a) 1X10^6, (b) 1X10^5, (c) 1X10^4 & (d) 1X0^3 CFU/larvae and incubated at 37°C for 7 days. The number of surviving wax moth larvae was determined daily. Controls were inoculated with Sterile 0.05% (v/v) Tween 20 (X).* A .fumigatus *Af293* (○), *Af1163* (□), *M13* (∆), *M15* (◊), *M20* (●), *M32* (■), *Ap12* (▲), *Ap15* (♦), *Ma1* (⬭), *J4* (▭), *J5* (△), *Ju1* (◇).

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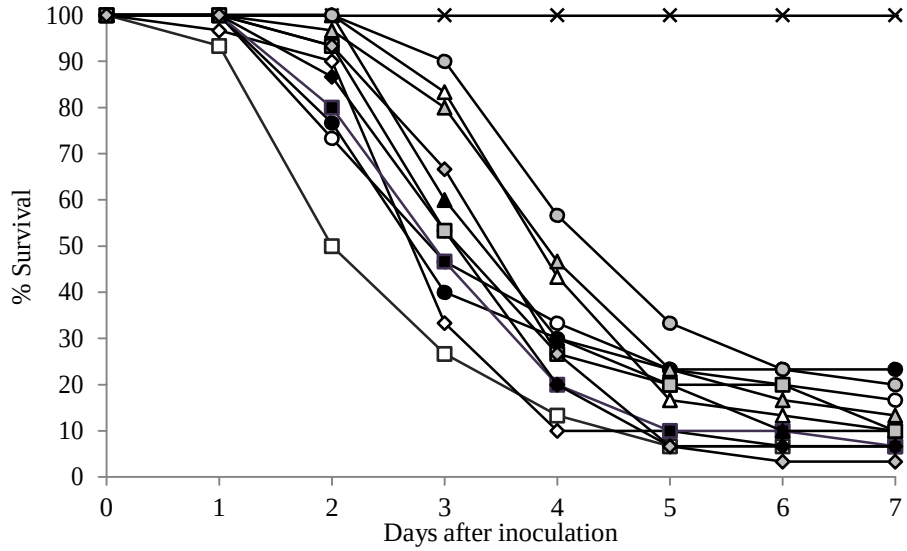
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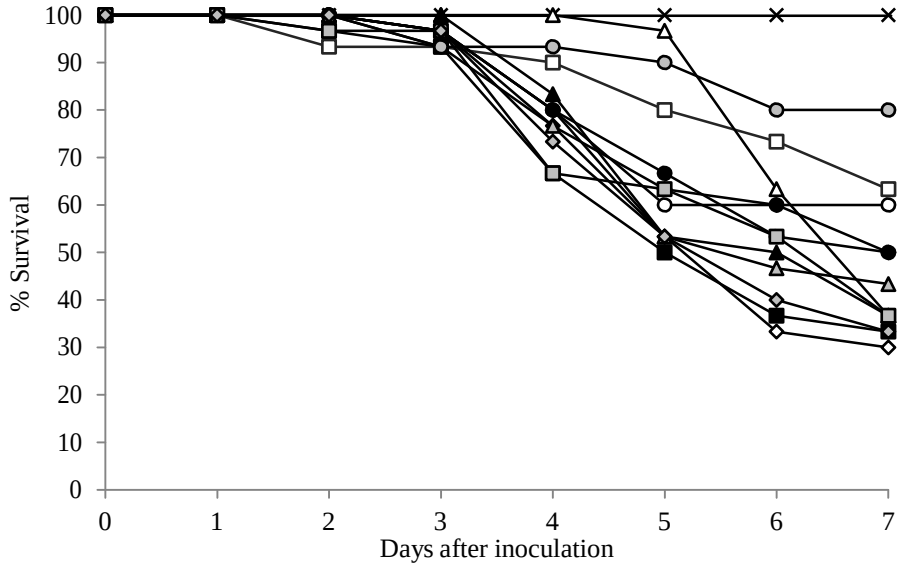
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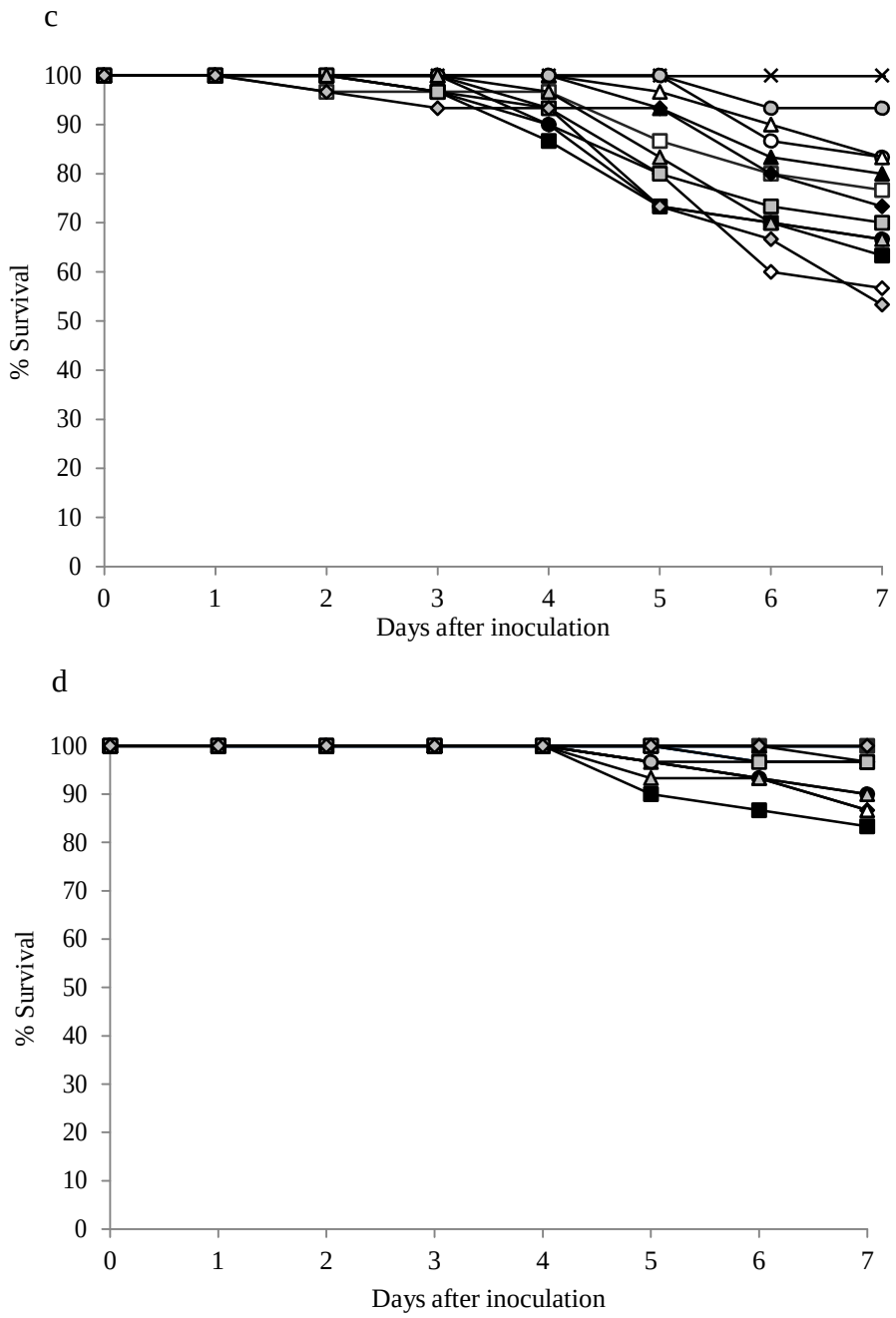

Figure 4.5. Influence of spore inoculums concentration on the survival of wax moth larvae. 30 wax moth larvae were inoculated with Dublin environmental isolates of A. fumigatus to give (a) 1X10^6, (b) 1X10^5, (c) 1X10^4 & (d) 1X0^3 CFU/larvae and incubated at 37°C for 7 days. The number of surviving wax moth larvae was determined daily. Controls were inoculated with Sterile 0.05% (v/v) Tween 20 (X). A. fumigatus Af293 (○), *Af1163* (□), *RB11* (∆), *RB12* (◊), *RB13* (●), *RB14* (■), *RB15* (▲), *RB16* (♦), *RB17* (◯), *RB18* (◻), *RB19* (△), *RB20* (◇).

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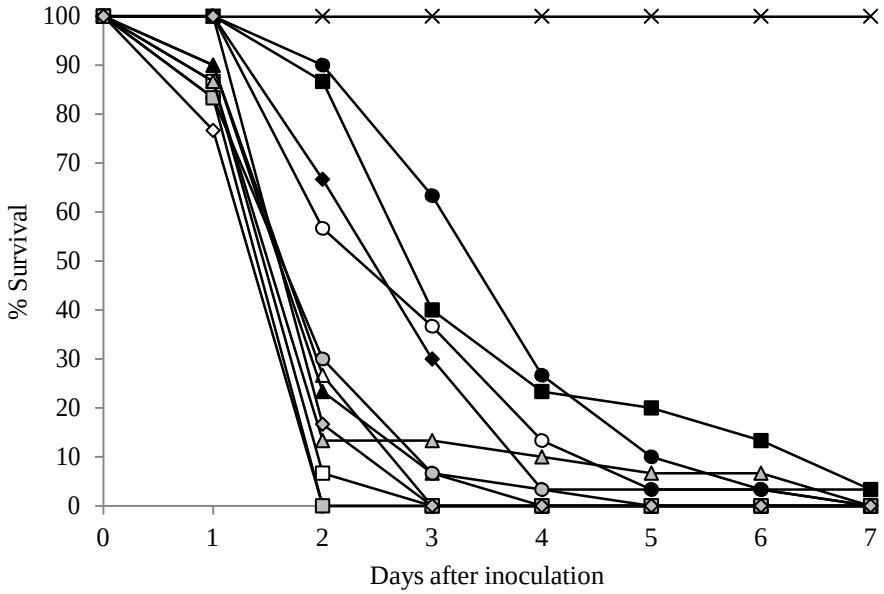
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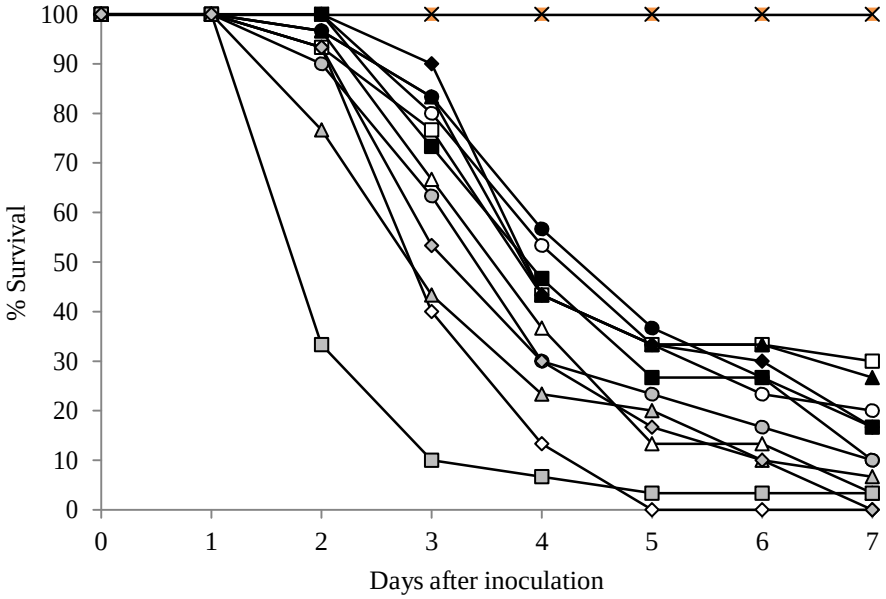
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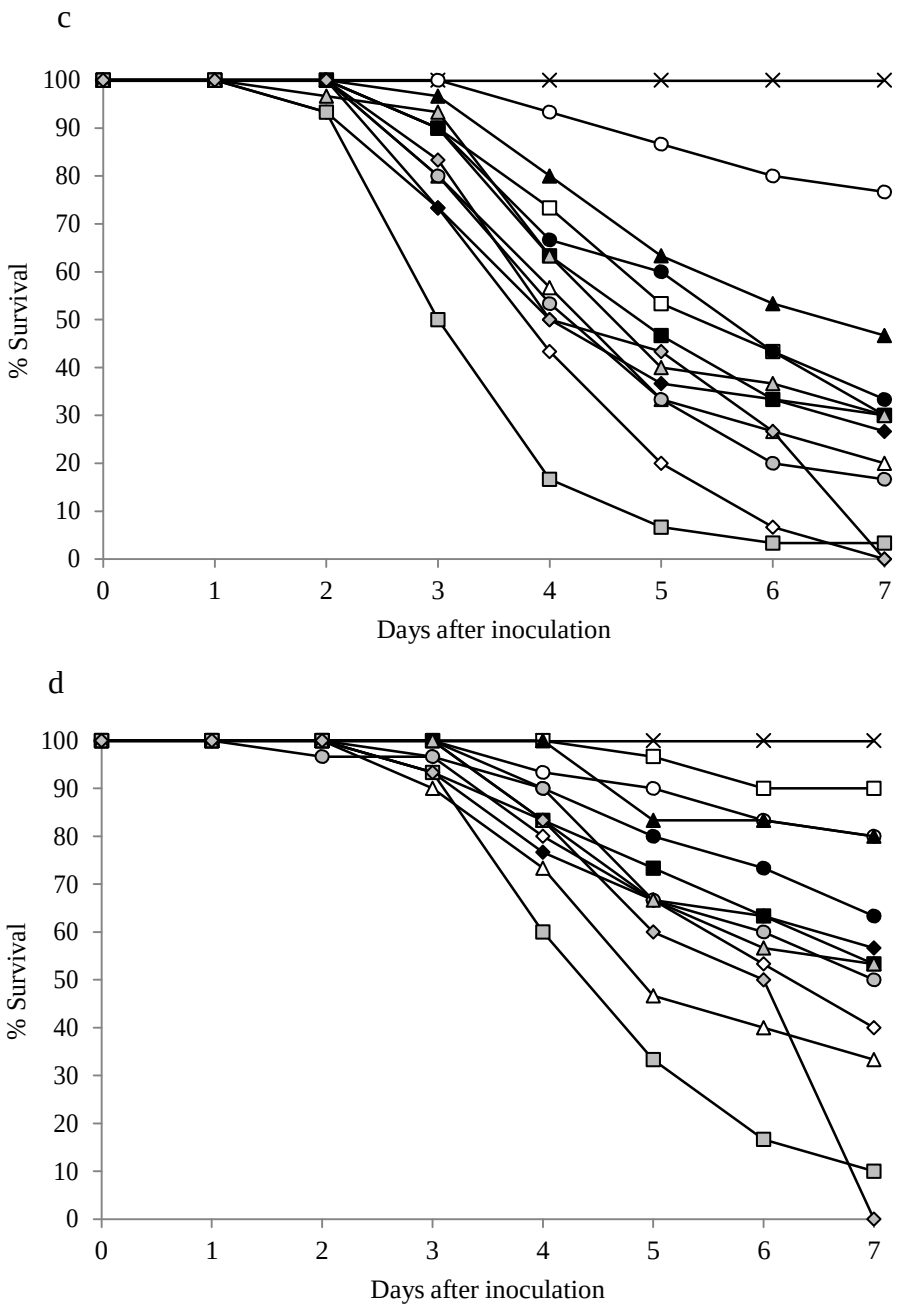
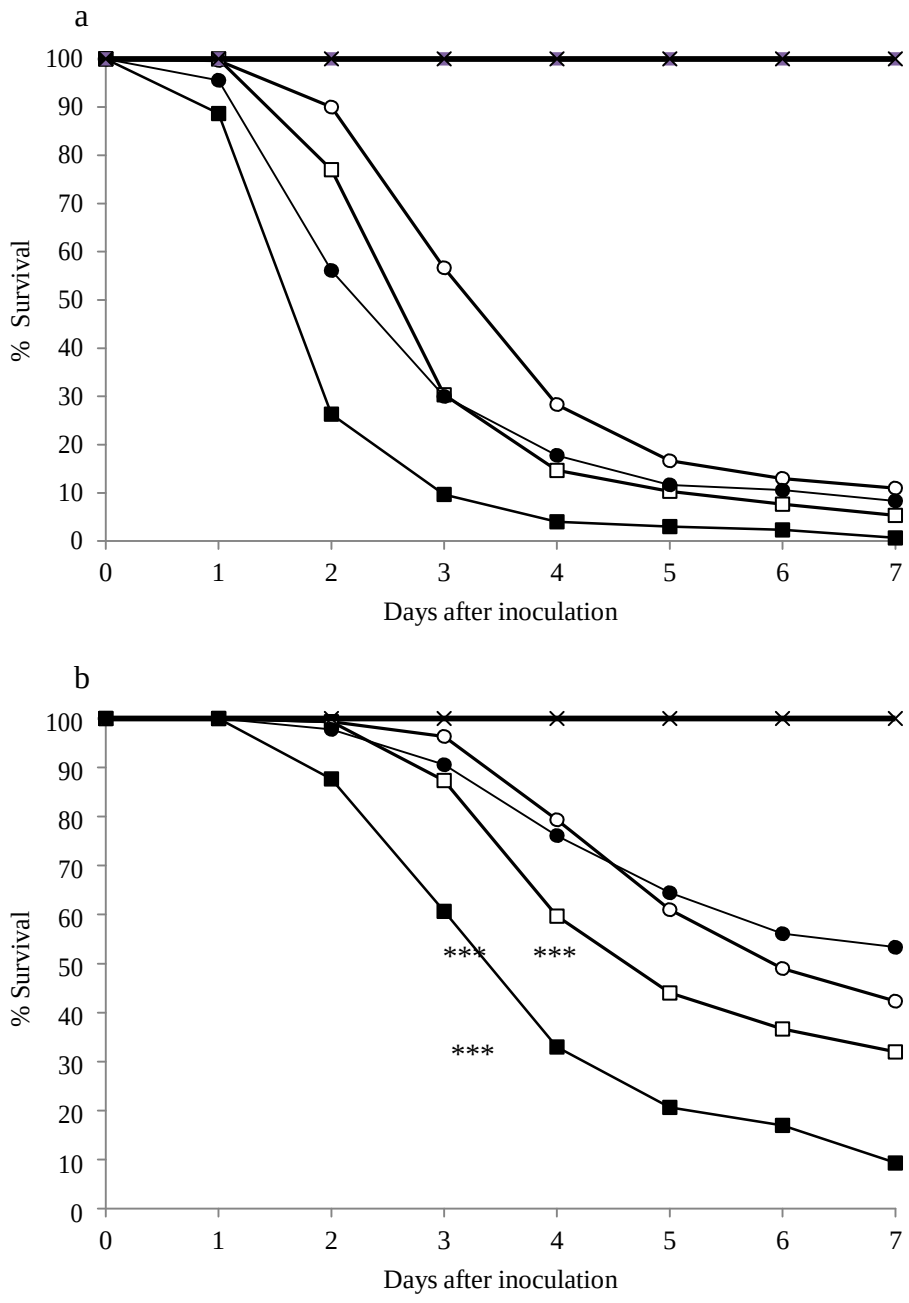


Figure 4.6. Influence of spore inoculums concentration on the survival of wax moth larvae. 30 wax moth larvae were inoculated with clinical isolates of A. fumigatus *to give (a) 1X10^6, (b) 1X10^5, (c) 1X10^4 & (d) 1X0^3 CFU/larvae and incubated at 37°C for 7 days. The number of surviving wax moth larvae was determined daily. Controls were inoculated with Sterile 0.05% (v/v) Tween 20 (X).* A. fumigatus *Af293* (○), *Af1163* (□), *Clin 1* (∆), *Clin 2* (◊), *Clin 3* (●), *Clin 4* (■), *Clin 5* (▲), *Clin 7* (♦), *Clin 9* (⬭), *Clin 11* (▭), *Clin 13* (◬), *Clin 15* (◇).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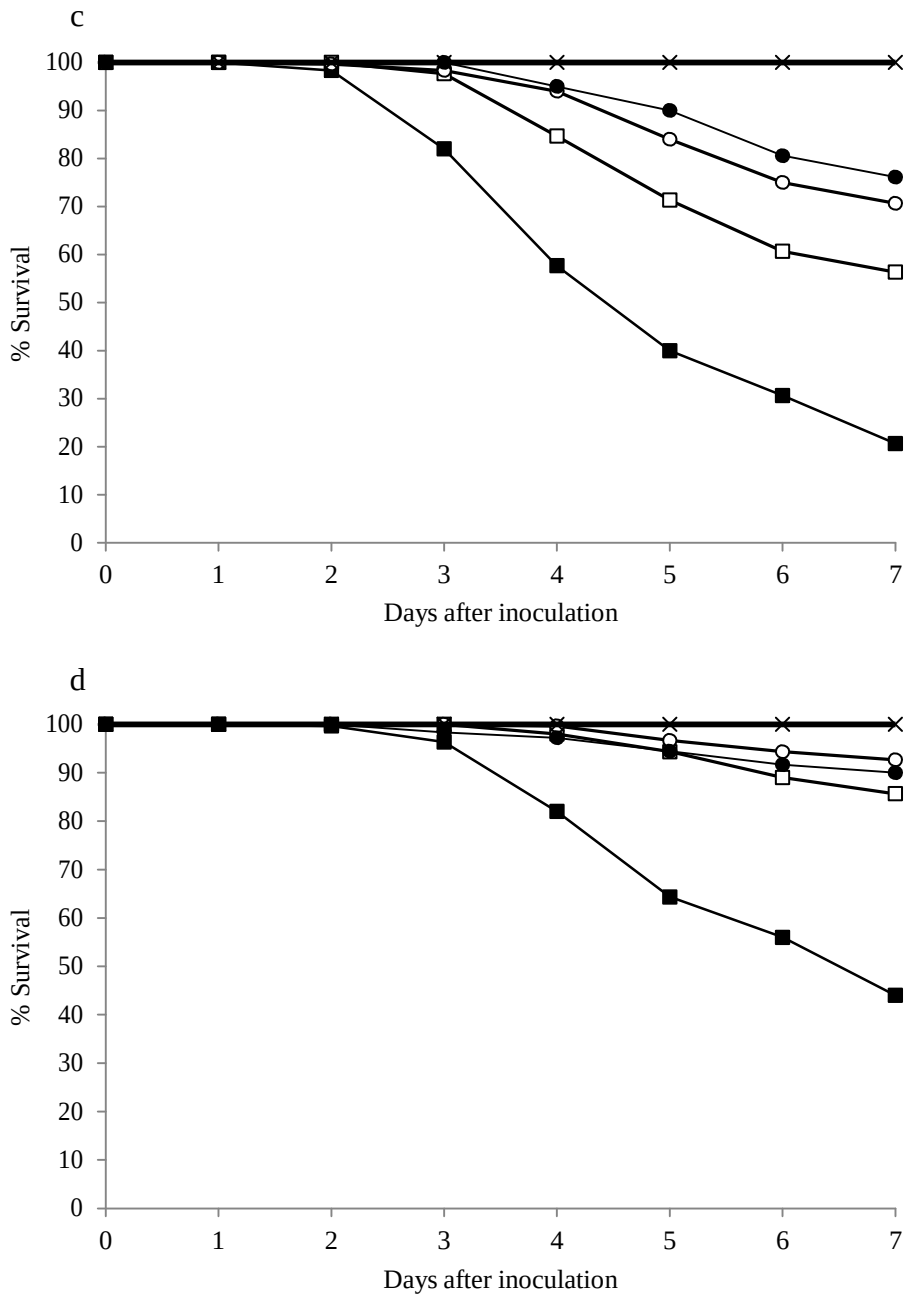

Figure 4.7. Combined Mann-Whitney analysis of the survival of G. mellonella inoculated with A. fumigatus *Manchester environmental (□), Dublin environmental (●), clinical (■) and strains Af293 and Af1163(○) inoculated with (a) 1X10^6, (b) 1X10^5 (c) 1X10^{4}and (d) 1X10^3 spores per larva over seven days. X = uninoculated control.*

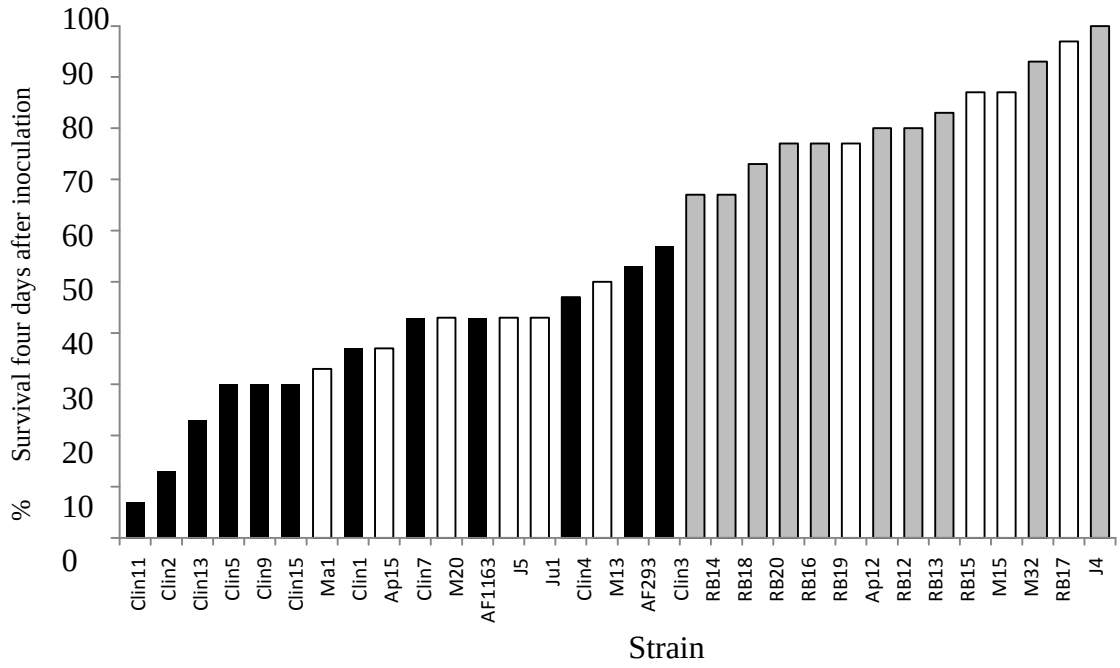

Fig 4.8. Percentage survival of wax moth larvae four days after inoculation with 1X105 spores per larvae various strains of A. fumigatus. *Clinical strains* (■) *Manchester environmental strains* (□), *Dublin environmental strains* (■).

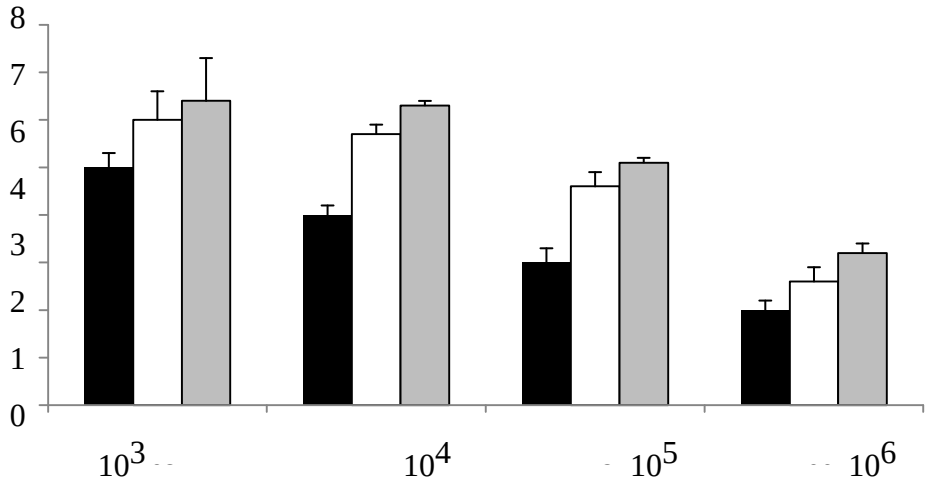

Figure 4.9. Kaplan-Meier approximation of the mean survival time of clinical (■)*, Manchester environmental* (□) *and Dublin environmental* (■) *isolates over seven days in wax moth larvae. Error bars represent SEM.*

4.5. Discussion

A variety of molecular typing methods have been developed over the past 15 to 20 years to investigate genetic diversity in the population of a wide variety of fungal species. Many of these techniques have also been used to study genetic diversity in the *A. fumigatus* population and include Random Amplified Polymorphic DNA (RAPD) (Anderson et al., 1996, Aufauvre-Brown et al., 1992, Mondon et al., 1995, O´Gorman et al., 2009), Restriction Fragment Length Polymorphism (RFLP) (Spreadbury et al., 1990), Restriction Enzyme Analysis (REA) (Denning et al., 1990), Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995), Sequence- Specific DNA Primer (SSDP) analysis (Mondon et al., 1997) and more recently Microsatellite Length Polymorphism (MLP) (Balajee et al., 2008, Bart-Delabesse et al., 1998, de Valk et al., 2007), Multi Locus Sequence Typing (MLST) (Bain et al., 2007) and Variable Number of Short Tandem Repeat typing (VNTR) (Vanhee et al., 2009). These methods have also demonstrated that there is a large genetic diversity within the *A. fumigatus* population (Balajee et al., 2008, Chazalet et al., 1998, Leenders et al., 1999, de Valk et al., 2009, Vanhee et al., 2009, O´Gorman et al., 2009) and have largely been used to investigate the epidemiology of isolates following clinical outbreaks of disease in hospitals (Alvarez-Perez et al., 2009, Bart- Delabesse et al., 1999, Vanhee et al., 2010, Guinea et al., 2011) but have also been used to study genotypic diversity of strains in avian disease (Arné et al., 2011 , Lair- Fulleringer et al., 2003, Olias a et al., 2011). The recent discovery of a functional sexual cycle in *A. fumigatus* probably accounts for the genetic diversity observed in the population (O´Gorman et al., 2009) and much of this diversity appears to be concentrated in discrete genomic islands within the genome that encodes genes involved in heterokaryon incompatibility and cell wall associated proteins that may be Involved in virulence (Fedorova et al., 2008). In addition, while the macromorphology of *A. fumigatus* strains appears to be largely similar within the population, micromorphology (such as conidia and vesicle size and shape) pathogenicity and enzyme secretion has been found to be variable (Alp and Arikan, 2008, Aufauvre- Brown et al., 1998, Ben-Ami et al., 2010, Birch et al., 2004, Chamilos et al.,

2007 Chamilos et al., 2010, Mondon et al., 1995, Mondon et al., 1996, Olias a et al., 2011, Rinyu et al., 1995). And limited studies on enzyme secretion has also demonstrated a range of expression levels and isoenzymes from different isolates (Alp and Arikan, 2008, Birch and Denning, 1999, Blanco et al., 2002, Lin et al., 1995, Rinyu et al., 1995, Rodriguez et al., 1996). In our study, RAPD typing also demonstrated a large degree of genetic variability within the airborne population of A. fumigatus collected monthly over a 12 month period from the same site in Manchester (Figs 4.4). Moreover, isolates typed from the same time point showed several genotypes indicating a mixture of sources contributed to the airborne population at any one time. This contrasted with the small number of isolates from Dublin which were also collected at a single time point from a single site (H. Fuller, personal communication) which with one exception showed identical genotypes with both primers (Figure. 4.4) indicating that the majority of airborne spores at the time of sampling may have arisen from a single source. That a range of genotypes were found at each monthly sampling point in this study is not unexpected as *A. fumigatus* spores are readily dispersed in the atmosphere due to their small spore size (O´Gorman, 2011) and samples were taken in the unclosed outside environment. Other studies that have typed A. fumigatus spores collected from the environment have also shown a high degree of genotypic diversity in the population, including the enclosed environments of hospitals where it might have been expected that the number of contributing sources may have been smaller (Araujo et al., 2010, Debeaupuis et al., 1997, Guinea et al., 2011, Menotti et al., 2005). Previous studies attempting to identify environmental sources of patient infection in hospital environments have generally found only a low number of patient isolate genotypes only some of which could be matched with an environmental phenotype, probably due to insufficient environmental samples being collected to capture the high level of genotype diversity reported (Araujo et al., 2010, Guinea et al., 2011). In addition, in those studies where environmental samples were taken at different time periods or locations within the same building, in only a few instances was an identical genotype recovered indicating a high degree of variability occurs at the same location over

time and at any one time in different locations (Araujo et al., 2010, Guinea et al., 2011). Many aspects of the pathogenesis of IA remain to be clarified, including the place of acquisition, the incubation period, the role of the virulence of the specific Aspergillus isolate and the possible contribution of more than one clone in human infections (Alvarez-Perez et al., 2009). While many studies have used mammalian models to investigate the impact of specific gene function on pathogenicity (Askew, 2008, Rementeria et al., 2005), there have been few studies that have compared the pathogenicity of different isolates of *A. fumigatus* and those that have report a range of pathogenicity between isolates (Jackson et al., 2009, Lionakis et al., 2005, Reeves et al., 2004). Invertebrate models of pathogenicity offer a cheaper and quicker alternative to animal models enabling large numbers of isolates to be compared (Chamilos et al., 2010, Kavanagh and Reeves e., 2004). In *A. fumigatus*, these models have included wax moth larvae and Toll-deficient mutants of *Drosphila* (Jackson et al., 2009, Lionakis et al., 2005, Reeves et al., 2004) and relative pathogenicity has been correlated to pathogenicity levels in mammalian models (Slater et al., 2011, , Chamilos et al., 2010). In this present study, a range in pathogenicity against wax moth larvae was apparent in both clinical isolates and environmental isolates. Moreover, as a group, the clinical isolates were significantly (of *p*value <0.5) more pathogenic than the environmental isolates. However, the environmental isolates showed an overlap in pathogencity with the clinical strains, suggesting that clinical isolates may represent the most pathogenic of the environmental isolates and that the infection process in patients may select for the most pathogenic strains present in the environment. Selection for the most pathogenic strains from the environment has been previously suggested as an explanation for why patients are often infected with only one and in those cases where co-infection is present, only two or three genotypes despite a highly diverse environmental population (Alvarez-Perez et al., 2009, Debeaupuis et al., 1997, Menotti et al., 2005, Guinea et al., 2011, de Valk et al., 2007, Tang et al., 1994). Moreover, in one study, while several genotypes were recovered from the respiratory tract of a patient, only one was recovered from invasive disease from solid organs suggesting further selection for invasion (de Valk et al.,

2007). As it is now becoming clear that pathogenicity in this opportunistic pathogen is polygenetic rather than being dependent on any one virulence factor (Askew, 2008, Rhodes, 2006), the broad diversity in the genotype of the population might be expected to give rise to a range in pathogenicity and ability to adapt and infect the lung. In addition, infection also depends on host factors which will also vary in differing individuals (Hartmanna et al., 2011) and selection in the lung from a diverse range of genotypes will therefore be multifactorial dependent on both *A. fumigatus* and host genotype.

4.6 Reference

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Chapter 5

Title: *In vivo* expression of *Aspergillus fumigatus* phospholipases during infection of wax moth larvae

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5.1. Abstract

The opportunistic pathogen *Aspergillus fumigatus* secretes a broad range of hydrolases thought to be important in host colonisation and tissue damage in the lung such as proteases and phospholipases. The genome of *A. fumigatus* is known to encode two major classes of phospholipases, phospholipase B and phospholipase C for which several studies have detected significant extracellular activity. In this study, we used the wax moth larvae insect model of infection to study the *in vivo* expression of phospholipases *Afplb1*, *Afplb3*, *AfplcA*, *AfplcB* and *AfplcC* relative to the expression of the housekeeping gene of actin by real-time PCR in five strains of *A .fumigatus* with differing levels of pathogenicity in this model. While the relative levels of expression for each phospholipase varied between strains, *AfplcC* was the most strongly expressed in all strains. Overlapping PCR was used to generate a gene replacement with the selectable marker Hygromycin B (Δ*Af*plcC). However, when thepathogenicity of this strain was compared to the parental strain in wax moth larvae, no difference in pathogenicity was observed.

5.2. Introduction

The opportunistic human pathogen *A. fumigatus* is considered to be the world's most harmful mould (Ben-Ami et al., 2010, McCormick et al., 2010, Pringle et al., 2005, Tekaia and Latge, 2005) and is a particular danger to immunocompromised hosts where it can cause a range of allergic, saprophytic and invasive diseases including allergic bronchopulmonary aspergillosis and aspergillosis. While other Aspergilli, notably *A. flavus* and *A.terreus* can also cause disease, *A. fumigatus* is responsible for the majority of Aspergillus infections (Rementeria et al., 2005). It is becoming increasingly clear that pathogencity in *A. fumigatus* is multifactorial and complex, involving the regulation and expression of a diverse network of genes controlling cell wall integrity, cell signalling, iron sequestration and cell stress amongst others, as well as an array of secreted hydrolases that are thought to play an important role in host tissue degradation and nutrition (Askew, 2008, Ben- Ami et al., 2010, Latge, 1999, Reeves et al., 2004, Robson et al., 2005, Schrettl et al., 2007, Tekaia and Latge, 2005, Tomee and Kauffman, 2000). The principal route of infection is by inhalation of airborne conidia where due to their small spore size, they are able to reach the alveoli and interact with lung surfactant. Lung surfactant is essential for maintaining gaseous exchange and is composed largely of phospholipid of which the majority is phosphatidylcholine (Morgenroth, 1988, Veldhuizen et al., 1998). Germinating conidia in the lung will therefore be in a phospholipid rich environment where secreted phospholipases may play an important function in establishing and developing infection and causing host tissue damage. *Candida albicans* and *Cryptococcus neoformans* encode genes for secreted phospholipase B (PLB) and in *C. neoformans* which encodes only one gene for PLB, disruption reduces virulence (Cox etal., 2001) In addition, disruption ofcnsec14, a phophatidylinositol transfer protein required for PLB secretion in *C. neoformans* is also attenuated in virulence (Chayakulkeeree et al., 2011). In *C. albicans*, up to five genes encode PLB, with plb1 the major PLB expressed during infection in a mouse model with disruption again reducing pathogenicity (Ghannoum, 2000,

Leidich et al., 1998, Mukherjee et al., 2001) while gene disruption of a cell surface-associated PLB has also been demonstrated to reduce pathogenicity (Theiss et al., 2006). However, *A. fumigatus* has in addition to two genes encoding putative secreted PLB (Af*plb1* and Af*plb3*), three genes encoding putative secreted phospholipase C (PLC) (Af*plcA*, Af*plcB* and Af*plcC*) and one putative secreted phospholipase D (PLD) (Li et al., 2012, Robson, 2009). In addition, activities corresponding to extracellular PLB, PLC and PLD have previously been reported (Birch et al., 1996, Li et al., 2012). In this study, we investigated the expression of phospholipases *in vivo* in a wax moth larvae (WML) model during infection with *A. fumigatus* strains with differing levels of pathogenicity. While *AfplcC* was by far the most highly expressed of all the phospholipases *in vivo* in all the strains, a Δ*AfplcC* knockout strain in *A. fumigatus* 1163 was not attenuated in pathogenicity compared to the parental strain.

5.3. Materials and Methods

5.3.1. Oganisms

Sequenced strains *AF*1163 and clinical isolates Clin5 and Clin11 were kindly supplied by Dr Caroline Moore and were obtained from the Wythenshaw hospital culture collection (University Hospital of South Manchester, Wythenshaw, Manchester, UK). Environmental strains M13 and Ap12 were previously isolated from the air from the University of Manchester campus (Alshareef, unpublished). Strains were maintained in 50% (v/v) glycerol at -80°C.

Escherichia coli strains were routinely maintained in LB-broth supplemented with 100 µg ml^{-1} ampicillin (prepared as 10 mg ml^{-1} filtered sterilized stock solution stored at -20^{o}C). Strains were stored on LB agar plates at 4°C for up to one month or at -80°C in LB broth containing 50% (v/v) glycerol.

5.3.2. Virulence determination in wax moth larvae

Wax moth larvae (*Galleria mellonela*) were obtained from Live Food Direct Ltd (Sheffield UK). Larvae (ca. 0.3-0.4 g in body weight) were supplied in boxes in wood shavings and after visual inspection and removal of dead larvae, were stored in the dark at 4°C for up to ten days. Wax moth larvae in groups of 30 were inoculated with 10 µl of an *A. fumigatus* spore suspension into the haemocoel through the last right or left proleg, using a Hamilton 1 ml gastight syringe (Fisher Scientific, UK) with a 10 µl repeating dispenser attachment (Jaytee Biosciences Ltd, UK). Spore concentrations of $1X10^{8,}$ to $1X10^{3}$ per ml were used to infect larvae and incubated at 37°C in the dark in Petri dishes (10 larvae per plate) and the mortality monitored daily for 7 days. Mortality was assessed by lack of movement in response to stimulation and by discoloration (melanisation) of the cuticle.

5.3.3. *In vivo* expression of phospholipases in infected wax moth larvae

For testing the relative gene expression in WML, we measured the virulence of tested isolates (Clin 5 and Clin 11 respectively) and Manchester environmental isolates (April 12 and March 13 respectively), along with Af1163.we tried to compare the hyper and hypo-virulent strains and the level of gene expression of the five genes. The mycelia growth was harvested after overnight incubation on orbital shaking for 18 h at 37°C by vacuum filtration and mycelium flash frozen immediately in liquid nitrogen. For wax moth larvae, individual larvae were flash frozen in liquid nitrogen and ground to a powder with a mortar and pestle in liquid nitrogen prior to RNA extraction. A NanoDrop 1000 Spectrophotometer (Thermo Scientific, UK) was used to measure RNA concentrations in samples. The measurement program was initialized with 1 µl of DEPC water loaded onto the pedestal and the machine set to zero. This was repeated a second time before the addition of 1 µl of the sample and the sample absorbance measured at 280 and 260 nm. The 260/280 nm ratio for all samples was between 1.8-2.0

Approximately ~50 mg of ground sample was transferred to an eppendorf tube and RNA extracted using a Qiagen RNeasy Plant mini kit (Qiagen, UK) according to the manufacturer's instructions. 6 µl of β-mercaptoethanol was added to 600 µl of buffer RLT before adding to the ground sample, vortexing and incubated for 2 min at 56oC. The whole lysate was transferred to a QIA shredder spin column, centrifuged for 2 min at 13,000 rpm and the supernatant collected. 500µl of ethanol was added, gently mixed by inversion, transferred onto an RNeasy spin column and centrifuged at 13,000 rpm for 1 min and the supernatant discarded. 700 µl buffer RW 1 was applied to the column, centrifuged at 13,000 rpm) for 1 min and the supernatant discarded. 500 µl of RPE buffer was then added to the column, centrifuged at 13,000 rpm for 1min and the supernatant discarded. Finally, RNA was eluted from the column by adding 50 µl RNase-free water directly the spin column and centrifuged at 13,000 rpm for 1 min. Then RNA concentration and purity was determined

using a Nano Drop 1000 spectrophotometer (Thermo Scientific, UK) and stored in aliquots at -80°C until required.

cDNA was generated using the QuantiTect Reverse Transcription kit (Qiagen, UK) according to the manufacturer's instructions. 2 µl of gDNA Wipeout buffer and 14 µl of RNase-free water was added to ca. 0.8 -1 µg of RNA in a 0.5 ml PCR tube and the sample incubated for 2 min at 42° C before placing on ice. 1 µl of Quantiscript Reverse Transcriptase, 4 µl of 5X Quantiscript RT Buffer, 1 µl of RT primer mix and 6 µl of master mix were added to the sample and incubated for 15 min at 42° C followed by 3 min incubation at 95°C to inactivate Quantiscript Reverse transcriptase. The sample was then diluted four-fold by the addition of 60 µl RNase- free water.

QRT-PCR was performed in triplicate for each sample using SYBR-Green to detect PCR products in 96 well plates according to the manufacturer's instructions (BioRad, UK). Controls in which DEPEC water replaced the sample and where no reverse transcriptase was added were included to check there was no contamination with genomic DNA.

2 µl of diluted cDNA was added to 18 µl of Master Mix (New England Biolabs, UK) which contained thermopol buffer (10x) for a volume of reaction 1X (2
µl), 0.4 µl dNTPs (10 mM, Bioline, UK), 0.6 µl of MgCl2 (Bioline, UK), 0.75 µl Syber Green (1/2000 dilution, Sigma-Aldrich, UK), 0.2 µl Thermostable Taq DNA polymerase (New England BioLabs., UK and 0.06 µl of forward and reverse primers

cDNA was generated using the QuantiTect Reverse Transcription kit (Qiagen, UK) according to the manufacturer's instructions. 2 µl of gDNA Wipeout buffer and 14 µl of RNase-free water was added to ca. 0.8 -1 µg of RNA in a 0.5 ml PCR tube and the sample incubated for 2 min at 42° C before placing on ice. 1 µl of Quantiscript Reverse Transcriptase, 4 µl of 5X Quantiscript RT Buffer, 1 µl of RT primer mix and 6 µl of master mix were added to the sample and incubated for 15 min at 42° C followed by 3 min

incubation at 95°C to inactivate Quantiscript Reverse transcriptase. The sample was then diluted four-fold by the addition of 60 µl RNase- free water.

QRT-PCR was performed in triplicate for each sample using SYBR-Green to detect PCR products in 96 well plates according to the manufacturer's instructions (BioRad, UK). Controls in which DEPEC water replaced the sample and where no reverse transcriptase was added were included to check there was no contamination with genomic DNA.

2 µl of diluted cDNA was added to 18 µl of Master Mix (New England Biolabs, UK) which contained thermopol buffer (10x) for a volume of reaction 1X (2
µl), 0.4 µl dNTPs (10 mM, Bioline, UK), 0.6 µl of MgCl2 (Bioline, UK), 0.75 µl Syber Green (1/2000 dilution, Sigma-Aldrich, UK), 0.2 µl Thermostable Taq DNA polymerase (New England BioLabs., UK and 0.06 µl of forward and reverse primers (50 µM). The PCR reaction was performed using a Chromo4 machine (BioRad, UK) according to the following protocol: step one initial denaturation at 94°C for 10 min for 1 cycle, step two: denaturation at 94°C for 30 s, annealing 60°C for 30 s, and extension for 72°C for 1 min for 35 cycles, step three: final extension 72°C for 5 min for one cycle only. Melting curves for each reaction were conducted at 60-90°C. Data from the QRT-PCR was analysed using Chromo4 software (BioRad, UK).

In order to quantify the expression of phospholipase genes in vivo in wax moth larvae, the Ct value for each phospholipase genes was compared to the Ct value for the housekeeping gene actin. The average Ct value for the phospholipase (CtPL) was deducted from the average Ct value for actin (CtAct) (mean at least three replicates) to give the value ΔCt, and converted to a fold change using 2ΔCt.

5.3.4. *Af*plcC gene knockout vector

For homologous recombination and replacement of the *Af*plcC gene with the selectable marker for hygromycin resistance, three overlapping amplicons consisting of the gene sequence for the hygromycin selective marker (primers 3 and 4) and upstream (primers 1 and 2) and downstream sequence (primers 5 and 6) of *Af*plcC gene were generated by PCR and the final construct generated by overlapping PCR using nested primers (primers 1N and 6N) (Table 5.2). The final construct consisted of the hygromycin resistance gene flanked by homologous sequence upstream and downstream of the targeted phospholipase gene enabling targeted gene replacement by homologous recombination following fungal transformation. A diagrammatic overview is shown in Fig 5.1.

gene	Oligonucleotides sequence	Amplicon Size (bp)	Accession number of the gene (*A. fumigatus* 1163)
Actin	TGCTCCTCCTGAGCGTAAAT (F)	80	AFUB_043700
	ACATCTGCTGGAAGGTGGAC (R)		
*plb*1	TGTTGGCTTTGTCATGGGTA (F)	100	AFUB_065820
	GATGTCGGTGAAGACGGATT (R)		
*plb*3	CCAATGGAGCTGGAGCTATC (F)	82	AFUB_034540
	TGCAGTAGACCACCGAGATG (R)		
*plc*C	CGGACGCTCTCTTCTTCAAC (F)	148	AFUB_090460
	ATGGAGTTGGTGTCGAGGTC (R)		
*plc*B	ACAGGTCGAGCAGAAGGGTA (F)	108	AFUB_016970
	GGGGTAAAGGGCTCAAAGTC (R)		
*plc*A	CGGCGAGGTCATCAACTACT (F)	143	AFUB_046870
	AAGTGCCGGACGTCAGATAG (R)		

Table 5.1. The primers used for RT-PCR of phospholipases B and C and actin from A. fumigatus 1163. The accession number for each gene in Af1163 is indicated in the last column. F=forward primer, R=reverse primer.

Fungal genomic DNA was extracted from mycelium grown for 16-18 h in Vogel's medium in liquid culture at 37°C on a rotary shaker at 250 rpm, harvested by vacuum filtration and flash frozen in liquid nitrogen. Frozen mycelium was ground in liquid nitrogen to a powder in a mortar and pestle pre-cooled to -20°C and genomic DNA extracted with a DNeasy Plant minikit (Qiagen, UK) according to the manufacturer's instructions and stored in DEPC water at -20°C until required.

Conventional PCR reactions were performed using amplification 0.2 µl Phusion polymerase with HF Buffer (New England BioLabs), 200 µM of each dNTP, 0.5 µM of each primer and ca. 30 template DNA. For generating amplicons upstream and downstream of the *Af*plcC gene, genomic DNA was used as the template, and for generating the hygromycin resistance amplicon, plasmid Pan7.1 was used as template. The following parameters were used for PCR: 30 s denaturation at 98°C, 35 cycles for 15 s at 98°C, 30 s annealing at 52°C and 40 s extension at 72°C with a final extension of 40 s at 72°C. PCR products separated on 0.8% (w/v) agarose gels in TE buffer at 80 V for 30 min, stained with ethidium bromide (10 mg/ml, Sigma) and visualized using a UVItec transilluminator. Bands were excised and extracted using QIAquick®Gel Extraction kit (Qiagen, UK) according to the manufacturer's instructions.To generate the Δ*Af*plcC amplicon for homologous recombination, ca. 0.15-0.2 µl each of the three purified amplicons were added to 5X Phusion polymerase HF Buffer, 0.02 U/µl Phusion polymerase (New England Biolabs, UK), 200 µM of each dNTP, 0.5 µM of µl. The following parameters were used for overlapping PCR: 40 sec denaturation at each of the nested primers (1N and 6N) in a volume mixture of 20

1	5`-GACAGTTGGAATTGCGGCCT-3`
2	3`-GCGCCCACTCCACATCTCCACTCGAGATGATGGACGCAGCTCT TACCCAT-5`
3	5`-ATGGGTAAGAGCTGCGTCCATCATCTCGAGTGGAGATGTGGAG TGGGCGC-3`
4	3`-TTTCTGAGCAGGTTTTGCATGAAATCTATAGAATCATCCTTATT CGTTGA-5`
5	5`-TCAACGAATAAGGATGATTCTATAGATTTCATGCAAAACCTGC TCAGAAA-3`
6	3`-ACCAAGCTGAGGAGCGAAGT-5`
1N	5`-CAAGCCAAGGAACGCCATGA-3`
6N	3`-CTGAGGGTGAGATGGAGCGT-5`

Table 5.2. Primer sequences used for AfplcC gene replacement with the hygromycin resistance marker. Primers 1 and 2 and primers 5 and 6 were used to amplify sequence upstream and downstream of AfplcC from genomic DNA. Primers 3 and 4 were used to amplify the hygromycin resistance gene from plasmid Pan7.1. Nested primers 1N and 6N were used for overlapping PCR to generate the knockout construct from the three amplicons.

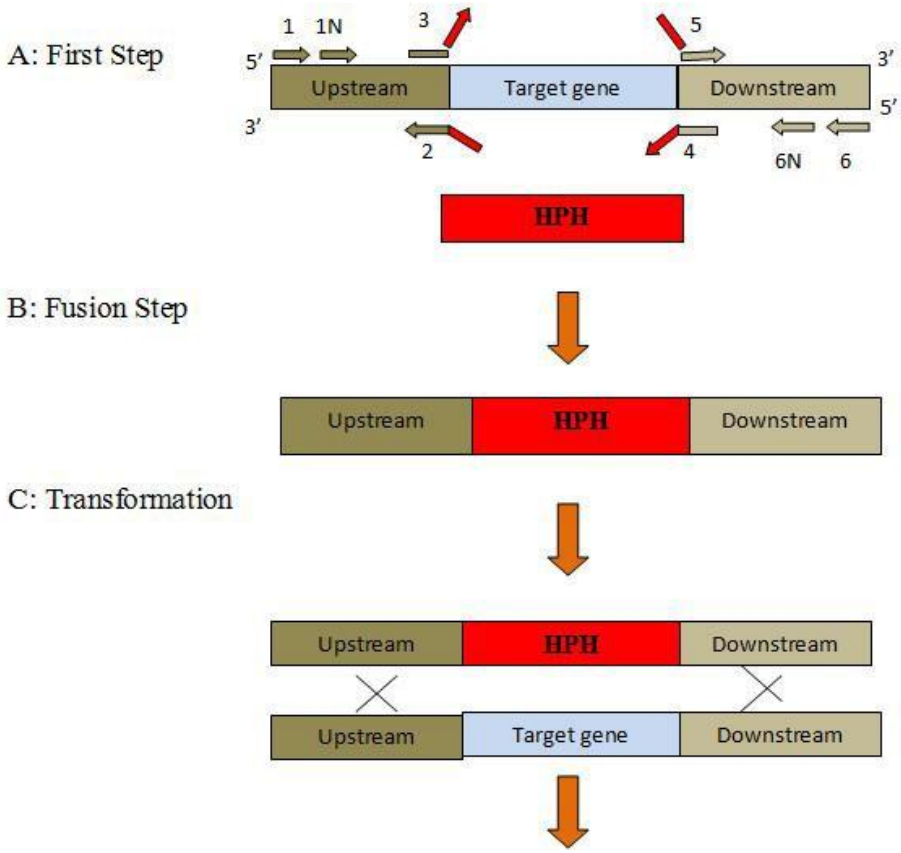

Figure 5.1. Diagrammatic overview of the overlapping PCR method for generating a targeted gene deletion. Numbers in A refer to the position of the primers used.

98°C, 35 cycles for 10 s at 98°C, 30 s annealing at 68°C and 40 s extension at 72°C with a final extension of 40 s at 72°C. PCR products separated on 0.8% (w/v) agarose gels in TE buffer at 80 V for 30 min, stained with ethidium bromide (10 mg/ml, Sigma) and visualized using a UVItec transilluminator. Bands were excised and extracted using QIAquick® Gel Extraction kit (Qiagen, UK) according to the manufacturer's instructions. As Phusion enzyme generates PCR products with blunt ends, it was necessary to add thymidine at the 5' and 3' ends to enable ligation into the cloning vector. Ca. 20 ng of PCR product was added to 3.55 µl buffer (160 mM (NH4)2SO4, 67 mM Tris-HCl, pH 8.8), 1.06 µl of 1X MgC12, 0.6 µl of 1X dNTP and 0.355 µl 1X Taq Polymerase (Bioline, UK) and run for 20 min for 72°C. After purification with a Qiagen QIAquick PCR purification kit according to the manufacturer's instructions, the amplicon was ligated into the pGEM-T vector (Promega, USA) to form vector PΔ*Af*plcC, cloned into *E. coli* JM109 competent cells and transformed cells were selected by blue/white selection according to the manufacturer's instructions. The fidelity of the insert was verified by sequencing in house.

An overnight broth culture (10 ml LB medium) was centrifuged at 5,000 XG and the vector isolated and purified using a Qiagen plasmid kit (Qiagen, UK) according to the manufacturer's instructions. Prior to fungal transformation, purified plasmid PΔAfplcC was linearised using the restriction enzyme BcuI (SpeI) (Fermentas, Life Sciences). 4 µl of 10X Tango buffer and 3 µl of enzyme were added to 30 µl of plasmid DNA and made up to a final volume of 40 µl with DEPEC water, vortexed briefly and incubated overnight at 37°C.

5.3.5. Fungal transformation

Eight Petri dishes containing 25 ml of SDB media were inoculated with 60 µl of a $1X10^7$ spore per ml suspension and incubated for 18 h at 37°C. After checking for confluent growth, mycelium was filtered through a sterile funnel lined with a double layer of sterile lens tissue (Whatman) and excess media removed by gently squeezing with a sterile plastic spreader. Mycelium was resuspended in 15 ml of protoplasting buffer (0.6 M KCl and 50 mM $CaCl_2$) containing 3% (w/v) Glucanex (NovoNordisk, Denmark) in a Corning tube at 30^o C with gentle shaking for ca. 3 h until protoplast formation was observed under a light microscope. Protoplasts were filtered through a sterile funnel lined with a double layer of lens tissue (Whatman), and the filtrate containing the protoplasts centrifuged at 3000 rpm for 15 min at 4°C. The protoplast pellet was gently re-suspended in 1 ml of ice cold protoplasting buffer and 100 µl of protoplasts used per transformation. 15 µl (~2 µg) of the linearised plasmid PΔ*Af*plcC was added to 100 µl of protoplasts, gently mixed and incubated on ice for 20 min. 1 ml of 60% (v/v) PEG (600) (Sigma, UK) at room temperature was slowly added and incubated at room temperature for 10 min to catalyse protoplast fusion and DNA uptake.

Malt extract agar containing 0.6M KCl was autoclaved at 121°C for 10 min, allowed to cool to ca. 55°C, before adding 2% (v/v) of a filter sterilised (0.22 µm) 50x stock solution of hygromycin B (Melford Laboratories Ltd) to give a final concentration of 200 µg/ml. 200 µl of protoplasts were gently mixed with 20 ml of medium, poured into a Petri dish and allowed to solidify. Plates were incubated at 37°C until growth was detected. If no growth was detected after 7 days the transformation was regarded as negative. Colonies were picked and transferred onto SAB agar containing hygromycin B (200 µg/ml) using sterile toothpicks. Genomic DNA was extracted from putative transformants and screened by PCR using nested primers 1N and 6N as previously described above.

5.3 Results

5.3.1. Pathogenicity of *A. fumigatus* strains in wax moth larvae

In order to investigate phospholipase expression during WML infection, real- time PCR was used to compare the relative phospholipase gene expression in WML infected with *A. fumigatus* strains of low to high virulence. One low virulent and one high virulent clinical and environmental strain (Clin5, Clin11, Ap12 and Mar13 respectively) and the sequenced strain *Af*1163 were used to infect WML with $1X10^5$ spores per larva and survival monitored over 7 days (Fig 5.2). Pathogenicity varied between the isolates with the order from most pathogenic to least being Clin 11 > Mar13 > Clin5 > *Ap*12 > *Af*1163. Uninfected control larvae remained viable over the 7 day period.

5.3.2. In vivo gene expression in infected wax moth larvae

The *in vivo* expression of Af*plb1*, Af*plb3*, Af*plcA*, Af*plcB* and Af*plcC* were quantified 1, 2 and 3 days after inoculation by real-time PCR. Three replicates were used on each day and each replicate consisted of three pooled larvae. Specificity of the primers was tested by undertaking PCR using cDNA and genomic DNA from a 24 h culture of Af1163 grown in Vogel's medium with 0.1% v/v lecithin replacing sucrose. Primers for phospholipases and actin (housekeeping gene) all gave single products of the predicted size (results not shown) and were confirmed by sequencing.

When gene expression for each phospholipase was determined relative to actin after 1, 2 and 3 days following infection, expression of *AfplcC* was by far the greatest in each strain (Fig 5.3, Table 5.3) compared to the other phospholipases and overall remained high on each day. By contrast the level of expression for the other

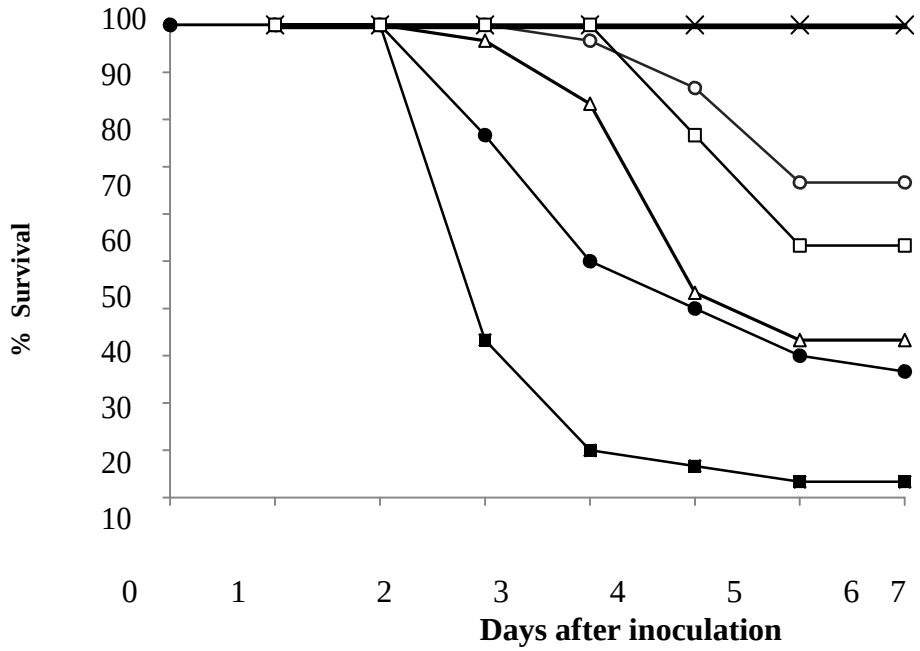

Figure 5.2. Percentage survival of wax moth larvae following inoculation with different isolates of A. fumigatus at 1×10^5 spores/larvae. 30 wax moth larvae were inoculated per isolate and percentage survival monitored daily 7 days at 37°C. Controls were inoculated with sterile 0.05% (v/v) tween 20 (X). A. fumigatus Af1163 (○), *Ap12* (□), *Clin5* (Δ), *M13* (●) and *Clin11* (■).

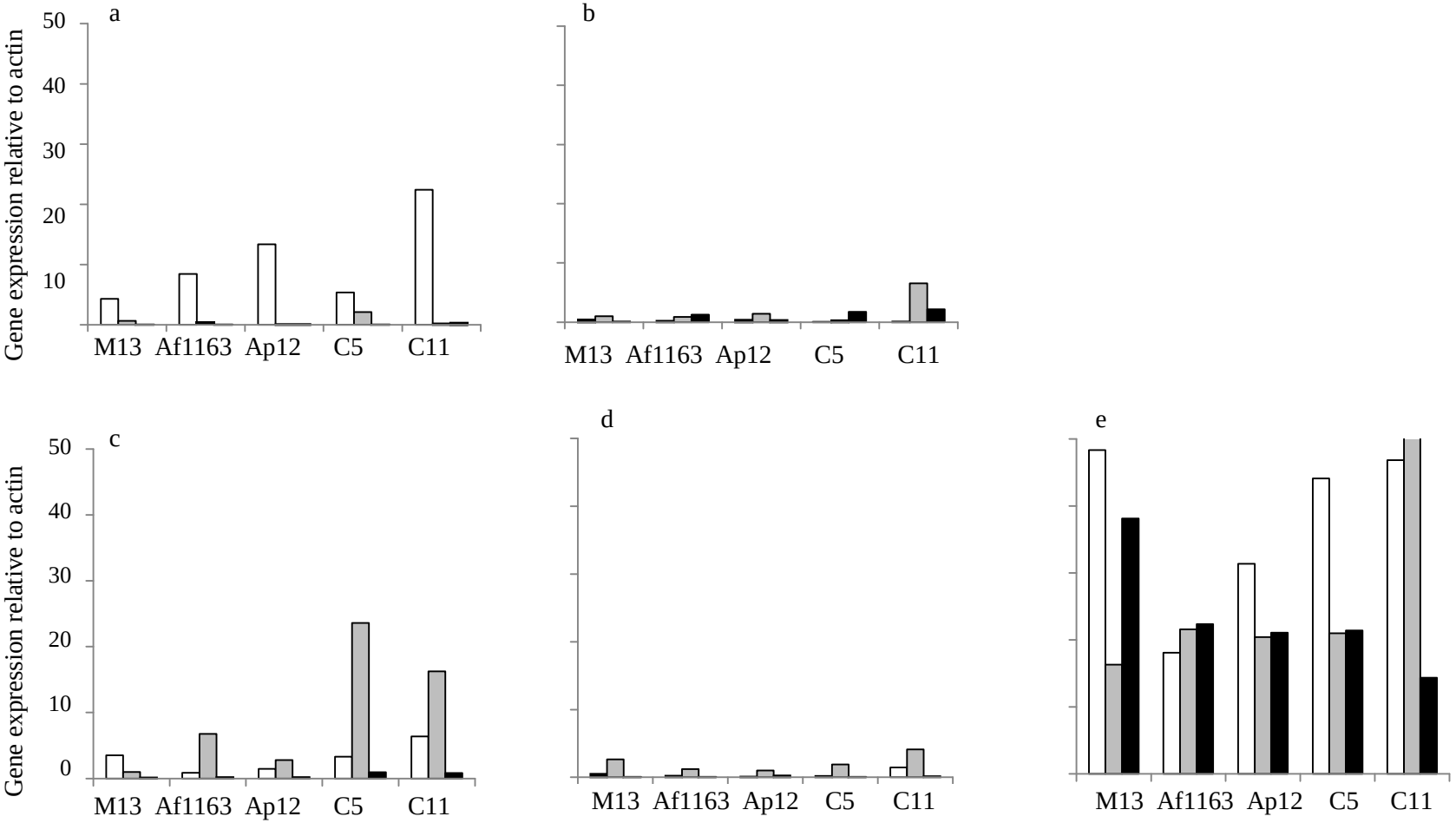

Figure 5.3. In vivo gene expression of Afplb1 (a), Afplb3 (b), AfplcA (c), AfplcB (d) and AfplcC (e) relative to actin, one (□), two (▒) and three (■) days after inoculation of wax moth larvae with 1×10^5 spores per larva. Results are the means of three larvae.

Strain	Gene				
	plb1	*plb3*	*plcA*	*plcB*	*plcC*
M13	0.04-4.4	0.05-1.0	0.15-3.5	0.06-3.9	16.3-48.3
Af1163	0.02-8.5	0.2-1.2	0.20-6.8	0.07-1.2	18.1-22.3
Ap15	0.02-13.4	0.3-1.4	0.20-2.8	0.27-1.0	20.4-31.4
C5	0.04-5.4	0.02-1.7	1.0-23.6	0.06-1.9	21.4-44.1
C11	0.2-22.4	0.06-6.5	0.80-16.3	0.12-4.1	14.4-59.0

Table 5.3. Range of expression of A. fumigatus phospholipases in wax moth larvae over three days post inoculation. Data is expresses as fold level relative to actin.

Phospholipases was more variable between strains, with Af*plb1* peaking on day 1 and declining thereafter, while *AfplcA* peaked on day 2. Af*plb3* and *AfplcB* were relatively weakly expressed compared to the other phospholipases. In order to evaluate if a relationship existed between phospholipase expression and pathogenicity in WML, the correlation coefficient between percentage survival of WML after seven days and mean gene expression over three days relative to actin and percentage survival of WML after seven days and peak gene expression value (highest) relative to actin was calculated for each phospholipase gene. In addition, the mean percentage survival after seven days, mean expression relative to actin over three days and peak gene expression value (highest) relative to actin were ranked highest to lowest and Spearman's Rank Order Coefficient (SROC) calculated (Tables 5.4 and 5.5). While there was no obvious correlation in the expression of phospholipases Afplb1, Afplb3 and *Af*plcA, there was a strong correlation of both mean expression and peak expression relative to actin with WML survival for both *Af*plcB and *Af*plcC with relative pathogenicity.

5.3.3. Gene replacement construct for AfplcC

As there was a strong correlation between pathogenicity and expression of *AfplcC* and as this gene was the most highly expressed during WML infection of all the phospholipases, a Δ*AfplcC* knockout strain was constructed in *A. fumigatus* 1163 to determine if pathogenicity was attenuated. Δ*AfplcC* was generated by homologous recombination with vector PGMT/*plc*::*hph* in which the selectable marker hygromycin B replaced the *AfplcC* open reading frame as illustrated in Fig 5.1.

	Strain					Correlation coefficient (r^2)	Spearman's rank correlation coefficient
	M13	1163	AP12	C5	C11		
Percentage survival	26.6	66.6	53.3	33.3	3.3		
Rank order	4	1	2	3	5		
Af*plb1*	1.7	3	4.5	2.5	7.6	0.25	
Rank order	5	3	2	4	1		-0.1
Af*plb3*	0.5	0.8	0.7	0.6	2.9	0.49	
Rank order	5	2	3	4	1		-0.03
Af*plcA*	1.6	2.6	1.5	9.3	7.8	0.32	
Rank order	5	3	4	1	2		-0.3
Af*plcB*	1.5	0.5	0.6	0.7	1.9	0.84	
Rank order	2	5	4	3	1		-1.0
Af*plcC*	34.3	20.7	24.3	28.8	40.1	0.98	
Rank order	2	5	4	3	1		-1.0

Table 5.4. Correlation between mean gene expression value relative to actin in vivo over three days and percentage survival of wax moth larvae after seven days.

	Strain					Correlation coefficient (r^2)	Spearman's rank correlation coefficient
	M13	1163	AP12	C5	C11		
Percentage survival	26.6	66.6	53.3	33.3	3.3		
Rank order	4	1	2	3	5		
Af*plb1*	4.4	8.5	13.4	5.4	22.4	0.21	
Rank order	5	3	2	4	1		-0.10
Af*plb3*	1.0	1.2	1.34	1.7	6.5	0.57	
Rank order	5	4	3	2	1		-0.40
Af*plcA*	3.5	6.8	2.8	23.5	16.3	0.21	
Rank order	4	3	5	1	2		-0.30
Af*plcB*	3.9	1.2	0.99	1.89	4.1	0.79	
Rank order	2	4	5	3	1		-0.90
Af*plcC*	48.3	22.3	31.4	44.1	59	0.99	
Rank order	2	5	4	3	1		-1.0

Table 5.5. Correlation between peak (highest) gene expression value relative to actin in vivo over three days and percentage survival of wax moth larvae after seven days.

Initially, three PCR's were performed, one for the *AfplcC* upstream sequence (primers 1 and 2), one for the downstream sequence (primers 5 and 6) and one for the hygromycin resistance gene (primers 3 and 4). A second nested PCR was performed with these products to generate the knockout cassette using primers N1 and N6 (Fig 5.4).

Linearised plasmid was used to transform fungal protoplasts, transformants selected on hygromycin media and homologous recombination and gene replacement screened by PCR (Fig 5.5) for the predicted amplicon size of ca 4.5 Kb (ca. 2.5 Kb for the untransformed parental strain). One strain (Δ*AfplcC*) was selected, gene replacement confirmed by sequencing and had an identical phenotype to the parental strain with no significant difference in colony radial growth rate on PDA or on Vogel's medium containing 1% glucose or 0.1% lecithin (results not shown). To investigate if the ΔAf*plcC* strain was attenuated for virulence in WML, WML were infected with $1X10^3$, $1X10^4$, $1X10^5$ and $1X10^6$ spores per larva of the parental Af116 strain and the ΔAf*plcC* strain and survival monitored for 7 days. While as expected, WML survival decreased with increasing spore concentrations, there was no significant difference between the parental and knockout strain (Fig 5.6).

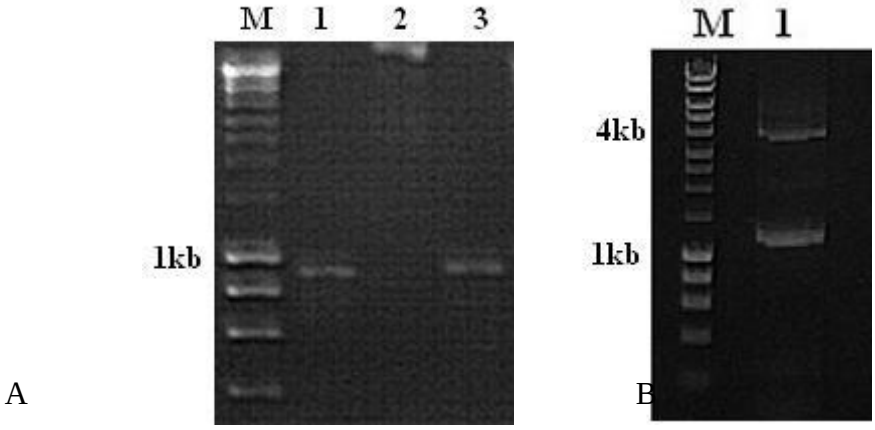

Figure 5.4. A: The PCR products of the upstream (lane 1, 874 bp) and downstream (lane 3, 783 bp) of the AfplcC gene. B: PCR products following overlapping PCR with the hygromyycin marker and upstream and downstream AfplcC PCR fragments. The predicted construct was size was 4 Kbp (lane 1). M= hyperladder I.

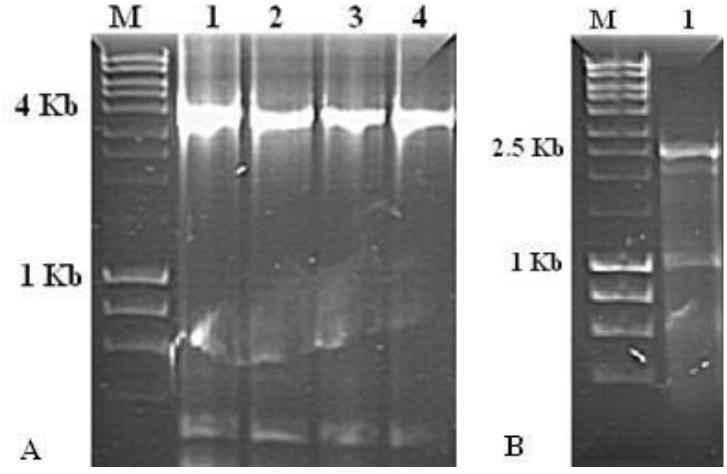

Figure 5.5. A: PCR screening of putative ΔplcC transformants (lanes 1-4). Homologous recombination and gene replacement of AfplcC with hygromycin was predicted to give a product of ca. 4 Kb. B: PCR with the intransformed wild type giving a predicted product of 2.5kb (lane 1). M= hyperladder I.

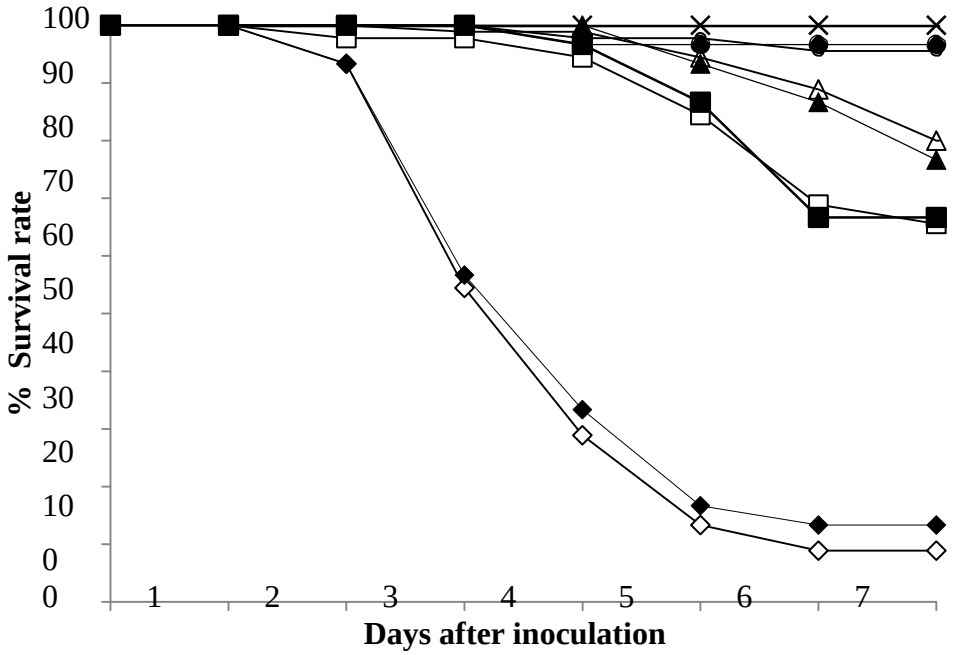

Figure 5.6. The percentage survival rate of WML infected with A. fumigatus Af1163 (open symbols) and ΔplC Af1163 (closed symbols) at 1X10^3 (○), 1X10^4 (Δ), 1X10^5 (□) and 1X10^6 (◊) spores per larva over 7 days. X uninfected larvae.

5.4. Discussion

Invertebrate models of microbial pathogenesis offer significant advantages to conventional vertebrate models. Due to both the high cost and ethical considerations, pathogenicity studies in animals are restricted to small numbers whereas, by contrast, insect models enable much larger numbers to be used allowing intra-strain variations in pathogenicity, drug efficacy and pathogenicity factors to be studied (Alastruey- Izquierdo et al., 2012, Chamilos et al., 2007 , Fallon J.P. et al., 2011, Fuchs and Mylonakis, 2006). For example Lutter et al., (2008) studied the variation in the pathogenicity of strains of *Psuedomonas aeruginosa* isolated from cystic fibrosis patients using a *Drosophila melanogaster* model and the same model was used to study intraspecific variation in pathogenicity in isolates of *A. fumigatus* and *A. terreus* (Ben-Ami et al., 2010). In addition, while invertebrates lack adaptive immunity, the innate immune responses in invertebrates and vertebrates is evolutionarily conserved, consisting of epithelial responses, phagocytosis and defensin release (Chamilos et al., 2007) and a positive correlation between pathogenicity in vertebrate and invertebrate models has been reported for *P. aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *A. fumigatus* (Brennan et al., 2002, Chamilos et al., 2010, Dunphy et al., 1986, Jander et al., 2000, Peleg et al., 2009, Slater et al., 2011). However correlations do not always exit. For example, while melanin deficient conidia of *A. fumigatus* were more susceptible to damage by human monocytes and were attenuated for virulence in a murine animal model (Jahn et al., 2002, Langfelder et al., 1988), they were hypervirulent in wax moth larvae (Jackson et al., 2009). Thus, changes in pathogenicity in invertebrate models cannot always be transposed to animal systems.

Due to the widespread distribution and persistence of airborne conidia of *A, fumigatus,* inhalation of conidia is the principle route of infection in susceptible hosts (O´Gorman, 2011). It has been suggested that secreted phospholipases may play an important role in pulmonary infection due to the high phospholipid content of alveolar surfactant (Ghannoum, 2000; Robson, 2009). In this study, we used the wax moth larval model to study the expression of secreted phospholipases in five strains of *A. fumigatus* with low to high relative pathogenicity during the early stages on infection. To date, there have been few studies that have investigated gene expression *in vivo* in wax moth larvae and only one that has investigated gene expression of phospholipases in *A. fumigatus* (Shen et al., 2004, Vickers et al., 2007). While the level of expression relative to actin varied between the strains for most of the phospholipases, there was no obvious correlation between expression levels and relative pathogenicity with the exception of Af*plcB* and Af*plcC* where a strong association between expression and WML survival was apparent (Tables 5.4 and 5.5) suggesting that PLC activity but not PLB activity may play a role in pathogenicity. For PLB expression, Af*plb1* was more strongly expressed than Af*plb3* with peak expression 1 day after inoculation. Shen et al (2004) also reported that Af*plb1* was more strongly expressed than Af*plb3* when *A. fumigatus* was grown in liquid medium in the presence of lecithin. Expression of Af*plcC* was considerabley higher than any other phospholipase, and coupled with the strong correlation with WML survival, suggested that it may play an important role in pathogenicity during infection. Therefore, a *ΔplC Af1163* was constructed by homologous recombination, replacing the *Af*plcC open reading frame with the selectable marker hygromycin in *A. fumigatus* 1163 and pathogenicity compared to the parental strain. However, no significant difference was observed and pathogenicity was not attenuated in this strain. Thus, PLCC alone does not play an important role in cell death, though it may be important later in cell decomposition. Alternatively, cell death and pathogenicity may depend collectively on expression of all the phospholipases and attenuation in virulence may only be

seen if several or all of the secreted phospholipases are deleted. A similar situation may also apply to proteases, where knockouts of single or multiple genes does not attenuate virulence (Jaton-Ogay K. et al., 1994, Monod et al., 1993, Monod et al., 1999, Monod M, 2009, Reichard et al., 1997, Tang et al., 1993) although *A. fumigatus* encodes for many more proteases than phospholipases (Monod M, 2009, Robson et al., 2005, Watson et al., 2012). However, deletion of a transcription factor regulating protease expression was also not associated with decreased virulence (Bergmann et al., 2009). In addition, regulation of extracellular hydrolases is also highly dependent on factors such as substrate composition, growth phase and extracellular pH (Bergmann et al., 2009, Farnell et al., 2012, Sriranganadane et al., 2010) and is also strain dependent (Birch et al., 2004) further complicating the assessment of extracellular hydrolases in pathogenicity. However, in *C. neoformans*, where only a single secreted phospholipase, PLB is present, disruption reduces virulence (Cox et al., 2001) and in *albicans* where only one secreted PLB appears to be significantly expressed, disruption also reduces virulence (Ghannoum, 2000; Leidich et al, 1998, Mukherjee et al, 2001). The lack of attenuation in virulence in this study when Af*plcC* was disrupted may reflect that *AfplcB* expression, which was also strongly correlated with WML survival, was sufficient to maintain pathogenicity. Multiple gene disruption in phospholipases will be required before the role of this class of hydrolase in pathogenicity can be fully assessed.

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Chapter 6

6.1. General discussion

The aim of this research was to monitor the airborne level of *Aspergillus fumigatus* in air in Manchester city over two years and examine the pathogenic potential and genetic variability of this population and compare with clinical isolates from patients and other environmental isolates (Dublin isolates). In addition, this study also aimed to investigate further the potential role of extracellular phospholipases in pathogenicity by monitoring the relative expression levels *in vivo* in infected wax moth larvae and by targeted deletion of most highly expressed phospholipase, *Af*plcC.

6.1.1. Environmental aspects

Levels of outdoor airborne fungi over a two year period showed significant seasonal variation and was strongly correlated with mean temperature with highest levels in the summer/Autumn. By contrast, airborne *A. fumigatus* spore concentrations were not seasonally associated and did not correlate with temperature. Instead, a low but persistent level of spores ranging from 3-20 CFU m^{-3} was observed over the two year period, consistent with a limited number of previous studies from urban environments (O´Gorman, 2011). While it is suggested that the seasonal increase in total airborne fungal spores, including *Aspergillus sp*. is due to increased fungal growth activity due to temperature and abundance of dead vegetation (Guinea et al., 2006, O´Gorman, 2011), it is unclear why *A. fumigatus* is not also affected in a similar manner, particularly as it is widely distributed in the environment and associated with decaying vegetation. While consistent with other studies, there have been very few studies examining the seasonal variation of outdoor airborne spore concentrations of *A. fumigatus* and most of these have been from urban settings. More long term studies from a variety of locations, including rural settings should be undertaken to determine if this persistent, non-seasonal pattern is a general phenomenon, or is environment dependent. For

example, urban sources of *A. fumigatus* may be from buildings and other environments that are not affected as much by external temperature changes.

6.1.2. The genetic variation in environmental and clinical isolates

Typing techniques to distinguish between *A. fumigatus* isolates were developed primarily for the purpose of outbreak investigation in hospitals and disease surveillance in humans. In our study, we used RAPD to investigate the genetic diversity amongst the environmental and clinical isolates and found that the majority of clinical isolates clustered into three clusters. However, the relationship between genetic relatedness and virulence to date is still a matter of controversy and it is unclear whether more virulent isolates are genetically distinct from other isolates, or whether infection by *A. fumigatus* is simply a matter of contracting infection from any environmental source (Thierry et al., 2010) .

The Manchester environmental isolates were found to be genetically diverse and diversity within the environmental population has been reported previously (Debeaupuis et al., 1997). However, Dublin isolates showed a lower genetic diversity compared to Manchester isolates and may be due to smaller sample size and that these isolates were all collected on a single occasion and therefore may have been derived from a limited number of sources, whereas over the two year period in Manchester, the source appeared to vary between sampling times.

There was a large variation in the pathogenicity within the Manchester environmental isolates as determined in a wax moth larvae model. Interestingly, when environmental and clinical isolates were compared, clinical isolates were the most pathogenic while the environmental isolates showed a broad spectrum of pathogenicity. Patients will be exposed to a genetically diverse spectrum of isolates from the environment while most studies have reported typically only one or a limited number of genotypes from infected the infected lung. Our present study supports the hypothesis that selection for the most pathogenic strains occurs within the lung environment in infected (Bart-Delabesse et al., 1999, Chazalet et al., 1998,

Leenders et al., 1999, de Valk et al., 2007, Araujo and Rodrigues, 2004, Guinea et al., 2010, Hartmanna et al., 2011). However, the pathogenicity of a much larger number of environmental and clinical isolates should be determined than undertaken in this current study to determine more confidently whether selection for the most pathogenic isolates from the environment occurs within patients. In addition, this would be strengthened by including other pathogenicity models, for example the *Drosophila melanogaster* model rather than basing pathogenicity on wax moth larvae alone.

6.1.3. Phospholipase gene expression

Extracellular phospholipases have been suggested to potentially play an important role in lung infection due to the high phospholipid content of the alveolar lung surfactant (Robson, 2009). In order to determine the potential relative role of the expression of this gene family during infection, real-time PCR was used to quantify the expression of the secreted phospholipases during wax moth larvae infection relative to the housekeeping gene actin. While all were expressed, only *Af*plcB and *Af*plcC were found to be correlated to pathogencity in wax moth larvae with *Af*plcC expressed far more highly. Therefore, a targeted gene disruption was undertaken for *Af*plcC and the pathogenicity of the Δ*Af*plcC knockout compared to the wild type. Despite the strong correlation between pathogenicity and *Af*plcC expression in wax moth larvae, the pathogenicity of the Δ*Af*plcC knockout strain was not attenuated compared to the wild type strain. However, as *Af*plcB was also correlated with pathogenicity, this gene and a double knockout strain should be generated and tested for attenuated virulence in order to determine the importance of this class of phospholipase. Although not correlated with pathogenicity, a third PLC, *Af*plcA may also have to be generated in a triple knockout as expression of this gene may compensate for the loss of the other two. In addition, double and triple knockout strains would have to be tested in an animal inhalation model as this model emulates lung infection and may show attenuated virulence more strongly than the wax moth lavae model.

6.2. Reference

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Appendix I

Table: Clinical isolates obtained from Wythenshaw hospital, Manchechester, UK. The patient sample site and hospital are shown. BAL = Bronchoalveolar lavag fluid and the site of isolation from the patient.

Code	Site of isolation	City/town	Hospital
15562	BAL	Newcastle	Freeman Hospital
15819	Sputum	Manchester	NMGU
15820	Sputum	??	
16258	Sputum	Liverpool	Alder Hey Hospital
16795	Sputum	Ashton-under-Lyne	Tameside
16916	Sputum	Manchester	Booth Hall Childrens Hospital
16975	Cough swab	Salford	Royal Manchester Childrens Hospital
17318	Sputum	Stockport	Stepping Hill Hospital
17406	Sputum	Manchester	Booth Hall Childrens Hospital
17597	Skin scrape	Salford	Salford Royal Hospital
17768	Sputum	Stockport	Stepping Hill Hospital
17796	Sputum	Liverpool	Alder Hey Hospital
17835	Sputum	Manchester	Booth Hall Childrens Hospital
17871	Sputum	Oldham	Royal Oldhan Hospital
17882	Ear swab	Kingston-upon-Thames, Greater London	Kingston Hospital
21407	Sputum	Manchester	Wythenshaw Hospital
21522	Sputum	Manchester	Wythenshaw Hospital
21636	Sputum	Durham	County Hospital Durham
21705	Sputum	Manchester	Wythenshaw Hospital
21732	BAL (Bronchial Alveolar Lavage)	Liverpool	Alder Hey Hospital
21932	Nasal secretion	Durham	County Hospital Durham
22020	BAL (Bronchial Alveolar Lavage)	Durham	County Hospital Durham
22178	PUS	Salford	Salford Royal Hospital
22216	Sputum	Chester	Countess of Chester Hospital
22236	Cyst capsule	Leeds	Leeds General Infirmary
22577	Tissue	Liverpool	Stepping Hill Hospital
22636	Trachea	Southport	Southport and Ormskirk Distric Hospital
22769	Tissue	Blackpool	Blackpool Victoria Hospital

22773	BAL (Bronchial Alveolar Lavage)	Liverpool	Alder Hey Hospital
22821	Sputum	Kingston-upon-Thames, Greater London	Kingston Hospital