Studies of clinical and environmental isolates of *Aspergillus fumigatus*

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Abstract

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Aspergillus fumigatus is a fungus of great environmental importance as well as being the most common filamentous fungal respiratory pathogen. Airborne *A. fumigatus* spores can reach potentially dangerous concentrations, particularly in the air around industrial composting sites. Drug resistant *A. fumigatus* infections are an increasingly common problem and the limited range of drugs available for the treatment of *Aspergillus* infections means that any resistance developing is a major cause for concern. Mycoviruses may offer an alternative approach to conventional treatment and exist in many fungal populations, including *A. fumigatus,* but there is little understanding of their importance or the influence they may have on their hosts.

Different air samplers used in bioaerosol studies were evaluated. An enrichment protocol for the isolation of mycoviruses from environmental samples was assessed as well as further characterisation and genome sequencing of a mycovirus of the *A. fumigatus* type strain NCPF7367. For clinical and environmental *A. fumigatus* collections drug susceptibility was assessed using the EUCAST protocol, virulence compared using a *Galleria* model and the mating type of the fungi determined by PCR.

Significant differences in sampler efficacy were observed. Putative mycoviruses were obtained from environmental samples and sequencing of the NCPF7367 virus genome suggests that this is a capsid-less virus. Increased azole resistance and virulence was found amongst compost-derived *A. fumigatus* relative to clinical isolates.

This study highlights the importance of sampler selection in bioaerosol studies. A novel enrichment procedure for mycovirus isolation from environmental samples appears promising and the genome sequencing sheds new light on the lifecycle of an *A. fumigatus* mycovirus. The study also suggests that compost-derived *A. fumigatus* may be less susceptible to azole drugs and more virulent than clinical populations. Drug resistance may be more geographically variable than previously thought and intermediate-level resistance appears not to be mediated by cyp51-A mechanisms.

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Declaration of joint efforts

My own work was central to the production and analysis of the data presented in this thesis; however, a number of colleagues were also involved in the production of data.

Members of the respiratory laboratory at Glenfield hospital were involved in initial inoculations of sputum culture plates and processing of sputum. The dsRNA sequencing was performed by Ian Adams at Fera Science Ltd. Abbie Fairs, Jack Satchwell and the research placement students in our lab; Tom Harding, Adam Roberts, Mahrukh Shameem and Satkarn Shergill helped with preparation of culture media and provided general laboratory assistance. Natalie Allcock and Stefan Hyman provided help with the electron microscopy. Catherine Pashley and Abbie Fairs helped with the identification of fungi and Jack Satchwell also helped with double-checking the MIC readings for the EUCAST work.

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Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
bp	Base pairs
°C	Degrees Centigrade
CF	Cystic fibrosis
CFU	Colony forming units
COPD	Chronic obstructive pulmonary disease
CSTEM	Cross-sectional transmission electron microscopy
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
ds	Double-stranded (DNA or RNA)
EDTA	Ethylenediaminetetraacetic acid
EUCAST	European Committee on Antimicrobial Sensitivity Testing
FEV$_1$	Forced expiratory volume in the first second
FVC	Forced vital capacity
GNB	Glycerol nutrient broth
HMG	High mobility group
ITS	Internal transcribed spacer region
kb	Kilobase pairs
kV	Kilovolts
MEA	Malt extract agar
ns	Not significant

NTA	Nanoparticle Tracking Analysis
NCPF	National Collection of Pathogenic Fungi
OD	Optical density
PBST	Phosphate buffered saline supplemented with 0.05% Tween-80
PDA	Potato dextrose agar
PGC	Potato dextrose agar supplemented with gentamicin and chloramphenicol
PGCF	Potato dextrose agar supplemented with gentamicin, chloramphenicol and fluconazole
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute liquid medium (RPMI-1640)
SD	Standard deviation
SDA	Sabauroud dextrose agar
SLM	Sabauroud liquid medium
SNP	Single nucleotide polymorphism
ss	Single-stranded (DNA or RNA)
TEM	Transmission electron microscopy
V	Volts
VLP	Virus-like particle
YMA	Yeast malt agar
w/v	Weight by volume
μm/mm/m	Micrometres/millimetres/metres
ng/μg/mg/g	Nanograms/micrograms/milligrams/grams
μl/ml/l	Microliters/millilitres/litres

Chapter 1 Introduction

1.1 Fungal taxonomy

Fungi are an abundant and diverse group of eukaryotic organisms, estimated to comprise at least 1.5 million, but probably as many as 3 million fungal species exhibiting great diversity (Hawksworth, 2012). The kingdom Fungi includes both macro and microorganisms ranging from microscopic spore forming filamentous fungi and yeasts to the enormous fruiting bodies of some mushrooms, which can be up to 15 kg in weight. Individual spores of some fungi can be <2 μm in diameter and yet some of the largest organisms ever recorded are the vast networks of subterranean hyphae of fungi such as the well-documented 'Humongous fungus', a forest floor dwelling species *Armillaria gallica* (Smith, Bruhn and Anderson, 1992). There are both marine and terrestrial fungal species and parasitic, saprophytic and symbiotic fungi (Moore *et al.*, 2011). Within the kingdom Fungi there are seven phyla; Chytridiomycota, Blastocladiomycota, Neocallastigomycota, Microsporidia, Glomeromycota, Basidiomycota and Ascomycota (McLaughlin *et al.,* 2009). The Ascomycota includes the important filamentous fungal genera *Penicillium* and *Aspergillus*. *Aspergillus fumigatus* is the main focus of this project.

1.2 Fungal reproduction

One of the most striking aspects of the fungal life cycle is that many fungi may reproduce both sexually and asexually. These different reproductive strategies are outlined below focusing primarily on ascomycetes and specifically *A. fumigatus*. Different terminology is used to distinguish fungi in the process of the different forms of reproduction; anamorph is the term used for the asexual form and teleomorph is the sexual form. *Aspergillus fumigatus* is the name given to the asexual, or anamorph, form of this species whilst the sexual, teleomorph, form is *Neosartorya fumigata* (O'Gorman *et al.*, 2009). Ultimately reproduction in the Ascomycota is based on the production of spores and these spores are known as conidia if produced as a result of the asexual reproduction cycle whilst spores produced as a result of sexual reproduction are termed ascospores. The sexual reproduction cycle in *A. fumigatus* was only seen relatively

recently (O'Gorman *et al.,* 2009) under carefully controlled laboratory conditions. By far the most common method of reproduction in this fungus is by asexual reproduction and the production of haploid conidia (Nieuwenhuis and James, 2016), but the discovery of a viable sexual reproductive cycle in *A. fumigatus* suggests greater potential for recombination and the introduction of greater genetic diversity into *A. fumigatus* populations than previously thought (Duarte-Escalante *et al.*, 2009). The sexual reproduction of fungi relies on a mating type system, in which two different mating type alleles are present within a population. The nature and naming of the mating types is highly variable between species, the a and α mating types of yeast being a well-studied example (e.g. Nasmyth, 1982).

In *A. fumigatus* mating type is governed by the MAT locus, which features one of two idiomorph alleles, MAT1-1 or MAT1-2, encoding high-mobility-group (HMG) domain or alpha box genes, respectively (Goldman and Osmani, 2007). These gene products act as transcription factors regulating distinct cascades of transcriptional control which allow mating type recognition, cell fusion and the instigation of fruiting body formation as well as many other cellular process required for sexual reproduction (Rydholm *et al.,* 2007).

The predominant reproductive strategy of the ascomycete fungi is asexual, via mitotic cell division of haploid hyphal cells or the mitotic production of conidia by a process of abstraction from specialised hyphal cells producing chains of haploid conidia (Geiser, 2009). For the sexual cycle to occur, colonies of two compatible mating types must be in close proximity. The development of specialised reproductive cells, the antheridium (male) and ascogonium (female), may then occur and it is the fusion of these haploid reproductive cells (plasmogamy) which begins the sexual cycle. The dikaryotic cell that results for this plasmogamy event then undergoes mitotic cell division to produce a fruiting body, the cleistothecium, composed of dikaryotic hyphae. On the outer surface of this structure the cells may develop into asci, in which the nuclei can fuse (karyogamy) to produce a diploid ascus, the zygote. This zygotic ascus then undergoes meiosis, producing four nuclei in the maturing ascus. A single round of mitosis and subsequent maturation then produces eight haploid ascospores within each ascus, containing four ascospores of each mating type. Upon release, these haploid spores may

then germinate, becoming haploid hyphal cells and re-enter the mitotic asexual reproduction cycle, forming mycelial haploid growth. These interwoven sexual and asexual life cycles are outlined in Figure 1-1. A further point of interest in *A. fumigatus* is that as well as the mating type genes, pheromone signalling genes have also been identified and appear to be well conserved (Rydholm, Dyer and Lutzoni, 2007). These would have no function in a purely asexual species and so the conservation of these genes suggests that *A. fumigatus* may reproduce via the sexual cycle more commonly than has so far been identified (Paoletti *et al.*, 2005).

1.3 Fungal ecology

Fungi are of great importance in the degradation of organic waste and thus play a crucial role in the functioning and sustainability of most ecosystems (Moore *et al*., 2011). Commercial uses of fungi are many and varied, ranging from the ancient use of yeasts in food and drink production to the use of fungal expression systems in biotechnology industries (Mattanovich *et al.*, 2012). In the natural world, many fungi co-exist with other organisms either as harmless commensals or as active symbionts, helping their associated organisms to thrive. An example of such a symbiosis with the animal kingdom is that some anaerobic fungi of the genus *Neocallimastix* exist in the digestive tract of their ruminant hosts and contribute crucial digestive processes which enhance the overall efficiency of the host's digestion (Wei *et al.*, 2016). In the plant kingdom many symbiotic relationships with fungi are known. Mycorrhizal symbiosis is one well-studied plant-fungus symbiosis in which fungal association with the root system of a plant allows greater uptake of water and/or soil nutrients for the plant, whilst providing the fungus with a steady supply of carbohydrates (Li *et al.*, 2006). In the human body fungi form a part of our normal flora and play many roles in maintaining the healthy equilibrium associated with our microbiota although such interactions are understudied, and considerably less is known about the fungal component than the bacterial component of our microbiome (Underhill and Iliev, 2014). Aside from these aspects of positive environmental, industrial and clinical importance, many fungi have the potential to be pathogenic to both plants and animals, as is discussed in sections 1.4 and 1.5.
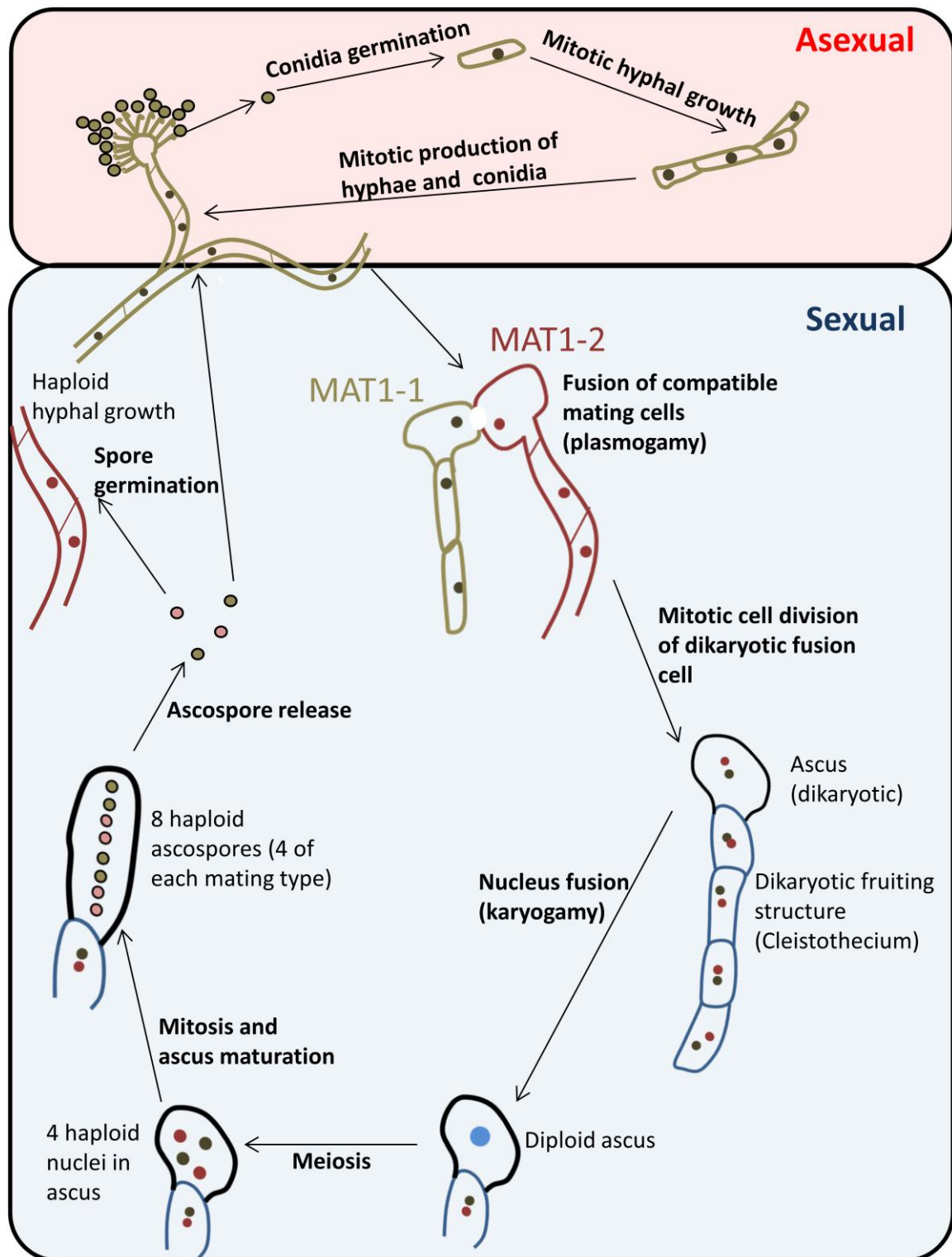
Figure 1-1: An outline of the life cycle of an ascomycete, showing stages of both sexual and asexual reproduction.

1.4 Fungi as plant pathogens

Much of the early mycological research was concerned primarily with plant pathogenic fungi and many of the principles of fungal biology and pathogenicity which were first elucidated in this research underpin our knowledge of human fungal pathogens (Sexton and Howlett, 2006). Throughout the course of infection, aside from causing physical damage to infected plants and thus directly causing a reduction in crop yield, many fungal plant pathogens also produce mycotoxins (Carris *et al.,* 2012). These secondary metabolites act as major virulence factors in the plant infection but can also then be transmitted up the food chain, being harmful to humans or other animals upon ingestion of the infected plant material (Gallo *et al.*, 2015). One well-studied example of this is the case of ergotism, a multi-symptomatic disease caused by the ingestion of wheat products contaminated with the alkaloid mycotoxins produced by the common wheat pathogen *Claviceps purpurea* (Coufal-Majewski *et al.*, 2016). Ergot poisoning has been a problem for mankind for centuries and has been studied extensively (e.g. Nicol, 1939) due to its importance in crop losses. Interestingly ergot contamination of wheat has even been recently reviewed as a potential cause of the circumstances and symptoms which led to the Salem witch trials of the 1690s, which are believed to closely match the symptoms of consumption of wheat products contaminated with ergot alkaloids (Mundra *et al.*, 2016). Clearly these secondary outcomes of fungal infection of crop plants leads to a further loss of edible crop yield- contaminated crops must be discarded even if the initial yield is not significantly affected. Treatment of crops with fungicides has thus become a common practice worldwide with triazoles being the most widely used systemic fungicides (Bowyer and Denning, 2014). In the UK, prothioconazole, epoxiconazole and tebuconazole are the three most commonly used fungicides (Price *et al.,* 2015), all of which are triazoles. The significance of this azole fungicide use is discussed further in section 1.9.

1.5 Fungi as human pathogens

Some fungi are important pathogens of humans and indeed many fungi have a role in both environmental and clinical settings. *Aspergillus* species play a role in environmental degradation of organic matter and nutrient cycling as well as having a

significant clinical presence as human pathogens (Paulussen *et al.*, 2016). Some fungi are able to act as pathogens of both plants and animals, as is the case with some species of the genus *Fusarium* (Guarro *et al.*, 2004). More commonly plant pathogenic fungi cause ill health in humans not by infection, but by the ingestion of mycotoxins produced by plant pathogens as outlined above. Most commonly this occurs with toxins produced by plant pathogenic species of the *Aspergillus*, *Claviceps*, *Fusarium* and *Alternaria* genera (Zain, 2011). The four most important genera of human pathogenic fungi are the yeasts *Candida* and *Cryptococcus* and the filamentous ascomycete genera *Trichophyton* and *Aspergillus* (Bowyer *et al.*, 2011)*. Candida* species include those associated with thrush and, far more seriously, candidaemia (most commonly *C. albicans*) (Arendrup, 2013). *Cryptococcus* species include important opportunistic pathogens such as *C. neoformans* which causes cryptococcosis in immunocompromised individuals which may progress to a form of meningitis. Another important *Cryptococcus* species is the more virulent *C. gatii* which can cause cryptococcosis and meningitis in otherwise healthy individuals with normal immune function (Patil *et al.*, 2013) but has a much more limited geographical endemic range. Filamentous fungal pathogens include dermatophyte fungi of the *Trichophyton* genus, which are responsible for skin infections such as athletes foot and ringworm (Kaufman *et al.*, 2007). Members of the genus *Aspergillus* can cause invasive infections in individuals whose immune systems are compromised, aside from being important allergens (Hope *et al.*, 2005). With the great increase in recent years in the number of people who have successful transplants, who receive cancer treatments and who live with AIDS there are many more individuals surviving with their conditions but with ineffective immune systems (Pfaller and Diekema, 2004). This has meant that the threat from such opportunistic pathogens has become an increasing problem. Particularly alarming is the very high rate of mortality caused by *A. fumigatus* infections in immunocompromised individuals (Richardson and Warnock, 2012). The biology and pathogenicity of *A. fumigatus* is considered in more depth in section 1.7. Another prevalent fungal pathogen of particular relevance to respiratory health is *Pneumocystis jirovecii*, the causal agent of *Pneumocystis* pneumonia (PCP). PCP is among the most prevalent opportunistic infections in HIV patients (Thomas and Limper, 2004) and indeed a sudden rise in PCP cases in the early 1980s was one of the earliest warning signs of the AIDS epidemic that was already underway (Masur *et al.*, 1981). *P. jirovecii* infection is

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also a common and serious complication in other immunocompromised patients; particularly haematology patients and transplant recipients (Cordonnier *et al.*, 2016) and has recently been recognised to have allergenic potential (Eddens *et al.*, 2016). Thus, like *A. fumigatus*, *P. jirovecii* may be an important fungal trigger of asthma symptoms in addition to its role as an opportunistic pathogen.

1.5.1 Immune responses to fungal infection

An overview of human immune responses to fungi is given below with particular emphasis on the immune system's response to *A. fumigatus*, along with a discussion of some of the features and virulence factors which enable *A. fumigatus* to be such a prolific and damaging opportunistic pathogen.

The innate immune system provides the first line of defence against fungal pathogens with physical barriers such as the mucus membranes and associated cilia action, as well as the protective surfactant within the lung helping to clear inhaled spores and prevent them from germinating successfully (Basset *et al.*, 2003).

When fungi are not fully cleared by such physical barriers, humoral immunity plays a crucial role in clearing fungal infections. The alternative complement pathway is an important mechanism by which fungal cells, once bound to C3, are bound by phagocytes and killed. C-reactive protein (CRP) is also able to bind to *A. fumigatus* cells and activates the complement system thus helping to clear the bound cells (da Silva Bahia *et al.*, 2003; Blanco and Garcia, 2008).

Phagocytes also play a vital role in fungal clearance. There is obviously a great deal of structural difference between fungal spores and actively growing fungal hyphae, and different immune cells appear to have distinct roles in dealing with these different physiological forms. Generally speaking, conidia are primarily killed by macrophages, and hyphal cells are killed by monocytes and polymorphonuclear cells (mostly neutrophils) as well as macrophages (Tomee and Kauffman, 2000). It should be noted however that the majority of *A. fumigatus* conidia evade immune recognition due to their hydrophobin surface coating (Aimanianda *et al.*, 2009). There has been shown to be a delay of a few hours associated with the macrophage response to fungal spore

challenge, and generally the effect is slow relative to macrophage clearance of other pathogens (Schaffner *et al.*, 1983). Also fungal spores have been seen to be able to germinate within macrophages after being engulfed (Gilbert, Wheeler and May, 2015). This time-delay and inefficiency associated with the macrophage response to fungal spores highlights the importance of neutrophils in the response to fungal pathogens. Neutrophils' role in fungal infection appears to be primarily to kill hyphal cells, although they are able to kill conidia too, and so have a role in killing conidia which macrophages fail to deal with (Diamond *et al.*, 1978; Shoham and Levitz, 2005). Many hyphae are too long and branching to be effectively engulfed by even the larger phagocytes and one method by which these large cells are dealt with is that neutrophils are able to adhere to the surface of hyphal cells. Once bound to a target fungal cell, they secrete reactive oxygen species (ROS) and may degranulate, releasing a cocktail of antimicrobial compounds directly onto the surface of the hyphae. This is an efficient process and is highly effective at clearing hyphal cells (Gazendam *et al.*, 2015).

Platelets are also an important aspect of the body's response to fungi, particularly as hyphal growth can directly invade blood vessels and lead to thrombosis or sepsis (Kamai *et al.*, 2006; Cheung *et al.*, 2009). Intravascular defence against fungal cells is thus highly important. Platelets bind to the surface of hyphae and become activated, damaging the fungal cell wall and releasing various cytokines in order to enhance neutrophil-mediated killing of the remaining cells (Speth *et al.*, 2014).

1.5.2 Fungal evasion of the immune system

Fungi secrete compounds which interfere with the processes essential for efficient cell-mediated immunity. For example aflatoxin and gliotoxin which are secreted by *A. fumigatus* both have immunosuppressant activity (Sugui *et al.*, 2007). Aflatoxin reduces the efficiency of phagocytosis and lowers the production of ROS by macrophages (Moon *et al.*, 1999; Bianco *et al.*, 2012). Gliotoxin inhibits the adhesion of macrophages to foreign cells and reduces phagocytosis (Coméra *et al.*, 2007).

The action of cilia is inhibited by some *A. fumigatus* compounds and the integrity of epithelia is also affected by some compounds produced by *A. fumigatus*, assisting in the early establishment of infection. (Amitani *et al.*, 1995).

Diffusible products released by *A. fumigatus* spores, conidial inhibitory factors (CIF), have general and wide-reaching immunosuppressive effects; reducing phagocytosis, superoxide production and H_2O_2 production (Nicholson, Slight and Donaldson, 1996). CIF has been shown to be released from *A. fumigatus* spores immediately in liquid suspension *in vitro* (Robertson *et al.*, 1987), so has been proposed to be part of the first line of *A. fumigatus'* defence against the immune system (Tomee and Kauffman, 2000). Another immune evasion molecule produced by *A. fumigatus* is *Aspergillus* diffusible product (AFD) which again is released by conidia (Mitchell *et al.,*1997) and has been shown, in a mouse model, to inhibit the production of pro-inflammatory cytokines and NF-κB (Slight *et al.*, 1996) and inhibit phagocytosis (Bertout *et al.*, 2002).

A. fumigatus also produces superoxide dismutase (SOD) which is likely to be an essential aspect of the avoidance of superoxide free radical-based degradation by phagocytes, particularly in the later stages of infection (i.e. post-germination). Gliotoxin and aflatoxin have also been shown to be produced at a later stage by *A. fumigatus* in *in vitro* growth models, being produced by hyphal cells rather than conidia (Lewis *et al.*, 2005) and are thus thought to contribute to the later and more long-term immune evasion of germinated *A. fumigatus*.

As the majority of infections occur via the inhalation of conidia one of the key sites in the early stages of infection is the lining of the respiratory tract. The ability of *A. fumigatus* to bind extracellular matrix proteins in the respiratory tract is thought to be crucial in the establishment of infection. Specifically laminin, fibronectin, fibrinogen and collagen binding by *A. fumigatus* allows infection by this route (Tronchin *et al.*, 1997; Bouchara *et al.*, 1999).

The fact that *A. fumigatus* can bind laminin, collagen, fibronectin and fibrinogen allows good adhesion to host basement membrane-associated matrices and so is likely to be important for assisting in tissue invasion (Tronchin *et al.*, 2008). Laminin is a major component of the matrix in the subendothelial basement membrane and so is exposed following initial damage to the structure of the endothelium. The laminin-binding protein is expressed on the surface of *A. fumigatus* conidia and so may be important in allowing spores to get a foot-hold early in the infection (Upadhyay *et al.*, 2009). Fibronectin and collagen are also integral components of the extracellular matrices of

the respiratory tract, and so *A. fumigatus* binding to these molecules is clearly advantageous in colonisation (Wasylnka and Moore, 2000). Fibrinogen binding occurs predominantly with hyphae rather than conidia, so is likely not involved in the initial establishment of infection (Fidel and Huffnagle, 2005). It should be noted that this capacity for fibrinogen binding has been shown to only be a feature of pathogenic *Aspergillus* species (such as *A. flavus, A. niger* and *A. fumigatus*) and so may be a significant virulence-determining factor (Bouchara *et al.*, 1988).

Toxin production is another key aspect of the pathogenic potential of *A. fumigatus*. *A. fumigatus* produces a number of extracellular secreted mycotoxins (Nieminen *et al.*, 2002; Kamei and Watanabe, 2005). Some good examples are fumagillin and helvonic acid which are able to inhibit cilia function (Lee *et al.*, 2016) and neutrophil function (Fallon *et al.*, 2010). *A. fumigatus* can also produce numerous ribotoxins which impair the function of host ribosomes, suppressing protein synthesis and thus having highly damaging and wide-ranging toxic effects (Lacadena *et al.*, 2007) and may play a role in mediating the airway inflammatory response to fungi (Álvarez-García *et al.*, 2010). Restrictocin, mitogillin and alpha-sarcin are all examples of potent ribotoxins secreted by *A. fumigatus* (Martinez-Ruiz *et al.*, 1999).

In addition to these toxins *A. fumigatus* also produces various extracellular enzymes, the primary function of which is in breaking complex molecules down to provide useable nutrients for the growing fungal cells. Aside from this nutritional role, many such enzymes are also important in assisting the invasion of tissue and evasion of the immune system (Farnell *et al.*, 2012). Nucleases, phosphatases, peptidases and proteinases are all secreted by *A. fumigatus* (Liu *et al.*, 2013). Serine proteinase secreted by *A. fumigatus* degrades the extracellular matrix of the respiratory tract, allowing colonisation and further lung damage (Iadarola *et al.*, 1998). Elastase activity has been shown as being higher in MAT1-1 isolates previously (Alvarez-Perez *et al.*, 2010). Elastin is a crucial component of the mucosal membranes in the respiratory tract, being an abundant component of the lamina propria (Starcher, 1986) and the role of elastase as a virulence factor in other respiratory pathogens is well known (Yanagihara *et al.*, 2003). The association between MAT1-1 and higher elastase activity has been proposed as one explanation for the apparent association between this mating type and

more invasive *Aspergillus* infections (Alvarez-Perez *et al.*, 2010). The MAT1-1 mating type in *A. fumigatus* has also been linked with increased pathogenicity in the *Galleria mellonella* virulence model (Cheema and Christians, 2011) which would appear to concur with these findings.

A study by Rhodes *et al.* (1988) found that in a collection of clinically-derived isolates of *A. fumigatus*, all of the isolates from invasive disease showed elastase activity, whereas the majority of those from non-invasive disease did not. It was also noted however that not all elastase-producing strains caused invasive disease (i.e. some of the non-invasive isolates were elastin producing strains) suggesting again that elastin may be an important virulence factor, but only in the context of a wider range of other virulence factors and host defence factors.

Another important factor is the basic physiology of the conidia themselves, as the very small spore size of *A. fumigatus* (2-4 μm in diameter) allows these spores to penetrate to the very lower airways. The outer coating of conidia is also highly hydrophobic, which is likely to help protect from innate host defences such as mucus and surfactant (Denning, 1998). In addition the thermotolerant nature of *A. fumigatus* allows it to survive and grow readily at body temperature, clearly an essential trait for a successful human pathogen.

1.6 *Galleria mellonella* infection model

The cost, difficulty and ethical issues associated with the use of vertebrate models to study the pathogenicity of fungi has led to the development and widespread acceptance of a model which uses an invertebrate organism instead; namely the larvae of the Greater Waxmoth, *Galleria mellonella*. This model has for many years been used as a pathogenicity model for the study of bacterial pathogens (Ramarao *et al.*, 2012) and has more recently been used to study fungal pathogens (Akhtar, 2014; Maurer *et al.*, 2015). Despite the obvious problem that the mammalian immune system is very different to that of the moth larvae, this model has been validated and shown to closely mirror the results obtained through a common mouse model of fungal pathogenicity (Slater *et al.*, 2011). Specifically, this model has been used recently to study variations in the pathogenicity of different *A. fumigatus* isolates (Fuchs *et al.*, 2010).

Upon infection there are distinct humoral and cellular mechanisms by which the *G. mellonella* immune system responds to foreign cells. The body cavity of *G .mellonella* contains haemolymph, analogous to blood, which contains different types of haemocytes-the immune cells (Kavanagh and Reeves, 2004). When surveillance haemocytes identify a foreign cell they recruit and activate phagocytic haemocytes, primarily plasmatocytes but also granulocytes to phagocytose the cell (Tojo *et al.*, 2000). In addition to this attempted phagocytosis, cascade pathways are also activated which lead to the formation of immune nodules. Nodule formation involves the release of adhesion and signalling molecules by haemocytes in response to recognition of a non-self cell. This in turn causes coagulation and accumulation of both phagocytic and granular haemocytes to the site of infection, encapsulating the invading cell in a matrix of haemocytes and adhesins to form a nodule (Lapointe *et al.,* 2012). The activity of *G. mellonella* haemocytes has been shown to be inhibited by the *A. fumigatus* mycotoxin fumagillin (Fallon *et al.,* 2011), which may be a key method of immune evasion by the fungus in invertebrate infections.

Alongside these cellular responses, the clotting of haemolymph and the activity of numerous antimicrobial peptides and enzymes within it form the basis of an effective humoral response (Jackson *et al.,* 2009).

1.7 *Aspergillus fumigatus*

Aspergillus fumigatus is a ubiquitous, thermotolerant saprotroph which, aside from being abundant at sites of organic degradation, is an opportunistic pathogen and is capable of causing serious disease in immunocompromised individuals (Hope *et al*., 2008). Indeed, *A. fumigatus* is the most common cause of invasive fungal infections in people whose immune systems are compromised (Ben-Ami *et al.,* 2010). *A. fumigatus* is not only limited to causing health problems in people with lowered immunity but also has an important role as a respiratory allergen. Sensitisation to *Aspergillus* antigens generally is associated with more severe asthma (Maurya *et al.*, 2005) and specifically, lung function is significantly worse in asthma patients who are sensitised to *A. fumigatus* than those asthma patients who are not *A. fumigatus* sensitised (Fairs *et al*.,

2010). A similar association between *A. fumigatus* sensitisation and greater impairment of lung function has been shown in COPD patients (Bafadhel *et al*., 2013).

Allergic bronchopulmonary aspergillosis (ABPA) is a damaging condition caused by hypersensitivity of the immune system to *Aspergillus* spores and is particularly prevalent amongst cystic fibrosis (CF) patients, where up to 15% of patients may be affected (Stevens *et al.*, 2003). Respiratory failure as a direct consequence of chronic infections accounts for over 90% of mortality in CF patients (O'Sullivan and Freedman, 2009). Although in the majority of these cases bacterial infections are responsible, colonisation of the airway with *A. fumigatus* is regularly seen in CF patients and is a significant cause of lung damage contributing to the pathology of this disease (King *et al.,* 2016).

Although most commonly associated with allergy or opportunistic pulmonary infections in the immunocompromised, *A. fumigatus* is also a leading cause of fungal keratitis in immunocompetent individuals (Leal *et al.*, 2012).

As a saprotroph, *A. fumigatus* obtains the nutrients it requires by degrading organic matter and so one common situation in which this fungus is abundant is in organic waste composting sites (1.8). In both small scale and industrial scale composting sites, *A. fumigatus* concentration in the maturing compost reaches very high levels and spores and hyphal fragments are released in high concentrations into the air (up to 3.68×10^{7} CFU/m^3 air) (Taha *et al.*, 2006) during the agitation and processing of the maturing compost (Crook *et al.,* 2008). The airborne spores from composting sites can be inhaled and due to their small diameter (~2-4 µm) these spores can penetrate to the very distal airways, through to the alveoli. The small nature of these airborne spores along with the various other virulence factors discussed in section 1.5.2 combine to allow *A. fumigatus* to infect, maintain growth inside the body, spread and cause disease. *A. fumigatus* is considered to be an emerging pathogen with the increasing frequency of *A. fumigatus* – associated disease over the last few decades being driven, at least in part, by a dramatic increase in the number of immunocompromised individuals. The increased frequency and rate of success of solid organ and bone marrow transplants and the increased survival of cancer patients following radio and/or chemotherapy are generally associated with long-term immune suppression for the patient. This along with the

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AIDS pandemic from the 1980s onwards has led to a much higher number of immunocompromised individuals worldwide currently than even twenty years ago (Low and Rotstein, 2011). A corresponding increase in the prevalence of many opportunistic pathogens has been seen, with the emergence of *A. fumigatus* infection typifying this (Pfaller and Diekema, 2004).

As an allergen, *A. fumigatus* causes or exacerbates diseases such as asthma, allergic sinusitis and alveolitis. These can all be caused by extracts or fragments of conidia or hyphal cells and can result from the inhalation of non-viable as well as viable spores (Green *et al.*, 2005). This at least partly explains why this aspect of *A. fumigatus* pathogenesis is more widespread than active infection. The predominant forms of disease associated with active *A. fumigatus* infection are; allergic bronchopulmonary aspergillosis (ABPA), aspergilloma and invasive aspergillosis (IA) (Latgé, 1999; Patterson and Strek, 2014). These diseases all involve infection with viable cells (almost always by inhalation of viable spores which then germinate in the airways), which progresses to mycelial growth within the patient. ABPA is an eosinophilic lung disease and is a known risk particularly for asthma and cystic fibrosis patients (Antunes *et al.*, 2010). Elevated *Aspergillu*s-specific IgE and eosinophilia are defining clinical characteristics (Walsh *et al.*, 2008). The underlying and long-term respiratory tract damage caused by these pre-existing conditions dramatically increases the prospect of an *Aspergillus* infection taking hold and being able to persist and grow within the lumen. ABPA results from non-invasive infection and the manifestation of disease is a result of a hypersensitivity reaction to the persisting allergen(s) with ongoing inflammation causing damage to the bronchial wall, bronchospasm and loss of smooth muscle (Zander, 2005). The severity of symptoms varies greatly depending on the starting state of the respiratory health of the patient and the success of antifungal therapy. The outcome can range from symptoms of exacerbated asthma and associated central bronchiectasis to extensive damage and fibrosis of the lungs. A 5-stage clinical staging system is currently used to classify the severity of ABPA, ranging from stage I (acute asthma symptoms) to stage V (Pulmonary fibrosis) (Grammer and Greenberger, 2009; Agarwal *et al.*, 2013; Denning *et al.*, 2014).

Aspergilloma is the growth and persistence of a 'fungus ball' composed of hyphal cells bound in a protein matrix. Pulmonary aspergilloma is by far the most common form of this disease as the fungus occupies existing cavities resulting from previous, underlying conditions (Howard *et al.*, 2013). Sporulating structures can be present on the outermost surface of the aspergilloma, which can then result in spores being released, with the potential to lead to fresh infections elsewhere (as well as acting as an internal source of allergenic spores) (Lydyard *et al.*, 2010). The aspergilloma itself, while often resulting in relatively mild symptoms, can disrupt local blood vessels leading to haemoptysis (Patterson and Strek, 2014). Treatment is generally reserved for symptomatic cases and is based either on surgical removal of the aspergilloma or antifungal therapy (Walsh *et al.*, 2008).

Invasive aspergillosis (IA) occurs almost exclusively in immunocompromised individuals (Steinmann *et al.*, 2014). Transplant recipients (solid organ and bone marrow) undergoing immunosuppressive therapy, leukaemia patients, AIDS patients and Chronic Granulomatous Disease (CGD) patients are particularly at risk as a result of their impaired immune function (Latgé, 1999). IA has a variety of forms differing in severity and time course of disease. Pulmonary IA (acute or chronic) is the most common form of IA, tracheobronchitis with *Aspergillus* invasion is seen most commonly in AIDS patients (Krenke and Grabczak, 2011) and disseminated IA involves spread of the fungus to sites other than the respiratory tract. This often involves infection of the heart but can affect the skin, eyes, kidneys or brain (Hope *et al.*, 2005). Due to the severity of IA and the difficulty in treating such disseminated disease, azole-based prophylaxis is currently recommended for high-risk patients (Walsh *et al.*, 2008).

1.8 Industrial composting

Industrial scale composting is an increasingly important aspect of the waste management process, and demand for composting sites is increasing (Gilbert *et al.*, 2011). Pressure to reduce the amount of waste going to landfill and the desire to convert waste into a useful product are driving this increase in the need for composting capacity in the UK.

The composting process relies on the activity of sequential populations of a variety of organisms. Consequently the microflora of developing compost is diverse, dense and shows successional changes in composition as the compost matures (Hansgate *et al.*, 2005). Initial degradation of the large, complex feedstock material by fungi and invertebrates is necessary to create the conditions required by the thermotolerant and thermophilic bacteria and fungi which are responsible for the majority of the rapid, full decomposition of the waste into thoroughly rotted mature compost. In industrial-scale composting there are 3 main types of composting system; open windrow, in-vessel composting (IVC) and aerated static pile (Stagg *et al.*, 2010). Of these, the open windrow system is by far the most common in use today in the UK (Pearson *et al.*, 2015). This is a very simple system, where the waste is shredded and arranged into long piles (windrows) and left outdoors for the composting process to work. In order to maintain sufficient oxygen levels for the microbes, these piles need to be turned regularly. In-vessel and aerated static pile systems remove this need for turning of the waste, by forcing air through the compost instead. Whichever system is used, the process essentially relies on creating conditions which allow the composting microbes to thrive.

Due to the density and variety of microorganisms present in maturing compost these sites typically release a high concentration of a diverse range of both viable and non-viable bioaerosols into the air, especially during certain tasks such as the turning and screening of the composting material (Crook *et al.*, 2008). It is known that some of the particles released are able to travel over considerable distance in the air (Cartwright *et al.*, 2009).

Although there is a diverse mix of bacterial and fungal bioaerosols associated with compost sites and the composition is highly variable from site to site and over time, there are certain species which are regularly among the predominant microbes found (Crook *et al.*, 2008, Le Goff *et al.*, 2010). The most abundant fungi are members of the *Aspergillus, Penicillium* and *Cladosporium* genera, and the most abundant bacteria belong to the *Bacillus*, *Micrococcus* and *Pseudomonas* genera (Crook *et al.*, 2008). Thermophilic actinomycete bacteria including *Thermobifida fusca*, *Saccharomonospora* spp. and *Saccharopolyspora* spp. are also part of the set of

regularly occurring predominant microorganisms (Le Goff *et al.*, 2010). In terms of particle sizes, the thermophilic actinomycetes commonly identified are typically ≤1 μm in diameter, and of variable length. The spores of *Aspergillus* species are 2-10 μm in diameter with *A. fumigatus* representative of the smaller end of this range (2-4 μm) and the spores of species such as *A. niger* being larger, typically around 3-10 μm (Campbell *et al.*, 1996). *Penicillium* spores are generally 2-5 μm in diameter (Schwab and Straus, 2004) with *Cladosporium* spores being typically larger, up to about 40x10 μm. Downwind of compost sites most bacteria found in air samples are within the 2-7 μm range, with the majority toward the smaller end of this range and the most abundant fungal spores being within the 3-7 μm range (Crook *et al.*, 2008). Clearly then, many of these bioaerosols released from composting sites are of an inhalable size and it is already known that some of the components of compost bioaerosols can have an impact on health (e.g. Taha *et al.*, 2007, Hope *et al.,* 2005). For example *A. fumigatus* is one of the most abundant fungi released from compost facilities and, as discussed in section 1.7, is able to cause serious disease in immunocompromised individuals and act as a potent allergen (Hope *et al.,* 2005). Table 1-1 highlights the level of *A. fumigatus* spores which can be found in the air at and around composting sites relative to levels seen in other industries known to be associated with bioaerosol release. From this comparison the very high level of *A. fumigatus* spore release from industrial composting sites is apparent.

Actinomycete bacteria make up a significant proportion of bacterial bioaerosols released from compost sites, and long-term exposure to these bacteria can also cause allergic responses and respiratory problems (Taha *et al.*, 2007). As well as viable microorganisms, there is also a considerable amount of endotoxin released from compost facilities at high concentrations and this is known to elicit strong immune responses and be able to cause health problems such as mucosal membrane irritation (Bünger *et al.*, 2007). With the understanding that exposure to microbes and other bioaerosols released from compost sites can lead to infection and cause allergic responses, current guidelines recommend that if a composting site is situated less than 250 m from the 'nearest sensitive receptor' (NSR), i.e. any workplace or dwelling which is not owned by the composting company, then site-specific bioaerosol risk assessments must be carried out before and during composting operations. In addition to this,

regular air sampling must also be undertaken at the NSR (Environment Agency, 2010). It has been shown however, that even at distances greater than 250m from the composting operations the bioaerosol load is often still greater than background levels (Stagg *et al.*, 2010). Obviously this depends on the site and varies greatly between sites, over time and depending on local weather conditions at the time of sampling. Due to the density of *A. fumigatus* present in developing compost and the diverse origins of feedstock materials into the composting process, much of which has a potential history of fungicide exposure, it has been suggested that large-scale composting operations may be one environment in which drug resistance is selected for in *A. fumigatus* (Snelders *et al.*, 2009). This would clearly heighten the risk associated with exposure to compost-derived *A. fumigatus* bioaerosols and is discussed in more detail in section 1.9 and chapter 5.

1.9 Drug resistance

1.9.1 Drug types- limited choice

As eukaryotes, fungal pathogens have much more in common with human cells than bacterial pathogens do. Consequently there are less suitable targets for drugs to act upon in fungal cells than bacteria because drugs interfering with fungal structures and processes are likely also to interfere with human cells. This limits the choice of antifungal drugs to those which target cellular components which are only found in fungi. This lack of drug targets is reflected in a relatively limited number of antifungal drugs. Furthermore, the majority of these drugs are based on very similar chemistry (the azoles, as discussed below). Due to this limited choice and similarity in the mechanisms by which these compounds work the emergence of strains of pathogenic fungi which are resistant to these drugs is clearly a cause for concern.

There are 4 main groups of antifungal drugs commonly used to combat fungal infections in humans; azoles, allylamines, polyenes and echinocandins (Lewis, 2011). The activity of azoles is based upon their interference with fungal cell membrane formation (Yoshida, 1988). These drugs inhibit the cytochrome P450 enzyme lanosterol 14 α-demethylase (encoded by the cyp51A gene) which is necessary for the production of ergosterol, a critical component of the fungal cell membrane (analogous to

cholesterol in our own cell membranes) (Weete, 1989). There are two distinct classes of azole compounds, the imidazoles and the triazoles. Both of these have similar structures, featuring a characteristic 'azole ring' structure (Mast *et al.*, 2013). The principal difference between the groups is that the imidazole ring contains three carbon and two nitrogen atoms whilst the triazole ring is composed of two carbon and three nitrogen atoms. As might be expected of such similar compounds these classes of drugs have similar modes of action, targeting the same enzyme. The key difference between the activity of the two classes is that a nitrogen atom at a different position on the azole ring is involved in the binding of iron in the cytochrome P450's haem group. Commonly used imidazoles for clinical use include clotrimazole and miconazole. Of the triazoles posaconazole, voriconazole, itraconazole and more recently isavuconazole have become in many situations the first line of defence in antifungal treatment (Basak *et al.*, 2016).

Allylamines also inhibit ergosterol synthesis but by interfering with a different enzyme important in the synthesis of membrane sterols, squalene epoxidase (Nowosielski *et al.*, 2011). Polyene antifungals bind to and partially crystallise ergosterol, damaging the membrane and echinocandin compounds interfere with the fungal enzyme 1,3-β glucan synthase, preventing the production of glucans which are necessary for the integrity of the fungal cell wall (Vanden Bossche, 1997).

Of these different drug classes by far the most commonly used are the azole drugs, specifically triazoles, which have become the mainstay of antifungal therapy (Basak *et al.,* 2016). Amphotericin B (a polyene antifungal) and caspofungin (an echinocandin) are among the more frequently used non-azole antifungals (Herbrecht *et al.*, 2005). Due to the almost exclusive use of triazoles in systemic antifungal therapy (Walsh *et al.*, 2008), as well as the high use of triazole fungicides in agriculture it is the triazoles which were studied in the drug resistance aspects of this project.

Table 1-1: *A. fumigatus* **bioaerosol levels identified in air sampled from various environments and industries. Note the very high** *A. fumigatus* **spore concentration in the compost site sample taken near an active windrow relative to the concentrations found in other industries.**

Environment/ industry	*A. fumigatus* level (cfu/m3)	Comments	Reference
poultry farm	80	*A. flavus* more prevalent than *A. fumigatus*	Sabino *et al.*, 2012
swine farm	100	*A. versicolor* more prevalent than *A. fumigatus*	Sabino *et al.*, 2012
garden waste collection	**10,000**	**Suggests** *A. fumigatus* **already prolific in waste early on, prior to composting**	**Nielsen *et al.*, 1997**
sewage system	270	Indoor, enclosed sewage treatment plant	Haas *et al.*, 2010
grass seed plant	800,000	Normally much lower level expected, this was a contaminated bag of grass seed	Madsen *et al.*, 2012
wood processing plant	only detected in 1 of 4 factories	Indicates low level of *A. fumigatus* bioaerosol in this industry	Sivrikaya and Kara, 2009
bamboo processing	N/A	*A. niger* prevalent in bamboo processing plant, *A. fumigatus* not detected	D'Elia, 2011
sewage system	540	outdoor	Haas *et al.*, 2010
compost, near windrow	**1,000,000**	**During typical daily turning activity**	**Clark *et al.*, 1983**
100m from compost	**>2,000**	**Downwind from composting activity**	**Crook *et al.*, 2008**
250m from compost	**>300**	**Downwind from composting activity**	**Crook *et al.*, 2008**

1.9.2 History and current state of drug resistance

As fungi are responsible for a significant loss of crops there has been a relatively long history of fungicidal drug use in agriculture which became more sophisticated and widespread in the first half of the 20th century, as our understanding of crop diseases grew (Russell, 2005). Enilconazole was one of the first azole fungicides to be developed, being introduced in the 1970s (Price *et al.*, 2015) and forty years on resistance is an established problem in dealing with plant pathogens, including resistance to azole drugs (Cools and Fraaije, 2008; Frenkel *et al.*, 2015).

The first azole introduced for clinical use was chlormidazole in 1958, which is often regarded as the beginning of modern antifungal therapy (Price *et al.*, 2015). Clinical azole resistance was first seen in *Candida* infections following azole therapy in the late 1980s/early 1990s (Fan-Havard *et al.*, 1991). The first recorded incidence of azole resistance in a clinical isolate of *A. fumigatus* was in the late 1990s, but was identified in a collection of isolates collected from patients in the late 1980s (Denning *et al.*, 1997). Within a decade of this work the prevalence of azole resistance increased to ~17% in some studies (Howard *et al.*, 2009). This increasing frequency of azole resistance in *A. fumigatus* (both clinical and environmental collections) has since continued (Brent and Hollomon, 2007; Bowyer *et al.*, 2011). The increase in the number of different azole compounds available for drug therapy (itraconazole was first licenced in 1997, voriconazole in 2002, posaconazole in 2006 and isavuconazole in 2015) seems not to have halted the increase in resistance (Verweij *et al.*, 2015). Cross resistance to multiple azoles is frequently observed, with the majority of resistant isolates being resistant to more than one azole (Snelders *et al.*, 2009, van Ingen *et al.*, 2015). In light of this, a switch to the use of azoles in combination with non-azoles such as amphotericin B or flucytosine is generally recommended if azole resistance is suspected to be likely (Verweij *et al.*, 2016). It has, however, also been shown that exposure to voriconazole can influence sensitivity to other, non-azole antifungal drugs. For example it has been observed that voriconazole exposure in *A. fumigatus* cultures caused a reduction in sensitivity to subsequent amphotericin B exposure (Rajendran *et al.*, 2015). As isavuconazole was only recently licensed for clinical use there is a lack of data on resistance to this drug, although it has already been shown that the most

common resistance mutations confer at least low level resistance to isavuconazole alongside other azole compounds (Seyedmousavi *et al.*, 2015).

With the increase in frequency of resistance being observed, there has been increasing demand for routine drug sensitivity testing, and clearly a uniform approach to testing for sensitivity to relevant compounds is desirable. In Europe, a method was developed as a collaborative effort involving many of the principal mycology research groups under the governance of the European committee on antimicrobial sensitivity testing (EUCAST) resulting in the protocol outlined in EUCAST definitive document E.DEF 9.1 (Rodriguez-Tudela *et al.*, 2008). Based on clinical outcomes associated with isolates tested by the EUCAST method, the committee have published a series of breakpoint values; *A. fumigatus* EUCAST MIC values to various azole drugs which are considered to be the point at which clinically relevant drug resistance is observed (Table 5-1). More recently culture-independent methods of resistance surveillance have highlighted that resistance may be more widespread than previously thought due to the culture rate being relatively low (Denning *et al.*, 2011). In the United States an alternative broth dilution method for testing antifungal susceptibility, the CLSI (M38-A) method, is more commonly used and there has been observed to be good concordance between this and the EUCAST method (Chryssanthou and Cuenca-Estrella, 2006).

1.9.3 Mechanisms of resistance.

The cyp51A gene encodes lanosterol 14α-demethylase, the cytochrome P450 enzyme which is the target of the azole antifungals (Bowyer *et al*., 2011). The cyp51 gene was first isolated and identified in *S. cerevisae* and was confirmed in *A. fumigatus* in 1990 (Ballard *et al.*, 1990). The first evidence that azole resistance in *A. fumigatus* was due to altered cyp51A configuration or expression was by Denning *et al.* (1997) in a study that identified that clinical isolates with high MIC values featured abnormal cyp51A expression. More recently it has been identified that different mutations of this gene are associated with different resistance phenotypes and with different origins of resistance. The two potential routes to resistance are that; azole therapy leads to evolution of resistance amongst fungi already present in the patient (Camps *et al.*, 2012b), or that

long-term background levels of azoles in the environment create a selective pressure which encourages the evolution of resistant genotypes in the environment which then subsequently infect individuals (Snelders *et al.*, 2008).

As azole-based chemicals are used as agricultural fungicides there is a clear selective pressure on *A. fumigatus* in the environment. Resistant strains are now thought to develop as a result of agricultural fungicide use in addition to arising in the clinical setting (Verweij *et al.*, 2009). In the Netherlands it was found that drug resistant isolates obtained from azole naïve patients and obtained from the environment were most commonly associated with a specific combination of mutations of the cyp51A gene (Snelders *et al.*, 2008). These mutations involve a 34 base pair tandem repeat in the promoter region of the gene, coupled with a single nucleotide polymorphism (SNP) which creates a leucine to histidine amino acid substitution at residue 98 (TR34/L98H). A common genotype identified in azole naïve and environmental samples is indicative of a common source and suggests selection for resistance in the environment and subsequent spread of this genotype due to the selective pressure brought about by azole use. Subsequently the TR34/L98H genotype has been found across the globe (as far-ranging as Australia (Kidd *et al.*, 2015), India (Chowdhary *et al.*, 2012), China (Liu *et al.*, 2015) and recently for the first time in the United States (Wiederhold *et al.*, 2016), as well as many other locations), generally considered to be indicative of a spread of resistance due to global azole use. Another combination of mutations has also now been identified to confer multi-azole resistance and appears also to be associated with an environmental origin, this is the TR46/Y121F/T289A genotype (Astvad *et al.*, 2014). This genotype has also now been observed globally (e.g. Vermeulen *et al.,* 2012; Hagiwara *et al.*, 2015; Liu *et al.*, 2015; Pelaez *et al.*, 2015)

It has been shown that exposure of *A. fumigatus* to agricultural fungicides can induce resistance to subsequent use of clinical azole compounds (Snelders *et al.*, 2012). Indeed it has been noted that the majority of patients from whom azole-resistant *A. fumigatus* is identified are azole naïve (van der Linden *et al.*, 2013). Clearly in these patients the resistance has not arisen during azole therapy, suggesting infection with a strain from the environment which was already resistant. Further interesting evidence for the environmental origin of resistance is the fact that in the USA, where agricultural

triazole use has been relatively limited, the TR34/L98H genotype and associated resistant phenotype is far less common (Pham *et al.*, 2014). Although not definitive proof in itself, this observation certainly fits in with the idea that resistance by this mechanism may arise due to the agricultural azole use that has become commonplace throughout Europe. Generally azole-based agricultural drugs have been used more in Europe than in the United States (Pham *et al.*, 2014). With a complex mix of both azole treated and untreated plant waste and highly concentrated and dense populations of *A. fumigatus*, composting sites may be one environment where azole resistance is selected for.

Conversely, in azole resistant *A. fumigatus* isolates obtained from patients who had received long-term azole therapy these TR34/L98H and TR46/Y121F/T289A genotypes appears not to be so prevalent and instead multiple different mutations (mostly SNPs) have been identified (Camps, *et al.*, 2012c and Howard *et al.*, 2009). This lack of a single mechanism amongst azole treated patients suggests that resistance developed independently in these cases, during the azole therapy. A more recent study on resistant isolates obtained from azole-treated patients used microsatellite genotyping to demonstrate that, although they may have some common resistance-inducing mutations, such isolates are genetically distinct from one another (Fuhren *et al.*, 2015). This lends weight to the theory that in these patients the resistance is developing independently within each patient during treatment, rather than an 'outbreak' of an already resistant strain.

This is a very different situation to that revealed by a genetic analysis of clinical and environmental isolates harbouring the TR34/L98H genotype which were observed not only to share the same genetic basis of resistance but also clustered together (and all clustered apart from non-resistant isolates) in analysis by microsatellite genotyping (Snelders *et al.*, 2009). The conclusion in this instance is that the resistant clinical isolates could well have developed their resistance in the environment and then infected and colonised patients as already-resistant strains.

Clearly then it is likely that both the clinical and environmental routes to resistance are viable and play a role in the observed proliferation of resistant genotypes of *A. fumigatus* in the clinical setting. Resistance developing in the clinical setting during

azole therapy leads to a range of different mutations in different individuals' isolates whereas resistance developing as a result of azole exposure in the agricultural setting produces less variation and the widespread TR34/L98H and TR46/Y121F/T289A resistance mechanisms.

1.10 Mycoviruses

Mycoviruses are viruses that infect fungi. Since the first report of a fungal virus by Hollings in 1962 many more have been identified, showing a great diversity in genome structure and organisation. As of the latest ICTV virus taxonomy report there were 92 mycoviruses identified (King *et al.*, 2012) of which 63 have double stranded RNA genomes, 28 are single stranded RNA viruses and one is a double stranded DNA virus. It is worth noting that since this report there have been some important additions to this list, including two *A. fumigatus* dsRNA viruses; AfuCV and AfuPV-1 and the dsDNA virus of *Sclerotinia sclerotiorum*, SsHADV-1, which are discussed later.

Although we now know that mycoviruses occur in many fungal populations, the functional relevance of the majority of these mycoviruses remains unknown (Son, Yu and Kim, 2015). Some viruses, however, are known to cause hypovirulence in their hosts (Pearson *et al.*, 2009).

The complex life histories of many fungi (often involving combinations of sexual and asexual reproduction at different times) has made the understanding of the role of these mycoviruses and the ways in which they may be transmitted from one cell to another somewhat challenging. Historically, it has been assumed that mycoviruses were entirely intracellular, with no part of their life cycles occurring outside of the host cell (van de Sande *et al,*. 2010) and thus having no stage where they exist as free, infectious particles able to infect new cells. It has long been known that in many cases mycoviral particles are produced upon infection and reside in the cytoplasm of an infected cell (e.g. Day *et al.*, 1977). Typically mycovirus particles are icosahedral in shape, with a diameter of around 20-50 nm (Kondo, Kanematsu and Suzuki, 2013). Although this represents the morphology of the majority of known mycovirus particles, it should be noted that great diversity does exist within mycoviruses (Nuss, 2010). One example of very different particle morphology is that of the ssRNA mycoviruses of the

Alphaflexiviridae family. These viruses form long and thin filamentous particles which can be around 700 nm long (Ghabrial *et al.*, 2015). Mycoviruses have been identified which form large icosahedral particles (e.g. ~80 nm diameter in the reoviridae) (Hillman *et al.*, 2004) as well as mycoviruses which do not form capsidated viral particles at all such as members of the narnaviridae (Dolja and Koonin, 2012).

The fungal cell wall is undoubtedly a major barrier to virus infection and this thick, chitinous cell wall had been thought to make extracellular fungal infection by viruses very unlikely. This view had led to the general assumption that virus propagation must be either via horizontal transmission through fusion of compatible fungal cell types and exchange of virus-containing cytoplasm or by vertical transmission during asexual, spore-forming reproduction- with virus particles becoming packaged into the progeny spores during the division of the parent cell's cytoplasm (Van Diepeningen *et al.,* 1997; Herrero and Zabalgogeazcoa, 2011). These methods of virus propagation do occur in nature and have proven to be a useful tool in studying these viruses in the laboratory, as cell fusion can be induced even in cell pairings that would normally not be compatible, by producing fungal cells which have had the cell wall removed (protoplasts) (Van Diepeningen *et al.* 1998). Such experiments have been used to identify the potential host range of some viruses of plant pathogenic fungi and to attempt to identify what effect, if any, some of these viruses have on their host(s). Although the fungal cell wall certainly does form a considerable barrier to mycovirus infection, viruses have been shown to be able to successfully infect bacteria which are protected by a capsule over 400 nm thick (Leiman *et al.*, 2007) proving that viruses are capable of overcoming such obstacles in their infection of host cells.

It is now also known that in some cases infectious viral particles are present extracellularly and can infect otherwise healthy, intact cells. Recent work on the plant pathogen *Sclerotinia sclerotiorum* has shown that purified virus particles obtained initially from an infected strain of this fungus can then be applied exogenously and are able to infect otherwise healthy strains and greatly reduce the virulence of this fungus (Yu *et al*., 2013). The ability of this virus (SsHADV-1) to infect and cause significant hypovirulence in otherwise healthy and highly virulent fungal cells is a striking demonstration of the potential of these viruses. This model serves as proof that

infection of healthy fungal cells by external, free virus particles is possible and has the potential to be used to help control an important agricultural pathogen.

A more typical method of transmission of a mycovirus is that if a fungal strain which is infected with a virus is compatible with a non-infected strain then vegetative fusion between the cells provides the pathway for the virus to infect new cells without needing to get through the barrier of the cell wall. This is the principle underlying the method of using mycoviruses as a form of biocontrol to deal with a plant pathogen, as in the cases of *C. parasitica* and *R. necatrix* (discussed in section 1.10.1). This mode of virus transmission has been shown to be possible in a controlled manner in *Aspergillus* species also, by making virus infected protoplasts from infected strains and fusing these cells with protoplasts of other, non-infected cells (van Diepeningen *et al*., 1998).

1.10.1 Mycoviruses of plant pathogens

Almost all of the early work on mycoviruses was concerned with plant pathogenic fungi. Only recently has their potential use against human pathogens begun to be explored (van de Sande *et al.*, 2010).

One of the best examples of a mycovirus infection proving to be of use in the control of a pathogen is the case of Chestnut blight, a transmissible fungal disease of Chestnut trees (Reviewed by Anagnostakis, 1982). The American Chestnut tree, *Castanea dentata*, was first noted to be suffering from a deadly fungal infection, Chestnut blight (caused by the fungus *Cryphonectria parasitica*) in 1904 in New York and the disease spread steadily, killing the majority of these trees in the United States throughout the first half of the century. Alarmingly, Chestnut blight was discovered in Europe in 1938, but although initially appearing to spread, it was noticed by Biraghi (1953) that some trees which were known to be infected appeared to be resistant to the disease. It was identified that these trees were infected with a hypovirulent form of *C. parasitica*. Over the next few years it was discovered that this hypovirulence could be transmitted to a previously virulent strain of the fungus and that diseased trees were able to recover following injection with hypovirulent strains of the fungus (Anagnostakis and Jaynes, 1973). It was also noted that all of the hypovirulent strains featured an uncharacterised dsRNA within their cytoplasm, and that it was the transmission of this dsRNA element

that appeared to confer hypovirulence (Day *et al.*, 1977). This is the dsRNA genome of *Cryphonectria hypovirus 1* (CHV-1) and its close relatives. The natural occurrence of *C. parasitica* strains carrying this virus within Europe likely prevented a widespread die-off of European Chestnut trees as was seen in America, where such a virus was not present. This is a striking example of the power of a mycovirus to attenuate the virulence of an otherwise highly pathogenic fungal host and the usefulness of the transmissible nature of mycoviruses from one strain to another. Transmission in this example is believed to occur primarily via cytoplasmic exchange following hyphal fusion (Turina and Rostagno, 2007), but it is possible that extracellular viral particles may occur in other fungus-virus relationships.

Interestingly in their analysis of the success of the use of virus-carrying strains of *C. parasitica* in biocontrol of chestnut blight, Milgroom and Cortesi (2004) note that in some locations the introduction of CHV-1 has proven successful but this is not always the case, and that in large parts of the United States it has proven to be ineffective. The main supposed reason for this failure is that the natural spread of viruses is required for effective biocontrol. Manually injecting cankers of infected trees with virus-infected C. *parasitica* strains is successful (Anagnostakis, 1982) but is obviously not possible over the large areas and vast numbers of trees involved. What would be required would be for the virus infection to spread within the tree populations as effectively as the fungus did in the first place.

Another example from the world of plant pathogens is that of a global fungal pathogen of many important crop plants, the white root rot fungus *Rosellinia necatrix*. This pathogen is prevalent worldwide (Pérez-Jiménez, 2006) and has been studied for many years with some strains noted to exhibit reduced virulence (Kanematsu *et al.*, 2004). Now viruses infecting *R. necatrix* have been found in over 5 virus families (Kondo *et al.,* 2013). Some of these virus infections have proven to be transmissible, as in *C. parasitica*, and may be associated with reduced virulence of the fungus (Sasaki *et al.*, 2006).

Such mycoviruses have been shown to have the potential to alleviate these otherwise devastating fungal infections of plants, even though the mechanisms of their activity is not fully understood.

1.10.2 Bacteriophages

Aside from their importance as infectious agents of plants and animals, viruses also play a central role in the life histories of many microorganisms. The precedent for studying fungal viruses is that bacterial viruses, bacteriophages, are known to play an important role in many bacterial species. Bacteriophages influence population dynamics, evolution and horizontal gene transfer (Hyman and Abedon 2012). Often bacteriophage genomes encode proteins which have a direct positive or negative bearing on the activity of their host (Casas *et al.*, 2011; Cumby *et al.*, 2012) and so mediate the host's virulence, growth and activity (Abedon and Lejeune, 2005). Bacteriophages are well studied and it has become clear that only by characterising and understanding the viruses that infect bacteria are we able to understand the full details of the life cycles of these bacteria.

Once bacteriophages have infected a bacterium they may undergo one of two possible infection types, namely lytic or temperate (Clokie *et al.*, 2011). In a lytic infection the phage genome is replicated and translated by the host and progeny viruses are produced (Abedon, 2008). The host cell is then lysed, releasing the progeny viruses which may then infect new cells. In a temperate, or lysogenic, life cycle the viral genome becomes incorporated into the host genome at which point it is known as a prophage (Fortier and Sekulovic, 2013). The virus may remain stable in this 'silent' state over many generations of the bacteria. Later (often as the result of some environmental stimulus) the viral genome may be expressed (Little and Michalowski, 2010). This switch back into a lytic phase means that new virus particles are produced and eventually the cell will lyse and release these progeny particles into the environment. Outside of a host cell viruses may be vulnerable to environmental stresses and unable to persist as stable, viable particles for a prolonged period of time (Jończyk *et al.*, 2011). If conditions are harsh (such as high temperature or UV radiation) then this instability is exacerbated. As mentioned, prophages may remain as DNA incorporated into their host's genome for many generations being copied and carried into the daughter cells as part of the bacterial genome with each round of bacterial cell division (McCarthy, Witney and Lindsay, 2012). Inevitably during the incorporation and subsequent replication of the viral genome into the host genome occasionally fragments of host genome become

incorporated into the viral genome (and *vice-versa*). By this transduction viruses can act as agents of horizontal gene transfer, as these fragments of bacterial DNA may then get incorporated into the genome of the cell which the virus subsequently infects (Mašlaňová *et al.*, 2013).

The potential use of bacteriophages as a means of biological control of pathogens has a long history, being recognised immediately upon the discovery of these viruses by Frederick Twort and Felix D'Herelle in 1915 and 1917, respectively (Reviewed by Keen, 2012). Their potential as therapeutic agents has gained renewed attention in recent years as antibiotic resistance among bacterial pathogens has become increasingly common (Reardon, 2014). With the current widespread concern about the increasing prevalence of antibiotic resistance and the lack of new antibiotic compounds in the research and development pipeline, increasing attention has begun to be paid to the idea of utilising bacteriophages as a natural and highly selective means of clearing bacterial infections. A clear parallel can be drawn with the increasing concern about drug resistance in fungi, yet our understanding of mycoviruses lags far behind our knowledge of bacteriophages. At the time of writing, a PubMed search for the term 'mycovirus' returns less than 1,500 publications (cf. >64,000 hits for the term 'bacteriophage') and of these, although they cover a period from the 1960s onwards, over a third are publications from the last 5 years. This highlights not only the paucity of research on mycoviruses historically but also the dramatically increased interest in this field in recent years.

1.10.3 Mycoviruses of human pathogens- *A. fumigatus*

Due to their ubiquity within bacterial populations in all environments, bacteriophages are the most abundant organisms on the planet (Clokie *et al.*, 2011). Viruses are present in almost all populations, including fungi (Herrero *et al.,* 2009; Ghabrial, 2013) and given this, it is not unreasonable to assume that viruses able to infect fungi may have a similar role in fungal populations to those infecting bacteria. Fungal strains infected with viruses are well documented; the ATCC and CABI type culture collections both have many filamentous fungi which are characterised as being infected with mycovirus (Table 1-2).

Table 1-2: The filamentous fungal type strains available from the ATCC and CABI culture collections which are known to harbour mycoviruses. Note that the majority of these viruses are of unknown composition, structure and host effect.

ATCC		
Strain ID	**Species**	**Virus**
10254	*Aspergillus aureus*	dsRNA mycovirus
201254	*Aspergillus niger*	dsRNA mycovirus
12761	*Ophiobolus graminis*	Unknown
22342	*Aspergillus inuii*	dsRNA mycovirus
12064	*Aspergillus heteromorphus*	dsRNA mycovirus
22343	*Aspergillus niger*	dsRNA mycovirus
28706	*Aspergillus alutaceus*	Unknown
201255	*Aspergillus niger*	dsRNA mycovirus
10577	*Aspergillus niger*	dsRNA mycovirus

CABI		
Strain ID	**Species**	**Virus**
104688	*Aspergillus foetidus*	Unknown
130408	*Aspergillus foetidus*	Unknown
143520	*Penicillium brevicompactum*	Unknown
146891	*Aspergillus niger*	Unknown
26210	*Penicillium chrysogenum*	Unknown
26211	*Penicillium chrysogenum*	Unknown
39824	*Penicillium brevicompactum*	Unknown
40233	*Penicillium rubens*	Unknown
41871	*Aspergillus foetidus*	Unknown
49137	*Acremonium chrysogenum*	Unknown
50566	*Aspergillus niger*	Unknown

Given the importance of *A. fumigatus* and considering the potential significance of mycoviruses in the life histories of their fungal hosts, it is worth looking at the prevalence and role of mycoviruses in this species. *A. fumigatus* mycoviruses have previously been identified (Anderson *et al.,* 1996, Bhatti *et al*., 2011b) and two *A. fumigatus* mycoviruses, AfuCV and AfuPV-1, have recently had their dsRNA genomes sequenced (Jamal *et al.,* 2010, Bhatti *et al.,* 2011a). This offers some useful insight into these viruses, as does recent work to attempt to identify the effect of infection with these two mycoviruses on the pathogenicity of *A. fumigatus*. Using two different strains of *A. fumigatus* which were either carrying a virus (AfuCV and AfuPV-1) or had been cured of their virus infection, Bhatti *et al.* (2011) reported significantly reduced sporulation in both strains when infected with the viruses. Despite this impaired sporulation upon virus infection, virus carriage did not give rise to any significant differences in murine virulence or the number of CFUs of *A. fumigatus* recovered from the mice. Özkan and Coutts (2015) injected virus infected and cured *A. fumigatus* isolates into the larvae of the Greater Waxmoth *Galleria mellonella*. There was no effect on fungal load or death rate upon infection of the fungus with either the chrysovirus (AfuCV) or the partitivirus (AfuPV-1) but infection with a different, uncharacterised *A. fumigatus* mycovirus (A78) gave rise to larger fungal colonies on AMM media and an increase in mortality of the infected larvae, suggesting increased pathogenicity of *A. fumigatus* when infected with this virus.

Another example of mycovirus effects upon their hosts is the killer yeast phenomenon. Some *Candida* dsRNA viruses are known to encode killer toxins, which are secreted by infected cells (El-Sherbeini and Bostian, 1987) and confer on their hosts the ability to kill non-infected competitors, thus helping the infected hosts to proliferate (and in so doing, perpetuating the virus population). Such mycovirus infections clearly have potential for use as a source of antifungal drugs, particularly as many such toxins show highly specific action upon fungal cells. (Weiler and Schmitt, 2003).These mycovirus-encoded toxins highlight the potential importance of study into the activity and survival strategies of these viruses.

1.11 Project overview

Originally this project was planned to be primarily a study of personal exposure to compost bioaerosols and the health effects of such exposure. The wind tunnel work presented in chapter 3 was intended to be preliminary to the on-site sampling planned for this original study but our external industry collaborator (SITA UK, the company responsible for the majority of industrial composting sites in the Midlands) withdrew from the project. Because of this, it was no longer feasible to perform the on-site exposure monitoring or assessment of compost site employee health and so a significant change of direction was decided upon. Alongside the sampler comparison work which had already begun the revised project focussed on mycoviruses, drug resistance rates and virulence and mating type differences between clinical and environmental *A. fumigatus* populations.

A. fumigatus is clinically relevant and is present at high concentrations in and around composting sites. There is growing recognition of the importance of environmental sources of drug resistance, with composting sites being one environment in which resistance may develop. We had access to clinical isolates obtained before, during and after an azole drug treatment trial (the EVITA3 study) as well as a collection of compost-derived isolates, allowing a direct comparative study of azole resistance, virulence and mating type differences in these two collections.

Mycoviruses have been proposed as a potential route to the development of future antifungal therapies, as seen with the use of bacteriophages in combating bacterial infections. In light of the increasing prevalence of azole resistance in *A. fumigatus* and the limited number of available treatment options it is clearly useful to assess any such potential alternative therapies. With *A. fumigatus* reaching such high densities in developing compost, this environment is worth studying as a potential source of *A. fumigatus* mycoviruses.

The hypotheses tested are outlined below in relation to the three results chapters presented in this thesis.

1.11.1 Hypotheses and aims

Hypothesis 1 - Bioaerosol sampling

Some air sampling techniques are more efficient than others and may be more appropriate for sampling the high levels of fungal spores in the air around composting facilities.

Specific aims

- To compare the collection efficiencies of a range of commonly used bioaerosol sampling devices in order to guide experimental design for future compost-site bioaerosol studies.

Hypothesis 2 - Mycoviruses

Mycoviruses are present within environmental populations of *A. fumigatus* and *A. fumigatus* mycoviruses can be isolated from raw environmental samples.

Specific aims

- To assess the validity of an enrichment procedure for the isolation of mycoviruses from environmental samples.
- To identify and characterise any mycoviruses present in compost samples collected.
- To characterise the mycovirus carried by *A. fumigatus* strain NCPF7367; to include both physical/structural analyses and sequencing of the dsRNA genome of this virus.

Hypothesis 3 - Azole resistance, pathogenicity and mating type in *A. fumigatus*

Antifungal drug resistance is more prevalent in compost derived *A. fumigatus* isolates than in clinical isolates and this resistance is associated with altered pathogenicity in these two populations of the fungus. Isolates obtained from patients following

antifungal drug treatment may also display higher antifungal drug resistance or greater pathogenicity. We also hypothesise that mating type differences between *A. fumigatus* isolates are associated with altered pathogenicity and source.

Aims

- To ascertain the prevalence of azole resistance within clinical and environmental collections of *A. fumigatus* and confirm if any resistance seen is due to the common TR34/L98H or TR46/Y121F/T289A mutations.
- To identify any difference in virulence between the two culture collections and establish if there is a correlation between virulence and azole susceptibility.
- To identify if one or other mating type is more prevalent in either of the two culture collections and see if mating type is associated with virulence and/or azole resistance.
- To compare azole drug resistance, pathogenicity and mating type of clinical isolates obtained before, during and after treatment with voriconazole.

Chapter 2 Materials and Methods

This chapter contains details of the methods used which are common to the different areas of laboratory work carried out over the course of the project. Detailed descriptions of chapter-specific methods are given in the relevant chapters (Chapters 3-5). All chemicals were obtained from Sigma-Aldrich, UK. Unless otherwise stated. Recipes for the media used are given in appendix 1.

2.1 Fungal culture

All culturing of fungi was performed under aseptic conditions in a class II biological safety cabinet.

2.1.1 Solid agar

Potato dextrose agar (PDA) was routinely used for the culturing of fungi on solid agar plates. For the preparation of agar plates, 400 ml of autoclaved, sterile potato dextrose agar was melted in a microwave until fully molten and then allowed to cool to ~50°C. At this point, any antimicrobial drugs required were added, to the appropriate final concentrations (see below). Approximately 20 ml was then poured into each of 20 90 mm triple-vented sterile Petri dishes (VWR ltd.) in a sterile class II biological safety cabinet. The plates were left to solidify and cool with the lids removed to reduce condensation and were then stored sealed in clean bags at 4°C until use. A sterility control plate was removed at random from the each batch, sealed with Parafilm (Bemis Inc.) and incubated at 37°C for 4 days to confirm the sterility of the batch. All plates were used within one month of being prepared. For the wind tunnel experiments, Malt extract agar (MEA) plates were used instead of PDA. These were prepared in the same manner as PDA plates, as per the recipe in appendix 1.

For routine culturing of *A. fumigatus* the PDA media was supplemented with 4 µg/ml gentamicin and 16 µg/ml chloramphenicol (to make PGC agar) to repress bacterial growth (Dolan, 1971). For initial culturing of sputum samples PGC agar was also supplemented with 5 µg/ml fluconazole (making PGCF agar) in order to repress yeast

growth as the raw sputum samples often contained a range of bacteria and yeast which otherwise can hinder the isolation of *A. fumigatus* (Randhawa *et al.*, 2005). Details of these antibiotics are given in Table 2-1.

Table 2-1: Concentrations of the antibiotic compounds added to growth media and the solvents in which the drug powders are dissolved.

Antibiotic	Stock concentration	Solvent	Volume added to 400ml culture media	Final concentration in media
Chloramphenicol	34 mg/ml	Ethanol	188.2μl	16 μg/ml
Gentamicin	50 mg/ml	Deionised water	32μl	4 μg/ml
Fluconazole	5 mg/ml	Dimethyl sulfoxide (DMSO)	40μl	5 μg/ml

Sub-culturing was performed by streak-plating isolates onto fresh PGC or PDA plates using a flamed wire loop to obtain axenic cultures of the *A. fumigatus* isolates from which stocks were then made. All *A. fumigatus* agar cultures were incubated on inverted plates, sealed with Parafilm at 37°C.

For long term stocks of isolates, a pure culture was prepared on PGC agar as outlined above and incubated until the culture showed good sporulation. 5 ml of Glycerol-nutrient broth (GNB, appendix 1) was then poured onto the culture. The spores were then disturbed using a sterile glass spreader to remove the spores from the surface of the culture and suspend them in the GNB. 2 ml aliquots of the GNB were then drawn off from the plate using a sterile Pasteur pipette and stored at -80°C in sterile 2 ml screw-capped cryotubes (VWR, Ltd.).

For bulk culturing from stocks to grow a large mass of fungi (for example for spore harvesting), 100 μl of spore stock solution was pipetted into the centre of a PGC agar plate and spread across the surface of the agar using a sterile glass spreader. The plates were sealed and incubated inverted at 37°C.

2.1.2 Liquid media

Sabouraud liquid medium (SLM) was used for the liquid culturing of *A. fumigatus* throughout this project apart from the growth in microtitre plates during the EUCAST testing, which used RPMI-1640 as the liquid growth medium. The EUCAST procedure is detailed in chapter 5.

Growth of fungi in liquid media can be a useful method for obtaining large volumes of fungal material. Two distinct methods of liquid culture were used in this project; static and shaking. In a static culture, approximately 20 ml of SLM was poured into a sterile Petri dish, fungal spores added and the plate sealed with Parafilm and incubated at 37°C without shaking. In this culture setup the fungi grow predominantly at the liquid-air interface and so a thick mat of fungal growth occurs at the surface of the media which sporulates heavily after 2-4 days incubation. For the shaking culture method the spores are added to approximately 50 ml of SLM in an Erlenmeyer flask and incubated at 37°C shaking at 150-200 RPM. Under these conditions the fungi grow as spherical balls of hyphae which sporulate much less heavily.

2.1.3 Spore harvesting

For much of the experimental work in this project fresh, pure suspensions of spores at a known concentration were required. The accuracy of this is essential in ensuring that correct inoculum densities are used. Pure cultures were grown by streak plating from the relevant stock onto PDA agar plates. Following incubation at 37°C for 2-4 days sporulation can be seen by colour developing in the growing culture.

To harvest the spores from this culture the spores were disturbed and collected by gently rubbing the surface of the culture with a sterile cotton swab soaked in phosphate-buffered saline supplemented with 0.05% Tween80 (PBST). The spores adhere to the swab and were then recovered and suspended by agitating the swab in a sterile bijou tube containing approximately 5 ml PBST. This suspension was then diluted and the spores counted (see 2.2) to establish the spore concentration.

2.2 Spore counting

Haemocytometer counts were used throughout the project. Diluting fungal spore
suspensions to a known density is an important part of many of the experiments used
and the accuracy of these spore concentrations is essential to ensure the consistency and
validity of the results obtained. A double-chamber haemocytometer with improved
Neubauer rulings (Hawksley, UK) was used. The use of a haemocytometer with
rhodium-coated grids greatly improved the ease and speed of counting. The chamber
was loaded with 10 µl of sample and counted immediately under light microscopy at
400x magnification. During counting, the purity of the spore suspension is also
monitored, and any hyphal structures noted. Generally in such spore preparations less
than 5% of the objects seen should be hyphal structures (Aberkane *et al.*, 2002). This
ensures the accuracy of the experiments, which assume that the spore concentration
accounts for the entirety of fungal material in the solution. In practice, the spore
harvesting method is highly selective for spores and hyphal cells were seldom seen.

The value obtained from the haemocytometer counts is used to calculate the
concentration of spores/ml. For some experiments such as the *G. mellonella*
inoculations (chapter 5), it was necessary to take into account the fact that not 100% of
the spores harvested were viable. In order to calculate the number of CFUs/ml that the
spores/ml represented it was necessary to make an appropriate dilution of the
suspension to 1×10^4 spores/ml. 10 µl of this dilution was then aliquoted onto the centre
of a PGC plate and spread to evenly cover the surface of the agar using a sterile glass
spreader. This was performed in triplicate and the plates sealed with Parafilm and
incubated inverted at 37°C. The plates would yield 100 colonies if the spore stock was
100% viable. From the number of colonies present after 24 hours incubation it is
possible to calculate the number of CFUs/ml represented by the original spores/ml
value counted.

2.3 Clinical isolate collection

2.3.1 Sputum induction and processing

All of the clinical isolates of *A. fumigatus* used in this project were obtained by culturing sputum samples. All clinical procedures were approved by the Leicestershire and Rutland ethics committee and all subjects gave written, informed consent to participate. Under aseptic conditions approximately 170 mg of sputum plug (±80 mg) was recovered from the raw sputum sample and spread evenly across the surface of a PGCF agar plate with a sterile glass spreader (Fairs *et al.*, 2010; Agbetile *et al.*, 2014). The plate was sealed with Parafilm and the plate incubated inverted at 37°C for 7 days, being monitored daily for growth. The point during incubation at which the plate was disturbed and individual colonies were subcultured varied depending on the rate of growth and number of colonies present so as to avoid overgrowth and the loss of smaller, slower growing species. Colonies were removed using a sterile wire loop and streak plated on PGC agar plates to obtain individual colonies. Once a pure culture was obtained, a needle-mount was prepared of the isolate for confirmation of identity by microscopy (see 2.4.2) and a stock culture was made in GNB and stored at -80°C.

2.3.2 Lung function

Post-bronchodilator lung function measures were taken 15 minutes after 220 μg of salbutamol was administered by inhalation. Forced expiratory volume in the first second (FEV1) and forced vital capacity (FVC) were measured with a spirometer by a qualified healthcare professional following the relevant guidelines (BTS/SIGN, 2008). Data was collated in Microsoft Excel and analysed further in Prism, V6.07 (Graphpad Software, Inc.).

2.4 Identification of *Aspergillus fumigatus*

2.4.1 Colony morphology

Macroscopic identification of *A. fumigatus* colonies based on colony morphology and growth characteristics on PGC agar was used as an initial identification method. *A. fumigatus* grows quickly on PGC agar and normally would produce a visible colony

within 24 hours of the start of incubation. The initial colony is white/pale light green and filamentous, with a characteristic darker blue-green colour developing as spores begin to be produced from 48 hours onwards (Figure 2-1). The underside of *A. fumigatus* colonies remains cream coloured. These characteristics are sufficient for the initial identification of probable *A. fumigatus* colonies from a mixed culture, which were then sub-cultured and their identity confirmed by microscopy and molecular means (sections 2.4.2 and 3.2.6)
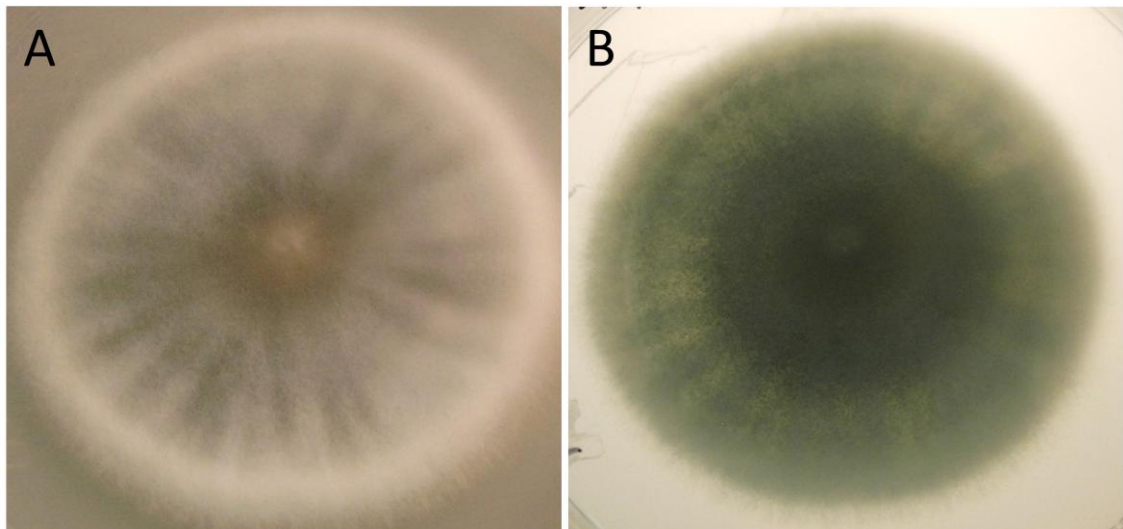
Figure 2-1: Photographs of *A. fumigatus* colony growth on PGC agar. A) After 24 hours incubation the colony has a pale, fluffy appearance with some light green colouration. B) After 72 hours incubation the sporulating topside of the culture shows a dusty, blue-green appearance.

2.4.2 Microscopy

Probable *A. fumigatus* isolates were further identified by light microscopy using wet mounts of sporulating hyphal growth with lactophenol cotton blue as the mountant and stain. Sterile mounted needles were used to pluck a small amount of material from the surface of a colony. This was gently smeared onto the surface of a glass microscope slide in order to tease apart the individual filaments. A small drop of lactophenol cotton blue stain was then applied, which stains all fungal cells by binding chitin. A glass coverslip was then gently placed onto the slide and the sample analysed under brightfield microscopy at 400x or 630x magnification. Fungal cells can clearly be

distinguished and confirmation of *A. fumigatus* identity is possible based on the structure and shape of the conidiophore, the spore-producing head of the hyphal growth. *A. fumigatus* characteristically features a single row of phialides covering the upper two-thirds of the vesicle, with no metulae present. The spores form in long chains from these spore head structures (Figure 2-2).
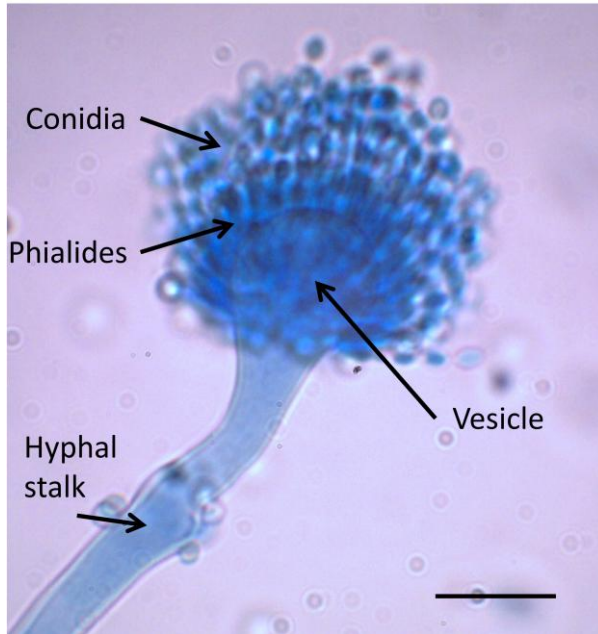
Figure 2-2: *A. fumigatus* **conidiophore structure seen at 630x magnification, stained with lactophenol cotton blue.** A single row of phialides branches from the small flattened vesicle, with conidia developing in chains from the phialides. No metulae are present and the phialides cover only approximately the upper two-thirds of the vesicle. Note the cotton blue stains all fungal structures giving good contrast. The bar represents 10μm.

2.5 DNA extraction

For the DNA extractions from fungi, a modified method based on the DNeasy Plant DNA extraction kit (QIAGEN) was used. The main modification to the published protocol for this kit is the addition of a bead bashing step at the beginning, to disrupt the cell wall and so improve the efficiency of DNA recovery.

A sample (~50 mg) of the fungal isolate was transferred from a fresh, sporulating culture to a 2 ml screw-cap cryotube containing 600 mg (±60 mg) of sterile, acid-washed glass beads (212-300 μm diameter, Sigma-Aldrich, UK) and stored at -20°C

until use. During the DNA extractions, all of the reagents and consumables used were provided in the DNeasy Plant DNA extraction kit (QIAGEN). To the samples, 400 μl of lysis buffer and 4 μl of RNase was added, briefly vortexed and then shaken vigorously for 2 minutes with a Biospec Mini-Beadbeater-16 at full power (3450 oscillations/minute) in order to disrupt the fungal cells. The remainder of the extraction procedure was performed as per the instructions provided by the kit manufacturer, with inclusion of the optimal centrifugation steps. Briefly, 10 minute incubation in a water bath at 65°C with frequent inversion of the tubes was followed by the addition of 130 μl of buffer AP2. This was vortexed briefly to mix, followed by 5 minutes incubation on ice to precipitate polysaccharides and proteins. Centrifugation for 5 minutes at 14,000 rpm was used to pellet the majority of cell debris and precipitates. The supernatant was then applied to the purple QIAshredder Mini-spin column and centrifuged at 14,000 rpm for 2 minutes to filter out remaining debris and precipitates. The flow-through from this step was recovered and the DNA precipitated, bound to the DNeasy mini-spin column, washed and eluted as in the protocol supplied in the kit. The elution was performed with 200 μl PCR-grade H_2O (in two steps of addition of 100 μl H_2O with 5 minutes room temperature incubation per step) and DNA samples were then stored at -20°C.

2.6 Gel electrophoresis

Agarose gels were prepared by dissolving agarose powder to 1.5% w/v in Tris-acetate-EDTA (TAE) buffer. The solution was heated in a microwave to fully dissolve and mix the agarose. Whilst still molten (at around 50°C) ethidium bromide (10 mg/ml) was added at 0.5 μl per 100 ml of agarose solution and mixed. The molten gel was poured into electrophoresis gel moulds and allowed to cool and solidify at room temperature. Once the gel was solidified and placed into TAE buffer in an electrophoresis tank samples were loaded into lanes alongside an appropriate reference ladder, either Hyperladder 1 kb (Bioline Ltd.) or PCRsizer 100 bp ladder (Norgen Biotek Corp.), depending on expected fragment sizes. The gels were then run at 100V for between 30 minutes and 1 hour and visualised on a UV transilluminator.

Chapter 3 Bioaerosol sampling

3.1 Introduction

3.1.1 Rationale

The purpose of this chapter of work was to compare commonly used sampling strategies in order to identify which methods might be best suited to sampling air at composting sites. The aim was to validate a method to use in future work to ascertain airborne *A. fumigatus* levels at and around the sites as well as to identify the personal exposure of employees working on the sites during different shifts and tasks. With a noted increase in the number of composting sites, reported breaches of Environment Agency guidelines for bioaerosol load downwind of composting sites and the acknowledged risk of bioaerosol exposure to compost site workers (Pearson *et al.*, 2015), it is important that appropriate bioaerosol monitoring practices are used.

3.1.2 Bioaerosol monitoring

The main approaches to air sampling for bioaerosols are based upon direct impaction of airborne particles onto agar, cyclone-based separation of particles, liquid collection, impaction onto an adhesive surface or filtration. There is also a choice of methods by which to analyse the samples once they have been collected. Culture (Predicala *et al.*, 2002), microscopy (Gillum and Levetin, 2007) and molecular methods (Diguta *et al.*, 2010) are all commonly used in bioaerosol studies. Different methods of capturing airborne particles suit themselves to different environments, as the bioaerosol load varies greatly between environments both in composition and abundance (Xu and Yao, 2013). Some techniques are more suited to sampling air with a high level of bioaerosols (such as the air at composting sites) whilst others are suited more to low-bioaerosol conditions (such as the air in a hospital ward).

Historically, the majority of aerobiological monitoring has been based on microscopy and culture and although valuable, these methods have significant limitations. Most fungal spores cannot be identified to the species level by microscopy and microscopic identification and counting requires a considerable amount of training and can be very time consuming. However, some samplers whose samples are analysed by microscopy

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(such as the Hirst sampler) can be left running for long periods of time and can give time-specific data which is not possible with many other sampling methods. It is widely accepted that the majority of fungi and bacteria cannot be cultured in lab conditions (Vanhee *et al.*, 2010) and so are undetectable by culture-based methods. If monitoring only by culture-based means, the total bioaerosol load is therefore likely to be greatly under-estimated. It is also worth noting that of course culture-based methods can only detect viable cells, whereas non-viable cells and cell fragments may still be clinically important, such as in allergy (Green *et al.*, 2005). Culturing can be either direct (such as agar impaction) or indirect (such as washing and serially diluting a sample collected either onto filters or into liquid). Indirect approaches have the advantage that the sample may be diluted before analysis in order to avoid sample overload, which can be a problem with direct impaction in highly contaminated environments such as downwind of a composting site (Thorne *et al.*, 1992). This is highlighted in the case of agar impactors, as discussed in 3.1.4. Also when using culture based methods faster growing fungi (such as *Rhizomucor* species) often overgrow and mask smaller, slower growing fungi, leading to a bias towards these faster growing fungi in the results (Taha *et al.*, 2007). An in depth and detailed study of bioaerosols should utilise a combination of these traditional methods as well as taking advantage of more recent developments in molecular techniques such as qPCR (Le Goff *et al.*, 2011) and other DNA-based analyses (e.g. Williams *et al*., 2001, Nieguitsila *et al.*, 2007). The range of potential sample analysis options is ultimately determined by the mechanism of sample collection (Table 3-1), and so deciding on a suitable sampling strategy is key to the development of an appropriate and effective experimental design. The principles of operation of the samplers used in this study are discussed below.

3.1.3 Filter samplers; the IOM and Button samplers

Filter sampling involves a vacuum pump drawing air through a filter assembly, which collects particulate matter in the air. One of the recognised drawbacks of filters is that they are a dry collection surface and cells can become desiccated on them, decreasing in viability over time (Wang *et al.*, 2015). Obviously this is not an issue if sampling for dust or other non-living particles (for which these samplers are also commonly used) but if collecting microorganisms this is a major issue and is particularly important to

consider if sampling over long periods of time and if analysing the samples by culture alone. Fungal spores are relatively resistant to desiccation (Platts-Mills *et al.,* 1998) but the effect can be profound if enumerating bacteria, especially Gram negatives (Eduard and Heederik, 1998). Indeed it has been demonstrated in some environments that with a 30 minute sampling duration and collecting mixed bioaerosols, only fungi were able to survive the desiccation (Wang *et al.*, 2001). It is primarily for this reason that the Association for Organics Recycling (AfOR) do not include filter samplers in their standard protocol for bioaerosol monitoring (Gilbert, 1999). It has also been shown that the small size and relatively low air flow rate of these samplers significantly reduces their collection efficiency and some have concluded that while they may be useful for qualitative sampling, they are not ideally suited to quantitative studies (Predicala *et al.*, 2002). Another drawback of filter sampling is that once the sample has been collected onto the filter, it must usually be washed off in order for appropriate dilutions to be made for subsequent analysis. This washing of particles from the filters is not 100% efficient and inevitably there will be a proportion of the particles which remain bound to the filter. One way of reducing the impact of both of these problems may be the use of filters made from solid gelatin instead of more traditional fibrous material such as quartz or polycarbonate. Gelatin filters have been shown to protect cells against desiccation during sampling (the gelatin itself is not as dry as other filter materials) (Nieguitsila *et al.*, 2011) and also these filters can be dissolved entirely during post sampling processing, thereby ensuring that there is no reduction in sample recovery during washing steps. A recent comparative study however demonstrated that the overall collection efficiency of gelatin filters was lower than polycarbonate (PC) or polytetrafluoroethylene (PTFE) filters (Wang *et al.*, 2015). An additional point to consider is that Li *et al*. (2000) found that the efficiency of the IOM filter sampler is, due to the directional design of the head (Figure 3-1, B), affected by wind direction and therefore is not well suited for use as a static sampler. This design is intentional as the IOM sampling head is designed to be worn on a lapel and used as a personal sampler, and so a directional airflow is desirable in order to accurately monitor the air that the wearer would be breathing in (i.e. in front of them). The more open face of the Button sampler (Figure 3-1, D) is designed to be less influenced by wind direction and so may be more suitable for static sampling in the field.

3.1.4 Agar impactor samplers; 'Andersen'-style samplers

The agar impactor used in this work (the Biostage 200 sampler, SKC Inc.) is a brand of a type of sampler known as an Andersen sampler, named for the original designer (Andersen, 1958). The principle of operation is the same for all such samplers, the main difference between samplers being the number and size of holes through which the air is passed and the sampling airflow rate. An agar plate is loaded into the sampler and air is drawn through the sampler nozzle by an external vacuum pump. The air passes through a metal stage which is held just above the surface of the agar plate. This metal stage has a precise arrangement of 200 fine holes in it, and the air passes through these holes under high pressure. Due to the high force of the air passing through and the close proximity to the surface of the agar, particles are deposited onto the agar as the air deflects across it and is drawn through the sampler to the vacuum pump. This results in 200 small impaction marks which can be seen on the surface of the agar plate. The plate is then incubated and monitored for fungal growth. The proportion of these marks on the agar surface which contain a colony forming unit is directly proportional to the CFU load in the air sampled and so based on the count and the volume of air sampled, it is possible to calculate, using probability tables (appendix 2, Macher, 1989) the concentration of spores in the air at the time of sampling. This sampler has become one of the predominant means of assessing bioaerosol load in outdoor environments (Xu *et al.*, 2013) and although effective, its use in areas with a high expected density of bioaerosols is limited because the high airflow means that sampling times often have to be reduced to one minute or less in order to prevent the plate becoming overloaded. This sampling method can therefore offer a good 'snapshot' of bioaerosol load at a particular point in time but may not be suitable for longer sampling periods such as measuring the exposure of a worker over the course of a shift for example. It should be noted also that there are variations on the principle of operation of this sampler, one example being the AirPort MD-8 sampler (Sartorius Ltd.) which operates in essentially the same way but has a considerably higher air flow, thus sampling a greater volume of air and increasing the sensitivity of the sampling. This is designed for use in environments such as hospital wards or clean laboratories where the expected bioaerosol concentration is low. Agar impaction samplers have also been shown to cause desiccation (Morris, 1995) and so this should also be considered when analysing

the results from such samplers. There is an additional issue of particle bounce, whereby a small proportion of particles in the air will bounce off the agar surface instead of settling (Cartwright *et al.*, 2009). This bounce effect is particularly pronounced with increased sampling time and is the primary reason sampling beyond ten minutes is not recommended. Again, this will contribute to the tendency to underestimate bioaerosol load and should also be considered in analyses.

3.1.5 Liquid impingement sampler; The CIP-10M

The CIP-10M microbiological air sampler (Arelco, France) collects airborne particles into liquid and is designed to be used both for personal and static sampling. The sampler has a round, rotating metal head which contains the collection fluid. In operation this sampling head spins, causing the liquid to rise up and coat the sides of the inside of the head due to centrifugal force. This rotating top piece of the sampler is designed with small aerofoils on the uppermost surface so that when rotating under high speed, air is drawn into the unit and directly onto this 'wall' of liquid. The airflow created is approximately 10 l/minute. Particles in the air are blown into the liquid, wherein they are captured as the air flows out of the device. This method is suggested to cause less cell damage (Görner *et al.*, 2006) as there is no sudden impact onto a solid surface thus increasing the viability of the captured cells. As the sample is collected directly into liquid, this method also does not suffer the same desiccation problems of filter samplers, nor the issues associated with particle retrieval from filters.

Although lower than many static samplers, the airflow rate of 10 l/minute is generally considered sufficient for static sampling (Schlosser *et al.*, 2012) and is also a rough simulation of human breathing rate so is suited to personal exposure sampling too. One advantage of the device is this suitability in both personal and static sampling regimes. Being able to use the same method for monitoring the bioaerosol concentration around a site and an individual's personal bioaerosol exposure offers the advantage that the samples are directly comparable. It is likely that they will both have suffered the same level of sample loss/inefficiency, so avoiding the introduction of bias into the analysis, as would undoubtedly happen if different samplers were used in these different situations.

In certain environments the CIP-10M has been shown to have a similar efficiency to agar bioimpactors (Görner *et al.*, 2006) although for fungi there was a slightly lower detection rate with the CIP-10M than with the bioimpactor used (the MAS-100 agar impactor with a flow rate of 100 l/minute, Merck Ltd.). It should be noted, however, that this study used pure water as the collection medium. It has been demonstrated that due to their hydrophobic nature spores of *Aspergillus* species (and many other fungi) have a tendency to clump together in water. This clumping would reduce the number of colonies seen by culture, as each colony could be the result of more than one spore on the same part of the agar. It is common practice when suspending fungal spores in water to add a small volume of Tween 80 into the water (typically 0.05%) (e.g. Silva *et al.*, 2001) as this mild detergent reduces the clumping of spores, alleviating this problem.

3.1.6 Rotating drum impactor; 'Hirst' microscopy sampler

The Hirst spore and pollen trap (Burkard, UK) is an impaction-based sampler which collects airborne particles onto a drum which holds a strip of Melinex polyester film (Tektra, USA) coated with a thin layer of Petroleum jelly (Vaseline, UK), and is the standard device used for pollen and spore monitoring by a number of organisations, including the UK pollen network (Lacey and West, 2006). The air is drawn into the sampler and onto the Vaseline-coated tape by an electric fan which can be mains or battery-powered (enabling the sampler to be used for fieldwork) and the tape is mounted on a clockwork mechanism which completes one full rotation every 7 days. As the air is blown over the tape particles in the air impact onto the Vaseline and become stuck so that as the tape moves, over time a trace is built up along the length of tape containing the particles in the air over that time. After sampling, the Vaseline-coated tape is removed and cut into sections to be mounted on microscope slides and stained for microscopic analysis. As the sampler runs continually and the drum revolves at a steady rate, the bioaerosol load in the air over time and at a specific time point can be calculated. Each 2 mm of the resulting slide-mounted tape represents an hour of sampling time.

3.1.7 Sampler comparisons

The samplers compared in this work are an 'Andersen' style agar impactor (the SKC Biostage-200, SKC Ltd., UK), a centrifugal liquid impinger (The CIP-10M, Arelco, Inc., France) and two filter-based personal (wearable) samplers (the Button and IOM samplers, SKC Ltd., UK). Each of these samplers was tested alongside the 'Hirst' rotating drum spore and pollen trap (Burkard Ltd, UK). Photographs of these samplers are shown in Figure 3-1 and details of their use are given in sections 3.2.1 to 3.2.4. Table 3-1 details the flow rates and potential methods of sample analysis for these and some other commonly used air samplers.

The hypothesis being tested in this work is that some air sampling techniques are more efficient than others and may be more appropriate for sampling the high levels of fungal spores in the air around composting facilities.
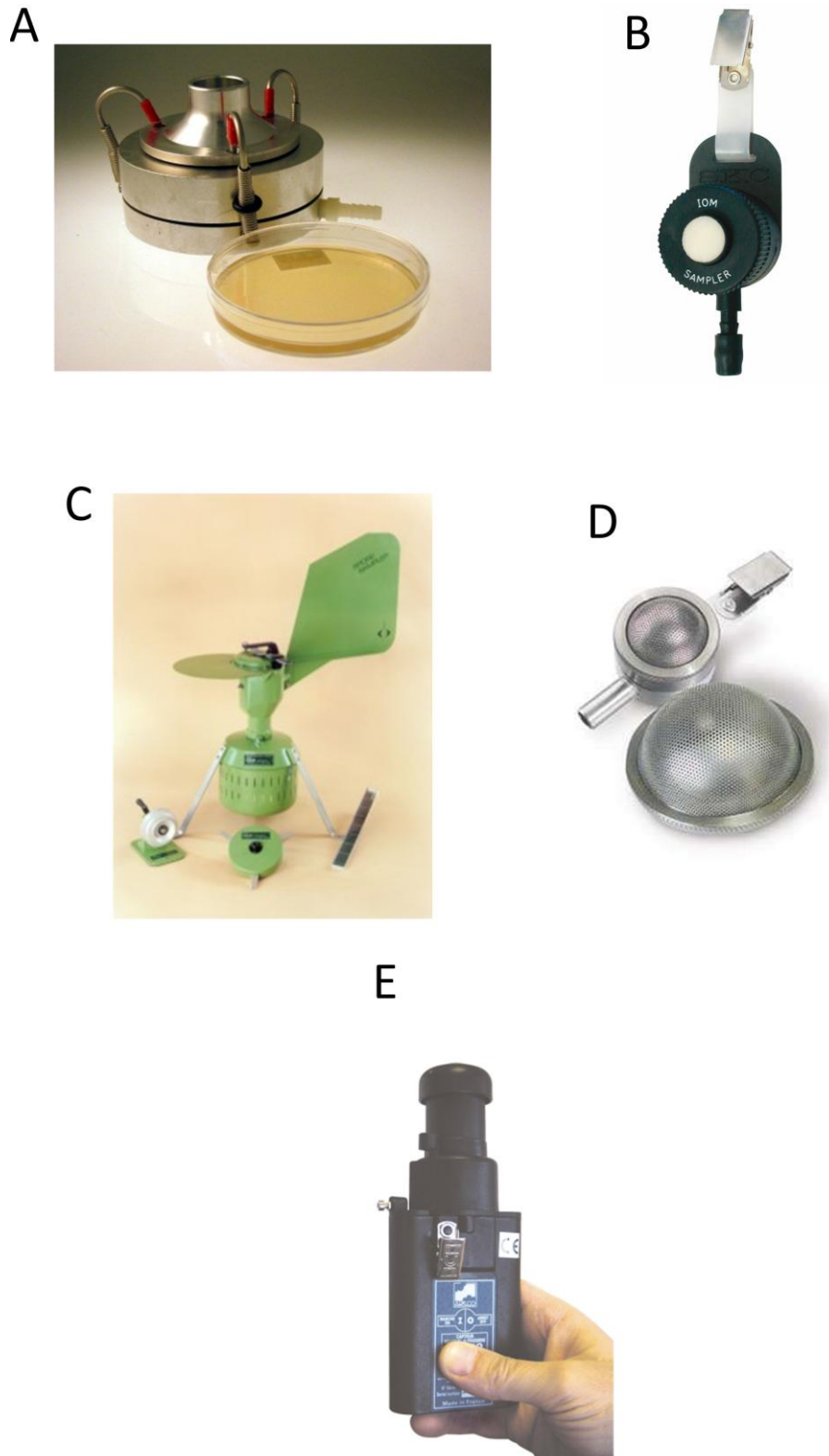
Figure 3-1: Photographs of the various air sampling devices used in this work. A) The SKC Biostage agar impactor. B). The IOM personal filter sampler C) The Hirst spore and pollen trap. D) The Button personal filter sampler. E) The CIP-10M centrifugal liquid sampler.

Table 3-1: A comparison of the air flow rates and sample analysis techniques of commonly used air samplers. Only the SKC Biostage, CIP-10M, Button, IOM and Hirst samplers are used in this work.

Sampler	Type	Flow rate (L/min)	Sample analysis method		
			Culture	Microscopy	Molecular
SKC Biostage	Andersen-type agar impactor (single stage)	28.3	Y	N	N
CIP-10M	Centrifugal liquid impinger	10	Y	N	Y
SKC Biosampler	Glass liquid impinger	12.5	Y	N	Y
Button	Filter	4	Y	N	Y
IOM	Filter	2	Y	N	Y
Burkard dry cyclone	Dry cyclone	16.6	Y	N	Y
Hirst	Rotating drum spore trap	10	N	Y	Y
Rotorod	Whirling arm trap	N/A	N	Y	Y
Air-O-Cell	Cassette	15	N	Y	N

3.2 Materials and Methods

3.2.1 Hirst spore and pollen trap

Fresh sample collection drums were prepared for each day of testing. For this, the drum was cleaned and a clean strip of Melinex tape (Tektra, USA) attached around the sampling surface, affixed in place by a small strip of double-sided adhesive tape. Petroleum Jelly (Vaseline, UK) was then melted over a Bunsen flame and the drum was lowered into the molten Vaseline whilst being rotating constantly at a steady speed in order to ensure even coating of the tape. Once cooled, excess Vaseline was removed using a razor blade and a blow-torch flame briefly passed over the rotating drum to smoothen the surface of the Vaseline film. The prepared drums were stored in secure metal cases before use to avoid damage to the smooth collection surface.

Upon use, the drum was loaded into the Hirst sampler and a line scored into the Vaseline film with a mounted needle to denote the start of the sampling trace. The airflow was checked with an airflow meter (Burkard Ltd., UK). The sampler was then left running continuously throughout the day, with the drum being manually moved on between experiments and the trace marked with a scratch by a mounted needle. The airflow was checked regularly. At the end of the wind tunnel experiments, the drum was retrieved from the sampler, placed back into the protective metal case and stored in a cool box to prevent spore germination. Once back in the laboratory, the Melinex tape coated in Vaseline was carefully peeled from the outer surface of the drum and was cut into sections, each individual section containing the trace for a different experiment. These sections of the trace were then mounted upside down on a glass coverslip with a small drop of lactophenol cotton blue stain (LCB) and then placed onto a glass microscope slide before being counted. All *Aspergillus/Penicillium*-type spores (Asp/Pen spores) were counted together in this work as it is not possible to distinguish these spores to a more accurate level than this by light microscopy alone.

3.2.2 Filter samplers; IOM and Button samplers

The filter-based samplers (IOM and Button) can be used with a variety of different filters to suit the intended use. Most commonly quartz filters are used for bioaerosol

monitoring (e.g. Crook *et al.*, 2008; Stagg *et al.*, 2010) and so were used throughout these experiments (SKC Ltd.). For both of these samplers, the mode of operation is the same. The front cover of the sampler was removed and a sterile filter disc was removed from its packaging with clean forceps. The filter was placed onto the support mesh and the front face of the sampler was attached, holding the filter in place. The air hose was connected to the sampler and the air pump switched on. The power of the pump was calibrated using an airflow meter (SKC Ltd.) and adjusted accordingly before use (to 4 l/minute for the button sampler and 2 l/minute for the IOM sampler). After the required sampling period the front face of the sampler was removed and the filter removed using clean forceps. The filters were immediately placed into 2 ml Phosphate buffered saline supplemented with 0.05% Tween80 (PBST) in a sterile bijou tube in order to prevent desiccation of the collected particles and stored in a cool box with ice packs whilst in transit back to the laboratory. The samplers were cleaned in between uses with ethanol wipes. Upon reaching the laboratory, the filters were washed and the sample serially diluted as previously described (Näsman *et al.*, 1999) and 100 µl of the resulting suspensions was plated onto malt extract agar (MEA), spread evenly with a sterile glass spreader and incubated at 30°C for up to 72 hours. Colonies were counted, morphology noted and identification confirmed by microscopy.

3.2.3 Agar impactor; SKC Biostage- 200

Agar plates prepared from MEA agar were used in this sampler. Due to the nature of the sampling mechanism, it is essential that the depth of the agar in the petri dish is uniform and precise, in order for the nozzles through which the air is passed to be in close proximity (but not in contact with) the surface of the agar plate. 25 ml of agar in a standard 90 mm diameter petri dish was the agar volume recommended by the manufacturer. The preparation of the agar plates was all performed in a sterile class II biological safety cabinet. Molten, sterile MEA agar at approximately 50°C was poured into a sterile 50 ml centrifuge tube to measure out 25 ml. This was then immediately poured into the petri dish and allowed to solidify and cool with the lid removed. As the presence of air bubbles on the surface of the agar can interfere with the operation of this sampler it was important to ensure that any air bubbles on the surface of the agar were removed by passing a Bunsen flame briefly over the bubbles. Once cool, the lids were

replaced, the plates sealed individually in zip-lock bags and stored at 4°C until use. Plates were always used within one month of preparation. In order to minimise contamination, the sampler was loaded only immediately prior to use. To load the sampler a single plate was removed from the bag, the lid being retained in the bag to maintain cleanliness. The plate was placed into the chamber of the sampler and the sampler top attached immediately. The air hose of the sampler was connected to the sampling pump, the sampler set in an appropriate position and the sampling pump turned on. After the allotted sampling time, the pump was switched off, the lid of the sampler unscrewed and the petri dish lid placed onto the agar plate. This was then sealed with Parafilm (Bemis Inc.), the relevant sample details written on the lid and the plate was stored in a cool-box whilst in transit back to the laboratory. Within 10 hours of sampling the sample plate was placed, inverted, at 30°C and incubated for up to 48 hours, being monitored for growth regularly. The number of colonies present within the 200 sampling marks on the agar surface was counted and the positive-hole correction table (appendix 2) was used in order to calculate the concentration of viable spores in the air sampled. Both parts of the sampler were cleaned thoroughly between uses with ethanol wipes and the air holes blown with a compressed air duster to ensure the holes did not become clogged.

3.2.4 Liquid collection; CIP-10M

2 ml of sterile PBST was pipetted into the metal rotating cup of the CIP-10M sampler immediately prior to use. The plastic head of the sampler was then attached and the sampler switched on. Due to the open nature of the rotating cup, it is important to keep the sampler level whilst loaded with PBST before use to avoid spilling the sampling liquid. Once the centrifugal cup is spinning and the PBST is being forced to the sides of the sampler head the sampler can be moved or turned upside-down and the liquid cannot spill out. Liquid samples were removed with a sterile pipette and stored in sterile bijou tubes, refrigerated during transit. Upon reaching the laboratory the samples were serially diluted and aliquots of 100 µl plated onto MEA agar plates, spread evenly with a sterile glass spreader and incubated at 30°C for up to 72 hours. The rotating cup was thoroughly washed with ethanol between uses and the sampling head cleaned with ethanol wipes.

3.2.5 Wind tunnel

Under controlled conditions in a wind tunnel with a known wind speed the samplers were run concurrently to ascertain their collection efficiency relative to one another. A wind tunnel at Rothamsted Research (Harpenden, UK) was used, the dimensions of which are 1 m x 1 m x 7 m. The tunnel has a variable wind speed up to 4 m/s. Temperature and humidity were measured but were not altered. A wind speed of 3 m/s was used throughout these experiments. The numbers of repeat runs of the wind tunnel with the different sampler combinations are given in Table 3-2. Note that as the Hirst sampler was run throughout all of the comparisons there are much more repeats of comparisons with this sampler than the other sampler-sampler comparisons.

Table 3-2: Number of repeat wind tunnel experiments of each sampler combination. The Hirst sampler was left running throughout the experiments as the standard against which the other samplers were compared.

	CIP	IOM	BUTTON	ANDERSEN
IOM	4			
BUTTON	4	6		
ANDERSEN	9	6	8	
HIRST	20	10	12	25

To release airborne spores in the wind tunnel, initial tests using passive release of spores from open, sporulating culture plates proved unsuccessful and so a method of active release by blowing dry spores into the air was used instead (Figure 3-2). Cultures grown on agar slants in 30 ml universal tubes were placed roughly central in the wind tunnel and an external air pump was used to blow a constant airflow through a tube through the lid of the universal tube. This airflow loosens and disperses the spores on the culture and the spore-containing air then passes out through another tube through the lid of the universal tube. Multiple tubes can be set up to be fed with high pressure air from a single pump, which can be operated from outside the wind tunnel, allowing

controlled release of the spores once the wind tunnel is running at the correct wind speed and once all of the samplers are running. With this method it was possible to set up 6 such spore dispersal tubes running from the same pump. 6 fresh sporulating culture tubes were used for each experiment.

Figure 3-3 shows an outline of the experimental setup used. The samplers being tested were placed toward the far end of the tunnel, approximately 6 metres downwind of the spore release. The airflow generated in the wind tunnel dispersed the spores in the air stream and carried them towards the samplers. The air containing the spores was then drawn into the samplers. Heavily sporulating, 4 day old cultures of *Penicillium chrysogenum* were used in these experiments as the conidia of this fungus are similar in morphology to the conidia of *A. fumigatus* (diameter of ~2-5 µm)), however, unlike *A. fumigatus* most *P. chrysogenum* do not grow at 37°C and it is rarely pathogenic. *A. fumigatus* is the primary fungus of interest in the present study and so it was important that the data collected in this work was directly applicable to the collection of *A. fumigatus* spores. The wind tunnel was turned on and gradually brought to a wind speed of 3 m/s at which point the samplers and the pump operating the spore dispersal were switched on. The samplers, wind tunnel and spore dispersal pump were then all run for 5 minutes. The spore dispersal pump was then switched off, the airflow stopped and the samples retrieved.

This was performed with combinations of all of the different samplers in order to directly compare the efficiency of the different samplers to one another. The experiment was repeated on three separate occasions, on different days using fresh spores and reagents. The Hirst sampler was included in every test, as a standard against which all of the other sampler combinations could be compared. The Hirst sampler was left running continuously throughout the day and the rotating drum manually moved on in between each experiment to ensure that each experiment produced a distinct sector on the resulting trace obtained from this sampler.

3.2.6 Sequencing

A small subset of colonies was chosen from plates of the Biostage sampler for DNA analysis to confirm their identity. It was not feasible at this stage of the study to conduct

a more thorough molecular identification of a large number of isolates, and so this small selection was chosen to represent colonies which would routinely be classified as *P. chrysogenum* or *Cladosporium* sp. By far the most commonly encountered colonies were those which appeared to be *Penicillium* sp. and *Cladosporium* sp. and so the purpose of this sequencing work was to gain some insight into the validity of the morphological identification being used. Based on their colony and microscopic morphology, three colonies identified as *Penicillium* and two colonies presumed to be *Cladosporium* were chosen. Fresh cultures of the colonies were streaked on PDA agar (2.1.1) to obtain pure, fresh colonies. DNA was then extracted from these isolates, as in section 2.5. Following DNA extraction, the Internal transcribed spacer region (ITS-1) of the nuclear ribosomal operon was amplified by PCR as previously described (White *et al.*, 1990) using primers ITS2 (5'-GCTGCGTTCTTCATCGATGC) and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG). PCR products were run on gel electrophoresis to confirm success of the PCR and the products sequenced by Sanger dideoxynucleotide sequencing using the Applied Biosystems 3730 sequencing machine at the Protein and nucleic acid chemistry laboratory (PNACL) at the University of Leicester. The resulting sequence data was analysed in FinchTV V1.4 (Geospiza Inc.) and sequence alignments performed using BLAST (NCBI).
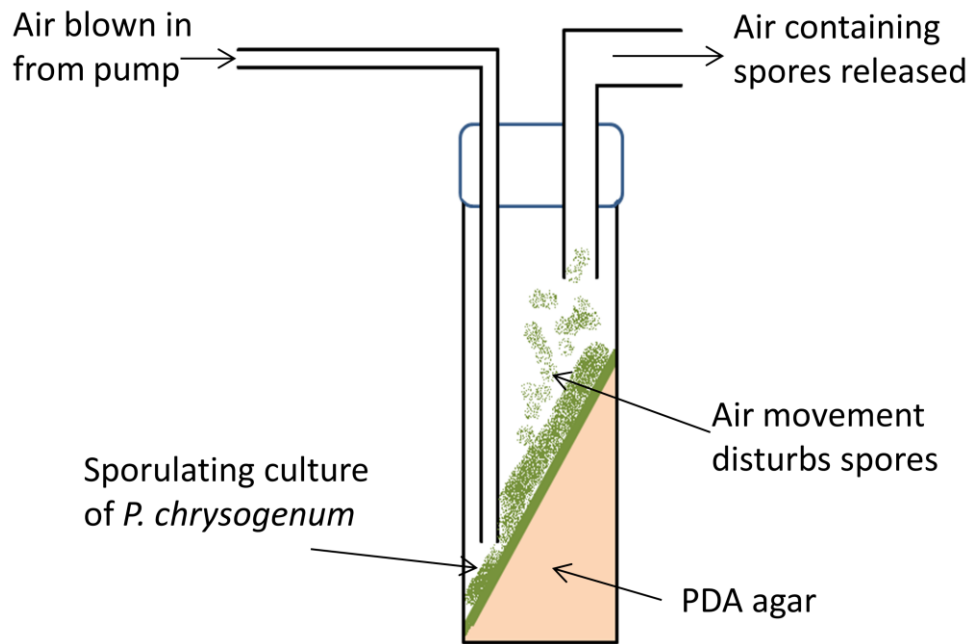
Figure 3-2: Diagram of the spore dispersal method used in the wind tunnel experiments. Heavily sporulating, 4 day old cultures of *P. chrysogenum* were used.
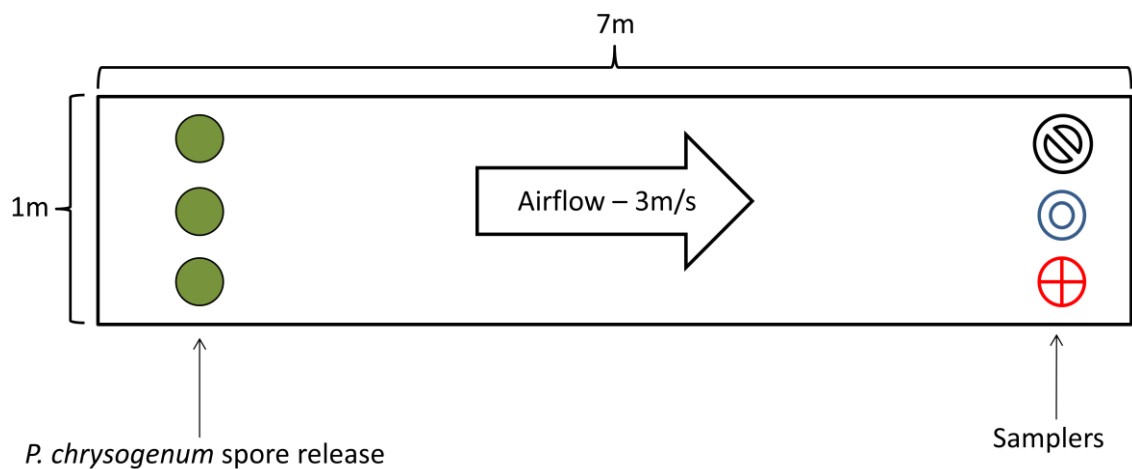
Figure 3-3: A diagram showing the layout of the experimental setup of the wind tunnel. Up to three samplers could be compared at any one time.

3.3 Results

3.3.1 Sampler comparisons

In these analyses, each data point represents an individual run of the wind tunnel with the relevant samplers running concurrently. The comparative tests were conducted using the Hirst sampler as a standard against which the other samplers were to be compared. Figure 3-4 shows correlations between Asp/Pen spore density as identified by the Hirst sampler and *Penicillium* CFU density detected by the other samplers being compared. Using Spearman rank correlation test, the correlation was significant in these comparisons for all samplers except the button sampler. (IOM; Spearman rank correlation coefficient r=0.8236, p=0.0047 **)(Biostage; r=0.7325, p<0.0001 ***) (CIP-10M; r=0.7979, p<0.0001***) (Button; r=0.1395, p=0.6673 ns)

For all of the samplers there was high variance observed and an apparently low collection efficiency relative to the number of spores observed using the Hirst sampler. The CIP-10M has strong correlation with the Hirst sampler (r=0.7979, p<0.0001, ***) with only the IOM sampler having a higher correlation coefficient (but lower level of statistical significance). These comparisons are also shown in Figure 3-5, showing each sampler's results as a percentage of the Hirst sampler's collection. In this analysis it can be seen that the CIP-10M shows significantly less variance than the IOM or Biostage samplers, but also that the overall collection efficiency, as a percentage of the spore count from the Hirst sampler is very low. The data for the Button sampler is excluded from this figure as the variance with this sampler is so high that the data for the other samplers becomes difficult to see. For reference, the analysis including the Button sampler data is included in Figure 3-6. In the sampler to sampler comparisons again each data point represents one run of the wind tunnel with the samplers side-by side and running simultaneously. None of the sampler pairings showed statistically significant correlation according to Spearman rank correlation test (Figure 3-7).

3.3.2 Sequence data

Three colonies which were identified morphologically and under light microscopy as *P. chrysogenum* and two blackish-brown colonies presumed to be contaminating

Cladosporium were sequenced. The sequence data from the three putative *P. chrysogenum* isolates all matched *Penicillium* species in BLAST analyses. The two black colonies were confirmed to be *Cladosporium* spp. By BLAST analyses.
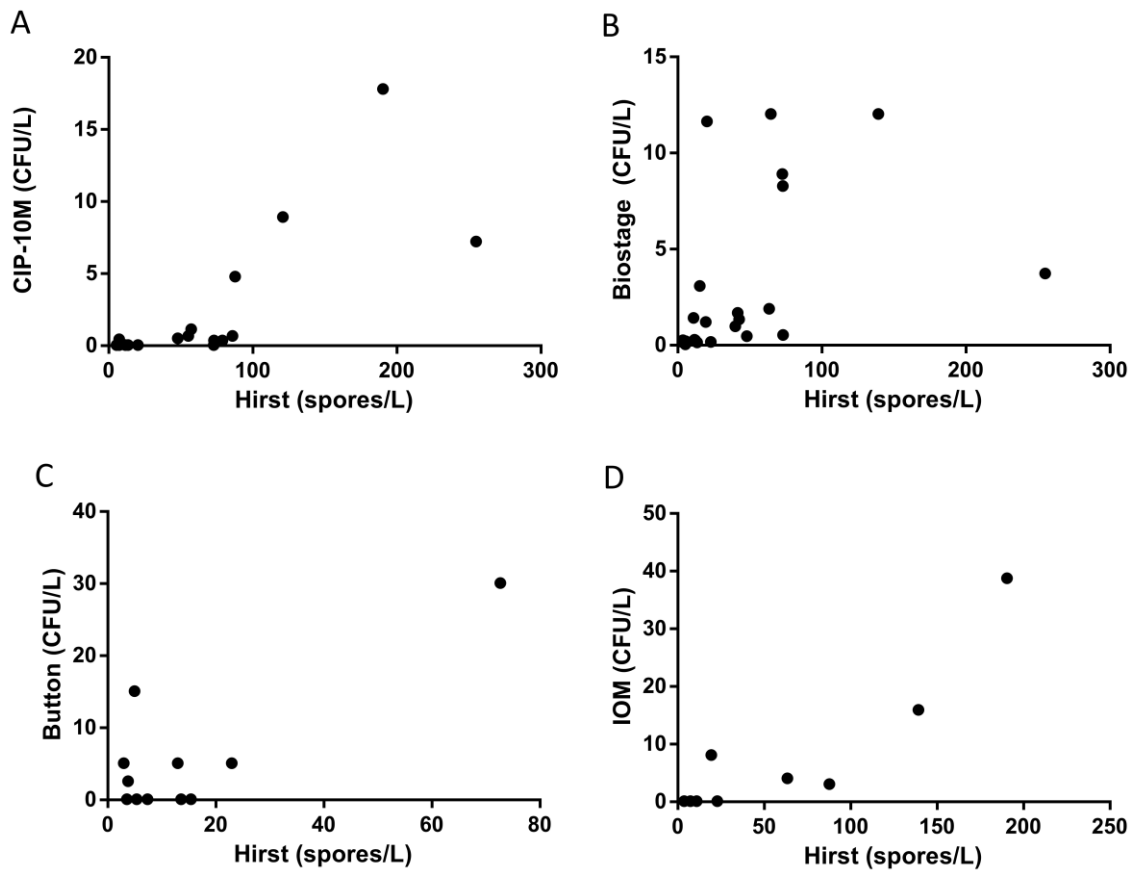
Figure 3-4: Correlation plots of each of the four samplers' data compared to the Asp/Pen spore counts from the Hirst sampler. A) Hirst and CIP-10M Spearman rank correlation coefficient r=0.7979, p<0.0001, ***). B) Hirst and Biostage r=0.7325, p<0.0001, ***. C) Hirst and Button r=0.1395, p=0.6673, ns. D) Hirst and IOM r=0.8236, p=0.0047 **. Note the consistently higher values obtained from the Hirst sampler and that the scale is given in spores per litre of air sampled for this sampler and CFUs per litre of air sampled for the other samplers.
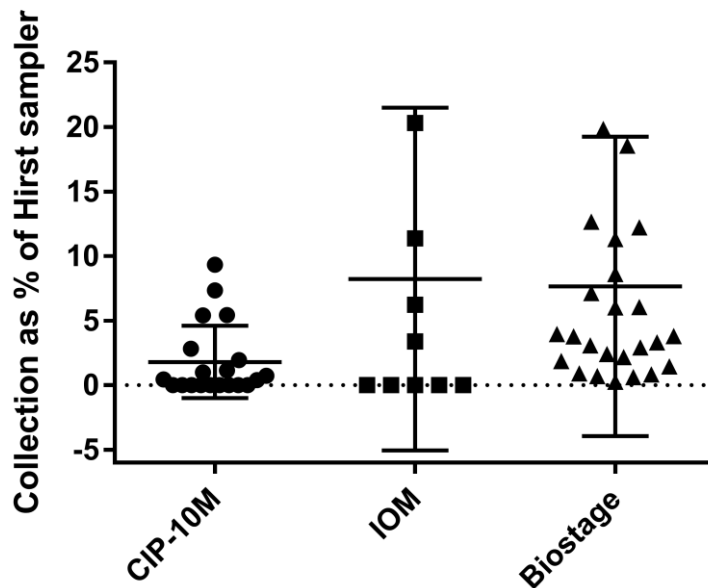
Figure 3-5: Comparison of the CIP-10M, IOM and Biostage samplers. Each comparison is shown as % of the Asp/Pen spore count obtained with the Hirst sampler. The CIP-10M has considerably lower variation than the IOM and Biostage but also a lower collection efficiency. The data from the button sampler is excluded from this analysis as the range was so large with this sampler that the scale was inappropriate for the other samplers. The bar and whiskers represent the mean and S.D.
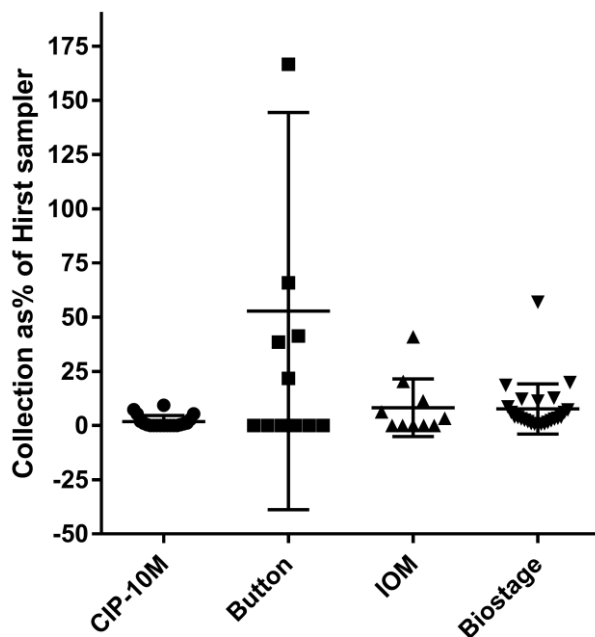
Figure 3-6: Comparison of four samplers. Shown as % of the Asp/Pen spore count obtained with the Hirst sampler. The data for the button sampler shows a very high level of variance. The bar and whiskers represent the mean and S.D.
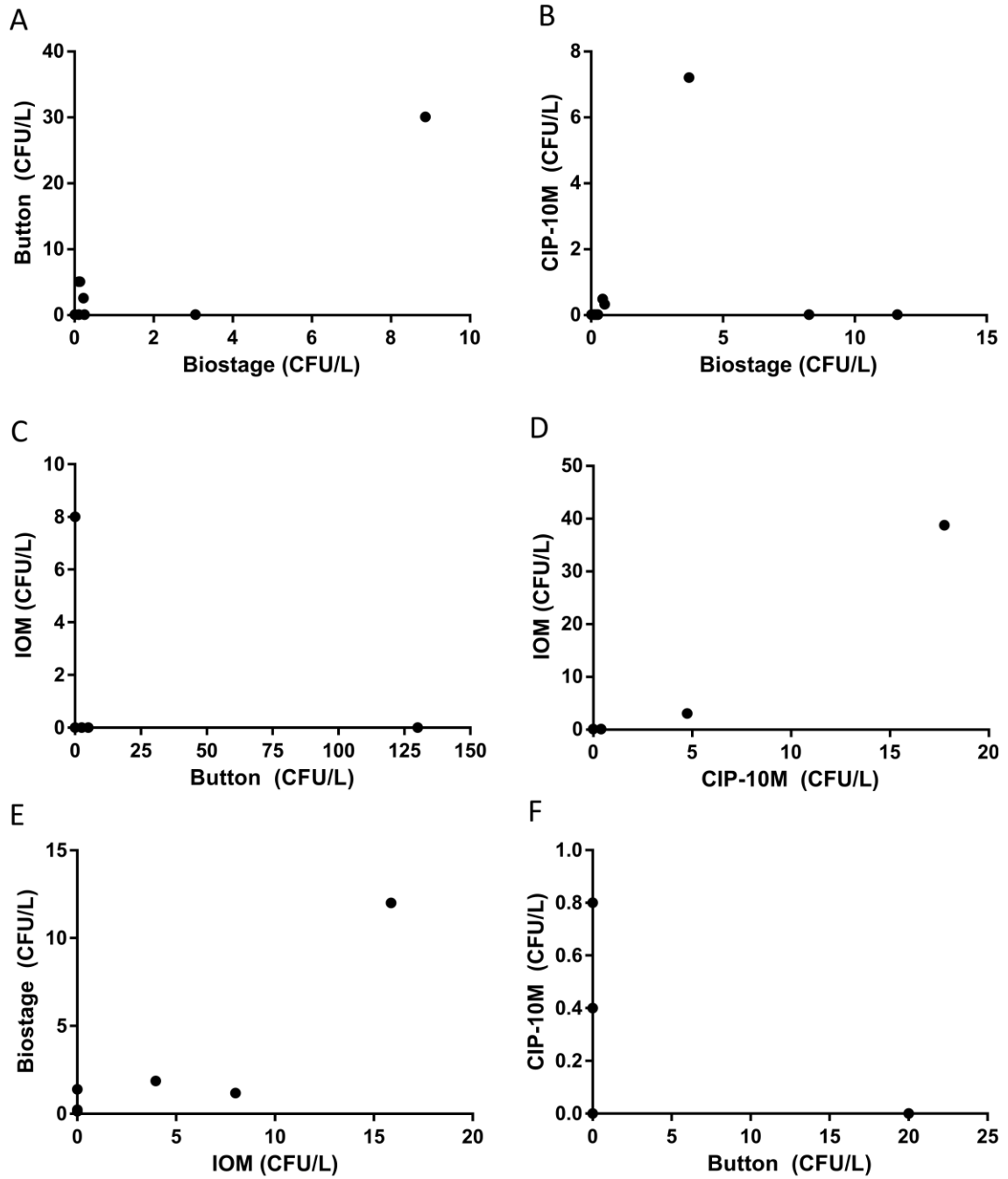
Figure 3-7: Correlation plots of the combinations of samplers tested. None of the correlations are statistically significant. A) Spearman rank correlation coefficient r=0.2698, p=0.5119, ns. B) r=0.2871, p=0.4603, ns. C) r=-0.4174, p=0.8333, ns. D) r=0.9487, p=0.1667, ns. E) r=0.6983, p=0.1667, ns. F) r=0.5443, p=0.75, ns.

3.4 Discussion

3.4.1 Wind tunnel and spore dispersal

Throughout the wind tunnel experiments there was a high background level of noise in terms of contaminating particles other than the spores that were intentionally released into the tunnel. A representative field of view of the samples obtained by the Hirst sampler is shown in Figure 3-8, highlighting the amount of particulate matter in the air beside the spores of *P. chrysogenum*. As has been mentioned, it is not possible to distinguish spores of *Aspergillus* species apart from *Penicillium* spores, let alone distinguish *P. chrysogenum* at the species level from spore morphology by light microscopy. This is one of the reasons for the much higher spore counts with the Hirst sampler (analysed by microscopy) than for the other samplers (analysed by culture). Despite this high background level of contaminating spores it is nonetheless possible to still compare the relative efficiencies of the different samplers- they are all still sampling the same environment and so direct comparisons can be drawn. It should be noted that the wind tunnel itself was old and had been used infrequently over the past two decades so these problems were perhaps unavoidable with this particular set-up. The spore dispersal system appeared to work much better than preliminary tests using passive spore release from open culture plates, but the high contamination level may mean that a higher level of *P. chrysogenum* spore release is necessary in order to overcome this background level of contamination.

It is clear however that there is considerable variation from one comparison test to another (with the Hirst sampler giving values of >250 spores/litre in some runs and <20 spores/litre in others), suggesting that the spore release is not consistent throughout. For the purpose of the direct comparisons this is not necessarily a problem as the different samplers in each run are still exposed to the same bioaerosol load as each other. The amount collected by each sampler relative to the other samplers' collection is the variable being studied in this work, the absolute spore number in the air is less important. Clearly though the greater control over the spore release the better, and so it would be useful in future to further refine the mechanism for releasing known quantities of spores into the air consistently.

An alternative for improving the efficiency and consistency of spore release could be to use a system like the particle-field and laboratory emission cell (P-FLEC; Chematec, Denmark) as used in similar studies looking at exposure to fungi cultured from damp buildings (Madsen *et al.*, 2016).
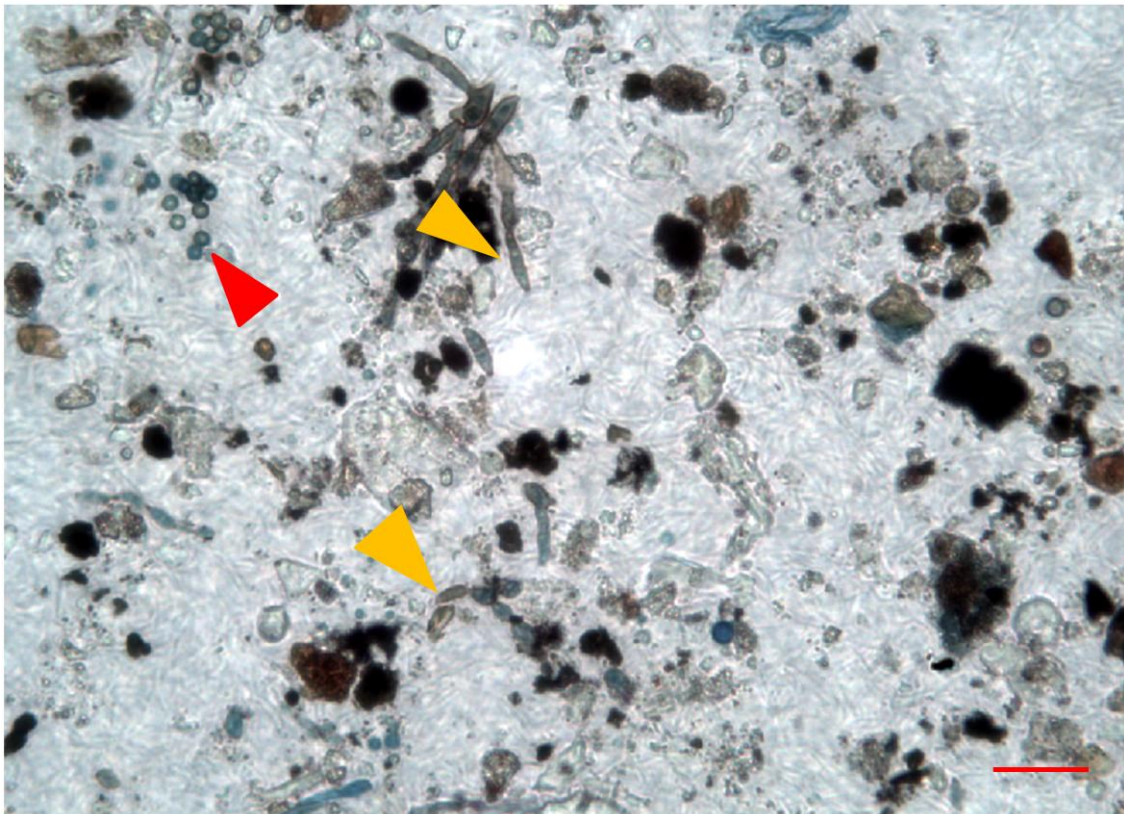
Figure 3-8: A typical field of view of the sample obtained by the Hirst air sampler during the wind tunnel experiments. Note the variation in spore/pollen types present. Red arrow denotes *Aspergillus/Penicillium* spores, Orange arrows show *Cladosporium* spores. The bar represents 20 µm.

3.4.2 Sampler comparisons

Despite the high level of contaminating spores in the wind tunnel it was still possible to directly compare the various samplers to one another. The first point to note is that the data for the Hirst sampler is given in spores per litre of air, rather than CFUs per litre of air as for the other samplers. As has been mentioned the spores counted will never have 100% germination rate and so one would always expect the spore count to be higher than the CFU concentration. In addition to this, *Aspergillus* and *Penicillium* conidia cannot be accurately distinguished from one another by light microscopy, and so are routinely counted together simply as *Aspergillus/Penicillium*-type spores (Fairs *et al.*, 2010). Again this goes quite some way to explain the significantly higher values obtained by the Hirst sampler than for the other samplers, for which only viable spores are counted and only *Penicillium* colonies are included. Further work to identify the percentage viability of the spores would be useful in order to see how much of the difference between the Hirst spores/l value and the other samplers' CFU/l value can be accounted for by this.

From the comparisons to the Hirst sampler, the CIP-10M and IOM showed the best correlation to the Hirst sampler, with correlation of the SKC Biostage and the Hirst also reaching statistical significance. The Button sampler data did not correlate with the Hirst sampler data. The high variability seen with the Button sampler (Figure 3-6) may, in part, account for this.

Comparisons between the test samplers did not reach statistically significant correlation for any of the sampler pairings (Figure 3-7). This is disappointing and highlights the large differences in results obtained from the different sampling methods available. This confirms the importance of careful consideration of sampling and analysis strategies before commencing sampling and the benefit of using, where possible, multiple different means of assessing bioaerosol load. It would be beneficial to run a larger number of repeats of the sampler pairings in order to more accurately assess the differences observed.

The Button sampler showed very high variability relative to the other samplers (Figure 3-6). Interestingly, the IOM and Button sampler showed the least level of concordance

of any of the sampler pairings (Figure 3-7, C). This is unexpected as these samplers are the most similar of any of the paired comparisons. These samplers both collect onto the same media (quartz filters) and the samples are treated in the same way and analysed by culture. These samplers are both similar in size, both being wearable samplers designed for personal exposure monitoring. The small differences in design would not be expected to create such dramatic differences in collection ability. The airflow of these two samplers is different with the Button sampler's airflow at 4 l/minute being twice that of the IOM sampler's 2 l/minute. It is unclear if this difference in airflow could account for such dramatic differences in collection efficiency.

It would be useful to continue this work with a higher number of repeat measures for these sampler comparisons to ascertain the validity of these findings. It would also be of interest to compare different types of filters in these samplers. Gelatin and polycarbonate filters are both commonly used and may offer improved maintenance of cell viability or collection efficiency in some circumstances (Wang *et al.*, 2015).

As for the Biostage sampler, agar impaction samplers' efficiencies are known to be influenced by; impaction stress (physical stress on particles due to fast collision with the hard agar surface), particle bounce (particles colliding with and bouncing off the agar surface instead of adhering to it) and desiccation stress during sampling (Griffiths and Stewart, 1999). Xu *et al.* (2013) showed that layering a small volume of mineral oil onto the surface of the agar plate prior to sampling significantly enhanced the collection efficiency and percentage viability of the particles collected and this may well be worth considering in future studies.

It is also worth bearing in mind that these experiments are concerned only with fungal spores, whereas in most sampling situations it may be important to know the samplers' collection efficiency for other particulate matter. It is known that the air around composting sites can also contain high levels of endotoxin, bacteria and 1-3β glucans (Wéry, 2014) which are likely to be of interest as well as fungi in clinical studies. This may also influence choice of sampler, for example endotoxin is associated with many respiratory/MMI symptoms (Rylander, 2002) and so the sampling strategy used in a broad study of the health of compost workers should take endotoxin level into consideration. Samples obtained from the CIP-10M sampler can be used in the LAL

assay to detect endotoxin levels, but an agar impactor's sample cannot. It should also be considered that molecular techniques for rapid identification and quantification of bioaerosol load are becoming more commonly used (Adams *et al.*, 2013) and so a sampling procedure which would allow for such techniques to be applied to the samples collected is desirable.

3.4.3 Sequence data

The five isolates tested by sequence analysis of the ITS region confirmed the macroscopic and microscopy identification, confirming that *Cladosporium* spp. were abundant in the samples. *Cladosporium* is a genus containing many common indoor and outdoor contaminant species, many of which are often found in buildings with fungal growth (Segers *et al.*, 2016). It is perhaps not surprising then that this fungus is present in the air flow of the wind tunnel. This sequence data goes some way to validating the results obtained from the colony counts, confirming that the *P. chrysogenum* isolates counted were indeed likely to be *P. chrysogenum*. Due to the relative genetic stability of the ITS-1 region (Pryce *et al.*, 2003) it is not possible to definitively say that the sequences obtained prove the colonies to be *P. chrysogenum* (rather than other, closely related *Penicillium* species) but confirmation to the genus level offers a suggestion that the identifications were correct. Obviously in future work it would be desirable to confirm a higher proportion of isolates by molecular means such as this, preferably including analysis of a more variable region such as the β-tubulin region which would offer greater distinction at the species level (Visagie *et al.*, 2014). Such analyses would allow a more detailed appreciation of the validity of the colony identification.

3.4.4 Conclusion

The original purpose of these sampler comparisons was to determine the most suitable approach for monitoring the bioaerosol exposure of people working on industrial composting sites. Due to unforeseen changes in circumstances, the company responsible for the composting sites withdrew from the project. This meant that air sampling to determine worker's exposure was no longer feasible and so the focus of the project changed. The move away from personal air monitoring meant that some of the

problems highlighted by these initial tests were never fully resolved. Nonetheless these preliminary experiments produced some interesting results, suggesting that a combination of sampling approaches would be desirable but that the CIP-10M sampler is likely to be most suitable for personal and static sampling if only one sampler type is to be used. The findings from this work support the hypothesis that some of the commonly used air sampling strategies are better suited to the efficient isolation and accurate quantification of compost site bioaerosols than others. With further method optimisation the wind tunnel setup could prove to be a useful tool in the planning of future bioaerosol monitoring work.

Chapter 4 Mycoviruses

4.1 Introduction

Viruses of fungi, Mycoviruses, are predicted to exist in between 30 and 80% of fungal species (Ghabrial *et al.*, 2015). Clearly they are relatively common but this somewhat vague figure also hints at the limited understanding of this branch of virology. Although the roles of mycoviruses are often unclear, and in many cases the functional relevance of infection remains unknown, it is likely that some of these viruses influence their hosts in ways which may be of interest or use. This is highlighted in chapter 1 with examples of mycoviruses of plant pathogenic fungi.

As has been discussed, *A. fumigatus* is an important human pathogen and drug resistance in *A. fumigatus* is an increasingly alarming and problematic occurrence (Denning and Perlin, 2011). Alongside the development of new antifungal drugs it is important to build a more complete understanding of the life history of *A. fumigatus* in order to be able to fully understand the mechanisms by which *A. fumigatus* is able to cause disease and thus how we may aim to combat *A. fumigatus* infections. Study of the mycoviruses carried by *A. fumigatus* is key to this understanding, not only able to offer insights into potential new drug targets but also as a potential means of direct biocontrol (van de Sande *et al.*, 2010) and as a useful laboratory tool for the manipulation and study of the fungus in detail (van Diepeningen *et al.*, 2008). The impact of mycoviruses on their hosts in some cases shows promise as potential antifungal treatments (Nuss, 2005).

Bacteriophages, viruses of bacteria, have been shown to have the potential to clear persistent, multi-drug resistant bacterial infections even when combination antibiotic therapy has failed (e.g. Morello *et al.*, 2011). Clearly this is a distant prospect for mycoviruses, but an understanding of mycovirus prevalence, role and mechanisms of survival and propagation have proven valuable in gaining insight into the fundamental biology of plant pathogens (Pearson *et al.*, 2009; Nuss, 2011), and may offer similar insight into fungal pathogens of man. With *A. fumigatus* being a most problematic fungal pathogen of humans (Dagenais and Keller, 2009), any work that may aid further understanding of *A. fumigatus* infections would be of value.

4.1.1 Mycovirus lifecycles

Unlike bacteriophages, mycoviruses have been generally shown to adopt predominantly temperate, intracellular life cycles with no free, infectious stage involved (van Diepeningen *et al*., 2006). Under experimental conditions however it has been shown that extracellular transmission is possible (van de Sande *et al*., 2010) and infection of healthy cells by exogenous application of free mycovirus particles has recently been demonstrated in the virus of the plant pathogen *Sclerotinia sclerotiorum* (Yu *et al*., 2013). There is further evidence of cell-free virus suspensions proving to be infectious under carefully controlled, specific laboratory conditions using protoplast fungal cells in both *Rosellina necatrix* (Sasaki *et al.*, 2006) and *Cryphonectria parasitica* (Hillman *et al.*, 2004). These examples provide further evidence that mycoviruses have more potential to infect via an extracellular route than has previously been thought.

Undeniably however, mycoviruses are most commonly propagated vertically through spore formation and cytoplasmic exchange during cell division and may be transmitted horizontally to a new host via the fusion of hyphae (anastomosis) and subsequent cytoplasm exchange. In all of these commonly observed scenarios transmission does not feature an extracellular phase. The apparent lack of extracellular transmission in most mycovirus life cycles may be due to the presence of the fungal cell wall which is an obvious barrier to such a route of infection (Nuss, 2010).

Whilst mycoviruses have been shown to be of great potential to reduce and alleviate the problems associated with some fungal pathogens almost all of this work has, until recently, been concerned with mycoviruses that target plant pathogenic fungi. If similar virus-induced hypovirulence occurs among fungal pathogens of animals then this could be of use in the treatment of such infections. With the narrow choice of antifungal drugs available and recent documented increase in drug resistance amongst *A. fumigatus* this issue is of increasing relevance to clinical practice.

4.1.2 Mycovirus diversity

There is a great deal of genome diversity amongst known mycoviruses, with ssRNA, dsRNA and DNA genomes all documented (King *et al.*, 2012). In terms of physical

presence there is similarly high variation, with some mycoviruses shown to be encapsidated icosahedral particles (Caston *et al.*, 2003; see Figure 4-1) and others existing solely as genetic elements (Magae, 2012). With such physical diversity, it is perhaps unsurprising that a variety of mycovirus life cycles is also seen. Whilst the majority apparently exist only intracellularly, transmissible only during cell division, sporogenesis and cell fusion (Hyman and Abedon, 2012), it has been demonstrated that mycoviruses can persist outside of the host and subsequently infect a new host cell (Yu *et al.*, 2013). In bacteriophage infections, both lytic and lysogenic lifecycles are well-documented (see 1.10.2). With some mycovirus infections demonstrated to kill their host cells upon infection (Xie *et al.*, 2006) and others having no known physiological impact on their hosts (Lin *et al.*, 2012), clearly there are similarly distinct life cycles which mycoviruses may follow. The majority of mycoviruses characterised to date are dsRNA viruses (ICTV, 2015) which form encapsidated icosahedral viral particles but which do not appear to persist outside of a host cell (Ghabrial and Suzuki, 2009).

The majority of *Aspergillus* mycoviruses studied to date appear to have little or no phenotypic effect on their hosts, although this research area is understudied and some mycoviruses have been shown to be associated with altered growth rate (Bhatti *et al.*, 2011). The primary aim of the work presented in this chapter was to assess the validity of a commonly-used bacteriophage identification and isolation procedure to be applied in the isolation of mycoviruses from environmental samples, to characterise any mycoviruses identified by this method, and to characterise the genetic composition and genome sequence of a mycovirus carried by the type strain *A. fumigatus* NCPF7367.

The hypothesis being tested in this work is that mycoviruses are present within environmental populations of *A. fumigatus* and that mycoviruses can be isolated from raw environmental samples by the use of an enrichment method similar to the methods used for the isolation of environmental bacteriophages.
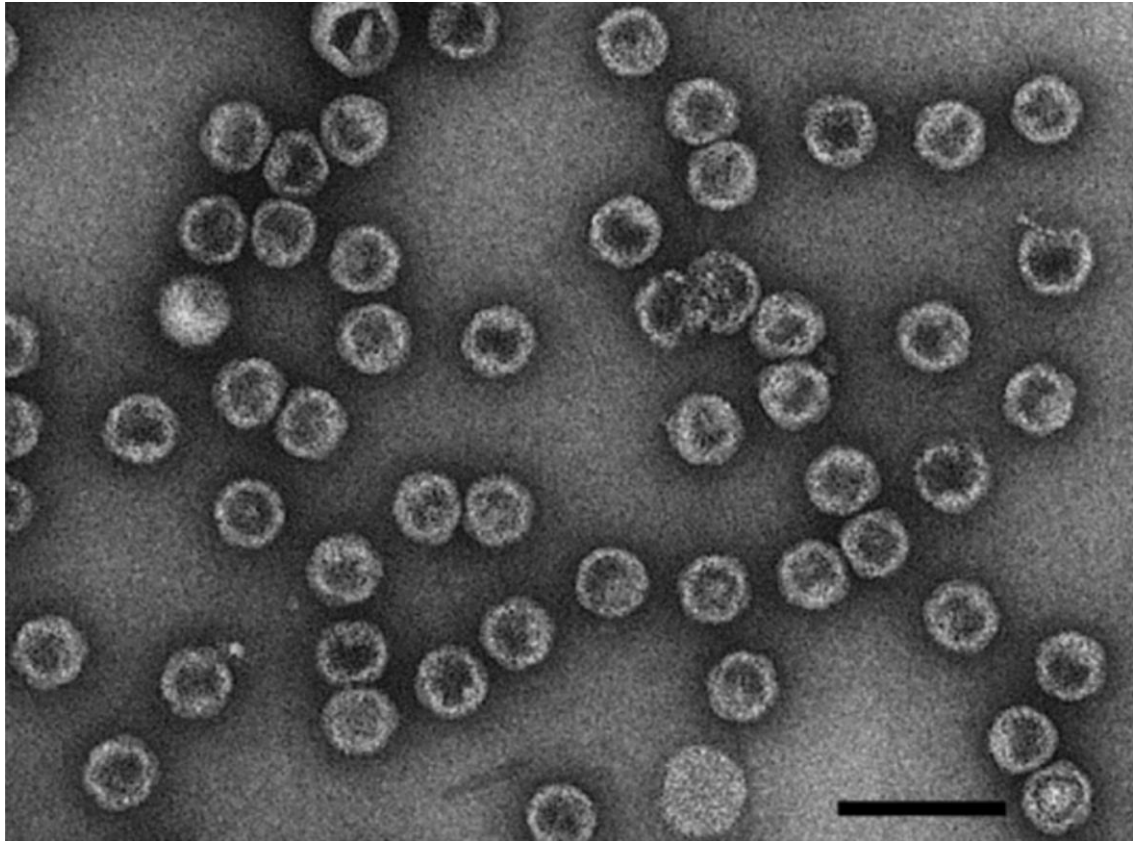
Figure 4-1: Particles of the *Penicillium chrysogenum* mycovirus PcV. The scale bar represents 100 nm. From Caston *et al.,* 2003

4.2 Materials and Methods

4.2.1 Strategy overview

Compost samples were collected (4.2.2) and the enrichment procedure was performed (**Error! Not a valid bookmark self-reference.**) in order to create a collection of enrichments from compost samples, some of which may contain mycoviruses. At first, established methods were then assessed for their suitability in identifying any mycoviruses in these enrichments. These methods are the single colony, plate reader and plaque assay (4.2.5-4.2.7). Following this the nanosight system (4.2.8) was used to screen the entire collection for mycovirus-sized particles, as this is a relatively high-throughput method. From the resulting data, any enrichments which appeared promising (i.e. those which contained an abundance of particles within the expected size range of mycoviruses) were further analysed by TEM (4.2.9) and dsRNA extraction (4.2.10) in order to further assess the presence or absence of mycoviruses within these enrichments.

The cellulose dsRNA extraction method (4.2.10) is not a high throughput method and requires a relatively large starting volume. With this in mind, two other dsRNA extraction procedures (4.2.11 and 4.2.12) were compared using the dsRNA-carrying *A. fumigatus* type strain NCPF7367. This was to assess if either of these methods would be suitable for screening the collection of putative virus enrichments for dsRNA.

Alongside this work, the mycovirus carried by strain NCPF7367 was studied in more detail. Density gradient ultracentrifugation was performed and Bradford assays, RNA quantification and CSTEM were used in attempts to isolate and characterise purified virus particles from this strain (4.2.13, 4.2.14, 4.2.15). The dsRNA genome of this virus was also sequenced (4.2.16) to gain further insight into the biology of this virus, and how it may relate to its important fungal host.

4.2.2 Compost site sampling

Compost samples were collected from composting sites of varying sizes, and feedstock around the UK between October 2011 and June 2013. For sample collection, a small hole was dug into the compost heap, between 10 and 30 cm deep. A thermometer was

inserted into the compost, the temperature measured and a sample of compost taken. Around 10 g of sample was taken at each sampling point. The samples were collected in sterile 50 ml centrifuge tubes and stored in the dark on an ice pack during transport back to the laboratory. The enrichment procedure was carried out as soon as possible, but always within 10 hours of sample collection.

4.2.3 Fungal identification and isolation

A culture-based approach was used to identify and isolate *A. fumigatus* from the compost samples. 1g of sample was weighed out into a sterile 50 ml centrifuge tube and 10 ml of filter-sterilised (0.22 μm pore size) PBST (0.05% Tween-80 in Phosphate buffered saline) was added. This was vortexed briefly before being rotated at 4 RPM at room temperature for 1 hour to fully disperse the sample. Any large floating pieces of debris were then manually removed using sterile forceps before 1/10 and 1/1000 dilutions of the suspension were prepared in PBST. The dilutions were then briefly vortexed and 100 μl of the dilutions pipetted onto PGC agar plates and spread using a sterile glass spreader. The plates were then incubated at 37°C for 5 days and growth monitored daily. Compost is a microbiologically rich and dense material and so it is expected that a high concentration of fungi and bacteria is likely to be present. It is for this reason that a 1/1000 dilution is included and that agar supplemented with gentamicin and chloramphenicol is used, in order to limit the growth of bacteria and aid the identification and recovery of fungal colonies (Dolan, 1971). Potential *A. fumigatus* colonies were confirmed by microscopy (as in 2.4.2), streaked to obtain pure cultures on PDA agar and stored at -80°C in glycerol nutrient broth (GNB) until use.

4.2.4 Enrichment

In order to isolate and amplify any *A. fumigatus* mycoviruses present in a sample of compost a virus enrichment procedure was used. This method is based on a bacteriophage enrichment protocol which is widely used for the study of phages obtained from environmental sources (Twest and Kropinski, 2009). The principle of this procedure is otherwise known as a host addition/virus amplification protocol (HAVAP) (Zemb *et al.*, 2008) but is hereafter referred to as enrichment. The raw sample is diluted, filtered and a small aliquot co-cultured with a panel of different

strains of *A. fumigatus*. This co-culturing dilutes everything in the original sample apart from any viruses which are able to utilise the *A. fumigatus* strain as a host. Any such viruses can infect the *A. fumigatus*, ultimately allowing the virus to replicate and leading to amplification of the virus population. If there is no viable match between virus and host *A. fumigatus* then the filtered product of this co-culture would contain only background levels of viral particles. This enrichment procedure is outlined in ==**Error! Not a valid bookmark self-reference.**== and was performed as follows; 3g of the raw compost sample was weighed into a sterile 50 ml centrifuge tube and 30 ml SM buffer (50 mM Tris-Cl, pH 7.5, 99 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin) was added. After vortexing the mixture was left rotating at 4 RPM at 4°C for 30 minutes. This was carried out in triplicate for each sample. The mixture was then centrifuged for 10 minutes at 10,000g to pellet compost debris and the majority of cellular matter. The supernatant was retrieved and at this stage the supernatants from the three repeats for each sample were merged and filtered through 0.45µm pore acrodisc syringe units (Pall Corp.) to remove remaining cellular material whilst retaining any virus particles present. Filtered supernatants were stored at 4°C in the dark until used for co-culturing.

For the co-culture part of the protocol, 3 ml of the relevant supernatant was added to a 10 ml shaking, exponential growth phase culture of *A. fumigatus* in Sabouraud liquid media (SLM) and incubated, shaking at 100 RPM at 30°C for 48 hours. During this co-culture, viable virus infection and propagation would lead to the production of progeny virus particles within the host cells, thus amplifying the viral population. Following this incubation, the culture was vortexed and centrifuged for 10 minutes at 10,000g to pellet hyphal cells. The supernatant was filtered through a 0.45 µm pore-size acrodisc filter and stored at 4°C in the dark. These filtered supernatants are the putative virus suspensions and contain any virus population which was able to utilise the *A. fumigatus* as a viable host for infection and replication. This procedure was performed at least three times for each compost sample obtained, each repeat using a different potential host strain of *A. fumigatus*. One of these host strains was always an *A. fumigatus* isolate derived from the same compost sample as the supernatant being enriched. As outlined in **Error! Not a valid bookmark self-reference.** this procedure relies on the presence of a viable host-virus pairing in the co-culture in order to achieve amplification of any virus present in the original sample. The resulting collection of these putative virus

enrichment suspensions was then tested by various means to identify which, if any, contained likely *A. fumigatus* mycoviruses. The methods of virus detection used are given in below.

4.2.5 Single colony assay

Due to the filamentous nature of *A. fumigatus* its growth over a period of days on solid agar is uniform and, if unimpeded, a single colony in the centre of an agar plate will grow out radially to eventually cover the plate. One method which has previously been used to identify if a solution is capable of restricting the growth of a fungal isolate is to grow a single colony in the centre of a plate and add the test solution to the growing edges of this colony (Yu *et al.*, 2013). For this the centre of a PDA plate was inoculated with *A. fumigatus* by dipping a pipette tip in a liquid suspension of *A. fumigatus* spores and then stabbing the pipette tip into the centre of the agar. The plate was then incubated upright at 37°C and after 2 days a single colony (~2cm in diameter) was observed. At this point, 10 μl of the putative virus suspensions being tested was dripped onto the very edge of the growth. Multiple suspensions were tested per plate, each being added to a specific sector of the plate and the position of each test sector recorded. One sector was left with no suspension added and another sector had neat, sterile SM buffer added- both as negative controls. The growth of the colony over the next 5 days was monitored to see if the growth was influenced by the addition of any of the suspensions added. A virus present which is able to infect and kill the fungus would result in a clear area where the fungal growth has not penetrated around where the virus-containing solution was applied (see Figure 4-3, A). Milder effects of virus infection such as reduced growth rate, reduced sporulation or altered pigmentation would also been seen by this method.

Aim: to amplify a viral population within a sample
using multiple *A. fumigatus* strains as hosts

Weigh out
3g of
compost
sample

Suspend in 30 ml
SM buffer & mix for
30 mins, 4°C.

Centrifuge (10,000g, 10 mins) to pellet debris and cells.
Filter supernatant (0.45 µm) to remove remaining
cells/debris whilst retaining virus-sized particles.

Inoculate into multiple
active-growth (early
exponential phase) cultures
of different potential host
strains of *A. fumigatus*.
Incubate, shaking 100 RPM
at 30°C for 48 hours.

If viable host: Virus may infect,
multiply and release progeny
upon cell death.

If non-viable host: Virus unable
to infect, cell growth continues
as normal. Any viral particles in
the sample may still be present
but will not be amplified and so
will be at very low concentration.

Vortex culture briefly and then centrifuge to pellet
fungal material. Filter supernatant, assign each
filtered supernatant a virus enrichment ID and
analyse for presence of virus particles.

**Figure 4-2: Outline of the virus enrichment procedure from compost samples
using multiple putative host strains of *A. fumigatus*.**
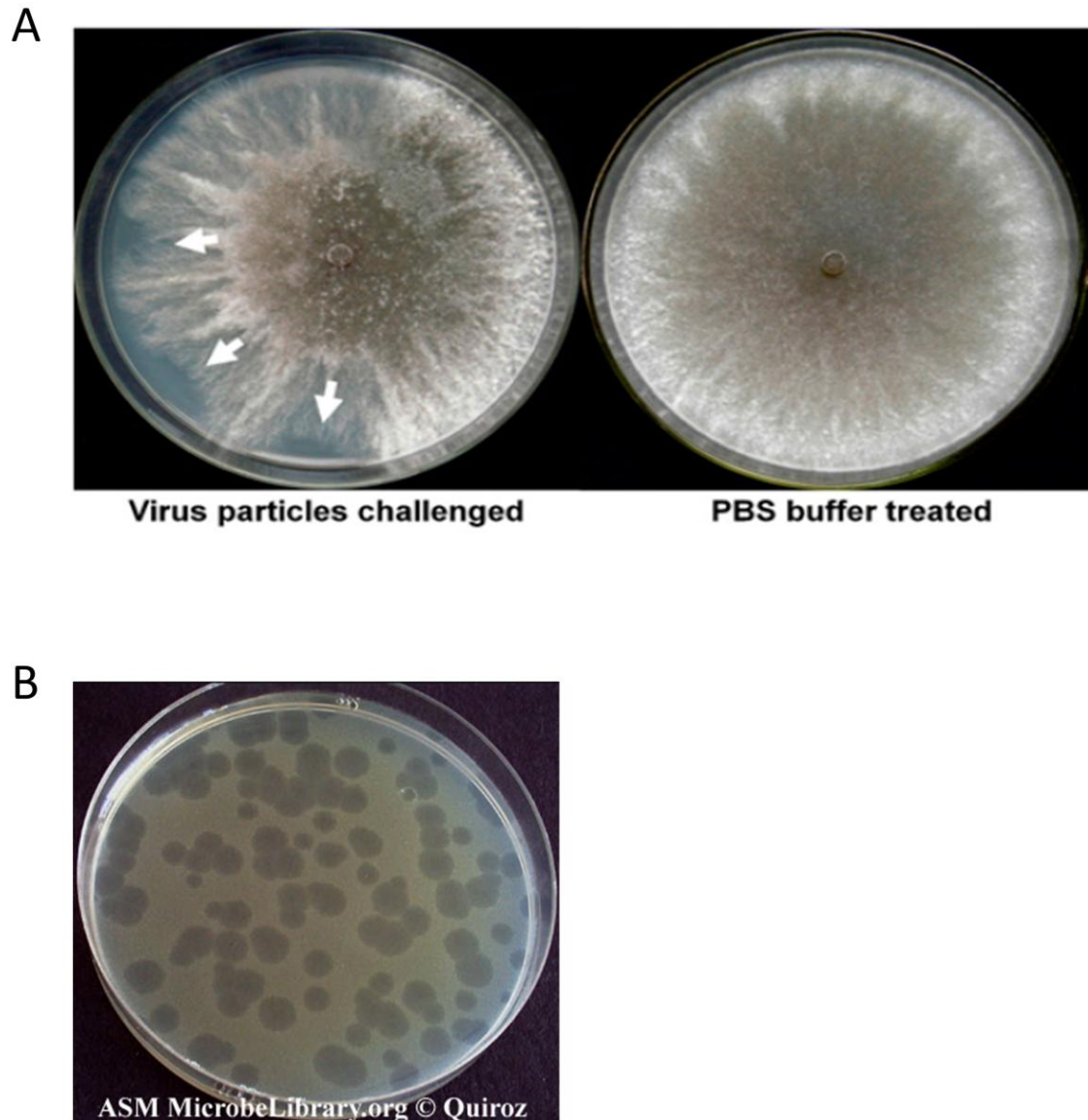
Figure 4-3: A) Example of the single-colony test showing clear sectors of inhibited growth following virus particle challenge compared to PBS-challenged control. In this case the host fungus is the plant pathogen *S. sclerotiorum*. White arrows indicate the points of virus challenge. Image from Yu *et al*., 2013.

B) An example of a plaque assay for bacteriophages using a lawn of host bacterial cells. Note the clear plaques in the lawn where lytic phages have resulted in lysis of the host bacteria. From American Society for Microbiology 'Microbe library'.

4.2.6 Plate reader

A comparison of growth curves produced by *A. fumigatus* strains grown in the presence and absence of an array of putative virus suspensions was used to attempt to identify any virus-containing suspensions which had an influence on the growth dynamics of the fungus. Initial methods trialled using 24 well plates and manually observing the growth for obvious differences after 24 and 48 hours proved to be too qualitative and variable. Instead, a method was used which involved multiple repeated cultures set up in SLM in 96 well plates and incubated in a plate reader set to record the optical density of each of the wells over a 48 hour period. For this, 40 µl of SLM was added to the wells and then 10 µl of the relevant spore suspensions added to the appropriate wells. To some of these wells, 10 µl of the virus enrichments being tested was then added. Control wells were inoculated with; no spores (media negative control), spores but no virus suspension (positive growth control), spores with 10 µl SM buffer instead of virus suspension (as a buffer control) and no spores but 10 µl of the virus suspension (as a further negative control as these suspensions should contain no fungi or bacteria). The plate was sealed with sealing film to avoid well-well contamination during the incubation and incubated at 37°C, shaking at 60 RPM in a plate reader (Varioskan Flash, Thermo-Fisher Scientific Inc.). Optical density readings at 405 nm were taken every hour for 48 hours. The raw data was exported into Microsoft Excel and growth curves plotted from this data using Prism V6.07 (Graphpad Software, Inc.). From these growth curves, comparisons were drawn to attempt to ascertain the effect of the addition of the panel of putative virus suspensions on the growth of the *A. fumigatus* strains tested.

4.2.7 Plaque assay

A commonly used technique for the isolation and quantification of phages is the plaque assay. The host bacterial strain is cultured as a lawn of growth, onto which the virus-containing solution is overlaid. This is then incubated and a viable virus able to infect and lyse the host cells will result in a clearing or plaque within the lawn of cells that grow (Figure 4-3, B).To test this procedure with fungi, a heavy spore suspension of *A. fumigatus* spores was poured onto a solid PDA plate and spread to form a confluent

layer. The putative virus suspension was then added to sectors of the plate and the plate was incubated upright at 37°C. Over 3-4 days the growth on the plate was monitored to see if there were any discernible growth differences between the sectors containing virus enrichments and the 'media only' portions of the plate (as well as SM buffer control sectors).

4.2.8 Nanosight

The Nanosight LM-10 microscopy system (Malvern Instruments Ltd.) uses a standard light microscope (20x) objective lens to track nanoparticles in a solution by monitoring patterns and movement of diffracted laser light. A laser beam is passed through a liquid suspension of the sample being tested and a digital camera detects the laser light which is diffracted by particles in the solution. In order to calculate the particle size distribution in the sample a video is recorded of these diffraction patterns and then the nanosight nanoparticle tracking analysis (NTA) software assigns each particle a number and tracks each particle frame-by-frame throughout the duration of the video. The distance moved by each particle is measured and based on Brownian motion kinetics and the viscosity of the buffer at the recording temperature, the sizes of the particles is calculated. This allows for rapid (in real time) screening of many samples to help screen out samples which have no virus-sized particles and allow further investigation into only the samples which show promising particle size distributions. In this work, a video length of 90 seconds was used for each sample, and the NTA software set to detect the lower range of particles sizes, with expected particles around 30 nm in diameter. Particle size distribution data was exported to Microsoft Excel, collated and size distribution graphs were plotted using Prism, V6.07 (Graphpad Software, Inc.). The size distribution data for each of the virus enrichment solutions was compared to the data from a control solution derived from the growth of the relevant fungal strain. These fungus-only controls are prepared exactly as each of the virus enrichments but instead of a putative virus suspension added during the co-culture step of the enrichment only sterile SM buffer is added to the culture. These represent the background particle size distributions of each host fungus.

4.2.9 Transmission electron microscopy

Following initial analysis of the data obtained from the nanosight, some of the virus enrichment solutions were visualised under TEM to identify the particles likely to be responsible for the peaks in particle size distribution profiles obtained with the nanosight system. Cell-free extract of a mature SLM culture of the virus-carrying *A. fumigatus* strain NCPF7367 was also visualised by this method.

Carbon coated copper grids were glow discharged briefly before 4 µl of the sample was added and allowed to adhere to the grid for 3 minutes. A small piece of filter paper (Whatman grade 1) was gently touched to the edge of the grid to wick off excess sample. The grid was then washed of excess sample by dropping 5 µl of ddH$_2$0 onto the grid and wicking off with filter paper. This wash step was repeated once. 4 µl of 1% Uranyl acetate was then added to stain the sample, and is wicked off with filter paper. This step is then repeated once and the grid allowed to dry briefly before being placed onto a clean sheet of filter paper in a clean small petri dish for storage until use.

The grids were analysed in a Jeol JEM-1400 Transmission Electron Microscope (Jeol Ltd.) at 80 kV. Representative images were captured digitally and analysed in ImageJ.

4.2.10 CF-11 Cellulose dsRNA extraction

Fresh cultures were grown in static liquid culture of SLM (see 2.1.2) and incubated at 37°C for 48 hours, non-shaking. The growth forms a thick, solid mat of growth on the surface of the liquid medium. This mat of growth was removed with sterile forceps and partially dried by placing onto a sheet of sterile paper tissue. The dried fungal mat was then frozen in liquid nitrogen and ground to a fine powder using a pre-cooled pestle and mortar, with more liquid nitrogen added as necessary to allow full grinding of the sample without thawing. The resulting powdered fungal tissue was weighed into a sterile 50 ml centrifuge tube.

To this powder, 4 volumes of 1X STE buffer (0.1M tris-HCl, 0.1M NaCl, 10mM EDTA, 1% SDS) was added and agitated gently on a rotator at 4 RPM at room temperature for 30 minutes. 0.5 volumes of phenol:chloroform (1:1) was added and the

mixture emulsified initially by shaking and then 30 minutes rotating at 4 RPM at room temperature. The mixture was then centrifuged at 10,000g for 10 mins and the upper aqueous phase collected. 0.8 volume of isopropanol was added to this, mixed and incubated for 30 mins on ice to precipitate nucleic acids. Centrifugation for 20 minutes at 4°C at 14,000g was then used to pellet the precipitated nucleic acids. The supernatant was carefully removed and the pellet resuspended in 1.3X STE buffer at 0.8 ml of 1.3X STE per 3 g of starting weight of powder. This was mixed thoroughly by shaking. The solution was then centrifuged for 20 minutes at 10,000g and CF11 cellulose microgranules added to the recovered supernatant (at 10 mg CF-11 per 3 g starting fungal material) and mixed for 30 minutes. Ethanol was then added dropwise to 20% volume with vigorous mixing. This was then mixed on a rotator at 4 RPM for 30 minutes at room temperature. Under this ethanol concentration, in STE buffer, RNA binds to cellulose. A centrifugation step of 2 minutes at 10,000g was used to collect the cellulose-bound RNA and the pellet was washed with 1X STE buffer supplemented with 16.5% ethanol. Centrifugation at 10,000g for 2 minutes was used to re-pellet the RNA-bound cellulose and then this washing step and centrifugation was repeated once. The supernatant was removed and the pellet air-dried. RNA was then eluted from the CF-11 cellulose by resuspension of the pellet in 1X STE buffer (with no ethanol added), at a ratio of 200 µl STE per 10mg of cellulose used. This was incubated at room temperature for 2 minutes, during which time the RNA becomes free from the complex with CF-11 and becomes resuspended in the solution. This was then centrifuged at 10,000g for 5 minutes to pellet the cellulose. The RNA-containing supernatant was carefully removed and digested with DNase (Promega) following the manufacturer's protocol (appendix 4). The final elution was into PCR-grade H_2O and the resulting purified dsRNA was stored at -20°C.

4.2.11 SV total RNA extraction kit (Promega) dsRNA extraction

The SV total RNA extraction kit from Promega uses 300 mg of liquid nitrogen frozen, powdered fungal material, prepared as in 4.2.10 as the starting material. 1 ml of RNA lysis buffer was added to the sample, vortexed briefly and mixed thoroughly to homogenise. 175 µl of the homogenate was pipetted into a sterile microcentrifuge tube and incubated at 70°C for 3 minutes to degrade the cellular structures remaining. This

was centrifuged at 13,000g for 8 minutes to pellet cellular debris. The supernatant was recovered and 200 µl of 95% ethanol added. Following brief mixing, this solution was pipetted directly onto the membrane of the spin column provided in the kit. This was placed into a collection tube and centrifuged at 13,000g for 1 minute. The flow through was discarded, the RNA-bound column washed with 600 µl of the supplied RNA wash solution and centrifuged again at 13,000g for 1 minute. A DNase treatment step was then performed; 80 µl of the supplied 'yellow core buffer', 10 µl 0.09M MnCl2 and 10 µl DNase solution was prepared, mixed thoroughly and 50 µl of this was pipetted onto the membrane of the spin column. A 15 minute incubation at room temperature allowed the DNase to degrade DNA present on the membrane and addition of 200 µl DNase stop solution quenches this reaction. Centrifugation at 13,000g for 1 minute removes the DNase and stop solution. A further wash step was then used, with 600 µl of RNA wash solution added and centrifuged at 13,000g for 1 minute. The flow through was discarded and the wash repeated with 250 µl of RNA wash solution, centrifuging again at 13,000g for minute. The spin column was then placed into a sterile microcentrifuge tube and the RNA eluted from the membrane by addition of 100 µl PCR-grade H_2O. 2 minute room temperature incubation was followed by centrifugation at 13,000g for 2 minutes to collect the eluted dsRNA into the tube. The RNA suspension was stored at -20°C.

4.2.12 CTAB modification of RNeasy RNA extraction kit (Qiagen) dsRNA extraction

This protocol again uses 300 mg of powdered tissue as the starting material. This powdered fungal material was added to 1 ml CTAB grinding buffer ($CTAB_3$, 1%Na_2SO_3, 2% Polyvinylpyrrolidone), mixed thoroughly and incubated at 65°C for 10 minutes. 1 ml chloroform:isoamylalcohol (24:1) was then added and the mixture shaken and mixed to an emulsion. Centrifugation at 13,000g for 10 minutes then separates the layers and 800 µl of the aqueous phase was carefully removed. To this, 800 µl of 4M LiCl was added, mixed thoroughly and incubated at -20°C for 1 hour to precipitate nucleic acids. Nucleic acids were then pelleted by centrifugation at 13,000g for 25 minutes. The supernatant was removed and the pellet resuspended in 50 µl PCR-grade H_2O. 350 µl of 'Buffer RLT' (from the QIAGEN RNeasy kit) was added along

with 250 µl ethanol. The solution was mixed and then pipetted onto an RNeasy spin column. This was then centrifuged at 8,000g for 15 seconds. The flow through was discarded and the membrane washed with 500 µl of 'Buffer RW-1' (from QIAGEN RNeasy kit), followed by centrifugation at 8,000g for 15 seconds. 80 µl of the supplied DNase was then added to the membrane and incubated at 30°C for 15 minutes. A further washing step again using 500 µl of 'Buffer RW-1' was followed by centrifugation at 8,000g for 15 seconds. Two further wash steps were then performed using 500 µl 'Buffer RPE' from the QIAGEN RNeasy kit with centrifugation at 13,000g for 15 seconds. The column was then dried by a further centrifugation at 8,000g for 1 minute before the column was placed into a sterile microcentrifuge tube and 50 µl PCR-grade H$_2$O was added to elute the remaining RNA. This was incubated at room temperature for 1 minute before centrifugation at 8,000g for 30 seconds to retrieve eluted RNA. The elution step was repeated once and the resulting RNA solution stored at -20°C.

4.2.13 Virus purification by PEG precipitation and ultracentrifugation

A 1 litre culture of the virus-carrying *A. fumigatus* strain NCPF7367 was prepared in SLM and incubated shaking at 150 RPM at 37°C for 5 days. The resulting culture was filtered through miracloth (Millipore Ltd.) to remove media. The fungal growth obtained was dried briefly on sterile paper tissues and weighed. The dried fungal mass was then frozen in liquid nitrogen and ground to a powder with a pre-chilled pestle and mortar. This powder was then suspended in 0.1M sodium phosphate buffer (pH7.4, supplemented with 3% Triton X-100) at a ratio of 45 ml buffer per 64 g starting weight of fungal tissue. The mixture was agitated for 10 minutes at room temperature before being clarified by centrifugation at 1,700g for 30 minutes at 4°C. The supernatant was recovered and PEG8000 added to 6% w/v and NaCl added to 1.5% w/v. This mixture was agitated gently overnight at 4°C in the dark to precipitate any viral particles before being centrifuged at 10,000g for 30 minutes to pellet any precipitate. The resulting pellet was then resuspended in 10 ml sodium phosphate buffer and ultracentrifugation at 86,000g for 2 hours was used to pellet viral particles. This pellet was then hydrated in 50 µl of sodium phosphate buffer, being left overnight at 4°C for the pellet to fully hydrate. The hydrated pellet was then resuspended in 300 µl Sodium Phosphate buffer

and layered onto a 10-40% sucrose gradient. The gradient was then centrifuged at 84,000g for 90 minutes to purify any viral particles obtained by the PEG precipitation at point of equivalence within the density gradient. 500 µl fractions of the resulting gradient were then sequentially drawn off carefully and pipetted into individual, sterile microcentrifuge tubes. Each of these fractions was then analysed by nanodrop for RNA content and by Bradford assay for protein content to identify which, if any, contain likely virus particles.

4.2.14 Bradford assay

Pre-prepared 'quick-start' Bradford reagent dye was used (Bio-Rad Ltd.) for the Bradford assay and the Bio-Rad BSA protein standard was used to prepare the standard curve against which the samples were compared to quantify the protein concentration in the fractions obtained from the sucrose gradient (4.2.13). In a flat-bottomed, clear 96 well plate (Appleton Woods), 250 µl of Bradford reagent was added to appropriate wells. 5 µl of sample was then added to relevant wells and 5 µl of the various standard dilutions added to other wells. Each sample was pipetted briefly to ensure adequate mixing and the plate was incubated at room temperature for 5 minutes. The absorbance at 595 nm was read for each well in a spectrophotometer (Tecan Ltd.), the data exported to Microsoft Excel and the protein concentration calculated in Prism, V6.07 (Graphpad Software, Inc.).

4.2.15 Cross-sectional TEM

For cross-sectional TEM, fungal cultures were grown as balls of hyphae. 400 ml of SLM was inoculated with 1,000 *A. fumigatus* spores and incubated at 37°C shaking at 100 RPM for 24 hours. This results in small (~5 mm diameter) balls of dense hyphal material. Individual hyphal balls were removed carefully with sterile forceps and placed into a 1.5 ml microcentrifuge tube with 1 ml PBS. The sample was centrifuged and washed in 1 ml PBS three times before 2 ml PBS supplemented with 10% BSA was added, shaken gently and left for 30 minutes at room temperature to infuse throughout the sample. The sample was then removed with tweezers and fixed by placing in 2.5% glutaraldehyde in 0.1M Sörensen's Phosphate Buffer (pH7.3) (SPB) and incubated at 4°C overnight. A 10 minute wash in 0.1M SPB was then repeated three times and a

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secondary fix performed by the addition of 1% aqueous osmium tetroxide to 1% of the total volume and potassium ferricyanide to 1.5%. This was incubated for 90 minutes at room temperature to fix before being washed with dH_2O and left for 10 minutes. The water was removed and the wash step repeated 3 times.

The fixed sample was then dehydrated by 30 minute washes through a series of progressing ethanol concentrations up to 100% (10%, 20%, 40%, 60%, 80%, 90% and 100% ethanol). Three further exchanges of fresh 100% analytical grade ethanol were used to ensure the sample was fully dehydrated. The ethanol is then replaced with propylene oxide and incubated at room temperature for 20 minutes, replacing with fresh propylene oxide after ten minutes.

The resin used was Spurr's Modified Resin (SM resin) and this was introduced step-wise in 90 minute wash steps of increasing SM resin concentration in propylene oxide. 3:1 propylene oxide:SM resin for 90 minutes followed by 1:1, then 1:3 before the final exchange for 100% SM resin. This was left for a further 30 minutes, replaced with fresh 100% SM resin and left overnight to fully penetrate into the sample. Three more changes with fresh 100% SM resin (each 90 minutes) were then performed before the final change into fresh resin in a BEEM capsule (BEEM Inc., USA). The resin was then polymerised by incubation at 60°C for 16 hours which fully solidifies the resin, embedding the sample within it. Following this incubation, the hardened resin capsule was released from the BEEM capsule using a bench press (BEEM, Inc.) and the excess resin trimmed away using a scalpel. The capsule is then ready for ultramicrotomy using a Reichert Ultracut E ultramicrotome (Reichert-Jung Inc.) to produce the cross-sectional slices through the resin and fungal cells.

The slices obtained from the ultramicrotome (90 nm in thickness) were then checked under light microscopy to identify those which appear to be of sufficient quality to use under TEM. These were then stained with 1% uranyl acetate and viewed with a Jeol JEM-1400 Transmission Electron Microscope at 80kV.

4.2.16 dsRNA sequencing

The dsRNA isolated from *A. fumigatus* strain NCPF7367 was purified and sequenced using the MiSeq RNA sequencing platform (Illumina Inc.). This sequencing was performed by Dr Ian Adams of the Food and Environment Research Agency, York, UK. Details of the MiSeq sequencing platform are included in appendix 3.

4.2.17 Analysis of genome sequence data

Analysis of the genome sequence data obtained from 4.2.16 was performed with the MEGA (v6) and Mauve (v2.4.0) software packages, and the NCBI's BLAST tool was used to search for similarities to previously published genomes.

4.2.18 Re-enrichments and time course experiments

Attempts were also made to re-enrich a small sub-set of the enrichments which originally gave promising nanosight data. These re-enrichment experiments involved growing the relevant host *A. fumigatus* strain in the original enrichment procedure. Serial dilutions of the putative virus suspension was then added to replicates of these *A fumigatus* cultures and the sample analysed by nanosight after 48 hours incubation as a dose-response experiment. A further re-enrichment was made, which was incubated for 7 days following addition of the putative virus suspension. An aliquot from this co-culture was removed every 24 hours and filtered before analysis by nanosight. This was a time course experiment intended to identify if the 48 hours being used routinely in the enrichment protocol was sufficient time for the virus infection and replication to take place.

4.3 Results

4.3.1 Virus identification method development

Initial attempts to perform plaque assays using fungi proved difficult as the filamentous growth of the fungus does not lend itself well to the formation of an even, confluent lawn of growth. Airborne spore production also confounded the assay, as spores released from the initial culture subsequently germinate and form satellite colonies of different density to the original culture. Figure 4-4 shows the typical growth of *A. fumigatus* observed in these preliminary tests showing the non-uniform growth across the surface of the agar. Single colony virus challenge testing was performed on a number of samples as shown in Figure 4-5. There was no impact of any of the virus enrichments tested in these preliminary tests. Finally, none of the preliminary tests carried out with the plate reader revealed any convincing evidence of altered growth of the host fungi. A problem with the plate-reader experiments was a high variability observed amongst repeats. Figure 4-6 shows typical growth curves produced by this method, and highlights the large variance between repeats.

4.3.2 Nanosight

In order to determine if virus particles were present in the enrichments and could be observed we used the nanosight system to assess particle size distribution profiles of the enrichment samples. This was to allow relatively rapid screening of all of the enrichments for virus-sized particles; thus allowing further study to focus only on those which contained particles within the correct size range. 128 enrichments were tested with the nanosight system alongside fungal baseline controls from all of the fungal strains which had been used in the preparation of these enrichments. Figure 4-7 shows an example graph featuring; A) an enrichment believed not to contain VLPs, B) a fungal baseline control and C) an enrichment showing a high density of particles within the appropriate size range for mycovirus particles. Overall, six samples (4.7%) appeared to have a significant abundance of mycovirus-sized particles, and the data from these samples, along with the 'background' abundance from the host fungal culture is shown in Figure 4-8. Note the low 'background' particle concentration and the significant peaks in abundance around 25-45 nm. Also note the baseline size

distribution curves from the fungus-only controls showing no such peaks. *A. fumigatus* strain '30', obtained from a small compost heap was the fungal strain used in the enrichment of samples V19 and V13, strain 'BGC' obtained from a larger composting site was used as the host strain for the enrichment of sample V17. The clinically-derived type strain NCPF7097 was used in the enrichments of V20, V57 and V84.

4.3.3 Dose response and time course re-enrichment

Attempts to repeat the enrichment of combinations of virus-host which initially gave promising nanosight data proved unsuccessful. Figure 4-9 shows a dose-response attempt and Figure 4-10 shows the time course experiment data. The high density of mycovirus-sized particles present in the original enrichment does not appear in any of these re-enrichment attempts. Compare to Figure 4-8 (B) and note that the high concentration of particles around 30 nm in diameter seen in the original V19 enrichment is not reproduced in these re-enrichment attempts.

4.3.4 Transmission electron microscopy

TEM was used to determine the morphology of the particles associated with the positive nanosight data (i.e. the particles which were within the mycovirus size range). Electron micrographs of virus suspensions V17, V19 and V20 are shown in Figure 4-11, with the appropriate fungus control samples for comparison. VLPs seen in these enrichments appear to be similar to mycovirus particles seen previously (see Figure 4-1). They appear as well defined and of a uniform size and shape, with a diameter of around 30-40 nm. There were no such particles observed in any of the fungal controls. Figure 4-13 shows electron micrographs obtained by TEM of the virus-infected type strain NCPF7367. No viral particles were seen.

4.3.5 dsRNA extraction

When products of the CF-11 cellulose dsRNA extraction protocol were run on gel electrophoresis multiple bands of dsRNA could be seen only in samples from virus enrichment V19 and in the positive control sample from NCPF7367 (Figure 4-12).

Figure 4-4: A typical plaque assay test plate inoculated with *A. fumigatus* strain NCPF7097. Note the non-uniform growth pattern. The lack of a confluent lawn of growth made this technique inappropriate for identifying mycoviruses by the plaque assay method.
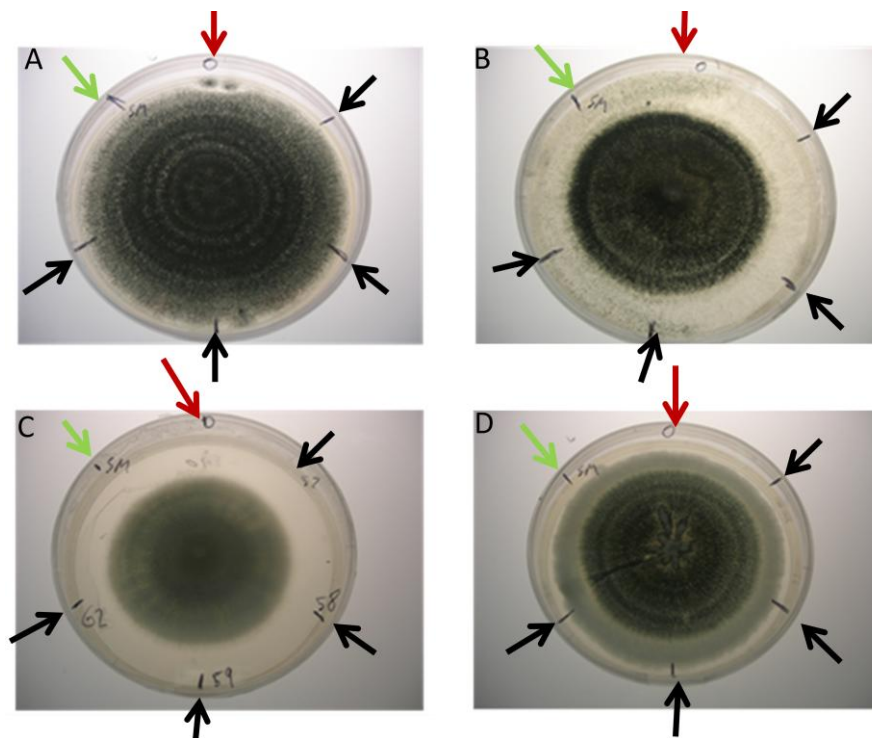
Figure 4-5: Four preliminary virus challenge tests shown 72 hours post virus challenge. The point of virus suspension challenge is shown by black arrows. SM control challenge is shown by green arrows and red arrows denote no-challenge control sectors. None of the virus challenge sectors show altered growth relative to the control sectors. *A. fumigatus* strains used were A) compost strain 'Af30', B) compost strain 'BGC' C) compost strain 'Af70' D) clinical strain NCPF7097.
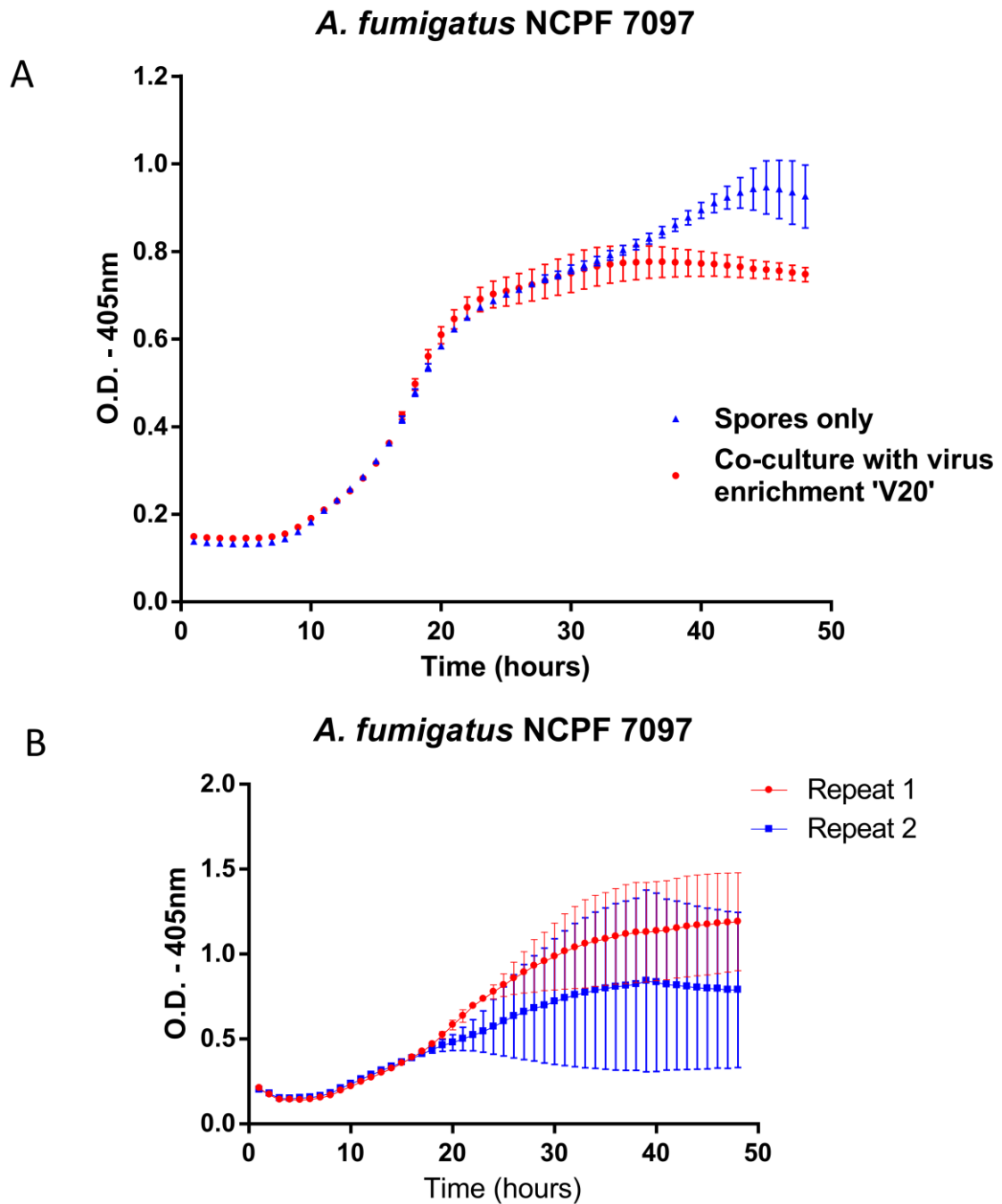
Figure 4-6: Growth curves produced from data obtained by the plate reader assay. A) A successful attempt with good reproducibility amongst the three repeats for each condition. This data would suggest different growth dynamics of the host fungus (*A. fumigatus* NCPF7097) when grown in co-culture with virus enrichment 'V20'. B) A typical further test of the assay, showing the very high variability between repeats. This is more typical of the data obtained, and the lack of reproducibility rendered this method unsuitable for screening the collection of enrichments for host effects.
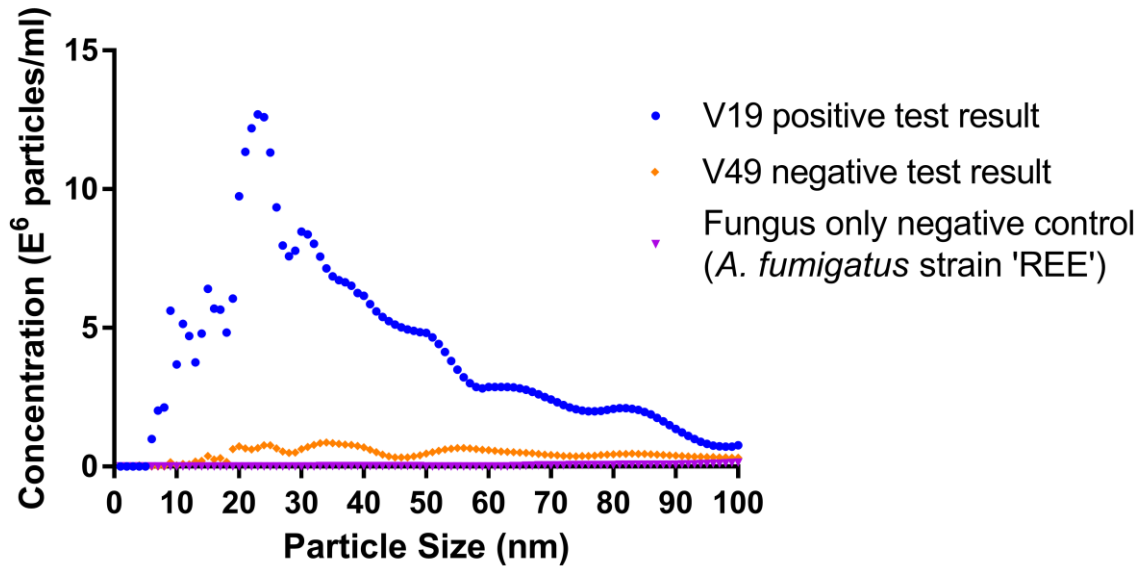
Figure 4-7: An example particle size distribution graph plotted from nanosight data. Shown in this example is data from one virus enrichment (blue) which contains an abundance of particles within the mycovirus size range of 25-40 nm, a virus enrichment (orange) which contained no such abundance of particles and a fungal control (purple) with no abundance of particles within the relevant size range.
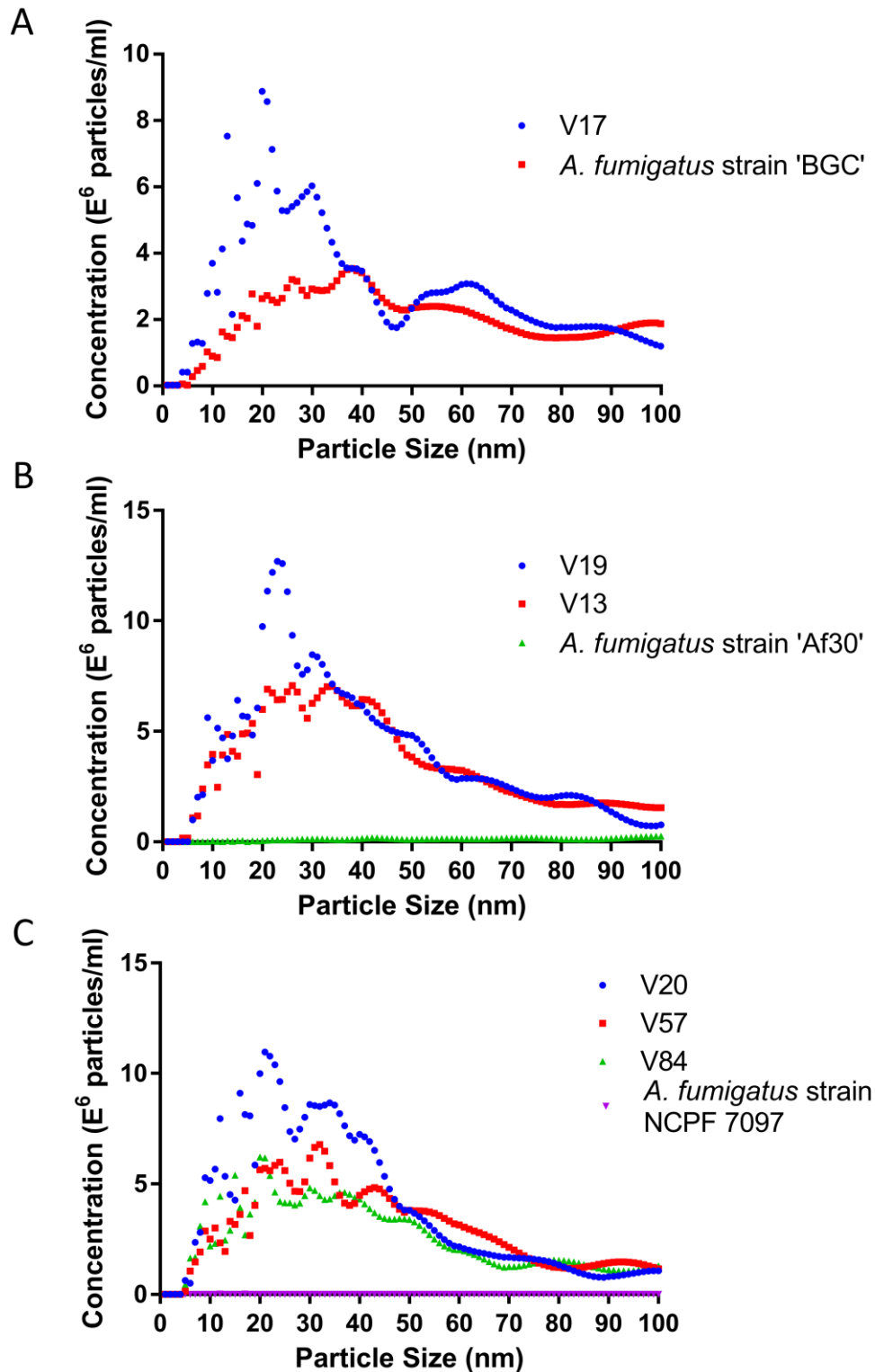
Figure 4-8: Particle size distribution profiles showing the 6 virus enrichments which produced significant peaks of particle abundance within the expected size range of mycovirus particles. Each of the virus enrichments is shown relative to the profile obtained from the fungal strain in which the enrichment was produced as a reference 'background' control. A) Enrichment V17, B) V13 and V19, C) V20, V57 and V84.

Re-enrichment of virus enrichment V19

- Af30 (fungus only)
- Af30+30µl V19
- Af30+300µl V19
- Af30+300µl V19 (repeat B)
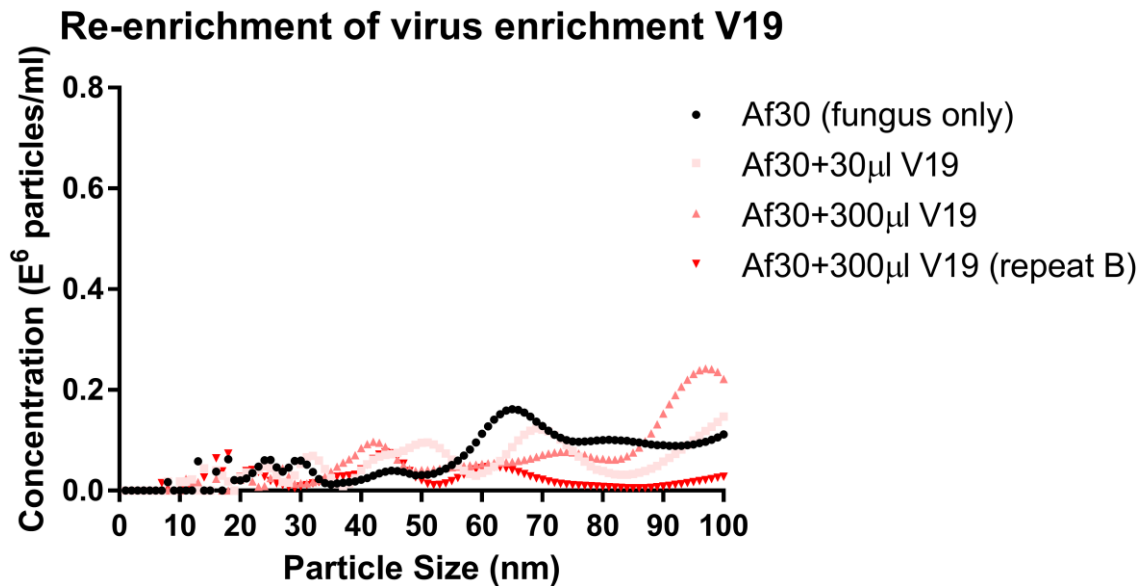
Figure 4-9: Particle size profiles for dose-response re-enrichment attempts. 30 µl and 300 µl of virus enrichment 'V19' was added to cultures of *A. fumigatus* 'Af30'. There was no abundance of particles within the size range of mycovirus particles observed following co-culture.

Timecourse re-enrichment test

- 48hrs
- 72hrs
- 96hrs
- 120hrs
- 144hrs
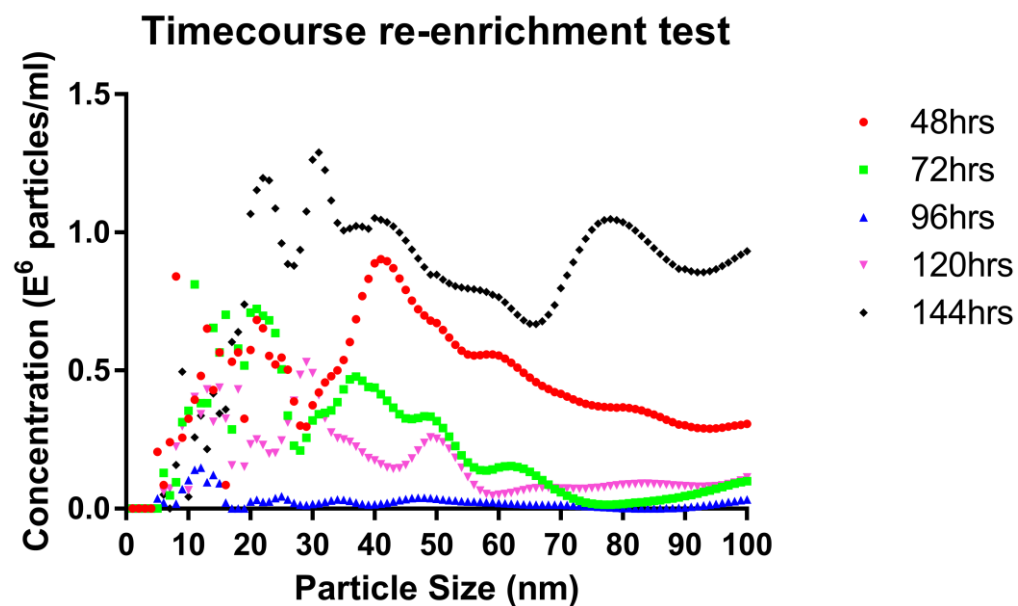
Figure 4-10: Particle size distribution curves for *A. fumigatus* strain Af30 inoculated with virus enrichment V19. Samples were removed from the co-culture over a 144 hour period. No significant peak in particle concentration around the expected virus particle diameter at any time point suggests that inability to reproduce the viral particle concentrations isn't due to an inefficiency with the 48 hour time point routinely used. Note the scale on the Y-axis covers a considerably lower range of particle concentration.
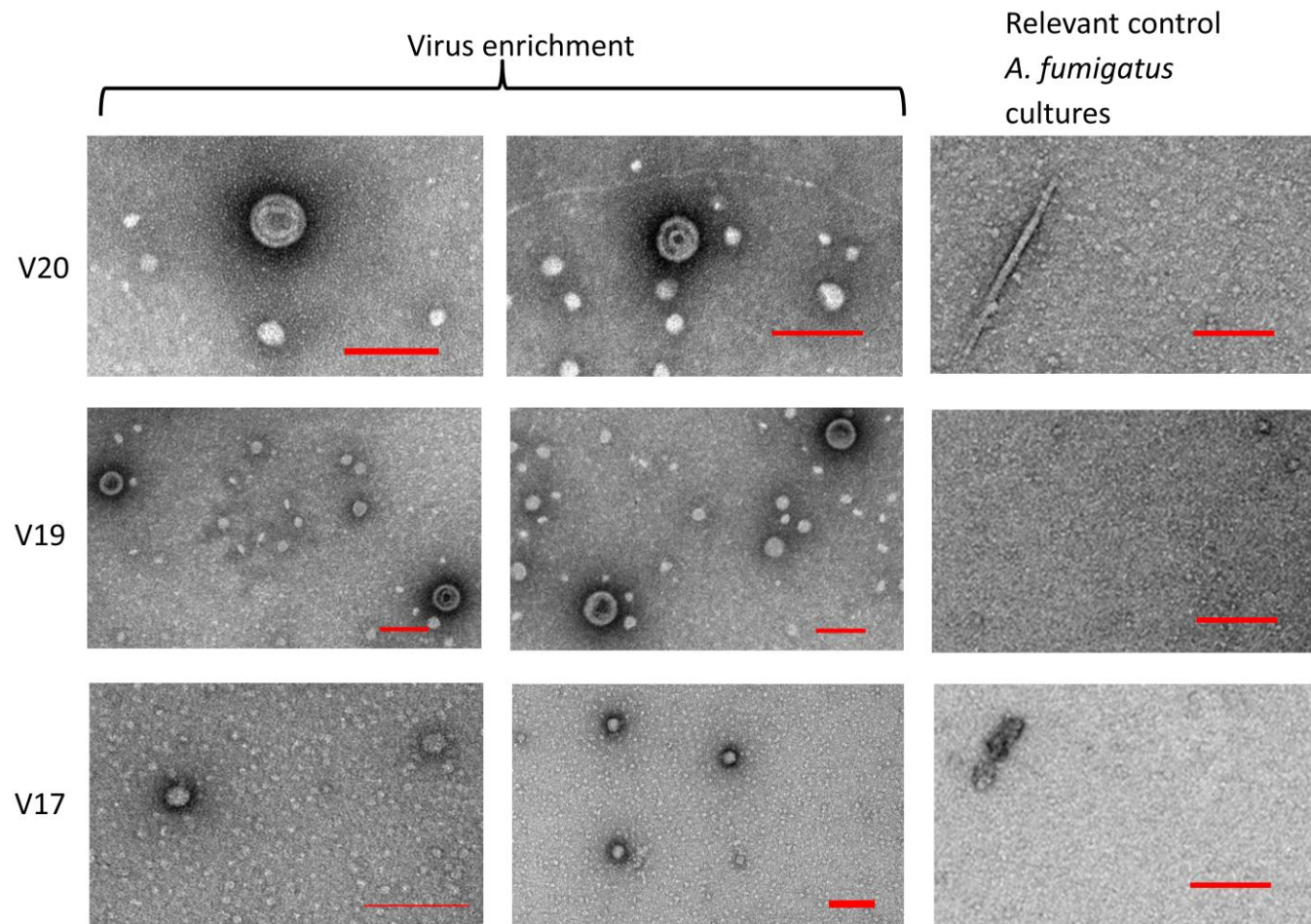
Figure 4-11: Representative electron micrographs of virus enrichment solutions V17, V19 and V20, with samples from their respective control *A. fumigatus* cultures. The bars represent 100 nm in each image.
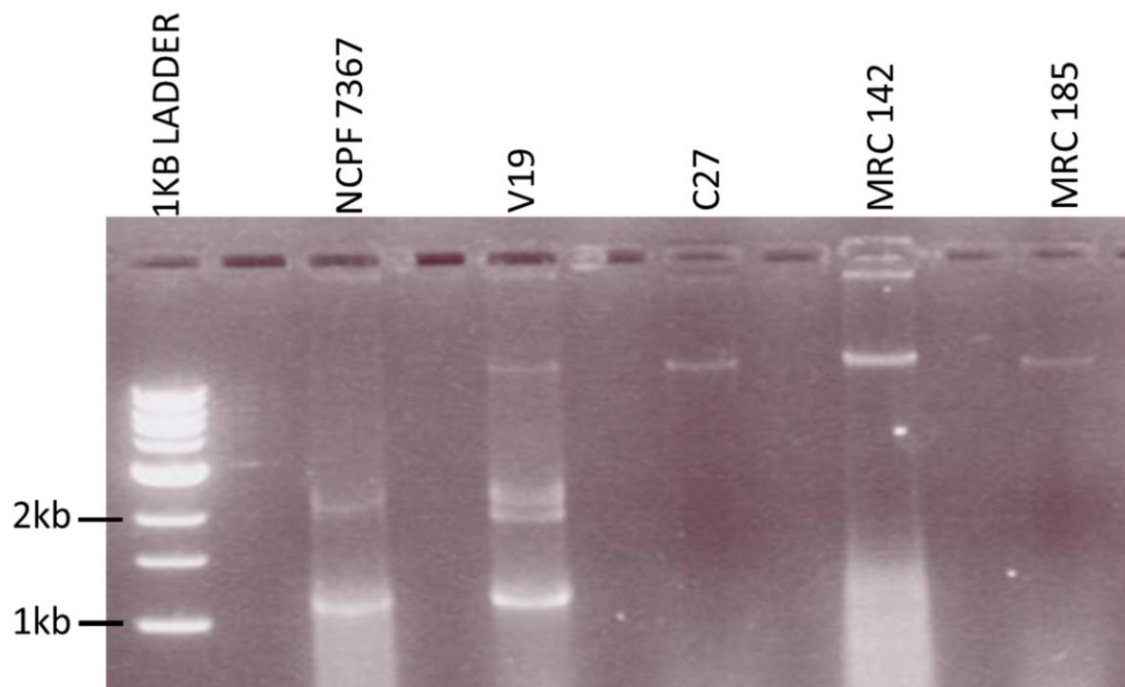
Figure 4-12: Electrophoresis gel showing samples obtained by the CF-11 cellulose dsRNA extraction protocol. *A. fumigatus* strain NCPF7367 and the virus enrichment sample V19 both contain clear bands of dsRNA between 1kb and 2.5kb in length. The other three samples C27, MRC142 and MRC185 are other *A. fumigatus* strains and appear not to carry dsRNA.
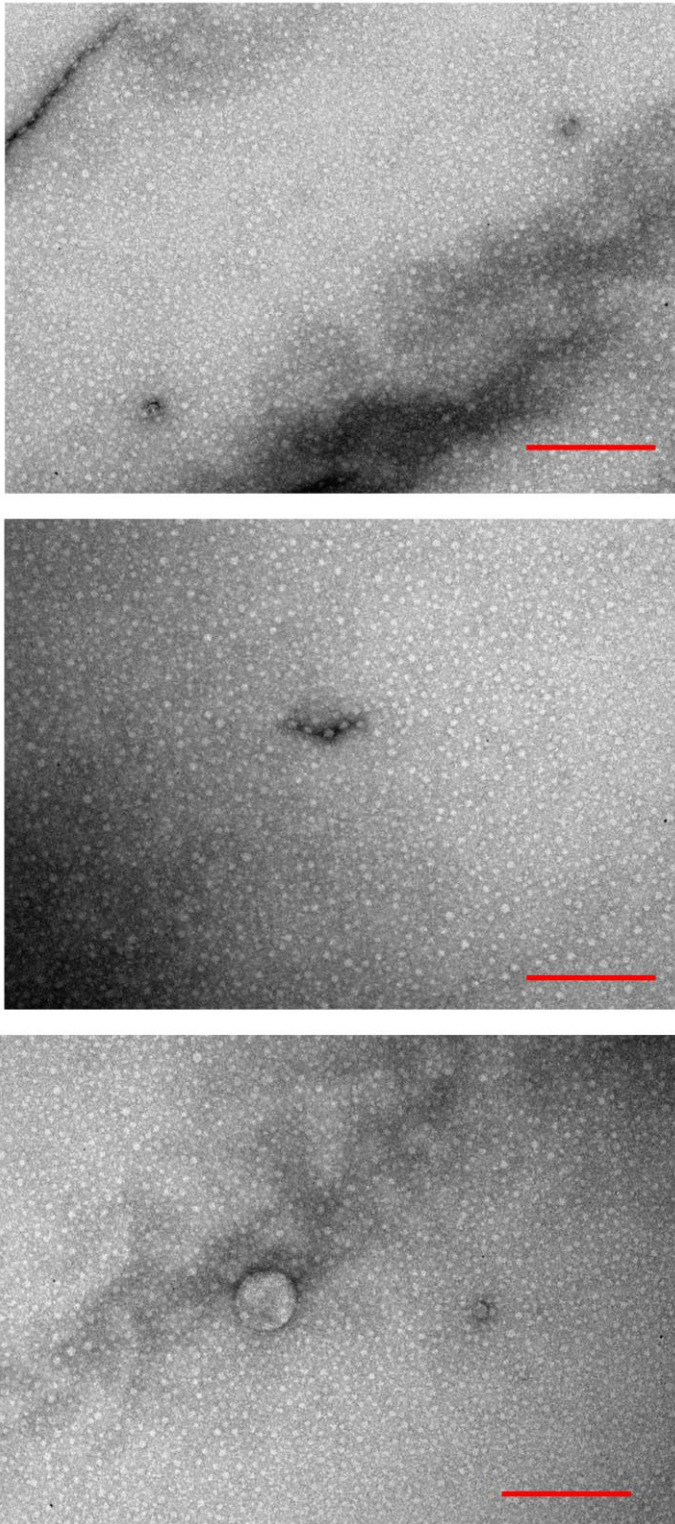
Figure 4-13: Electron micrographs of cell-free extract of the virus-carrying *A. fumigatus* strain NCPF7367. No viral particles were observed. The bar represents 200 nm in each image.

4.3.6 dsRNA extraction kit tests

Neither of the RNA extraction kits tested was able to detect dsRNA in the virus-infected *A. fumigatus* strain NCPF7367 (Figure 4-14). NCPF7367 was the positive (virus-carrying) control and NCPF7097 was the negative (virus-free) control. The gel electrophoresis photographs along with the nanodrop data for these two *A. fumigatus* strains show no clear isolation of dsRNA from the samples. There was also a relatively high concentration of DNA present in the extraction products of both strains, despite a DNase treatment step in both protocols.

4.3.7 *A. fumigatus* NCPF7367 virus particle purification

Following ultracentrifugation through a sucrose density gradient, the aliquots removed sequentially from the column were analysed for nucleic acid content with the nanodrop system and protein content using the Bradford assay. The data for these assays for each of the fractions of the sucrose gradient are shown in Figure 4-15. There was no fraction which contained an abundance of both protein and nucleic acid.

4.3.8 Cross-sectional TEM

Cross-sectional TEM of the virus-carrying isolate of *A. fumigatus* NCPF7367 showed no obvious viral particles. The cells remained intact throughout the processing and the resin had embedded throughout the sample effectively. The staining was uniform and gave sufficient contrast. Cell organelles could be seen, as could the septa between individual hyphal cells. No virus-like particles were observed in either strain. Representative images are shown in Figure 4-16 toFigure 4-18.
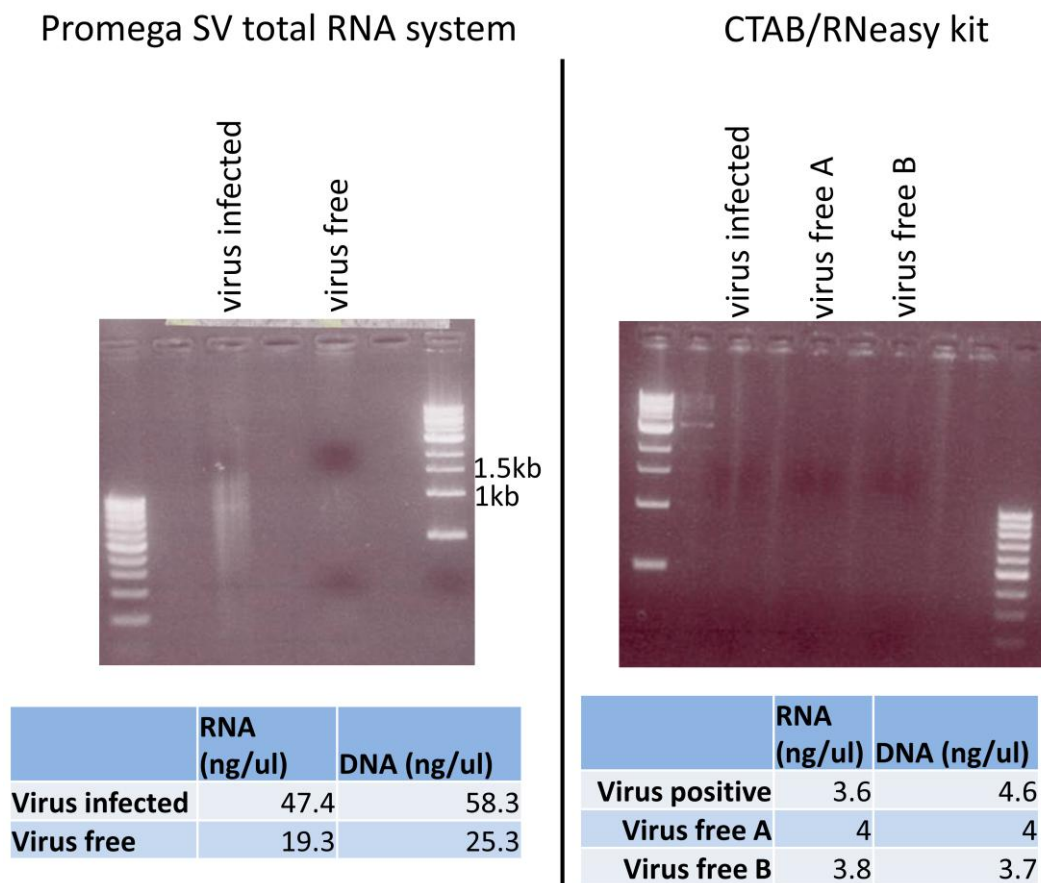
Figure 4-14: Gel electrophoresis photographs of the products of the SV total RNA isolation kit (Promega) and the CTAB modification of the RNeasy RNA extraction kit (QIAGEN). Note the lack of any clear bands in the gel photographs. The data from analysis of these samples with the nanodrop system is also shown, again there is no clear isolation of RNA in the positive control relative to the virus-free negative controls, and a considerable concentration of DNA in the samples.
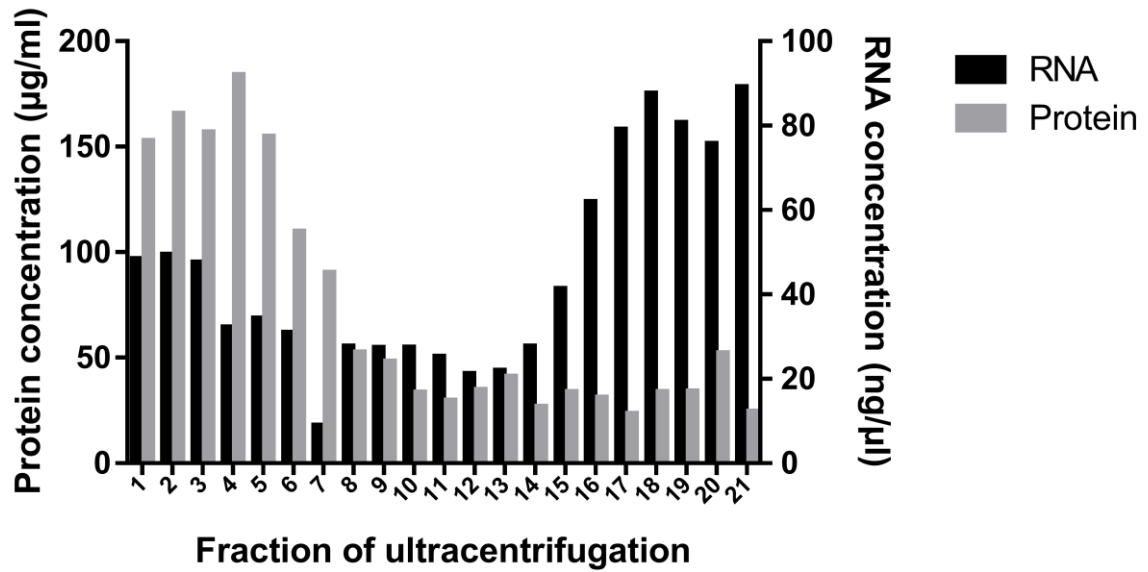
Figure 4-15: RNA and protein concentration of the 21 aliquots removed from the column of sucrose gradient following density gradient ultracentrifugation of the putative virus suspension obtained from the virus infected strain NCPF7367. None of the gradient fractions contains an abundance of both protein and nucleic acid.

Figure 4-16: Transmission electron micrographs taken through cross-sections of fungal hyphae of the virus-carrying *A. fumigatus* strain NCPF7367. There are no virus particles seen. The bar in the upper image represents 2 μm and in the lower image the bar represents 1 μm.
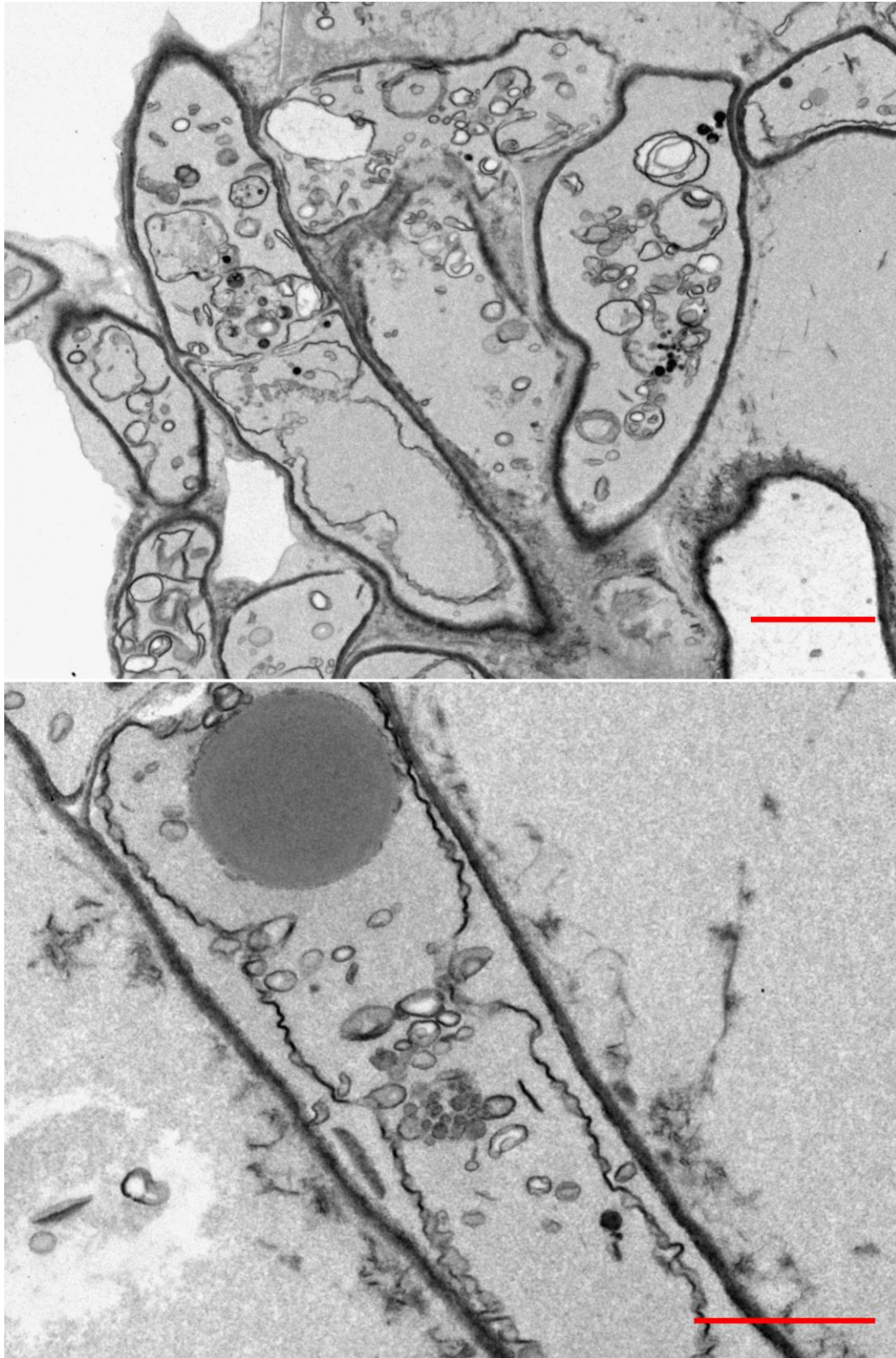
Figure 4-17: Transmission electron micrographs taken through cross-sections of fungal hyphae of the virus-free *A. fumigatus* strain NCPF7097. There are no virus particles seen. The bar in the upper image represents 2µm and in the lower image the bar represents 1µm.
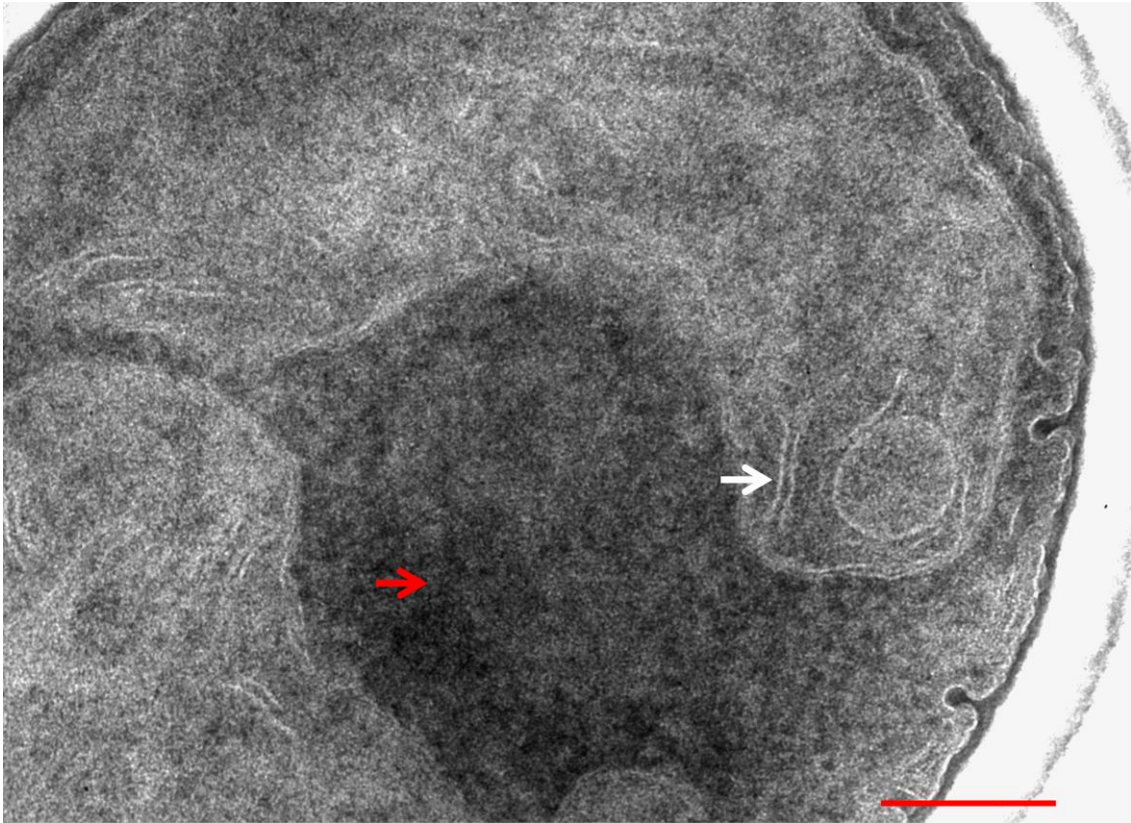
Figure 4-18: Higher magnification electron micrograph typical of the cross sections through cells of the virus-carrying *A. fumigatus* strain NCPF7367. Mitochondrial membranes can be seen (white arrow) and the cytoplasm appears slightly granulated (red arrow) but there are no visible viral particles. The bar represents 200 nm.

4.3.9 *A. fumigatus* **NCPF7367 dsRNA sequence**

The sequence data obtained from the virus genome isolated from NCPF7367 showed the genome structure of this virus to consist of four distinct genome segments. The sizes of these segments are 2309, 2195, 1920 and 1301 base pairs which roughly concurs with the banding pattern observed on gel electrophoresis following the dsRNA extraction from this strain (Figure 4-12).

The sequences of these four genome segments, aligned with the four segments of the viral genome obtained from *A. fumigatus* strain NCPF 7367 and published by Kanhayuwa *et al*. (2015) are shown in figures 4-19 to 4-22. The genome sequence data obtained in the present study for dsRNA segment 1 featured 99% identity to the segment 1 sequence published by Kanhayuwa *et al.* (2015) with 100% coverage of the sequence. Segments 2 and 3 both matched with 99% identity with 99% coverage and segment 4 was a 99% identity match but with only 86% coverage, due to the considerably longer 5' UTR in in our sequence data.

Analysis of this genome sequence data using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the largest of the four fragments encodes an RNA-dependent RNA polymerase (RDRP) enzyme, a typical feature of dsRNA viruses. The next largest genome segment features a region with homology to a putative temperature tolerance-associated domain. Segment 3 features a putative methyl transferase domain. The product and function of the smallest of the fragments is unclear. Notably, none of the fragments encode a protein with homology to any structural genes (i.e. capsid proteins).

An outline schematic of the genome segments, showing the size and position of the open reading frames (ORFs) and the putative protein domains mentioned above is shown in figure 4-23.The amino acid sequence of each of the ORFs, aligned to the previously published sequences for which there was similarity as shown by the BLAST analysis are shown in figures 4-24 to 4-27.

At the amino acid sequence level, there was 99% identity over 100% coverage of all of the ORFs derived from our sequences compared to the sequences published by

Kanhayuwa *et al.* (2015). The identity and coverage statistics for the BLAST analysis of the protein sequence of each ORF (as shown in figures 4-24 to 4-27) is summarised in table 4-1.

Table 4-1: Summary of the identity and coverage statistics of the BLAST searches of the amino acid sequence alignments of the four segments shown in figures 4-24 to 4-27.

Segment	Sequence	% Identity	% Coverage
Segment 1	NCPF7367 virus (Kanhayuwa *et al.* , 2015)	99	100
	BDV-1	54	98
	CCV-1	43	99
	A. tenusissima virus	49	62
Segment 2	NCPF7367 virus (Kanhayuwa *et al.* , 2015)	99	100
	BDV-1	46	99
	CCV-1	32	97
	Alternaria sp. viral protein	39	99
Segment 3	NCPF7367 virus (Kanhayuwa *et al.* , 2015)	99	100
	BDV-1	43	94
	CCV-1	26	90
	M. lini virus	40	91
Segment 4	NCPF7367 virus (Kanhayuwa *et al.* , 2015)	99	100
	BDV-1	54	88
	CCV-1	35	87

```
              *        20         *        40         *        60         *        80         *        100        *        120
A :  ------------------------------------------------------------------------------------AGTCGAACATGACGCCCTCCTGATCAGTGTCGTGGAGGTACTGACACC :  48
B :  CCCCCGCCCGGCGGGGGGGGCACGCGGATCGACAACGCGCGCCCCTCCCGCCGGGCCGGCTTGTGTGCGGTGATTAGTCGAACATGACGCCCTCCTGATCAGTGTCGTGGAGGTACTGACACC : 126

       *        140        *        160        *        180        *        200        *        220        *        240
A :  ACTTCTCAACGGTACATTCATAAGCCCACTGAGCGGCGGGCGTCAGCTCCCCGGTGAAGAGGTGCATGCCGGTGGAGACCGAGGTGTGCAGGCGTCCCGGGTCGTGGTCGCCGTCTCGTGGGGTGT : 174
B :  ACTTCTCAACGGTACATTCATAAGCCCACTGAGCGGCGGGCGTCAGCTCCCCGGTGAAGAGGTGCATGCCGGTGGAGACCGAGGTGTGCAGGCGTCCCGGGTCGTGGTCGCCGTCTCGTGGGGTGT : 252

       260        *        280        *        300        *        320        *        340        *        360        *        3
A :  GGAGCGAGAGCAGGGCGCGCCGGTCGATGACGGTCTCGGAGAGCGCCCTGTAGACGGCGAGCAGCGCGTCCTGTTCGCGGTTCCCGAACATGCTCTGGGCCGTGTCCTCGAGGACTTTCTTGGCGG : 300
B :  GGAGCGAGAGCAGGGCGCGCCGGTCGATGACGGTCTCGGAGAGCGCCCTGTAGACGGCGAGCAGCGCGTCCTGTTCGCGGTTCCCGAACATGCTCTGGGCCGTGTCCTCGAGGACTTTCTTGGCGG : 378

       80         *        400        *        420        *        440        *        460        *        480        *        500
A :  CGTCGGTGAGACCGGCGGCCTCGAGGGAGACATTGAGGGACCGGTCAATCTCATTCAGGATGGCGTAGACAATGGGGTCTGTGCCCATGTGGTCGACCATCATTGCGCGCACGTAGAGCTTGTCGA : 426
B :  CGTCGGTGAGACCGGCGGCCTCGAGGGAGACATTGAGGGACCGGTCAATCTCATTCAGGATGGCGTAGACAATGGGGTCTGTGCCCATGTGGTCGACCATCATTGCGCGCACGTAGAGCTTGTCGA : 504

       *        520        *        540        *        560        *        580        *        600        *        620        *
A :  CTACTGGGTCGTGACGGGGCCGTCCGGCGAGTTTGGCGAACAGGGAGTTGTGGCTGCGCGTAATGAGAAGACCATGTGGCGTCCTGACCAAGGACCGGGAGCAGAACCGCACGCGCCCAAGCTTGA : 552
B :  CTACTGGGTCGTGACGGGGCCGTCCGGCGAGTTTGGCGAACAGGGAGTTGTGGCTGCGCGTAATGAGAAGACCATGTGGCGTCCTGACCAAGGACCGGGAGCAGAACCGCACGCGCCCAAGCTTGA : 630

       640        *        660        *        680        *        700        *        720        *        740        *
A :  CAGTGACCTCAGTCTCATCCACCTTAAGCTTCATGCCGAAACGAGCGAGGAACTCGGCATGCTTTTCAGGGTCGTAGGCGAGGCCCAGCACTGAGAAGGGTGCTCAGGAAGAGCTGGTTGTCTC : 678
B :  CAGTGACCTCAGTCTCATCCACCTTAAGCTTCATGCCGAAACGAGCGAGGAACTCGGCATGCTTTTCAGGGTCGTAGGCGAGGCCCAGCACTGAGAAGGGTGCTCAGGAAGAGCTGGTTGTCTC : 756

       760        *        780        *        800        *        820        *        840        *        860        *        880
A :  CGTAAGAGATGAGAGTGCCATCGACCGCATCGGCGAGAAGCTCGGCTGCGCCATGGTGCGTAAGTGAGGTCTCCTCGACGGCCCGTGCTATGGCGTGACCTATGACCAAGAGGTTGTTCCCCGTAG : 804
B :  CGTAAGAGATGAGAGTGCCATCGACCGCATCGGCGAGAAGCTCGGCTGCGCCATGGTGCGTAAGTGAGGTCTCCTCGACGGCCCGTGCTATGGCGTGACCTATGACCAAGAGGTTGTTCCCCGTAG : 882

       *        900        *        920        *        940        *        960        *        980        *        1000
A :  TGTTGACAAGCGAGGTGTAGAAGGAACCGCTTGGCATGCACCCGTAGAGCTTGACGAAGTACCCCGCGGGATGCTCGACGAGCTTCTCAAGGAGTTGCCGCCGGGCGACCCTGTACATCAGGCCCC : 930
B :  TGTTGACAAGCGAGGTGTAGAAGGAACCGCTTGGCATGCACCCGTAGAGCTTGACGAAGTACCCCGCGGGATGCTCGACGAGCTTCTCAAGGAGTTGCCGCCGGGCGACCCTGTACATCAGGCCCC : 1008

       *        1020        *        1040        *        1060        *        1080        *        1100        *        1120        *
A :  TCGTAGCGAGGCCCTCAGGGGACAATGCAGACTTGTCGACGGCGTCCTCGAGGAGGTCAATGGAGTACTTGATGAGCGCCTCGCACATGTTGGCATCCCACTTGGTAATGTCGCTCATGATGAGCC : 1056
B :  TCGTAGCGAGGCCCTCAGGGGACAATGCAGACTTGTCGACGGCGTCCTCGAGGAGGTCAATGGAGTACTTGATGAGCGCCTCGCACATGTTGGCATCCCACTTGGTAATGTCGCTCATGATGAGCC : 1134

       1140        *        1160        *        1180        *        1200        *        1220        *        1240        *        1260
A :  GCGCTCCGTCGGGAGTGGAGAAGCGCCCGTCGAAAGCCCTCTCAACCAGGTCCATGGAATTTCCTCGCCCTGCCCCTGCCGGGTCCGAACCCAGGTCCGTGGGTGGGGTCGTTGTCCATGAGGAAGT : 1182
B :  GCGCTCCGTCGGGAGTGGAGAAGCGCCCGTCGAAAGCCCTCTCAACCAGGTCCATGGAATTTCCTCGCCCTGCCCCTGCCGGGTCCGAACCCAGGTCCGTGGGTGGGGTCGTTGTCCATGAGGAAGT : 1260

       *        1280        *        1300        *        1320        *        1340        *        1360        *        1380
A :  GGGAAATGTCGTAGTGGAGGAAGCTGGCGAGGGCGTAGTTCACCGGTGAAAGGTTGAATATAAAGCGCGGGACGGGCGCTTGGCGAACTCCGTCGACGATCTTGGCTGCTTTAGGCTCGCGCTTGC : 1308
B :  GGGAAATGTCGTAGTGGAGGAAGCTGGCGAGGGCGTAGTTCACCGGTGAAAGGTTGAATATAAAGCGCGGGACGGGCGCTTGGCGAACTCCGTCGACGATCTTGGCTGCTTTAGGCTCGCGCTTGC : 1386

       *        1400        *        1420        *        1440        *        1460        *        1480        *        1500        *
A :  CGAACGTGAGCGTGACCTGGGTCGTGGTGTGTCAACCCAACCTGGGGGGGGGGCGCCCGCGCGCCACCTGGACCCCCGCGCTGGCGTACCGAAGGAGGGGAGGAGGACATGGTGGCGATGAGGCGGGGGT : 1434
B :  CGAACGTGAGCGTGACCTGGGTCGTGGTGTGTCAACCCAACCTGGGGGGGGGGCGCCCGCGCGCCACCTGGACCCCCGCGCTGGCGTACCGAAGGAGGGGAGGAGGACATGGTGGCGATGAGGCGGGGGT : 1512

       1520        *        1540        *        1560        *        1580        *        1600        *        1620        *        16
A :  CACGTCGGTCCTCGGCGCCGAGTGAGCGATATTCCCCGGCGACCCCTTCTTTATGATGGCAAGGAGATTGTCAGGCTGCGCATCGGAGAGGTCCTGGCCTTGACCTTGCAGCCCTTCCTTCGCC : 1560
B :  CACGTCGGTCCTCGGCGCCGAGTGAGCGATATTCCCCGGCGACCCCTTCTTTATGATGGCAAGGAGATTGTCAGGCTGCGCATCGGAGAGGTCCTGGCCTTGACCTTGCAGCCCTTCCTTCGCC : 1638

       40         *        1660        *        1680        *        1700        *        1720        *        1740        *        1760
A :  AAAGCCGGGCGAAGACGGTCTGGGCGGCACGCATGGCGTCATCGGTGGGCCCGTCGACACTGCGCGTGCGCTCATGGACATACTCGAGGAAGCGCGGGTGGAGCGTGTCCGGGTCATGAGTGGTGA : 1686
B :  AAAGCCGGGCGAAGACGGTCTGGGCGGCACGCATGGCGTCATCGGTGGGCCCGTCGACACTGCGCGTGCGCTCATGGACATACTCGAGGAAGCGCGGGTGGAGCGTGTCCGGGTCATGAGTGGTGA : 1764

       *        1780        *        1800        *        1820        *        1840        *        1860        *        1880        *
A :  AGTCGCCGAGGTCAATGGACTCCTTGGCACGTTTCCTGTAACCCACCGGGTCGCGGGTGCGCTGGGAGACCTTGTTAATCGCCGAGATTAGAGGAGCGGTGAGGGGCCTCTCAGGGTTGGGGGGTC : 1812
B :  AGTCGCCGAGGTCAATGGACTCCTTGGCACGTTTCCTGTAACCCACCGGGTCGCGGGTGCGCTGGGAGACCTTGTTAATCGCCGAGATTAGAGGAGCGGTGAGGGGCCTCTCAGGGTTGGGGGGTC : 1890

       1900        *        1920        *        1940        *        1960        *        1980        *        2000        *
A :  CGCCGCCAAAGGAGTAGGGGTGGGGAGCTGGTACTGCGAGATGTGGAACGTCGGGTCCTAAGTTTGGAGGCCTTTTCCACGCAGCGTCGAGACGGTGAGAGTCGCGGACGAAGGCACGGGGCC : 1938
B :  CGCCGCCAAAGGAGTAGGGGTGGGGAGCTGGTACTGCGAGATGTGGAACGTCGGGTCCTAAGTTTGGAGGCCTTTTCCACGCAGCGTCGAGACGGTGAGAGTCGCGGACGAAGGCACGGGGCC : 2016

       2020        *        2040        *        2060        *        2080        *        2100        *        2120        *        2140
A :  CGAGCTCGCTGGCGTTTTTGGCACCATCGAACTGCCTGAGTTCGCGCAACAAGTCCCCATCAGCATAGGACTTGAGCCGAAAGGCGCGGGCGCGCGCTTGTGGGGCCGGACGACGGGATCGGAAC : 2064
B :  CGAGCTCGCTGGCGTTTTTGGCACCATCGAACTGCCTGAGTTCGCGCAACAAGTCCCCATCAGCATAGGACTTGAGCCGAAAGGCGCGGGCGCGCGCTTGTGGGGCCGGACGACGGGATCGGAAC : 2142

       *        2160        *        2180        *        2200        *        2220        *        2240        *        2260
A :  CAGTGGGGACACCGTGGACCGGGACGGAGATGGGGGCGGAGGTTGGCCGACGACGAAAGACATTGTGTGATTGACCTGCAGTAAGCCACCCTTGAGGGTGCGGCGGGCGTACCACGAGACAATGT : 2190
B :  CAGTGGGGACACCGTGGACCGGGACGGAGATGGGGGCGGAGGTTGGCCGACGACGAAAGACATTGTGTGATTGACCTGCAGTAAGCCACCCTTGAGGGTGCGGCGGGCGTACCACGAGACAATGT : 2268

       *        2280        *        2300        *        2320        *        2340        *        2360        *        2380        *
A :  CGGAGACGTAGGAGGCGGTAGGGGCGGGAGCGTTGCTGGGCGCACCGCTGACGACGAGTTTCAAGGTCTCTGGGACCCCTATATCGGGATTGGCGACATTGGAGCACACAAGCGGAGA------- : 2309
B :  CGGAGACGTAGGAGGCGGTAGGGGCGGGAGCGTTGCTGGGCGCACCGCTGACGACGAGTTTCAAGGTCTCTGGGACCCCTATATCGGGATTGGCGACATTGGAGCACACAAGCGGAGAAGTTTCC : 2394

       2400
A :  -------- :   -
B :  TTTTATTCC : 2403
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Figure 4-19: Sequence alignment dsRNA segment 1. Differences in sequences are shown by red lettering. Note the larger 5' and 3' UTR regions in sequence B. A) NCPF7367 virus sequence data obtained in the present study, B) NCPF7367 virus sequence data published in Kanhayuwa *et al.*, 2015 (Genbank accession HG975302).

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              *        20         *        40         *        60         *        80         *       100         *
A : ------------TTTGTTCTGGGCAAGCACGCTGCAACGTCGAGCAACTCCCATATAGGGGACGATGAGACATGGCGGACCTAACCCGTCTTAGGAATATTGTTCTGTCGGGC : 101
B : TGAATAAAAGATTTGTTCTGGGCAAGCACGCTGCAACGTCGAGCAACTCCCATATAGGGGACGATGAGACATGGCGGACCTAACCCGTCTTAGGAATATTGTTCTGTCGGGC : 112

        120         *       140         *       160         *       180         *       200         *       220
A : GATATGAAAGTCGCCCTCACTGCAGCAATTCTGTACCACCTCTCCCCCAACCCCTCCGCGATCACCGATTACGGGGTGGATGAGGTCAAGAGCGTGGTGTCATGGCTCGTCT : 213
B : GATATGAAAGTCGCCCTCACTGCAGCAATTCTGTACCACCTCTCCCCCAACCCCTCCGCGATCACCGATTACGGGGTGGATGAGGTCAAGAGCGTGGTGTCATGGCTCGTCT : 224

              *       240         *       260         *       280         *       300         *       320         *
A : CCTCGCCCGGGATGTTCTACGGCGACTTGTTGCCGCCACTGGAATCACCCCTGACATCCTCTCTACTTTCCTTCCCGCGCTCGATGATCAAGCCTGCGCCGACGCCGCCTC : 325
B : CCTCGCCCGGGATGTTCTACGGCGACTTGTTGCCGCCACTGGAATCACCCCTGACATCCTCTCTACTTTCCTTCCCGCGCTCGATGATCAAGCCTGCGCCGACGCCGCCTC : 336

        340         *       360         *       380         *       400         *       420         *       440
A : TCTTGCCAACGCCATGGCGACCCCCGATGCCGAGTGGGACCGCGAGCACCGCAACCGTTTCACGTCCGCACTCGCGCACCACTCCGCCGACGCTGCTAGGCTCGCCGCCCAG : 437
B : TCTTGCCAACGCCATGGCGACCCCCGATGCCGAGTGGGACCGCGAGCACCGCAACCGTTTCACGTCCGCACTCGCGCACCACTCCGCCGACGCTGCTAGGCTCGCCGCCCAG : 448

              *       460         *       480         *       500         *       520         *       540         *       560
A : TCGTCGGCTGAGTTCGCTGCGGTCGACGCCATGGAAGAGGCTGGCTACGAGAATCAGGACCACGCTCGCACCGCGGTCAACGAGCTCAACCGCGTCTATGGCAATACCTACC : 549
B : TCGTCGGCTGAGTTCGCTGCGGTCGACGCCATGGAAGAGGCTGGCTACGAGAATCAGGACCACGCTCGCACCGCGGTCAACGAGCTCAACCGCGTCTATGGCAATACCTACC : 560

              *       580         *       600         *       620         *       640         *       660         *
A : GGGTCTTCACTCGTCAACACGCCCGCTCCCGCGTGGCCATCGCCGCCTCTTTCAAGATCGATGACCACGGCACCCTCTTCCACGTCCTCACCCCCTGGTGCCTCTCCCGAAC : 661
B : GGGTCTTCACTCGTCAACACGCCCGCTCCCGCGTGGCCATCGCCGCCTCTTTCAAGATCGATGACCACGGCACCCTCTTCCACGTCCTCACCCCCTGGTGCCTCTCCCGAAC : 672

        680         *       700         *       720         *       740         *       760         *       780
A : ATCGGCCATGATCTACGTCGCTGTCATCGCCAGGCATGTGAGGCGTCGCCACATCGAGGAGCGCGCCTCCAGATTGGCACGCACTGCCCTGACCTACGCCGCTCGCGCCGGC : 773
B : ATCGGCCATGATCTACGTCGCTGTCATCGCCAGGCATGTGAGGCGTCGCCACATCGAGGAGCGCGCCTCCAGATTGGCACGCACTGCCCTGACCTACGCCGCTCGCGCCGGC : 784

              *       800         *       820         *       840         *       860         *       880         *
A : TTAGAGGCTGTACGCGCCATCATAGAGCCCGCCATTGCCATAGTGACAAGTTTCCGTTACGACCCTGCCAAGATCGCCTTCGTGGACGATTTTGACCGGGTCAGGGAGTCCA : 885
B : TTAGAGGCTGTACGCGCCATCATAGAGCCCGCCATTGCCATAGTGACAAGTTTCCGTTACGACCCTGCCAAGATCGCCTTCGTGGACGATTTTGACCGGGTCAGGGAGTCCA : 896

        900         *       920         *       940         *       960         *       980         *      1000
A : TCACTACGAGGAGTCCTCCAACGCTTGTCGCCTTCGCTTGCGTCCATATGTGCGGGAACCAGGACGTCGTTTCCTTGGCGGCGCGACTCCGCATGGAGTCGCTACCACAACT : 997
B : TCACTACGAGGAGTCCTCCAACGCTTGTCGCCTTCGCTTGCGTCCATATGTGCGGGAACCAGGACGTCGTTTCCTTGGCGGCGCGACTCCGCATGGAGTCGCTACCACAACT : 1008

              *      1020         *      1040         *      1060         *      1080         *      1100         *      1120
A : CCATGAGACCCCGCCAGCCGTCGCCGAGTACACCGACCACGTCGACGACGAACAGTCCGCCCTCCTCATGTTCTCTCGCGTCCTCAAGAGACTGAGACATGAGCGCGACAGG : 1109
B : CCATGAGACCCCGCCAGCCGTCGCCGAGTACACCGACCACGTCGACGACGAACAGTCCGCCCTCCTCATGTTCTCTCGCGTCCTCAAGAGACTGAGACATGAGCGCGACAGG : 1120

              *      1140         *      1160         *      1180         *      1200         *      1220         *
A : GGGGTGCACGGCCCCCGCCTCAACAAGGATCCCATGCTCGGCTACATAGCCCGGGGAGTACGCCGTTCCGAGAGGAACACCATGGCGTGCATCAACAGGGTTGAGGAGGCCG : 1221
B : GGGGTGCACGGCCCCCGCCTCAACAAGGATCCCATGCTCGGCTACATAGCCCGGGGAGTACGCCGTTCCGAGAGGAACACCATGGCGTGCATCAACAGGGTTGAGGAGGCCG : 1232

        1240         *      1260         *      1280         *      1300         *      1320         *      1340
A : TGTCCTTTCTGCGCTCGAAGGGGGTCGACACCCTCAATACACTGTTCGTCATCGAATGGGGCGGTGAATTTGACTATCCTACCGTCATGGCTGCCCTCGCCGTTGCCAGGTT : 1333
B : TGTCCTTTCTGCGCTCGAAGGGGGTCGACACCCTCAATACACTGTTCGTCATCGAATGGGGCGGTGAATTTGACTATCCTACCGTCATGGCTGCCCTCGCCGTTGCCAGGTT : 1344

              *      1360         *      1380         *      1400         *      1420         *      1440         *
A : GGATGTCTGTGTCGACATTGGTCCCTCGGGCATTGACCTACCCGGCGCCGACGCCAAGGCCGAGGATGGCACCGAAGAGTACAACTACTCTTTGCTGCTCGCCTCAGCCGAG : 1445
B : GGATGTCTGTGTCGACATTGGTCCCTCGGGCATTGACCTACCCGGCGCCGACGCCAAGGCCGAGGATGGCACCGAAGAGTACAACTACTCTTTGCTGCTCGCCTCAGCCGAG : 1456

        1460         *      1480         *      1500         *      1520         *      1540         *      1560
A : GAGCGGCGCATGCCTCGTATGCCCAGGGTGCCCTATCCGTCCGACGAGCCCCTTGAGGCCAAACTGCGGCGCGTCGCGCAGTTCTACTCTCTCTCGGGCTCTGCCAATATAG : 1557
B : GAGCGGCGCATGCCTCGTATGCCCAGGGTGCCCTATCCGTCCGACGAGCCCCTTGAGGCCAAACTGCGGCGCGTCGCGCAGTTCTACTCTCTCTCGGGCTCTGCCAATATAG : 1568

              *      1580         *      1600         *      1620         *      1640         *      1660         *      1680
A : CATACTCAGCGGGGGGGTCACCCAAAAGAGGGGGTTCCTGTCAACATCTGTATCGACACCGCCAACAGGCTTAATGCCATACGCAATGTCACCCCGGACGTTAACGTCGT : 1669
B : CATACTCAGCGGGGGGGGTCACCCAAAAGAGGGGGTTCCTGTCAACATCTGTATCGACACCGCCAACAGGCTTAATGCCATACGCAATGTCACCCCGGACGTTAACGTCGT : 1680

              *      1700         *      1720         *      1740         *      1760         *      1780         *
A : TTATGCCTGCGCCGAAGCTCTCCTCCCCCCCCCTCTGCGGGCACGGACTCGAGCTCGGGCCCGACGACTACCTTCAGTGCGCGTCCCTGGTCGATGAGGATTGCCCCGCCTGC : 1781
B : TTATGCCTGCGCCGAAGCTCTCCTCCCCCCCCCTCTGCGGGCACGGACTCGAGCTCGGGCCCGACGACTACCTTCAGTGCGCGTCCCTGGTCGATGAGGATTGCCCCGCCTGC : 1792

        1800         *      1820         *      1840         *      1860         *      1880         *      1900
A : AATGCTCACTACCGCGCCACCGCCGTCCTCTCTGCTGCCTGCGCTGACAGACAGGTGCGCATCGTTAAGCCAAGATCCGCCTACGCTCATAACGGGCACCTGTCCTTGGAGC : 1893
B : AATGCTCACTACCGCGCCACCGCCGTCCTCTCTGCTGCCTGCGCTGACAGACAGGTGCGCATCGTTAAGCCAAGATCCGCCTACGCTCATAACGGGCACCTGTCCTTGGAGC : 1904

              *      1920         *      1940         *      1960         *      1980         *      2000         *
A : TCGTCCCGGGCCACCCTACATGCGTGGACGACACTCTCCTCACCGTCGACTCCATGGTCGCGGCCAATGTCCTCCGCAACCACGATTGGGGCGATGAGCCCGTTACTCCACC : 2005
B : TCGTCCCGGGCCACCCTACATGCGTGGACGACACTCTCCTCACCGTCGACTCCATGGTCGCGGCCAATGTCCTCCGCAACCACGATTGGGGCGATGAGCCCGTTACTCCACC : 2016

        2020         *      2040         *      2060         *      2080         *      2100         *      2120
A : AGCCGGCGGCAACCCGACATCGCCTACCCTCGCCCGCGTCACAGCGGAGAACATGCGCACTGTGTACTCCGCTCTCAATAACGGCGCCGTGGGGCCCTTCGACTATGACGAC : 2117
B : AGCCGGCGGCAACCCGACATCGCCTACCCTCGCCCGCGTCACAGCGGAGAACATGCGCACTGTGTACTCCGCTCTCAATAACGGCGCCGTGGGGCCCTTCGACTATGACGAC : 2128

              *      2140         *      2160         *      2180         *      2200         *      2220         *
A : ATGGCGTCAATTGCCGCTTCAATAGCGCCGTAGACTGCGCTGCATCGTGCTTGACCAGCGTCCGACGGGGAGATCGG----------------------------- : 2195
B : ATGGCGTCAATTGCCGCTTCAATAGCGCCGTAGACTGCGCTGCATCGTGCTTGACCAGCGTCCGACGGGGAGGGGCTTAATTGCCCCCCCCGCCGGGCCTGGGG : 2233
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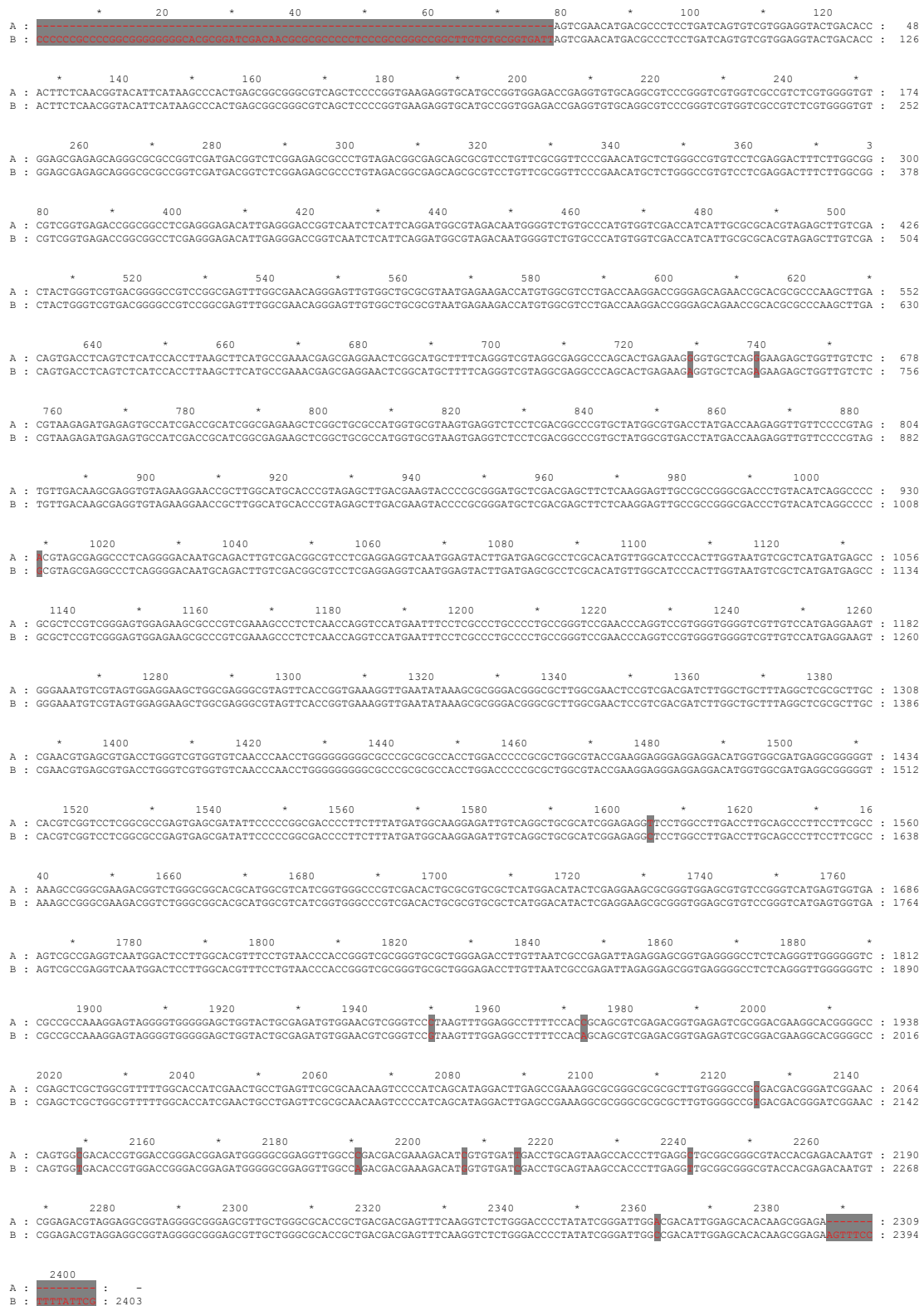
Figure 4-20: Sequence alignment dsRNA segment 2. Differences in sequences are shown by red lettering. A) NCPF7367 virus sequence data obtained in the present study, B) NCPF7367 virus sequence data published in Kanhayuwa *et al.,* 2015 (Genbank accession HG975303).

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            *         20         *         40         *         60         *         80         *        100         *
A : --------------AGAACGCTTGCATGCTCCAACGCCCCGCAACCACCATGCTAGCGAGGAAAAGGGGAGTGCGGTCCGTCCCGTCAACCGCATCAAGGGGGCCTCCAC :   96
B : CGAATAAAAGGAAACTAGAACGCTTGCATGCTCCAACGCCCCGCAACCACCATGCTAGCGAGGAAAAGGGGAGTGCGGTCCGTCCCGTCAACCGCATCAAGGGGGCCTCCAC :  112

      120         *        140         *        160         *        180         *        200         *        220
A : CATCGACGAGGCGACCATCTGAGACACCGTCCCACCCGTCAACTTCGGGGGTGTCGAACCTGGCGCTCTCGGTGCGGTCCCGCTCCACCACCACAGTCGCCGCCTCTGGACC :  208
B : CATCGACGAGGCGACCATCTGAGACACCGTCCCACCCGTCAACTTCGGGGGTGTCGAACCTGGCGCTCTCGGTGCGGTCCCGCTCCACCACCACAGTCGCCGCCTCTGGACC :  224

            *        240         *        260         *        280         *        300         *        320         *
A : ATCGGGCGAAAAGGTCGTTCTGCCCTTCTCGCTGTTCGAGTTCGGCTTCCCACCCGACATGCCTTCCTCTCCGCTACCGGAGGACTCTCAATTCAAGGATGAGGGTGGCCAG :  320
B : ATCGGGCGAAAAGGTCGTTCTGCCCTTCTCGCTGTTCGAGTTCGGCTTCCCACCCGACATGCCTTCCTCTCCGCTACCGGAGGACTCTCAATTCAAGGATGAGGGTGGCCAG :  336

      340         *        360         *        380         *        400         *        420         *        440
A : CGGTTCATGGCGAGCGAGGCTGGTACAATCCTGCGCAGGGATGATCGGAGGTACGCTGCGCTACAGCGCCAATTCATCGTGCGCAGCGTCCGCCTTACCGGCTCTTCCATCC :  432
B : CGGTTCATGGCGAGCGAGGCTGGTACAATCCTGCGCAGGGATGATCGGAGGTACGCTGCGCTACAGCGCCAATTCATCGTGCGCAGCGTCCGCCTTACCGGCTCTTCCATCC :  448

            *        460         *        480         *        500         *        520         *        540         *
A : TGGTCCTCGGGTCCGGGTCGTCGAAATCCATAATTCCCCTCCTCAGGAGGGGGGTCGCAACCGCCACCTTCGTCGACACCTCCCAGGCTGCCCTCGACCGCATGAGGCGCAA :  544
B : TGGTCCTCGGGTCCGGGTCGTCGAAATCCATAATTCCCCTCCTCAGGAGGGGGGTCGCAACCGCCACCTTCGTCGACACCTCCCAGGCTGCCCTCGACCGCATGAGGCGCAA :  560

            *        580         *        600         *        620         *        640         *        660         *
A : CCTCACCGAGGCCGGGATAACCGCCACCGTCGACGCCCAGTTCGTCTGCTGCGACGCCTGGGATTGGATCTCCGGTGATGACCAGCCCCTGTACGACGTCGTCATCGCGACC :  656
B : CCTCACCGAGGCCGGGATAACCGCCACCGTCGACGCCCAGTTCGTCTGCTGCGACGCCTGGGATTGGATCTCCGGTGATGACCAGCCCCTGTACGACGTCGTCATCGCGACC :  672

      680         *        700         *        720         *        740         *        760         *        780
A : AAGTGCCTCGGGCTGATCTTCTCTAGCGACCCGGCCCAGCGCGACGTGCAGTCGCTCCTCGACTACTGCTCTGCCATCCTCCGGGATGACGGATCCGTGTTCGTCGACCACC :  768
B : AAGTGCCTCGGGCTGATCTTCTCTAGCGACCCGGCCCAGCGCGACGTGCAGTCGCTCCTCGACTACTGCTCTGCCATCCTCCGGGATGACGGATCCGTGTTCGTCGACCACC :  784

            *        800         *        820         *        840         *        860         *        880         *
A : ACCTCGCTTTCGCCTCCCTTCCCCACGGTACGAGGGTCGCGTCCGCAGTAGAGCCCGAGTTGTTCGACCTGGCGACCATTGCTGGCCGCTACGCTGACGACGTCGCGTACAA :  880
B : ACCTCGCTTTCGCCTCCCTTCCCCACGGTACGAGGGTCGCGTCCGCAGTAGAGCCCGAGTTGTTCGACCTGGCGACCATTGCTGGCCGCTACGCTGACGACGTCGCGTACAA :  896

      900         *        920         *        940         *        960         *        980         *       1000
A : TGCTGAGGTTGACCACCAGGACTTCGATCGGGTTGCCTCCTTTGTCTCTTCTGCGGCCGCGCACCTTGTCCAGGTGTGGCAGTTCTTCCATTACCGCCTCAAGAACGTCGGC :  992
B : TGCTGAGGTTGACCACCAGGACTTCGATCGGGTTGCCTCCTTTGTCTCTTCTGCGGCCGCGCACCTTGTCCAGGTGTGGCAGTTCTTCCATTACCGCCTCAAGAACGTCGGC : 1008

      *       1020         *       1040         *       1060         *       1080         *       1100         *       1120
A : CGAGCCAAGCCCGGGGCAACGTTCTCGCTCCATAAAGCGCCATGCCCCACGGAATTCCCGACACCGCCCGCATTGGAATTCGACGCCCTCGCTGATGCCATGTACCCCGTCA : 1104
B : CGAGCCAAGCCCGGGGCAACGTTCTCGCTCCATAAAGCGCCATGCCCCACGGAATTCCCGACACCGCCCGCATTGGAATTCGACGCCCTCGCTGATGCCATGTACCCCGTCA : 1120

            *       1140         *       1160         *       1180         *       1200         *       1220         *
A : ACGGGCGTGGTGTCAAGCGCATCCCCACCGCCTCTGACATCAAGGGGCATCCTTACGCCACCGCGCTCGTCAAGTATGATGGTGAGCATGGGGTCCTTGTGCTCGATGGCGC : 1216
B : ACGGGCGTGGTGTCAAGCGCATCCCCACCGCCTCTGACATCAAGGGGCATCCTTACGCCACCGCGCTCGTCAAGTATGATGGTGAGCATGGGGTCCTTGTGCTCGATGGCGC : 1232

      1240         *       1260         *       1280         *       1300         *       1320         *       1340
A : TAACGCCACCTTTATATCTGGCCGTTACCGCTTCGCACGCCAGCTGCACCTCTCAGTCCAGCCCGTTCTGGCCTGTACTGCCGAACTGGTGTCAGTTTCCCCGCAGACCTCC : 1328
B : TAACGCCACCTTTATATCTGGCCGTTACCGCTTCGCACGCCAGCTGCACCTCTCAGTCCAGCCCGTTCTGGCCTGTACTGCCGAACTGGTGTCAGTTTCCCCGCAGACCTCC : 1344

            *       1360         *       1380         *       1400         *       1420         *       1440         *
A : GTGCTCATCATCACGGGGCTTATCTCACTCGGCGATGCCTTCGCCGACCCGCTCGATTACGAGGCCCTCCGTCCCCTCGTTCCCACCCTCGAGCGCCTTGCCCCATCCGGGA : 1440
B : GTGCTCATCATCACGGGGCTTATCTCACTCGGCGATGCCTTCGCCGACCCGCTCGATTACGAGGCCCTCCGTCCCCTCGTTCCCACCCTCGAGCGCCTTGCCCCATCCGGGA : 1456

      1460         *       1480         *       1500         *       1520         *       1540         *       1560
A : TCGTTCCCACGATCCCCGAGCACGTTCGGCTAGTCAAGGGCTCCGCGGTCCACTTCTTTGGTCCCCACGGGTCAGTCCTCCGCGCCCCCGTTGACGGTGTCCAAGTCAACAC : 1552
B : TCGTTCCCACGATCCCCGAGCACGTTCGGCTAGTCAAGGGCTCCGCGGTCCACTTCTTTGGTCCCCACGGGTCAGTCCTCCGCGCCCCCGTTGACGGTGTCCAAGTCAACAC : 1568

            *       1580         *       1600         *       1620         *       1640         *       1660         *       1680
A : CGGCCGGCAAGGCTGGTGTGTTCATCAAGCCTGCCGCTGCCTGCACCGTCGACGCCACGACCAATGATGCTTGTGACGCCATCGCTGATGCATACGCTGCCCTTGGGCTTTCC : 1664
B : CGGCCGGCAAGGCTGGTGTGTTCATCAAGCCTGCCGCTGCCTGCACCGTCGACGCCACGACCAATGATGCTTGTGACGCCATCGCTGATGCATACGCTGCCCTTGGGCTTTCC : 1680

            *       1700         *       1720         *       1740         *       1760         *       1780         *
A : GCGATGCCCTACGTCCAACCAGGGGAGGGGGACAACATATACGAGTTCCACCGCGTTCGGGAACGCACGTCTGGCGCCCAGGACGCCCACGGCCTGACAAGAACCGCAGCG : 1776
B : GCGATGCCCTACGTCCAACCAGGGGAGGGGGACAACATATACGAGTTCCACCGCGTTCCGGGAACGCACGTCTGGCGCCCAGGACGCCCACGGCCTGACAAGAACCGCAGCG : 1792

      1800         *       1820         *       1840         *       1860         *       1880         *       1900
A : ATAAGCCTGGCACCGTGGTCCACACGGTCGCTGCCAGCATCATGGCGGAGCAACTAGCCCTCACCAGTGACGTCGCCGCCCTCGTGTCCAAGATCTTCCGGTGAGTGGAACG : 1888
B : ATAAGCCTGGCACCGTGGTCCACACGGTCGCTGCCAGCATCATGGCGGAGCAACTAGCCCTCACCAGTGACGTCGCCGCCCTCGTGTCCAAGATCTTCCGGTGAGTGGAACG : 1904

            *       1920         *       1940         *       1960         *
A : TTGGAGATGCTCGCGTCCCGACGCGGCGGGGA------------------------------- : 1920
B : TTGGAGATGCTCGCGTCCCGACGCGGCGGGGGGGGGCCCTGGCCCCCCCCCCGCGCCGGGGCGGGGG : 1970
```

Figure 4-21: Sequence alignment dsRNA segment 3. Differences in sequences are shown by red lettering. A) NCPF7367 virus sequence data obtained in the present study, B) NCPF7367 virus sequence data published in Kanhayuwa *et al.,* 2015 (Genbank accession HG975304).

Figure 4-22: Sequence alignment dsRNA segment 4. Differences in sequences are shown by red lettering. A) NCPF7367 virus sequence data obtained in the present study, B) NCPF7367 virus sequence data published in Kanhayuwa *et al.,* 2015 (Genbank accession HG975305).
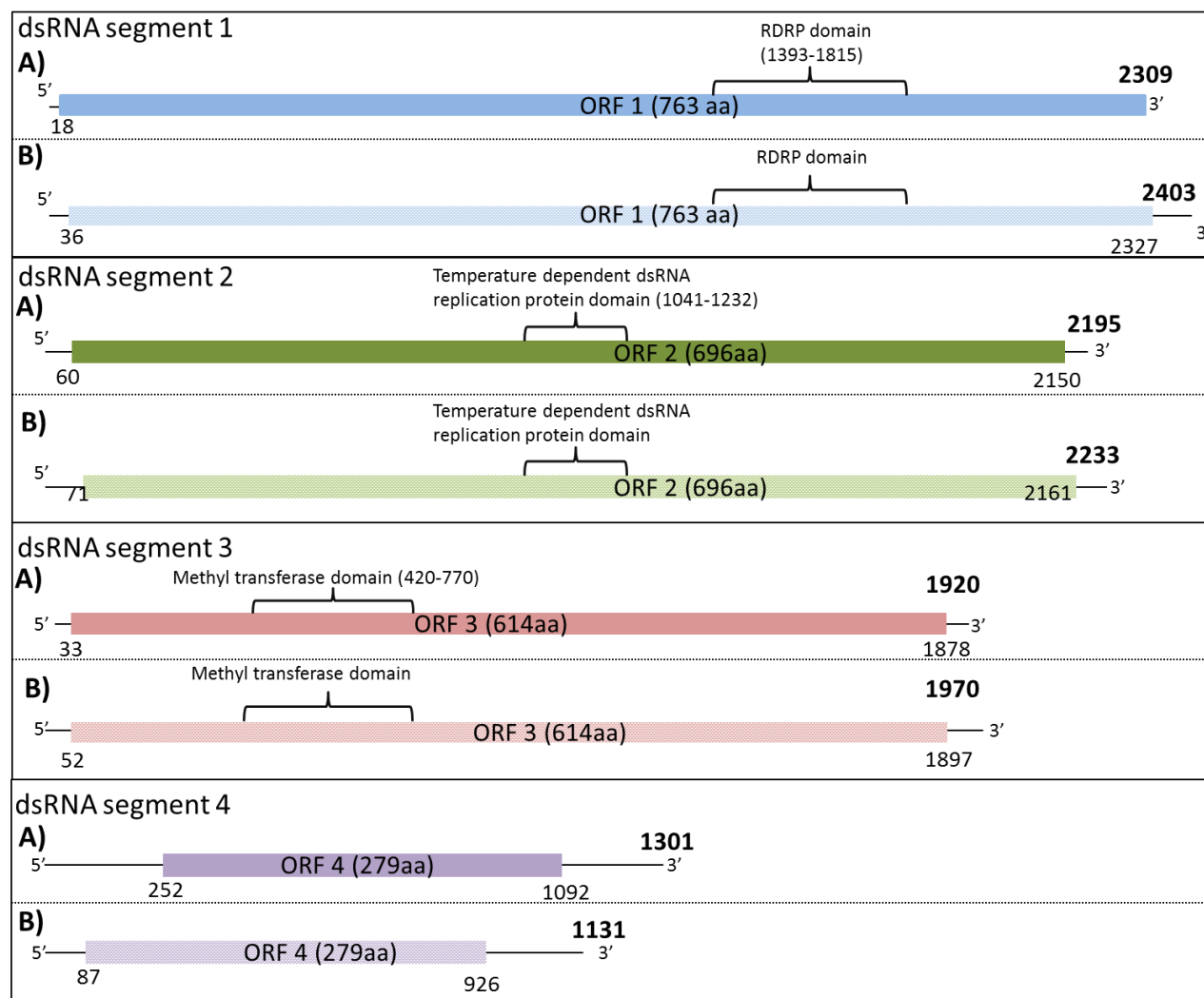
Figure 4-23: Outline of the four segments of dsRNA identified from genome sequencing of the virus carried by *A. fumigatus* strain NCPF7367. The size and position of the putative ORFs on each segment are shown, as are the regions on segments 1, 2 and 3 which feature high similarity to known protein domains. A) derived from the sequence data obtained in the present study, B) derived from the sequence data published by Kanhayuwa *et al.* (2015).

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            *        20         *        40         *        60         *        80
A : MSSNPDIGVPETLKLVVSGAPSNAPAPTASYVSDIVSWYARRSLKGGLLQVNHTMSFVVGPTSAPISVPVHGVATGSDPVVAAP :  84
B : MSANPDIGVPETLKLVVSGAPSNAPAPTASYVSDIVSWYARRNLKGGLLQVDHTMSFVVWPTSAPISVPVHGVTTGSDPVVTAP :  84
C : -----------LQVNFAPRTSATPQAVTDTIGEMVSWSLGRGLRGGTRLSDRVIAFQLD-DDRVAEVPVAGNCTGTEPTISAA :  71
D : ----------------------------------------------------------------------------------- :   -
E : ----------------------------------------------------------------------------------- :   -


         *        100        *        120        *        140        *        160
A : QARARAFRLKSYADGDLLRELRQFDGAKNASELGPRAFVRDSHRLDAAVEKASKLRDPTFHISQYQLPHPYSFGGGPPNPERPL : 168
B : QARARAFRLKSYADGDLLRELRQFDGAKNASELGPRAFVRDSHRLDAAVEKASKLTDPTFHISQYQLPHPYSFGGGPPNPERPL : 168
C : HARARAFRVKPLADGGLIAALREYDGTRNESDMAKRAFVRDSSRLAEAVKKAAMLKNPTISISHYQLPDPFSFGGGPPEQERPL : 155
D : ----------------LESLRRFDTPSDE-KTSRRSFTRDSHRLDDAVLKAASLKDPSFRIYPLQRETPLSFGGGPPGEERPLK :  67
E : ----------------LEALRGFDGVRDE-NTKPKEFIRDSHRLDEAVAKAVTLKDPSFTIYPLQRSTPMSFGGGPPGDERPLK :  67


         *        180        *        200        *        220        *        240        *
A : TAPLISAINKVSQRTRDPVGYRKRAKESIDLGDFTTHDPDTLHPRFLEYVHERTRSVDGPTDDAMRAAQTVFARLWRRKGCKVK : 252
B : TAPLISAINKVSQRTRDPVGYRKRAKESIDLGDFTTHDPDTLHPRFLEYVHERTRSVDGPTDDAMRAAQTVFARLWRRKGCKVK : 252
C : RSLAAAAAHKAARYVPPHRRYLQHANEAINLGDFTTHDPDTLHPRFLEYVHERVTAVDDRTDRAMRVATTALSRLWADKFSPPA : 239
D : PTGLVTAGRMAKAAGTDELYRREMATALKMGDSTTHDPDSLHPRFLEYVHERTRSVPEKENKALAWAVTVMKELWRNQGIRAEA : 151
E : PFGLVMCDRAKPGRGDDVLYQREMKQALQMGDFTTHDPDTLPPRFLEYVHERVRELPSRESHAIHWAQRVMRKIWARRGVYAEA : 151


         260        *        280        *        300        *        320        *
A : ARNLSDAQPDNLLAIIKKGSPGEYRSLGAEDRRDPRLIATMSSSLLRYASAGVQVARGRPPPGWVDTTTQVTLTFGKREPKAAK : 336
B : ARSLSDAQPDNLLAIIKKGSPGEYRSLGAEDRRDPRLIATMSSSLLRYASAGVQVARGRPPPGWVDTTTQVTLTFGKREPKAAK : 336
C : ARGLSDIDPANLRPLLNNGSPGEYRLLGAADRLDDRLVDTMAASLARYSSAANATLKTGRVPTWIETTTQPVLTFGKKEPKAXK : 323
D : RGIDSAEPDVLSSLMNPGSAGEYAEMGATNRKDPEVLELLSKSIKRYYKAGHAMATRGRRAPWADFTQQPVMSFGKKEAKAAKL : 235
E : RGIDSAEAGPLSLMMNPGAPGEFSEAGMTNRKDPEVVEVLSQGVKRFFKAGHAMATRGRRPRWVDFTQQPTVCFGKKERKAAKM : 235


         340        *        360        *        380        *        400        *        420
A : IVDGVRQAPVPRFIFNLSPVNYALASFLHYDISHFLMDNDPTHGPGFGPGRGRARKFMDLVERAFDGRFSTPDGARLIMSDITK : 420
B : IVDGVRQAPVPRFIFNLSPVNYALASFLHYDISHFLMDNDPTHGPGFGPGRGRARKFMDLVERAFDGRFSTPDGARLIMSDITK : 420
C : MRGGERVAPVPRLIFNVSPINYALAVFLHGDLSKKLQEFDPTHGPGFGPGRGRATVFLDLLERAAGGGVFNRAGA-FSMSDIEK : 406
D : VNGVRTPPVPRFIFNPSPVNYALAAFLHGDLSHQLQTRDPTHGPGFGPGRGKAWKFLDKVAAHLLP-GKAELSCKAIMSDIAKW : 318
E : NGTTREAPIPRFIFCPSPVNYAQGAFLHSDISHQLQQKDPTHGPGFGPGRGRAWKLLDKVSKHLSSDGTATLDCDAIMSDIAKW : 319


            *        440        *        460        *        480        *        500
A : WDANMCEALIKYSIDLLEDAVDKSALSPEGLATWGLMYRVARRQLLEKLVEHPAGYFVKLYGCMPSGSFYTSLVNTTGNNLLVI : 504
B : WDANMCEALIKYSIDLLEDAVDKSALSPEGLATRGLMYRVARRQLLEKLVEHPAGYFVKLYGCMPSGSFYTSLVNTTGNNLLVI : 504
C : WDANMCEALMGYAFDLLESAVDTSQLTDHARATRALMVRVARRQLMEKLVEHPSGYLVWLSGCMPSGSYYTSLINTIGNDLLVL : 490
D : DANMSEALLSATFDLMESVVDKSSLDATGRATRRIMADVAKRQLMVKLIEHPSGYLLELFGCMPSGSFYTSCVNTIGNDLLALS : 402
E : DANMSEALISASFDLLESAVDKSKLDATGRATRTLMLDATKRQLMVKVLEHPSGYFLEMYGCMPSGSFYTSLLNTVGNDLLALS : 403


            *        520        *        540        *        560        *        580
A : GHAIARAVEETSLTHHGAAELLADAVDGTLISYGDNQLFPEHPFSVLGLAYDPEKHAEFLARFGMKLKVDETEVTVKLGRVRFC : 588
B : GHAIARAVEETSLTHHGAAELLADAVDGTLISYGDNQLFSEHLFSVLGLAYDPEKHAEFLARFGMKLKVDETEVTVKLGRVRFC : 588
C : GHIADRAMTTLAMDEEAVAQQLARCAGGTLVSYGDNQLISEELFLSLGLTYDADAHAEFLSRFGMKLKIEETKVSPWVGDARFC : 574
D : LLGVTLMEQGVELSDVSPISVAQQASSDLVSYGDNQLIFDSLFSRFGVSYSLERHEAHLAAFGMKLKVDETGVSSQLGDVRFCS : 486
E : LLAMLIMESGQDLLDIDIDLVAESAEPDLASYGDNQLVFSSLFSRFSTTYSVERHAAHLAVFGMKLKVDETDVT---------- : 477


            *        600        *        620        *        640        *        660        *
A : SRSLVRTPHGLLITRSHNSLFAKLAGRPRHDPVVDKLYVRAMMVDHMGTDPIVYAILNEIDRSLNVSLEAAGLTDAAKKVLEDT : 672
B : SRSLVRTPHGLLITRSHNSLFAKLAGRPRHDPVVDKLYVRAMMVDHMGTDPIVYAILNEIDRSLNVSLEAAGLTDAAKKVLEDT : 672
C : SRAAVQTPYGLIVTRPHTSIYQKLAGRPRHDPAIDKLYVRALMMDHMGTDPIAFEIMDSVDSMVDVDLSTVGLTPAVRSVLTDA : 658
D : RGALLTPHGLAIVRSHTSIFHKIGGRAEVDPVINKLYVRALMVDLLGTDPILYHGLQHLEASIDVPGDARLSEHRLHKMVEPFA : 570
E : ----------------------------------------------------------------------------------- :   -


         680        *        700        *        720        *        740        *
A : AQSMFGNREQDALLAVYRALS-ETVIDRRALLSLHTPRDGDHD-PGRLHTSVSTGMHLFTGELTPAAQWAYECTVEKWCQYLHD : 754
B : AQSMFGNREQDALLAVYRALS-ETVIDRRALLSLHTPRDGDHD-PGRLHTSVSTGMHLFTGELTPAAQWAYECTVEKWCQYLHD : 754
C : AKSFYGDASDDSLSRVLEALS-TSRVDRRALLSLHTPRTGAEGSVAKLGHAPQLGGPLLTNVLTPAAEWVAELGPKQWIEYLER : 741
D : KKLYGNSSPESVASFVSLLKSPSAPDRGVLLSLRLPADAPGA-LKRFGTGLTLGSGKATTGLDDVGRWLLDLSPADYWKYVNDT : 653
E : ----------------------------------------------------------------------------------- :   -


         760        *
A : TDQEGVMFD-------- : 763
B : TDQEGVMFDRDRPRSR- : 770
C : TGQMGVLTD-------- : 750
D : GQTSIIYN--------- : 661
E : ---------------- :   -
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Figure 4-24:4

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           *        20         *        40         *        60         *        80
A : MADLTRLRNIVLSGDMKVALTAAILYHLSPNPSAITDYGVDEVKSVVSWLVSSPGMFYGDVVAATGITPDILSTFLPALDDQAC :  84
B : MADLTRLRNIVLSGDMKVALTAAILYHLSPNPSAITDYGVDEVKSVVSWLVSSPGMFYGDIVAATGITPDILSTFLPALDDQAC :  84
C : MADLTRLREIALSGDLNTTFVGAALFHLSESPTASPHYTAEAAQAAVEFLSTAPVPVMGEIVGPLSQGALHFQRVKQGMTMSDY :  84
D : MADLARLRHMLLDPTESAVLLASMCHHLIPDPTSVDLFDYSFPPRARDWIISAPFPLFGPAIPASKSSFEAILEPLRAMEYNSL :  84
E : MADLTRLRTILLEHGSSVVPVGSAVYHLTKSPVSHVLVDANLVSEVLAWMAAAPGPFFGDLISSAGITPADCAQLLEDLTDKQA :  84

           *       100         *       120         *       140         *       160
A : ADAASLANAMATPDAEWDREHRNRFTSALAHHSADAARLAAQSSAEFAAVDAMEEAGYENQDHARTAVNELNRVYGNTYRVFTR : 168
B : ADAASLANAMATPDAEWDREHRNRFTSALAHHSADAARLAAQSSAEFAAVDAMEEAGYENQDHARTAVNELNRVYGNTYRVFTR : 168
C : ARAATLANAMATPDSEWDRAGRNTFASRLVEHRPDAAQHAAQSSAEFAALDALTDAGYENMQHAANELNLVRRTKAHYFKHHVR : 168
D : DMAAVLMRSMRVADNPFAKVTRGETTGDVAQSRAGAAQHAAQSPIEFAIYDVLRDAGYTSVSDALSKLQSFLTSTKAFVHPITR : 168
E : AEAATMVNAMTIADDEWDRGTRDQYTTATVLHERDAAKIAAQSSAEFAALDALTAAGYAGVDDAVGALSVMRRTFKHNYKVHVR : 168

           *       180         *       200         *       220         *       240         *
A : QHARSRVAIAASFKIDDHGTLFHVLTPWCLSRTSAMIYVAVIARHVRRRHIEERASRLARTALTYAARAGLEAVRAIIEPAIAI : 252
B : QHARSRVAIAASFKIDDHGTLFHVLTPWCLSRTSAMIYVAVIARHVRRRHIEERASRLARTALTYAARAGLEAVRAIIEPAIAI : 252
C : QFARSRHAVSCRFDMSPGVIGAILSPWCLSRQSAYAYVAVMLAHIRKDVRTQRAARIAAGTVSSYSARSSLEAVRRVVEPAIYI : 252
D : QFSRHRSAALFLINKPGARGPTAFLTPFFTSKEATKLFAAVVFRSDILTSLSRLNAARGVTVLGYAARASLEAVRRIVEPAHAI : 252
E : QFARSRHCVVARFVRSDGAVRIVITPWCLSRRNAQAYAALCMADWHRHMVEMRTINRARTVATFAERTSLQAVRDIIAPALALL : 252

          260         *       280         *       300         *       320         *
A : VTSFRYDPAKIAFVDDFDRVRESITTRSPPTLVAFACVHMCGNQDVVSLAARLRMESLPQLHETPPAVAEYTDHVDDEQSALLM : 336
B : VTSFRYDPAKIAFVDDFDRVRESITTRSPPTLVAFACVHMCGNQDVVSLAARLRMESLPQLHETPPAVAEYTDHVDDEQSALLM : 336
C : LSTYTFDPRRLAFCADDGRVMETIQTRSPHVLAAFAAVCAAGTSDVVMDLAAMRSASVPADRPEPQVLTEYEERLDNEESAMLL : 336
D : VNEYSFDPQTLSFIDPAGDPTYHITDRTPSVVVAFGRVLSGGSVDACSKASDLRTSLDIPGGLPDVAREYFSSTGNVEDTIRSA : 336
E : TRYSYDATNMAFVDGSGRPVQKVGARAPYVAVAFAAVFSSGAGDATLGLARARKAAADTLTDAVKLKTATDYLEMADSPFAGLA : 336

          340         *       360         *       380         *       400         *       420
A : FSRVLKRLRHERDRGVHGPRLNKDPMLGYIARGVRRSERNTMACINRVEEAVSFLRSKGVDTLNTLFVIEWGGEFDYPTVMAAL : 420
B : FSRVLKRLRHERDRGVHGPRLNKDPMLGYIARGVRRSERNTMACINRVEEAVSFLRSKGVDTLNTLFVIEWGGEFDYPTVMAAL : 420
C : FSRALRHYRAERDASVPGPRLNKDPTLGYVVRAIKRIERNTQACIQRILEVVSTARHRGVDMTNTLVFVEWGGGIDYAQVIGAI : 420
D : ATALSDFRSFRDSHPGGHELNKDSGLSFFSRDRATRDRNTAACITRVEEVACLLRSQGRDIARMAFVVEWGGPLNNDSILAALA : 420
E : LFSRALAEYRDKRHASPWGDRVNKGAMLGYVARERDKLTRNTAACINRIEEIAALLAMKGHHKDSTLYVVEWGGRVSAHAVLGA : 420

           *       440         *       460         *       480         *       500
A : AVARLDVCVDIGPSGIDLPGADAKAEDGTEEYNYSLLLASAEERRMPRMPRVPYPSDEPLEAKLRRVAQFYSLSGSANIAYISG : 504
B : AVARLDVCVDIGPSGIDLPGADAKAEDGTEEYNYSLLLASAEERRMPRMPRVPYPSDEPLEAKLRRVAQFYSLSGSANIAYVSG : 504
C : ALAKIDVAVDVGQSGTDVPGMDVVGEDDDPTCSIQLLASAGRRNLPRMPIVSYAASDTLESKISGLSELYTGEGAGIIYVSGG : 504
D : LTGATACVDVGPGAFKVGDSSEELATENDKGAYVCLIPRASTRGLPRMPIVGYESGDSVTQRLDRLLSFISGPDNYPPVAYITG : 504
E : AATAKIDIALDVAGSGVDVPGIDVYADDDDPPHHYQLYLASARRRMLPRMPSVPYRAGTPLIEKLSLVYESVGGRASDYKLAYV : 504

           *       520         *       540         *       560         *       580
A : GVTQNEGVPVNICIDTANRLNAIRNVTPDVNVVYACAEALLPPLCGHGLELGPDDYLQCASLVDEDCPACNAHYRATAVLSAAC : 588
B : GVTQNEGVPVNICIDTANRLNAIRNVTPDVNVVYACAEALLPPLCGHGLELGPDDYLQCASLVDEDCPACNAHYRATAVLSAAC : 588
C : VVRDEGTPVSVCADASARLNAVFVASERSNVLMGVAEALLPPLCDHAADVSVDDYLDSLSQVEEDCKACEAHYRSVVLLSTLCV : 588
D : GSTTSGAAPADIIGITQARAALLTDYLASGSFFCGDILIPDVCRHPPDGTDDECLACANFDYSVFTVGDVCQLDGFSLVKPRAS : 588
E : NGGMSQLAETPVSVCTDSNARMAAMDGARLTLPIPFYSTEVLLPPACHHIVDMDPDEYLDDFGTGNPNCFQCEAHYRALSAVGD : 588

           *       600         *       620         *       640         *       660         *
A : ADRQVRIVKPRSAYAHNGHLSLELVPGHPTCVDDTLLTVDSMVAANVLRNHDWGDEPVTPPAGGNPTSPTLARVTAENMRTVYS : 672
B : ADRQVRIVKPRSAYAHNGHLSLELVPGHPTCVDDTLLTVDSMVAANVLRNHDWGDEPVTPPAGGNPTSPTLARVTAENMRTVYS : 672
C : RDGVRVVKPRSSYGHNGHVSMEFIRGEGRLFEDTAATFDSMVACNVLRNYPWVEDPITPVGGGNPTSPPYSALMADVITHIYRL : 672
D : YAHNLHLSLEWIHGAAREFSESRTLLALDSVVATNVARNADWGDFVPRDGPRRPHHPSYGLKVKLIIEDVYRSMAEGA------ : 666
E : ALDRPGVRLTKPRSVFAHNALFGLEWIGTDSAMEDSLETLDAAIACNALRNYEYPNPPPDPTPGKDLSSPDLLELMQDSVRQVY : 672

          680         *
A : ALNNGAVGPFDYDDMASIAASIAP : 696
B : ALNNGAVGPFDYDDMASIAASIAP : 696
C : LSGGRAAPLDLLDL---------- : 686
D : ------------------------ :   -
E : TLLAGPFAGPL------------- : 683
```

Figure 4-25: Amino acid sequence alignment of ORF2 predicted from the RNA sequence of dsRNA segment 2. Green shading denotes >50% alignment over all of the sequences compared, red lettering with grey shading denotes differences between the amino acid sequence obtained in the present study and that published by Kanhayuwa *et al.,* 2015. A) NCPF7367 virus sequence data obtained in the present

study, B) NCPF7367 virus sequence data published in Kanhayuwa *et al.,* 2015 (Genbank accession HG975303), C) BDV-1 genome segment (Genbank accession: AKE49496), D) CCV-1 genome segment (Genbank accession: NC_024705), E) *Alternaria* sp. viral dsRNA (Genbank accession: ACL80752).
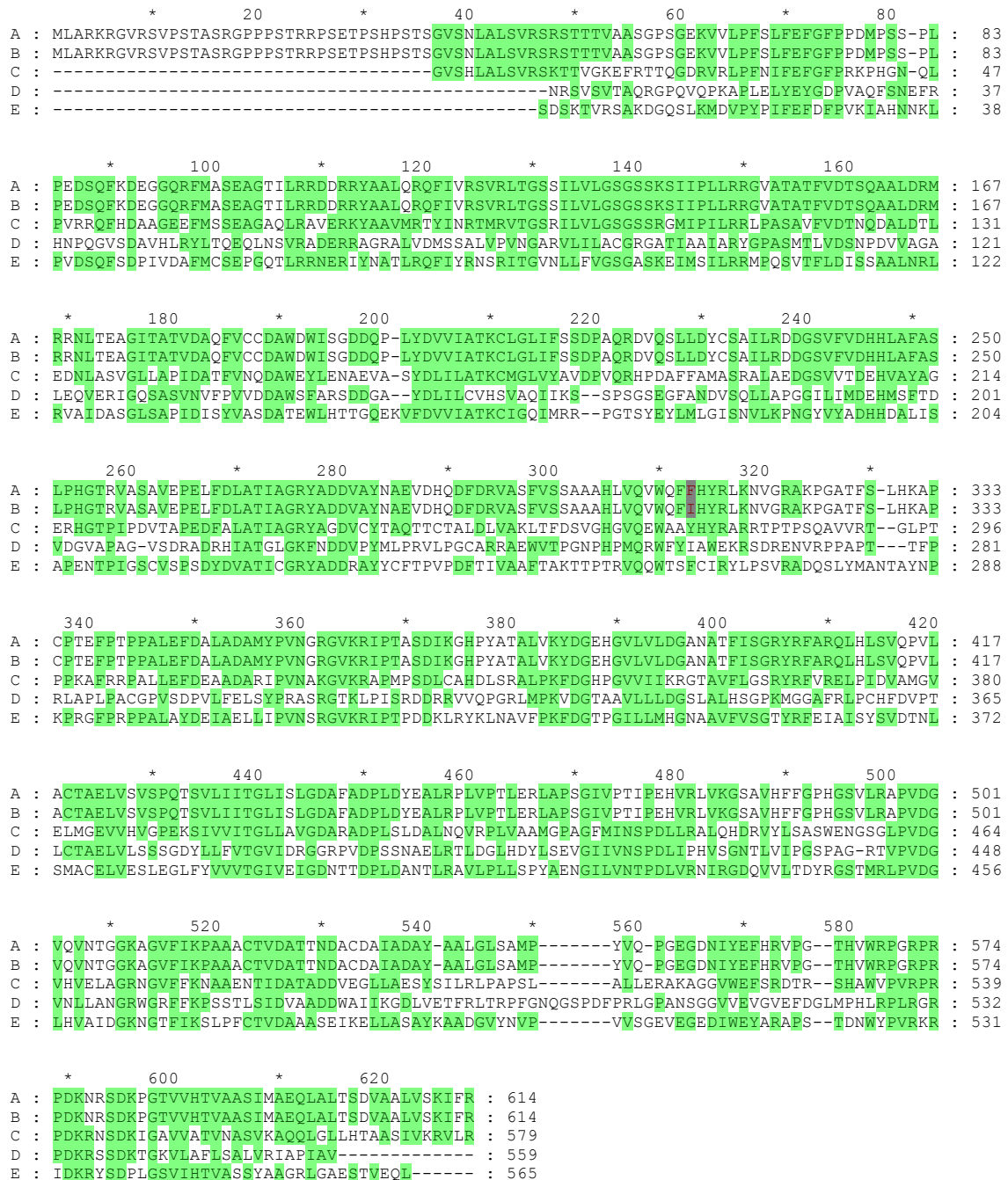
```
            *         20        *         40        *         60        *         80
A : MLARKRGVRSVPSTASRGPPPSTRRPSETPSHPSTSGVSNLALSVRSRSTTTVAASGPSGEKVVLPFSLFEFGFPPDMPSS-PL :  83
B : MLARKRGVRSVPSTASRGPPPSTRRPSETPSHPSTSGVSNLALSVRSRSTTTVAASGPSGEKVVLPFSLFEFGFPPDMPSS-PL :  83
C : -----------------------------------GVSHLALSVRSKTTVGKEFRTTQGDRVRLPFNIFEFGFPRKPHGN-QL :  47
D : -----------------------------------------------NRSVSVTAQRGPQVQPKAPLELYEYGDPVAQFSNEFR :  37
E : -----------------------------------------------SDSKTVRSAKDGQSLKMDVPYPIFEFDFPVKIAHNNKL :  38


        *         100       *         120       *         140       *         160
A : PEDSQFKDEGGQRFMASEAGTILRRDDRRYAALQRQFIVRSVRLTGSSILVLGSGSSKSIIPLLRRGVATATFVDTSQAALDRM : 167
B : PEDSQFKDEGGQRFMASEAGTILRRDDRRYAALQRQFIVRSVRLTGSSILVLGSGSSKSIIPLLRRGVATATFVDTSQAALDRM : 167
C : PVRRQFHDAAGEEFMSSEAGAQLRAVERKYAAVMRTYINRTMRVTGSRILVLGSGSSRGMIPILRRLPASAVFVDTNQDALDTL : 131
D : HNPQGVSDAVHLRYLTQEQLNSVRADERRAGRALVDMSSALVPVNGARVLILACGRGATIAAIARYGPASMTLVDSNPDVVAGA : 121
E : PVDSQFSDPIVDAFMCSEPGQTLRRNERIYNATLRQFIYRNSRITGVNLLFVGSGASKEIMSILRRMPQSVTFLDISSAALNRL : 122


        *         180       *         200       *         220       *         240        *
A : RRNLTEAGITATVDAQFVCCDAWDWISGDDQP-LYDVVIATKCLGLIFSSDPAQRDVQSLLDYCSAILRDDGSVFVDHHLAFAS : 250
B : RRNLTEAGITATVDAQFVCCDAWDWISGDDQP-LYDVVIATKCLGLIFSSDPAQRDVQSLLDYCSAILRDDGSVFVDHHLAFAS : 250
C : EDNLASVGLLAPIDATFVNQDAWEYLENAEVA-SYDLILATKCMGLVYAVDPVQRHPDAFFAMASRALAEDGSVVTDEHVAYAG : 214
D : LEQVERIGQSASVNVFPVVDDAWSFARSDDGA--YDLILCVHSVAQIIKS--SPSGSEGFANDVSQLLAPGGILIMDEHMSFTD : 201
E : RVAIDASGLSAPIDISYVASDATEWLHTTGQEKVFDVVIATKCIGQIMRR--PGTSYEYLMLGISNVLKPNGYVYADHHDALIS : 204


        260       *         280       *         300       *         320        *
A : LPHGTRVASAVEPELFDLATIAGRYADDVAYNAEVDHQDFDRVASFVSSAAAHLVQVWQFFHYRLKNVGRAKPGATFS-LHKAP : 333
B : LPHGTRVASAVEPELFDLATIAGRYADDVAYNAEVDHQDFDRVASFVSSAAAHLVQVWQFIHYRLKNVGRAKPGATFS-LHKAP : 333
C : ERHGTPIPDVTAPEDFALATIAGRYAGDVCYTAQTTCTALDLVAKLTFDSVGHGVQEWAAYHYRARRTPTPSQAVVRT--GLPT : 296
D : VDGVAPAG-VSDRADRHIATGLGKFNDDVPYMLPRVLPGCARRAEWVTPGNPHPMQRWFYIAWEKRSDRENVRPPAPT---TFP : 281
E : APENTPIGSCVSPSDYDVATICGRYADDRAYYCFTPVPDFTIVAAFTAKTTPTRVQQWTSFCIRYLPSVRADQSLYMANTAYNP : 288


        340       *         360       *         380       *         400       *         420
A : CPTEFPTPPALEFDALADAMYPVNGRGVKRIPTASDIKGHPYATALVKYDGEHGVLVLDGANATFISGRYRFARQLHLSVQPVL : 417
B : CPTEFPTPPALEFDALADAMYPVNGRGVKRIPTASDIKGHPYATALVKYDGEHGVLVLDGANATFISGRYRFARQLHLSVQPVL : 417
C : PPKAFRRPALLEFDEAADARIPVNAKGVKRAPMPSDLCAHDLSRALPKFDGHPGVVIIKRGTAVFLGSRYRFVRELPIDVAMGV : 380
D : RLAPLPACGPVSDPVLFELSYPRASRGTKLPISRDDRRVVQPGRLMPKVDGTAAVLLLDGSLALHSGPKMGGAFRLPCHFDVPT : 365
E : KPRGFPRPPALAYDEIAELLIPVNSRGVKRIPTPDDKLRYKLNAVFPKFDGTPGILLMHGNAAVFVSGTYRFEIAISYSVDTNL : 372


        *         440       *         460       *         480       *         500
A : ACTAELVSVSPQTSVLIITGLISLGDAFADPLDYEALRPLVPTLERLAPSGIVPTIPEHVRLVKGSAVHFFGPHGSVLRAPVDG : 501
B : ACTAELVSVSPQTSVLIITGLISLGDAFADPLDYEALRPLVPTLERLAPSGIVPTIPEHVRLVKGSAVHFFGPHGSVLRAPVDG : 501
C : ELMGEVVHVGPEKSIVVITGLLAVGDARADPLSLDALNQVRPLVAAMGPAGFMINSPDLLRALQHDRVYLSASWENGSGLPVDG : 464
D : LCTAELVLSSSGDYLLFVTGVIDRGGRPVDPSSNAELRTLDGLHDYLSEVGIIVNSPDLIPHVSGNTLVIPGSPAG-RTVPVDG : 448
E : SMACELVESLEGLFYVVVTGIVEIGDNTTDPLDANTLRAVLPLLSPYAENGILVNTPDLVRNIRGDQVVLTDYRGSTMRLPVDG : 456


        *         520       *         540       *         560       *         580
A : VQVNTGGKAGVFIKPAAACTVDATTNDACDAIADAY-AALGLSAMP-------YVQ-PGEGDNIYEFHRVPG--THVWRPGRPR : 574
B : VQVNTGGKAGVFIKPAAACTVDATTNDACDAIADAY-AALGLSAMP-------YVQ-PGEGDNIYEFHRVPG--THVWRPGRPR : 574
C : VHVELAGRNGVFFKNAAENTIDATADDVEGLLAESYSILRLPAPSL-------ALLERAKAGGVWEFSRDTR--SHAWVPVRPR : 539
D : VNLLANGRWGRFFKPSSTLSIDVAADDWAIIKGDLVETFRLTRPPFGNQGSPDFPRLGPANSGGVVEVGVEFDGLMPHLRPLRGR : 532
E : LHVAIDGKNGTFIKSLPFCTVDAAASEIKELLASAYKAADGVYNVP-------VVSGEVEGEDIWEYARAPS--TDNWYPVRKR : 531


        *         600       *         620
A : PDKNRSDKPGTVVHTVAASIMAEQLALTSDVAALVSKIFR : 614
B : PDKNRSDKPGTVVHTVAASIMAEQLALTSDVAALVSKIFR : 614
C : PDKRNSDKIGAVVATVNASVKAQQLGLLHTAASIVKRVLR : 579
D : PDKRSSDKTGKVLAFLSALVRIAPIAV------------- : 559
E : IDKRYSDPLGSVIHTVASSYAAGRLGAESTVEQL------ : 565
```

Figure 4-26: Amino acid sequence alignment of ORF3 predicted from the RNA sequence of dsRNA segment 3 Green shading denotes >50% alignment over all of the sequences compared, red lettering with grey shading denotes differences between the amino acid sequence obtained in the present study and that published by Kanhayuwa *et al.,* 2015.. A) NCPF7367 virus sequence data obtained in the present study, B) NCPF7367 virus sequence data published in Kanhayuwa *et al.,* 2015 (Genbank accession HG975304), C) BDV-1 genome segment (Genbank accession: AKE49497), D)CCV-1 genome segment (Genbank accession: NC_024706), E) *M. lini* viral dsRNA (Genbank accession: CAA45724).

```
                *          20            *          40            *          60            *          80
A : MSPAPILSAEQAAHLAQLTVDDVSLVIKLASLGLKAKEIQQYAAEIAEGGDPEIDLAPGAPRPVVIHAWSFVKDRGQYHSTYGLS :  85
B : MSPAPILSAEQAAHLAQLTVDDVSLVIKLASLGLKAKEIQQYAAEIAEGGDPEIDLAPGAPRPVVIHAWSFVKDRGQYHSTYGLS :  85
C : -----LLTKEQASLLAALGEGEVAAIIKLASLGLRPTGILEYARAVDGDEVPAPPTPNTAAKPVTIVAWSFLKDRGQYHATYGLS :  80
D : ------LTSREAKLLMQLPDEAIAGILKLASHGLKPAGIIEYASAVAKDQEPAPFPALTGPKPLTIQLHSFLADPRQYSDTYSLA :  79

                *          100           *          120           *          140           *          160           *
A : SAQAGELLDLLHTDPKDAVKRIQAIVSERLRLRGSSRPVVVDLEGAPSSTSAKGGAPPSGDGGLSLLRQEIKRNSGVYGSYRFTA : 170
B : SAQAGELLDLLHTDPKDAVKRIQAIVSERLRLRGSSRPVVVDLEGAPSSTSAKGGAPPSGDGGLSLLRQEIKRNSGVYGSYQFTA : 170
C : APRAGELLELLLTDADAAAREIHTIVADALRQRGSPRPVHVTLDGVPSRKKAGGEGAPTGDGGLGLLSQEIASNPGTYGSYRFVP : 165
D : MDVVERLRALEMSDRDEARKELAAVISATPRIAASGKPVRVVDDGAPGGAPGSAQPLAGASSVTKSLAAEIASNSTLYGSYKFDA : 164

                180           *          200           *          220           *          240           *
A : YPTSRPGGQAFAVGLGGGLYAFGQSKAVAVAAARIARVKGRSDDLVRPYIHFWMEGSSPRAASDIPAKV-WDGRLGPHTEPPAEP : 254
B : YPTSRPGGQAFAVGLGGGLYAFGQSKAVAVAAARIARVKGRSDDLVRPYIHFWVEGSSPRAASDIPAKV-WDGRLGPHTEPPAEP : 254
C : EQTGQPGARAYRVRLGGGLYVFAQSKKLATAAARITRVKGRSDPLVRDFVAFWATGSSAKFGGEIPDKVTFDGRQGPN------- : 243
D : FETQLGVTERFAVDLGENFYVVTFSKKNAIAIARLVCVHGRSYASAAPYVVYVDSAGRMLRGGDIDSRR-GDCRRGPR------- : 241

                260           *
A : ATKAKTEAPTTSGGRASTPKG : 275
B : ATKAKTEAPTTSGGRASTPKG : 275
C : -------------------- :  -
D : -------------------- :  -
```

Figure 4-27: Amino acid sequence alignment of ORF4 predicted from the RNA sequence of dsRNA segment 4. Green shading denotes >50% alignment over all of the sequences compared, red lettering with grey shading denotes differences between the amino acid sequence obtained in the present study and that published by Kanhayuwa *et al.,* 2015. A) NCPF7367 virus sequence data obtained in the present study, B) NCPF7367 virus sequence data published in Kanhayuwa *et al.,* 2015 (Genbank accession HG975305), C) BDV-1 genome segment (Genbank accession: AKE49498), D)CCV-1 genome segment (Genbank accession: NC_024707).

4.4 Discussion

4.4.1 Mycovirus enrichment from compost samples

Overall, the development of the procedure for enriching environmental samples for *A. fumigatus* mycoviruses, an approach which was based on our experience with phages, yielded some interesting and promising results. This is the first time such a virus enrichment idea has been tried in the search for mycoviruses and the data show some convincing evidence for mycoviruses having been found but despite this promising start, clearly further optimisation is needed in order to realise the potential of such an approach.

In order for enrichment to be successful; the virus must be capable of infecting an otherwise healthy host fungal cell, this infection must lead to the replication of the viral genome, expression of viral proteins and the formation of progeny virus particles and the progeny virus particles must then be released from the host cell. If successful then progeny viral particles may be detected in the cell-free suspension produced from the culture. We therefore applied the various methods outlined in order to determine whether or not there was a detectable abundance of putative mycoviruses within the resulting collection of enrichments.

The few samples which yielded convincing data for the nanosight, electron microscopy and RNA extractions support the idea that such viruses may exist. A full study of the dsRNA profiles of all of the 128 virus enrichments would have been ideal. However the protocol we successfully used for identification of dsRNA in the type strain NCPF7367 was not suited to processing large numbers of samples and our attempts to use the two commonly used RNA extraction procedures for small starting quantities of sample were unsuccessful suggesting that this may be more challenging than previously thought (Figure 4-14).

There have been prior studies in which environmental *A. fumigatus* isolates were screened for the presence of viral dsRNAs (e.g. Bhatti *et al.*, 2012) but in future it may also be of interest to screen raw compost samples for viral genomes in order to build a more complete picture of the prevalence of these mycoviruses within compost. Also,

screening all of the compost-derived *A. fumigatus* isolates used throughout this project would be helpful in future in order to ascertain the frequency of virus carriage in this population relative to a non-compost collection of *A. fumigatus*. Previously it has been suggested that the frequency of virus carriage may be higher in environmental *A. fumigatus* populations (Bhatti *et al.*, 2012).

It should be recognised that all of the mycovirus enrichments used throughout this work were obtained using a relatively limited range of host strains (with each sample co-cultured with 3-5 strains of *A. fumigatus*). If mycoviruses have very narrow host ranges then this approach would underestimate their prevalence. The identification of an intrinsically susceptible strain of *A. fumigatus* would be a major step forward in terms of optimising this procedure. In similar phage work it is known that such amenable reference strains may be difficult to find but are invaluable in developing efficient and sensitive enrichment protocols for the isolation of lytic phages (Nale *et al.*, 2016). For mycoviruses, one possibility to assist in this could be the use of protoplasts instead of 'normal' healthy cells. The absence of the cell wall in protoplasts has been shown to make fungal cells more amenable to viral infection (Kondo *et al.*, 2013) and so would presumably increase the chance of any viruses present in the samples being able to infect and propagate during the co-culture.

One of the reasons that compost samples were used in this work was that it is known that developing compost contains high densities of *A. fumigatus* and so may be a potential source of the viruses able to infect the *A. fumigatus*. It is worth considering however that compost can be quite an extreme environment (the temperature inside a large, active compost heap can reach over 70°C) which doesn't necessarily lend itself well to viruses persisting outside of host cells. It is known that environmental conditions can influence the life cycle of phages, able to trigger the switch between a temperate and a lytic cycle (Williamson and Paul, 2006) and this may also be the case with mycoviruses. If so then the relatively harsh conditions encountered inside an active compost heap may not encourage mycoviruses to adopt a lifecycle which involves surviving outside of the host cell. For this reason, it would be interesting to continue the idea of using enrichment to study viral populations using samples obtained from other, less extreme environments also. Degrading leaf litter also has a high

abundance of *A. fumigatus* (Song *et al.*, 2010) but is not associated with such harsh conditions, and so may be a suitable target for looking for such viruses in future studies.

4.4.2 Virus identification method development

The single colonies generally grew well but no effects were observed with the addition of any of the virus enrichments tested (Figure 4-5). The low prevalence of mycoviruses and their unknown but possibly narrow host range means that the single colony approach is not a suitable method for screening a large library of samples. The single colony approach is time consuming and the determination of growth can be qualitative (with patchy growth occurring with some strains). Although this approach is seemingly unsuited to the identification of new mycoviruses, it is certainly a promising method for the determination of host effects and host range of any mycoviruses identified, as demonstrated by Yu *et al.*, 2013 (Figure 4-3, A).

From the work to optimise the plaque assay, growth curve comparisons and the single-colony experiments the general conclusion is that these methods were also not suitable for screening the enrichment collection to identify mycoviruses. The results however do suggest that some of these approaches may be suitable for studying the effects of mycoviruses on their host and for ascertaining the host range of any mycoviruses identified.

In particular, if the variability issues of the growth curve experiments using the incubating plate reader could be addressed then this could be a promising way to identify growth-rate changes associated with possible mycovirus infections. This method could be a high-throughput and novel way to test multiple virus-host combinations relatively simply and with quantitative results. Traditionally colony growth rates on solid agar have been used for this purpose (Tsai *et al.*, 2004) but this method is time consuming and it can be difficult to achieve sufficient accuracy when manually measuring multiple fungal colonies. The example of growth curve comparisons shown in Figure 4-6 (A) highlights that this method could be a useful tool in the study of such effects. Again, the findings here suggest that this could be a useful tool to study viruses once they have been identified even though it maybe not an ideal means to identify them in the first place.

These initial tests proved unpromising and so it was instead decided to concentrate on a higher-throughput method of looking for VLPs using the nanosight procedure.

4.4.3 Nanosight

The Nanosight proved to be much more suitable for the rapid screening of the collection of enrichments to help focus only on those samples which were likely to contain virus particles. This method is relatively high-throughput and is sensitive. The main downside to this approach is that the resulting data does not indicate what the particles are, just whether or not there is an abundance of virus-sized particles present. Nonetheless, it proved to be an effective way of screening the large collection of virus enrichments and narrowing this collection down to include only samples which contain particles within the expected size range of mycoviruses. Of the 128 enrichments produced 6 (4.7%) showed significant peaks of particle concentration within the range of 25-45 nm in diameter (see Figure 4-8). In the relevant control samples (obtained from the fungal culture used in the enrichment, but grown as a pure culture, without putative virus suspension co-cultured) there were no peaks within this size range. Together these findings do suggest that there is a population of virus-sized particles being amplified as a result of the co-culturing enrichment procedure. This suggests that these enrichments are worthy of further study. This is the first time, to my knowledge, that the nanosight system has been used to identify possible mycoviruses in fungal samples. The use of the nanosight system proved to be a quick method of screening out any enrichments in the collection which clearly had no evidence of virus-sized particles and thus was invaluable in this work and may prove to be a useful tool in future mycovirus studies.

4.4.4 Re-enrichment

As can be seen in Figure 4-9 andFigure 4-10 the initial attempts to further amplify the population of probable viruses in the enrichment 'V19' proved unsuccessful. The lack of peaks of particle concentration in the re-enrichments suggests no successful virus propagation in the host cells.

It is assumed that most mycoviruses do not routinely feature an extracellular phase in their life cycle (van Diepeningen *et al.*, 2006), being believed to propagate mostly via hyphal anastomosis, mitotic asexual cell division, or spore production (Ghabrial *et al.*, 2015). As none of these require the ability to persist in the environment outside of a host cell, it is likely that such viruses may be very sensitive to their external environment. An explanation for the lack of successful re-enrichment following a brief period in storage could be explained by the early findings of El-Sherbeini & Bostian (1987) who found that cell-free preparations of some yeast dsRNA viruses became non-infectious following a short period of storage at 4°C. This may explain why, after a period of storage at 4°C in cell-free buffer it would appear that any viruses present initially became unviable. Indeed it is certainly possible that the SM buffer (which is routinely used for the storage of phages) may not be the ideal storage medium for dsRNA viruses and that this may explain why they appear to be no longer viable. In contrast however, some other mycoviruses have been shown to remain stable and viable as isolated particles outside of the host cell (Hillman *et al.*, 2004). It is of course likely that different mycoviruses have greatly different abilities to persist as purified particles under laboratory conditions. Further work to elucidate the longevity of mycoviruses of human pathogens and to optimise storage media and conditions will be an important step in the development of successful mycovirus studies.

4.4.5 Transmission electron microscopy

TEM performed on enrichments V17, V19 and V20 showed the presence of virus-like-particles (Figure 4-11) which appear to be similar in size and shape to mycoviruses previously reported (Pearson *et al.*, 2009). These findings, together with the lack of similar structures seen in the fungal-control samples would appear to concur with the nanosight data and further suggests that these samples do contain mycovirus particles. Unfortunately owing to financial and time constraints it was not possible to perform TEM analysis on all of the other enrichment samples. Clearly this would be an important next step in the full evaluation of the enrichment procedure. In addition, following the cross-sectional transmission electron microscopy (CSTEM) work with *A. fumigatus* strain NCPF7367 (4.3.8) it would be of interest to perform CSTEM on the

fungal products of the co-culturing used in the production of these virus enrichments in order to see if viral particles can be identified within these cells.

4.4.6 dsRNA extraction

Enrichment V19, which showed the highest particle abundance on the nanosight data, was tested for the presence of dsRNA using the CF-11 cellulose dsRNA protocol (4.2.10). The multiple bands of dsRNA present (Figure 4-12) is indicative of the presence of a dsRNA mycovirus genome. The apparently multipartite nature of this genome, with three or four segments, is concurrent with the presence of a mycovirus, although it is not clear to which family such a mycovirus may belong. Four genome segments are a feature of the chrysoviridae, however the genome segments of this family range from 2.9-3.6kb (Nuss, 2010), considerably larger than the dsRNAs present in this sample. The genome segment sizes of the partitiviridae, in the range of 1.4-3 kb, more closely match the segments observed but the partitiviridae feature bipartite genomes and there are clearly three (and possibly a faint 4th) bands seen on the gel. It has been shown that an individual fungus may be infected concurrently with more than one mycovirus (Wang *et al.*, 2014). Indeed some mycovirus infections may require a so-called 'helper' virus to be carried by the host in order to be able to be propagated (Rodríguez-Cousiño *et al.*, 2011). Such a multiple infection would explain this pattern of dsRNA bands that would appear not to fit in with the virus genome descriptions of any of the currently recognised mycovirus families.

Viewed alongside the nanosight data and the particles observed on TEM, this finding does strongly suggest the presence of a mycovirus in this enrichment, and offers evidence of the success of the enrichment procedure. Overall these findings strongly support the hypothesis that *A. fumigatus* mycoviruses are present and may be isolated from compost samples by enrichment. Unfortunately it was not possible to perform this dsRNA extraction protocol for any of the other virus enrichments samples due to a lack of starting material – the CF-11 protocol requires a reasonably large starting volume. It is for this reason that attempts were made to identify an RNA isolation procedure for use with small starting volumes (discussed below, 4.4.7). This method was also used to isolate and purify the dsRNA genome of the mycovirus which is known to be carried by

A. fumigatus strain NCPF7367. The multipartite dsRNA genome of this virus is also shown by the gel electrophoresis image in Figure 4-12.

4.4.7 dsRNA extraction kit tests

The purpose of testing these RNA extraction kits was to find a way of screening all of the virus enrichments for dsRNA. For many of these enrichments only a small volume (<1 ml) was available and the previously mentioned lack of success in re-enriching (4.4.4) to further amplify any viruses present ruled out the possibility of using a large volume procedure such as the CF-11 cellulose protocol. It was necessary therefore to attempt to identify a method that would be able to detect dsRNA from a small volume in a relatively high-throughput manner.

Two commonly used commercially available dsRNA kits were tested, the SV total RNA isolation kit (Promega UK) and a modification of the RNeasy RNA extraction kit (QIAGEN). The lack of bands seen on the gels (Figure 4-14), along with the nanodrop data suggest that these RNA extraction kits are not sensitive enough to detect the dsRNA genome of the mycovirus infecting *A. fumigatus* strain NCPF7367. This perhaps is not surprising given the low starting volume used in these protocols and the possibility that virus titre may be relatively low in such apparently asymptomatic virus infections (Roossinck, 2010). These findings effectively ruled out the possibility of using one of these relatively high-throughput RNA extraction methods as a means of screening the collection of enrichments for viral dsRNA genomes. The apparent false negative results obtained for strain NCPF7367 also suggest that dsRNA genomes and thus mycovirus carriage in fungi is likely to be underestimated when similar approaches are used to determine mycovirus presence.

4.4.8 *Aspergillus fumigatus NCPF7367* virus particle search

In the initial attempts to more fully characterise the nature of the dsRNA virus carried by NCPF strain 7367 there were no virus-like particles seen under T.E.M (Figure 4-13) suggesting that the virus may be completely intracellular. Consequently cross-sectional TEM (CSTEM) was performed on this strain alongside *A. fumigatus* strain NCPF7097 as a negative control (this strain is known not to carry a mycovirus). The CSTEM

(Figure 4-16 toFigure 4-18) showed no evidence of viral particles within the cells of NCPF7367 either. Previously it has been noted that mycovirus infection may be associated with accumulations of VLPs in the cytoplasm, granulated cytoplasm and apparently damaged nuclear and mitochondrial membranes (Zhang *et al.*, 2009). No such observations were apparent in the comparison of CSTEM images obtain from the virus infected and virus-free NCPF isolates 7367 and 7097.

In addition, the fractions resulting from the sucrose gradient assay had their protein concentration measured by the Bradford assay and their RNA concentration measured using the nanodrop. Any viral particles present would be expected to be purified during the PEG precipitation steps of the procedure and would then be separated out into a band on the density gradient during ultracentrifugation. The expected result of this would be that there would be a band of virus particles at some point in the gradient, where both RNA and protein would be at a considerably higher concentration. This was not observed (Figure 4-15) which suggests an absence of viral particles in the NCPF7367 culture, in concordance with the findings of the TEM and CSTEM work on this strain, and subsequently backed up by the genome analysis (4.4.9).

4.4.9 NCPF7367 virus genome sequence

The data obtained from the MiSeq protocol was analysed using the BLASTx sequence alignment search tool (NCBI). Subsequently the genome sequence of this virus was also sequenced and published by Kanhayuwa *et al.* (2015), whose results confirmed the findings and conclusions from our own sequence analysis as well as further describing the infectious nature of this dsRNA genome. The genome segment sequence alignments shown in figures 4-19 to 4-22 show the high level of concordance between the sequences obtained in the present study and those published by Kanhayuwa *et al.* (2015). Such high identity matches over all of the dsRNA segments suggest that the two sequence sets are indeed the genome sequence of the same virus, as may be expected as the host strain *A. fumigatus* NCPF7367 is a well characterised and stable type strain with an apparently stable and consistent carriage of this virus.

At the time of analysis each of the four segments showed sequence homology to one of four genome segments of a virus of *Cladosporium cladosporioides*, a common

environmental mould, and to one of four genome segments of a mycovirus of the widespread plant pathogen *Botryosphaeria dothidea*. The genome segments of the *Cladosporium cladosporioides virus 1* (CCV-1) covers GenBank accession numbers NC_024704 to NC_024707 and the genome segments of the *Botryosphaeria dothidea virus 1* (BDV-1) cover GenBank accessions AKE49495 to AKE49498. In addition to the similarity to these multi-segmented mycovirus genomes, each of the segments also showed some homology to other known or putative mycoviruses as outlined below. A schematic overview of the size and position of the ORFs within each genome segment and the position of the protein domains mentioned is shown in figure 4-23. The ORFs predicted from each genome segment appear to be complete, as confirmed by protein analysis by Kanhayuwa *et al*. (2015) which demonstrated that the proteins produced from each genome segment match the protein size predicted from the amino acid sequences of these putative ORFs.

The largest segment (Segment 1) features a typical RNA dependent RNA polymerase motif categorised as being an RDRP motif in the reverse transcriptase-like superfamily. This showed homology to the largest, RDRP-containing genome segments from CCV-1 (Genbank accession: NC_024704) and BDV-1 (accession AKE49495) as well as to an RDRP-containing genome segment of a mycovirus of the plant pathogen *Alternaria tenuissima* (Genbank accession AJP08049). Alignment of these amino acid sequences to the sequence of the ORF predicted from the RNA sequence of segment 1 obtained in this study is shown in figure 4-24.

The next largest fragment (Segment 2) contains a region which appears to share sequence homology to a protein domain previously found to play a role in temperature-tolerance in an *S. cerevisiae* mycovirus (Vermut *et al.,* 1994). This segment shares sequence homology to CCV-1 segment 2 (Accession NC_024705), another segment of the BDV-1 genome (Genbank accession AKE49496) as well an uncharacterised sequence from a different *Alternaria* sp. dsRNA sequence (Accession ACL80752). The function of this gene product remains unknown. Figure 4-25 shows alignment of these amino acid sequence matches to the putative ORF encoded by segment 2.

The next smallest segment (Segment 3) contains a methyltransferase domain and shows homology to segments of CCV-1 (Accession NC_024706) and BDV-1(accession

AKE49497) genomes as well as a dsRNA fragment previously identified in the plant pathogen *Melampsora lini* (Accession CAA45724). RNA virus methyltransferase enzymes are RNA capping enzymes, adding a guanylyl group to the 5' end of the $+^{ve}$ strand of the RNA molecule and have been previously identified in RNA mycovirus genomes (Howitt *et al.*, 2006). The alignment of these amino acid sequences to the ORF predicted from the sequence data of segment 3 is shown is figure 4-26.

The smallest genome segment (Segment 4) shares only significant homology to the smallest genome segments of CCV-1 (accession NC_024707) and BDV-1 (accession AKE49498) and is of unknown function. The protein produced from this RNA would be a Proline, Alanine and Serine rich protein (PASrp). It has subsequently been proposed (Kanhayuwa *et al.*, 2015) that this protein may have a function involved in genome-coating and thus in offering protection for the genome. It is noted by Kanhayuwa *et al.* also that PASrps are almost always encoded by viruses with no capsid, which further supports this theory. In addition, they show that this protein is seen associated with these dsRNA genome fragments in Atomic Force Microscopy (AFM) analysis. This suggests that while not having a capsid, this virus genome may instead be afforded some protection by close association with this PASrp. Figure 4-27 shows the alignment of these amino acid sequences to that of the ORF predicted from the sequence data obtained from segment 4.

Crucially, none of the proteins encoded by any of these genome segments bear similarity to any structural viral proteins (capsid proteins etc.) and so it is now assumed that this virus in fact doesn't produce infectious viral particles and instead exists solely as a genetic element (albeit maybe in close association with the abovementioned PASrp). Indeed this is backed up by further work by Kanhayuwa *et al.* (2015) which showed that the purified genome alone was infectious and was able to propagate throughout generations of a previously virus-free isolate. The adoption of a life cycle which involves the genome itself acting as the infectious stage may be a strategy to overcome the cell wall as a barrier to the traditional route of viral particle-borne infection.

This finding that the virus encodes no capsid protein and presumably exists instead as 'naked' dsRNA is consistent with the earlier negative findings from the TEM, CSTEM

and protein work. An abundance of virus particles was not found by any of these methods as there are no virus particles produced by this mycovirus. This finding also raises the point that non-particle-forming mycoviruses would of course not be identified by the nanosight system used to screen compost enrichments for viruses in the present study.

Screening both for viral particles and for viral genomes (RNA and DNA) is necessary in order to more accurately determine the prevalence of mycovirus carriage in fungal populations. Limiting the search to only one of these fields will inevitably lead to underestimating the prevalence of such viruses. There was a focus on dsRNA in this project as the majority of known mycoviruses have dsRNA genomes. It should be considered, however, that the mycovirus recently observed to have potential for use as a biocontrol agent against *S. sclerotiorum* has a DNA genome (Yu *et al.*, 2013), and so would be undetected in a purely dsRNA-based search strategy. This further highlights the importance of using a combination of approaches in the search for mycoviruses and designing mycovirus identification strategies as broadly as possible to take into account the great diversity in structure and form seen amongst mycoviruses. Assays to identify sub-genomic DNA fragments amongst fungal isolates, such as the approach used in the initial identification of the S. *sclerotiorum* mycovirus (Yu *et al.*, 2010), should therefore be considered in future studies alongside dsRNA and viral particle screening.

Chapter 5 Azole resistance, pathogenicity and mating type in *Aspergillus fumigatus*

5.1 Introduction

5.1.1 Resistance

The frequency of azole resistant isolates of *A. fumigatus* being detected is increasing (Howard *et al.*, 2009; van der Linden *et al.*, 2015; Verweij *et al.*, 2016). The origins of this resistance are largely unknown, and there are questions about the prevalent mechanisms of resistance in different settings. This study of the sensitivity profiles to a range of azole drugs in clinical and environmental sample collections aims to provide further insight into the prevalence, mechanisms and origin of resistance within these distinct populations of *A. fumigatus*.

As early as 1997 multiple mechanisms of azole resistance had been postulated (Denning *et al.*, 1997). At this time only one azole drug, itraconazole, was licensed for therapeutic use in the UK (with Amphotericin B being the only other licensed antifungal available for treatment of *Aspergillus* infections) but the introduction of other azole drugs and the subsequent use of combination drug treatment seems not to have prevented the proliferation of resistance, including multi-drug resistance.

The most common resistance mechanism is TR34/L98H; a point mutation leading to an amino acid substitution at L98H in the cyp51A gene alongside a 34 base pair tandem repeat in the promoter region of this gene (Verweij *et al.*, 2009). The cyp51A gene encodes 14α sterol demethylase - the target enzyme of azole drugs and an important enzyme in the production of ergosterol in fungal membranes (Kalb *et al.*, 1987).

5.1.2 Pathogenicity

A. fumigatus is known to be able to cause a range of invasive infections (Dagenais and Keller, 2009). Infection with *A. fumigatus* generally occurs by the inhalation of airborne conidia (O'Gorman, 2011). Aside from the allergenic potential of these spores (Edwards *et al.*, 2012), they may go on to germinate within the lung. Such a germination event may result in colonisation of the respiratory tract (de Vrankrijker *et*

al., 2011) and may result in the growth of a fungus ball, or aspergilloma within the lung itself (Guazzelli *et al.*, 2012; Howard *et al.*, 2013). Less commonly and with far more serious implications for the patient, the mycelial growth of the fungus may invade local tissues. This may then lead to invasive aspergillosis (Denning, 1998; Dagenais and Keller, 2009) either near the site of infection (most usually the lung) or if the tissue invasion reaches the blood stream then disseminated aspergillosis can occur. In this scenario sites of fungal growth and damage can range to almost anywhere within the body, including the brain (Hope, Walsh and Denning, 2005). As the most common mechanism of infection involves the inhalation of spores from the environment it has been suggested that infection itself acts as a selective process. The clinical population can thus be considered a sub-population of the global environmental population, with those isolates more readily able to instigate viable infection being selected for (Alshareef and Robson, 2014).

An invertebrate model of pathogenicity using the Waxmoth *Galleria mellonella* as the test organism allowed all of the isolates tested in the drug resistance screening work to also be tested for pathogenicity. As these collections include a large number of clinical and environmental isolates, this pathogenicity testing was to help identify the range of virulence profiles amongst different *A. fumigatus* populations.

5.1.3 Mating type

Having been considered entirely asexual for around the first 150 years of our knowledge of *A. fumigatus* this fungus was demonstrated in 2009 to be able to undergo sexual reproduction (O'Gorman, Fuller and Dyer, 2009). Although this stage of the *A. fumigatus* life cycle appears to be very sensitive to environmental conditions and is presumed only to be a rare occurrence it nonetheless shows that *A. fumigatus* has greater potential ability to adapt to its conditions than previously thought and to accelerate population-wide phenotypic changes (such as resistance to antifungal drugs) via the recombination events associated with meiosis-driven sexual reproduction. Sexual reproduction in *A. fumigatus* is governed by a mating type system similar to that found in other sexual fungi (Pyrzak, Miller and Miller, 2008). The MAT locus features one of two idiomorphs; the MAT1-1 and MAT1-2 alleles. These encode either an

alpha domain or an HMG domain respectively, both of which are transcription factors which ultimately determine sexual compatibility and control the switch from asexual reproduction to the sexual reproduction cycle (Varga, 2003).

The use of the MAT mating type locus as a means of genotyping isolates is a useful tool as it not only gives insight into the population structures of *A. fumigatus* but may also shed light on the questions around whether one or other mating type is more likely to be associated with different environments or growth characteristics. For example the fact that one of the mating types has previously been shown to be associated with higher activity of elastase, considered to be an important virulence factor in respiratory pathogens, suggests that there may be a bias towards invasive disease and MAT1-1 (Alvarez-Perez *et al.*, 2010).

The hypothesis underlying the work presented in this chapter is that antifungal drug resistance is more prevalent in compost derived *A. fumigatus* isolates than in clinical isolates and this resistance is associated with altered pathogenicity in these two populations of the fungus. Isolates obtained from patients following antifungal drug treatment may also display higher antifungal drug resistance or greater pathogenicity. It is also hypothesised that mating type differences between *A. fumigatus* isolates are associated with altered pathogenicity and isolate origin.

5.2 Materials and Methods

5.2.1 Clinical isolates

The clinical isolates used in this work were isolated from sputum obtained from patients by the protocol outlined in 2.3.1. The sputum samples were taken routinely from patients enrolled in the 'Effectiveness of Voriconazole In the Treatment of Aspergillus Associated Asthma' (EVITA3) study at Glenfield hospital, Leicester, UK between 2009-2013. This study involved the treatment of subjects with either voriconazole or a placebo compound (Agbetile *et al.*, 2014). The longitudinal nature of the study makes it possible to use isolates obtained before, during and after azole therapy for the subjects in the treatment group, as well as comparing the clinical group as a whole to the environmental isolates. Lung function data collected for each subject was used to identify any correlation between severity of disease and the characteristics of the *A. fumigatus* cultured from sputum (in terms of azole sensitivity, virulence in *Galleria* pathogenicity model and mating type). Sputum samples were incubated at 37°C on PGCF agar plates and monitored for 7 days. *A. fumigatus* colonies were counted and sub-cultured to obtain a pure isolate which was initially stored on agar slants at 4°C until being transferred to -80°C in Glycerol Nutrient Broth (GNB) for long term storage. The 80 clinical isolates used in this work were from 44 individual subjects, of which 17 were in the placebo group and 27 were treated with voriconazole.

5.2.2 Environmental isolates

The environmental isolates were obtained either from air samples collected on compost sites by use of the Andersen air sampler as per current guidelines (AfOR, 2009) or from solid compost samples collected using the procedure detailed in 4.2.2. The raw compost sample is weighed and suspended in phosphate buffered saline supplemented with 0.05% Tween 80 (PBST), agitated gently, diluted and an aliquot of the resulting mixture is plated out onto multiple PGCF agar plates, incubated at 37°C for up to 5 days. Probable *A. fumigatus* colonies are confirmed by needle-mount microscopy and sub-cultured to obtain pure cultures. Pure isolates are then stored at -80°C in GNB until use.

5.2.3 EUCAST plate preparation

Drug sensitivity testing was performed by the broth microdilution method published by The European Committee on Antimicrobial Sensitivity Testing (EUCAST) in the protocol EUCAST E.Def 9.2 (Arendrup *et al.*, 2014). Sensitivity to three commonly prescribed clinical azole antifungal drugs; voriconazole, itraconazole and posaconazole was tested. EUCAST publish breakpoint values for these drugs- the minimum inhibitory concentration (MIC) values at which an isolate can be considered resistant to clinically-relevant concentrations of the drug. The clinical breakpoint values for *A. fumigatus* for these azole compounds are shown in Table 5-1.

The plates containing the dilutions of the antifungal compounds were prepared in batches and stored at -80°C until use. The growth media used in the EUCAST testing is RPMI-1640 containing 2% glucose (appendix 1). This liquid medium was prepared initially at double concentration and the concentration of the drug in each well of the 96 well plate is prepared to be double the required testing strength, in a total volume of 100 µl. All of the initial dissolving and dilution steps in the preparation of the drug suspensions were carried out in sterile glass bijou tubes, as there has been some suggestion that plasticware used in these stages may bind some of the compounds and thus reduce the overall available concentration of drug in the final dilutions (Caroline Moore, personal communication). For the preparation of the batches of plates the azole compounds in pure, powdered form were dissolved in DMSO to a concentration of 1600 mg/l and left shaking at room temperature in the dark for 1-4 hours to ensure the powder fully dissolved. Serial dilutions were then made in DMSO down to 3.125 mg/l, from which further 1/100 dilutions were made in 2x RPMI. This gives working solutions of 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06 and 0.03 mg/l which were then aliquoted into the wells of a flat-bottom, sterile, polystyrene 96 well plate (Appleton woods). The plates were then sealed individually into plastic bags and stored upright at -80°C. Upon inoculation of the spore suspension into the plates, the addition of 100 µl of the spore suspension into each well brings the RPMI, test drug and spore density to the required final testing concentration.

Each time a new batch of plates was prepared, one test plate was inoculated with 6 control strains of fungi; 3 *Candida* yeasts (*C. krusei* strain ATCC 6258, *C. krusei* strain CL3403 and *C. parapsilosis* strain ATCC 22019) and 3 filamentous fungi (*A. fumigatus* strain NCPF7097, *A. fumigatus* strain NCPF 7100 and *A. flavus* strain CM1813) whose antifungal sensitivities are well documented and known to be stable and reproducible. The use of these control strains is to provide initial confirmation that the dilutions and pipetting have been carried out accurately and that the wells of the plates at this stage contain the correct working concentration of the drug. Each further time the plates were used a single control strain was inoculated alongside the isolates being tested, in order to confirm that the drug's potency isn't reduced following storage at -80°C and during the freeze-thaw cycle.

Table 5-1: Breakpoint MIC values for the three azole compounds used in this work for four common *Aspergillus* species. The S≤ and R> columns for MIC values denote sensitivity or resistance at the given values. (N= No breakpoint values published) (From EUCAST, 2015).

Antifungal agent	MIC Breakpoint (mg/L)							
	A. flavus		*A. fumigatus*		*A. nidulans*		*A. terreus*	
	S≤	R>	S≤	R>	S≤	R>	S≤	R>
Itraconazole	1	2	1	2	1	2	1	2
Posaconazole	N	N	0.126	0.256	N	N	0.126	0.256
Voriconazole	N	N	1	2	N	N	N	N

5.2.4 EUCAST plate inoculation

The inoculation of plates containing each of the drugs being tested was carried out at the same time, from the same spore suspension for each isolate. All isolates were grown on PDA agar plates at 37°C for 2-4 days before fresh spores were harvested (Section 2.1.3). The spore suspension was counted using a haemocytometer (Section 2.2). From this suspension a dilution is made in PBST to give 3.5×10^5 spores/ml. 100 μl of this

suspension was added to each of the relevant wells of a EUCAST drug dilution plate pre-prepared as in 5.2.3. The negative control well of the same row of the EUCAST plate was inoculated with 100 µl of the same batch of PBST used in the spore dilutions and the positive control well was inoculated with 100 µl of the spore suspension, but contained only the RPMI-1640 medium, with no drug added.

The inoculum density and spore viability was confirmed by plating out a further dilution of the inoculum. 10 µl of the inoculum was added to 2 ml PBST and 100 µl of this was then pipetted onto the centre of a sterile PDA plate and a sterile glass spreader was used to spread the suspension evenly over the surface of the agar. The plate was incubated at 37°C and the number of colonies counted after 24-48 hours. Between 100-250 colonies corresponds to $2\text{-}5\text{x}10^5$ CFU/ml in the suspension used for EUCAST plate inoculation, which is the inoculum density range over which the EUCAST method is validated. Taking the 50% dilution into account, as 100 µl of spore suspension is added to 100 µl of drug dilution in the well of the plate, this equates to a final inoculum in the wells of $1\text{-}2.5\text{x}10^4$ CFUs.

5.2.5 cyp51 PCRs and sequencing

In an attempt to characterise the genetic basis of any resistance seen, a region of the cyp51A gene was sequenced for a sub-set of samples to identify if any of three well characterised mutations in this gene, previously implicated in azole resistance (Chen *et al.*, 2005), are present.

For the isolates selected for cyp51A genetic analysis total genomic DNA was extracted (see section 2.5) and two PCRs were used to amplify the part of the promoter region of cyp51A in which the TR34 and TR46 mutations occur and a region in the coding sequence of this gene in which the L98H and Y121F mutations occur. The PCR primers used were primers P-A7 (5'- TCATATGTTGCTCAGCGG -3') and P-A5(5'-TCTCTGCACGCAAAGAAGAAC-3') (Mellado *et al.*, 2001) which amplify the region (Fragment A) of the promoter where the TR34 and TR46 mutations occur (638bp in the wild-type, 672bp or 684bp if the TR34 or TR46 mutations are present) and CYP1-L (5′-CACCCTCCCTGTGTCTCCT-3') and CYP1-R (5′-AGCCTTGAAAGTTCGGTGAA-3') (Chen *et al.*, 2005) which amplify a 576bp

fragment (Fragment B) of the coding region of the cyp51A gene (including the site of the L98H and Y121F mutations). The fragments from these two primer pairs overlap, allowing the sequence data to be merged to create one continuous sequence of the region covering these three mutation sites. To account for the potential for PCR-induced errors in the sequence data each PCR was performed twice independently and PCR success was confirmed by gel electrophoresis (see 2.6). Each PCR product was then sequenced in both directions using the forward and reverse primers in order to give full read coverage and ensure fidelity once the resulting sequences were aligned to one another and compared.

The PCR reaction mix was as follows; a final concentration of 0.5 µM of each primer was used in 1x QIAGEN PCR buffer, 0.2mM dNTPs, 1 unit HotStarTaq, 5 µl DNA, made up to 25 µl total volume with PCR-grade H_2O. PCR cycling conditions were as follows; initial melting at 95°C for 5 minutes followed by 30 cycles of [30s denaturing at 95°C, 40s annealing at 59°C, 110s extension at 72°C] and a final extension step of 72°C for 10 minutes.

The PCR products were run on 1% agarose gels to confirm PCR success and were then cleaned up using the spin-column Jena PCR purification kit (Jena Bioscience GmbH). The DNA concentration was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific Ltd.) and from this data appropriate dilutions were made to achieve a concentration of 20 ng/10 µl. This was sequenced by the Protein and Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester using the Applied Biosystems 3730 Genetic Analyser system. The resulting sequence data was analysed using the FinchTV software (Geospiza Inc., Version 1.4.0) and alignments performed using the MEGA software package v6.0. The sequences obtained were aligned to one another to merge then compared to four previously published cyp51A sequences with Genbank accession numbers; AF338659, EU807920, KJ210331 and KP270713.

5.2.6 *Galleria mellonella* **virulence study**

Prior to testing a wide range of isolates for virulence in *G. mellonella*, initial experiments were carried out to determine a suitable dose and methodology. A dose-

response experiment using the reference strain *A. fumigatus* NCPF7097 was performed. Spore stock of this strain was recovered from -80°C, plated out onto PDA and incubated for 3 days at 37°C. The spores were then harvested in PBST (see 2.1.3). The resulting spore suspension was removed from the plate with a Pasteur pipette and centrifuged for 10 minutes at 1500g. The pellet was then resuspended in 5 ml PBST and vortexed. A 1/100 dilution (in PBST) of this suspension was counted on a haemocytometer (see 2.2) to ascertain the concentration of spores/ml. The spore suspension was then diluted to a concentration of 1×10^8 spores/ml. A further dilution was made to achieve a concentration of 1×10^4 spores/ml, of which 10 µl was spread onto a PDA agar plate (in triplicate) for enumeration of CFUs. 100% viability of the spores in this suspension would yield 100 colonies on these plates. The plate was incubated at 37°C for 24 hours and the 1×10^8 spores/ml stock was stored in the dark at 4°C during this time. From the CFU count the viability of the spore suspension was calculated, and based on this the number of CFUs/ml in the original 1×10^8 spores/ml stock was calculated. Appropriate dilutions were then carried out in PBST to yield 1×10^7, 1×10^6, 1×10^5, 1×10^4 and 1×10^3 CFU/ml suspensions. 10 µl of each of these suspensions injected into the larvae gives the following doses; 100000, 10000, 1000, 100 and 10 CFUs. Each of these inocula was injected into groups of 10 larvae in triplicate as detailed below. The larvae were then monitored daily for 7 days and the survival curves plotted.

Throughout this work, final instar *Galleria mellonella* (Greater Waxmoth) larvae were used (Livefoods UK Ltd.). All larvae were used within one week of delivery, and only those larger than 1cm in length and which appeared healthy (with no patches of dark colouration or any obvious signs of ill-health) were used. The larvae were stored at 4°C in the dark until the day of the inoculations when they were removed from the fridge and left for ~1 hour in the dark at room temperature, whilst the spore dilutions were made. This allows the larvae time to acclimatise to the warmer temperature and to wake up. It also ensures more efficient injections and causes less damage to the larvae, as they are very soft and prone to damage if injected before warming to room temperature. The spore suspension was vortexed thoroughly before being drawn into the syringe (Beckman U-100 insulin syringe; 28G needle). 10 larvae were then injected using a Tridak Stepper repetitive pipette (Tridak LLP), set to dispense 10 µl volumes. The

spore suspension was injected directly into the haemocoel through the last left proleg of the larva. The injections were carried out immediately once the syringe was filled with the spore suspension and a fresh syringe was used for each repeat group of 10 larvae, to prevent the spores clumping at the bottom of the syringe. Three control groups of larvae were used; No intervention, Needle injury but without injection and injection of 10 μl PBST. Each group of 10 larvae was placed in a sterile Petri dish and incubated in the dark at 37°C for 7 days, with the number of larvae alive scored for each group daily. Dead larvae were removed as they were counted, and were stored individually in 1.5 ml Eppendorf tubes at -80°C for potential future work to enumerate fungal load in these larvae. Some larvae can appear to be dead (blackened colour, no movement) but are in fact still alive and so it is important when counting alive/dead larvae daily to gently prod any suspected dead larvae and look carefully for a response to this touch. Figure 5-1 shows larvae in varying stages of health following inoculation. It is also worth noting that a small number of larvae, in both control and treatment groups, began to pupate at around 6 days of incubation. Any such larvae could still be scored for alive/dead as they remained responsive to touch.

The data was analysed and survival curves plotted using the Kaplan-Meier method. Three distinct analyses were performed; the survival curves analysed for significant difference to one another, the time taken for 50% of the larvae injected to die, and the percentage survival 72 hours post-infection.

5.2.7 Mating type AFM-PCR assay

The two idiomorphs of the mating type locus, MAT1-1 and MAT1-2 can be distinguished by a multiplex PCR approach, the AFM PCR assay (Paoletti *et al.*, 2005). Three primers are used, one is complementary to a region found in both mating types and each of the other two primers is only complementary to either the MAT1-1 or MAT1-2 idiomorphs. The primers are; AFM-1 which is complementary only to MAT1-1 (5' CCTTGACGCGATGGGG GG3'), AFM-2 which is complementary only to MAT1-2 (5' CGCTCCTCATCAGAACAACTCG3') and the common primer AFM-3 (5' CGGAAATCTGATGTCGCCACG 3') (Paoletti *et al.*, 2005). The primer pairings produce fragments of different lengths, so mating type can be easily determined by gel

electrophoresis, shown in Figure 5-2. DNA was extracted from the fungal isolates using the QIAGEN DNeasy plant mini-kit, as outlined in section 2.5. The PCR reaction mixture was as follows; 1x QIAGEN PCR buffer, 0.2mM dNTPs, 0.25 µM of primers AFM1 and AFM2, 0.5 µM of the common primer AFM3, 1 unit HotStarTaq DNA polymerase (QIAGEN) and 5 µl sample DNA, made up to 25 µl total volume with PCR-grade H_2O. The PCR cycling conditions used were; initial melting at 95°C for 15 mins followed by 35 cycles of [30s at 95°C, 30s at 60°C, 1 minute at 72°C] and a final extension step of 5 minutes at 72°C.

5 µl of PCR products were run at 100v, 250mA in a 1.5% agarose gel for 40 minutes, visualised with a UV transilluminator and electrophoretic mobility of the fragments was quantified against PCRsizer 100bp ladder (Norgen Inc.). A representative gel photograph is shown in Figure 5-2. The banding patterns observed corresponding to the mating types MAT1-1 and MAT1-2 are shown.

5.2.8 Statistical analysis

All raw data was collected in Microsoft Excel and statistical analyses performed using Prism, V6.07 (Graphpad Software, Inc.). Parametric data was analysed by unpaired t-tests and non-parametric data was analysed by Mann-Whitney and Kruskal Wallis tests. Spearman's rank correlation test was used for correlation analyses and Kaplan-Meier statistics were used for the plotting of survival data, with the Mantel-Cox Log-rank test being used for analysis of survival curves. Fisher's exact test was used for analysis of the mating type data.
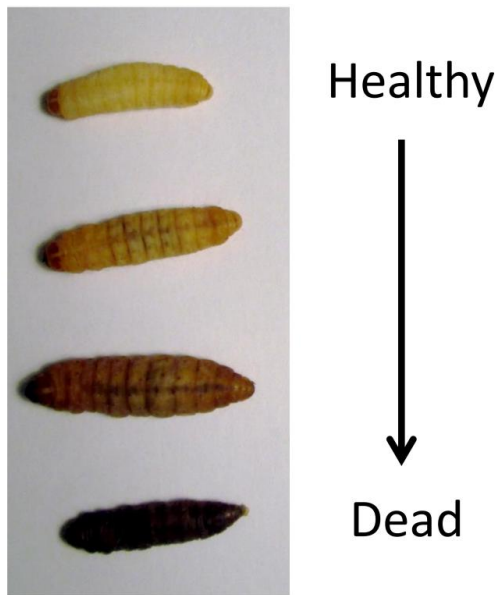
Figure 5-1: *G. mellonella* **larvae in different states of health.** Note the characteristic darkening of the larvae as health deteriorates and the shrunken, black appearance of the dead larva.
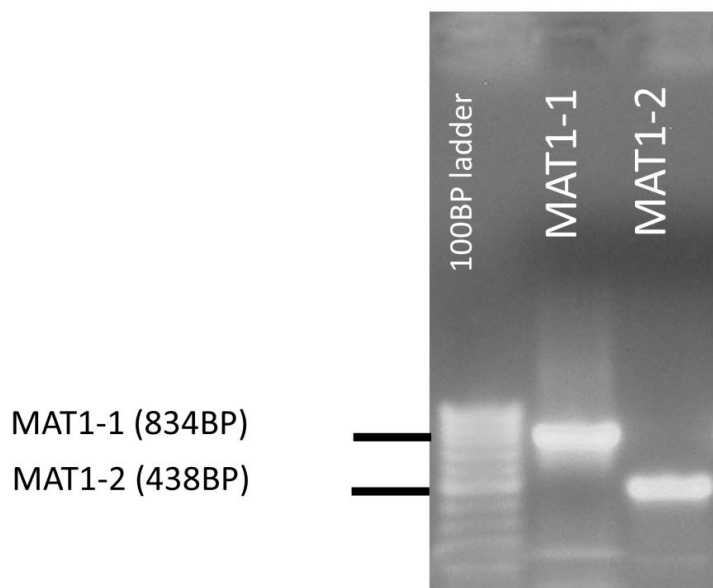
Figure 5-2: A representative gel electrophoresis image showing the MAT1-1 and MAT1-2 fragments produced by the AFM MAT PCR assay. 1.5% agarose gel run for 40 minutes at 100v.

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5.3 Results

5.3.1 Antifungal susceptibility

None of the samples tested by the EUCAST procedure gave MIC values above the resistance breakpoint value for either voriconazole (2 mg/l) or itraconazole (2 mg/l). One environmental isolate had a MIC value of 0.5 mg/l for posaconazole, above the breakpoint value of 0.25 mg/l. The MIC values for all isolates obtained for the three azole compounds are shown in figuresFigure 5-3 Figure 5-5. From these data it can be seen that some isolates produced a MIC value which is at the level of the breakpoint value. These isolates are classed as showing intermediate resistance (see **Error! Reference source not found.**). Seven Isolates showed intermediate resistance to voriconazole, of which six also showed intermediate resistance to posaconazole. Five isolates showed intermediate resistance to itraconazole, of which two also showed intermediate resistance to posaconazole and thirty-two isolates showed intermediate resistance only to posaconazole. There were no samples which showed intermediate resistance to both voriconazole and itraconazole. These data are summarised in Figure 5-6.

It can be seen from Figure 5-3 toFigure 5-5 that the majority of higher MIC values are in the environmental isolate group rather than the clinical isolate group. This is highlighted in Figure 5-6 also. This trend of higher MIC values, thus lower susceptibility to all three drugs in the environmental isolates is statistically significant (Mann-Whitney test; Voriconazole: p=0.0287, itraconazole: p<0.0001, posaconazole: p<0.0001) and is shown in Figure 5-7.

A correlation between the MIC values for each combination of the drugs was found to be statistically significant, as shown in Figure 5-8.

The clinical isolates can be divided into fungi derived from azole naïve and azole treated patients. There is no significant difference in MIC values to voriconazole between the isolates from voriconazole naïve and voriconazole treated patients. Conversely, there was a significant difference in both itraconazole MICs and

posaconazole MICs between voriconazole-naïve and voriconazole-treated samples (Mann-Whitney p=0.0194 and p=0.0315 respectively). This is shown in Figure 5-9.

Of the seven isolates which showed intermediate resistance only to voriconazole one was a clinical isolate. There was one clinical isolate which had an intermediate resistance MIC value for itraconazole out of a total of four and seven out of the thirty-five intermediate isolates to posaconazole were clinical isolates. In total, forty-six isolates were at or above the intermediate-resistance threshold for one or more drugs.

Of the seven voriconazole intermediates none were also intermediate for itraconazole and six were also intermediate to posaconazole. Of the five itraconazole intermediate resistant isolates, two were also intermediate resistance to posaconazole. Of the forty-two isolates showing intermediate resistance to posaconazole, eight were also intermediate resistance to another drug (six voriconazole, two itraconazole). From this analysis there would appear to be no compelling evidence for cross resistance amongst these intermediate resistant isolates.
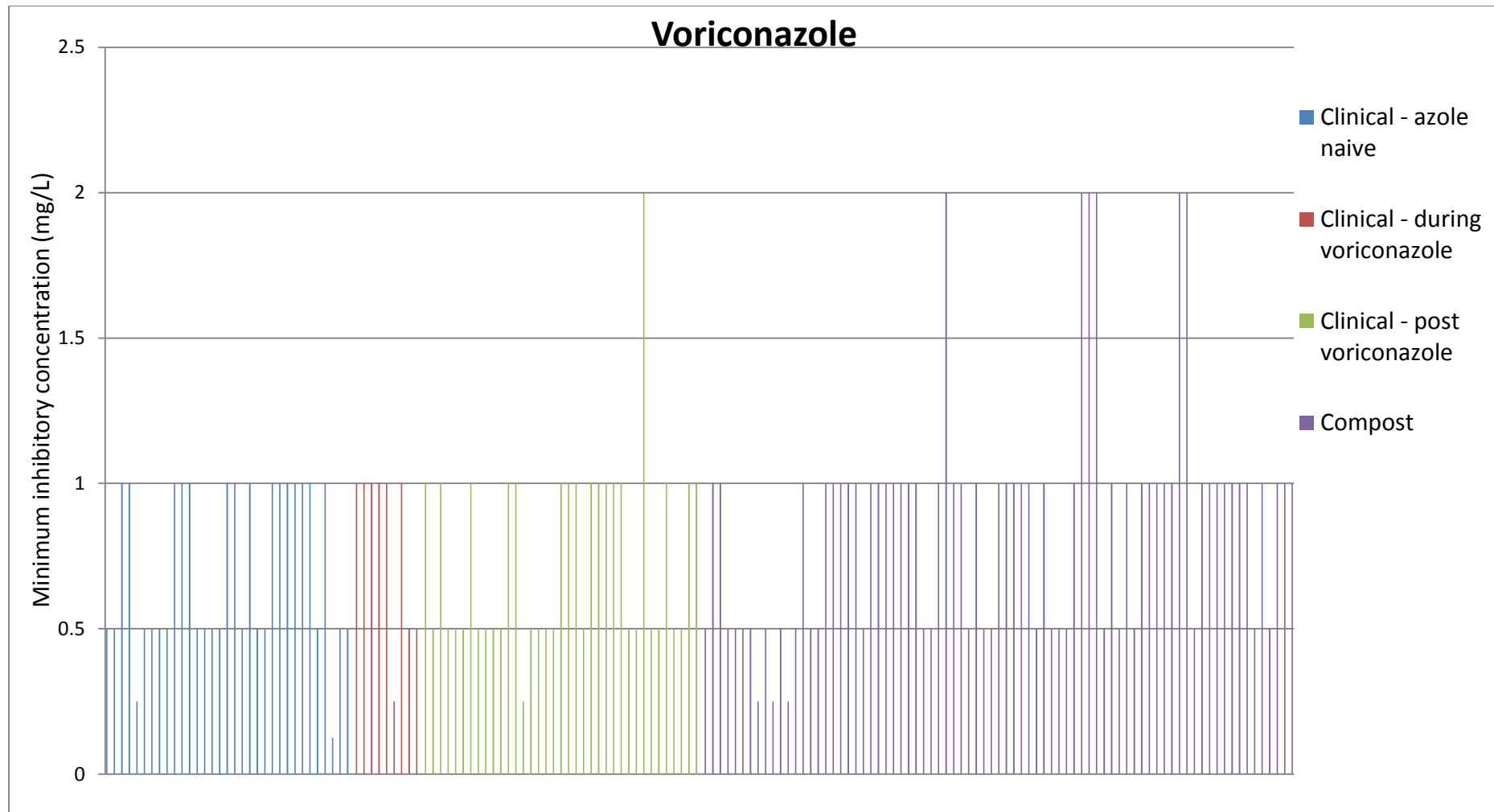
Figure 5-3: MIC values for voriconazole amongst the clinical and environmental *A. fumigatus* isolates tested
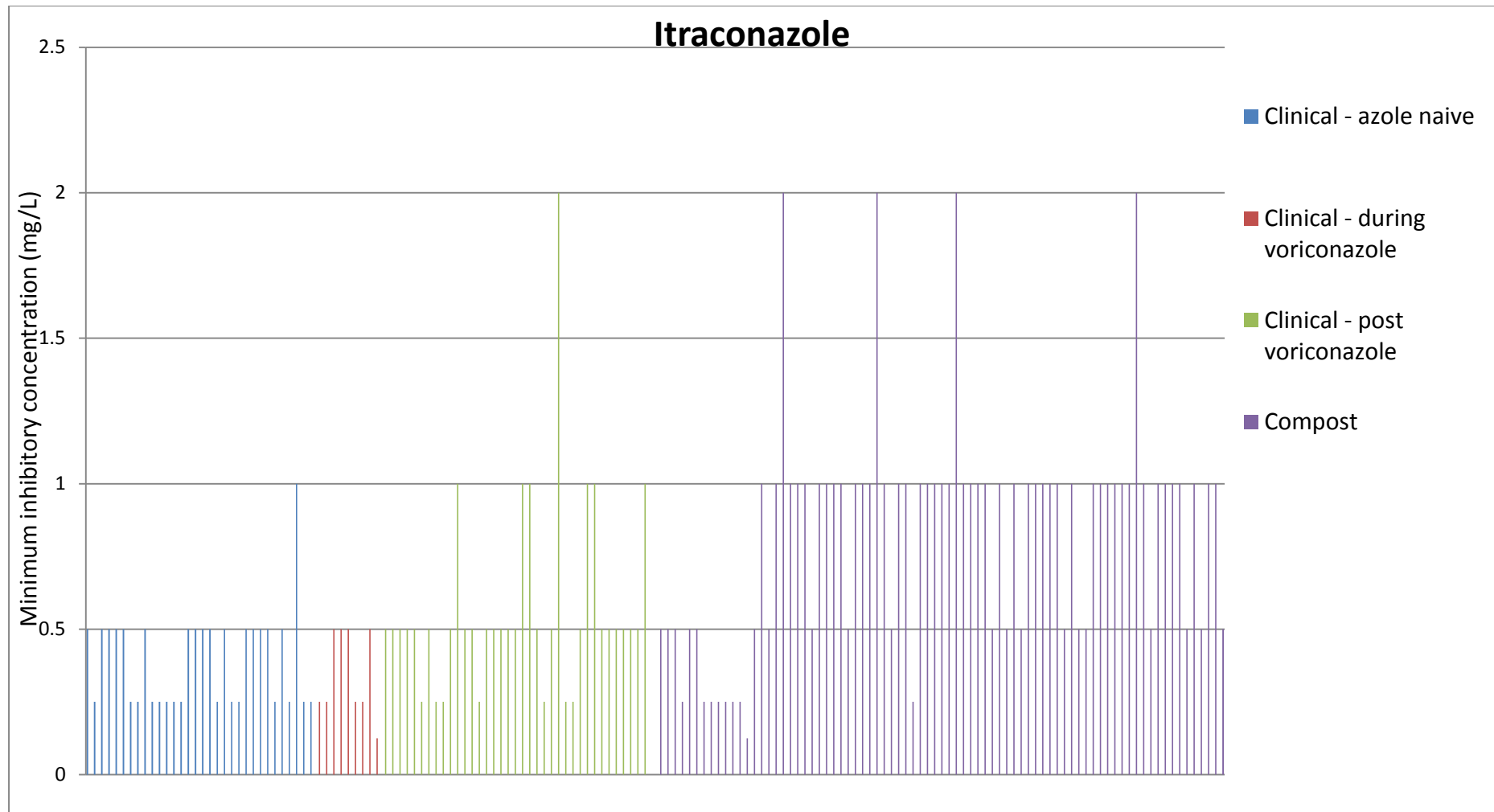
Figure 5-4: MIC values for itraconazole amongst the clinical and environmental *A. fumigatus* isolates tested
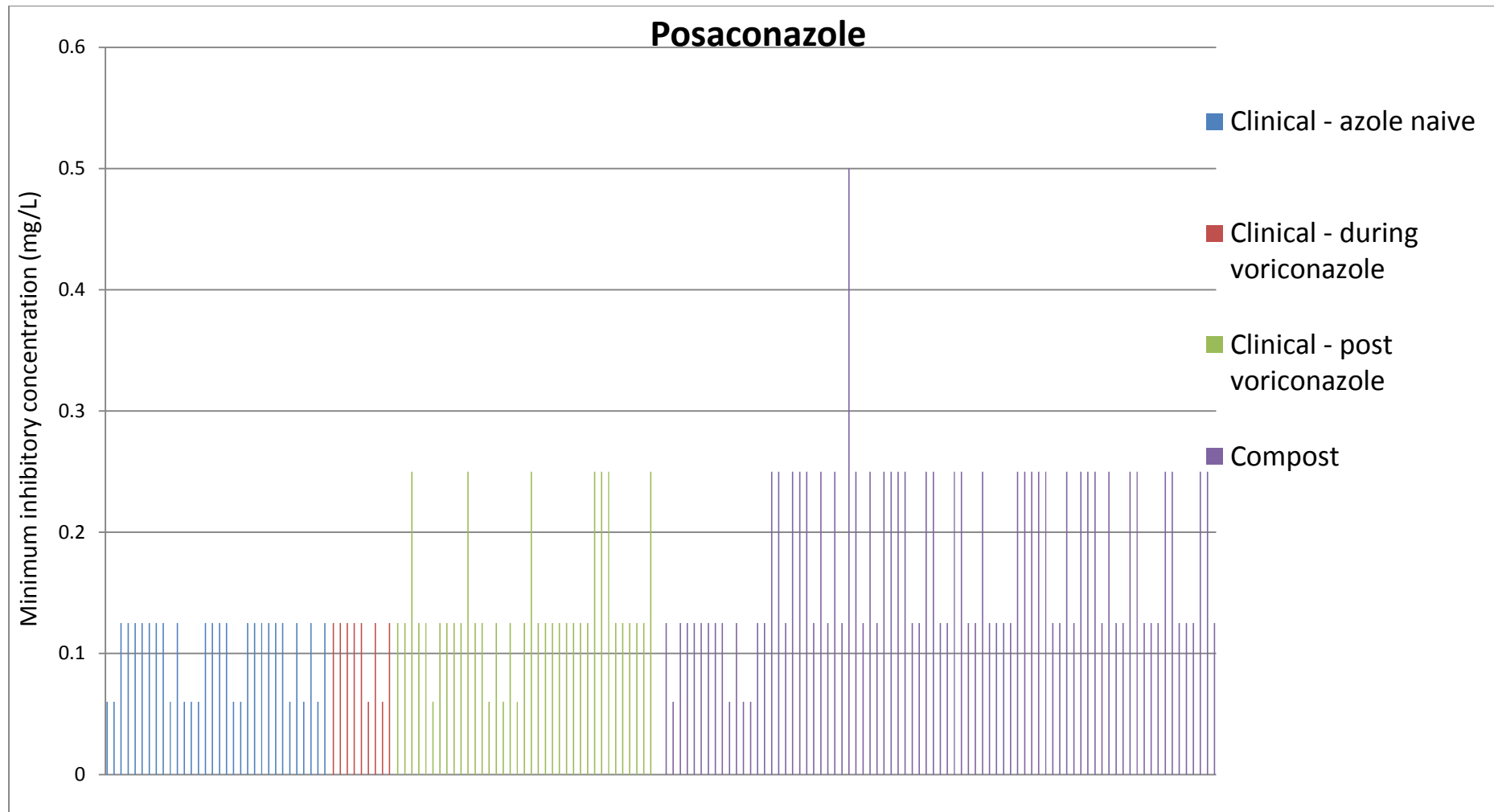
Figure 5-5: MIC values for posaconazole amongst the clinical and environmental *A. fumigatus* **isolates tested**
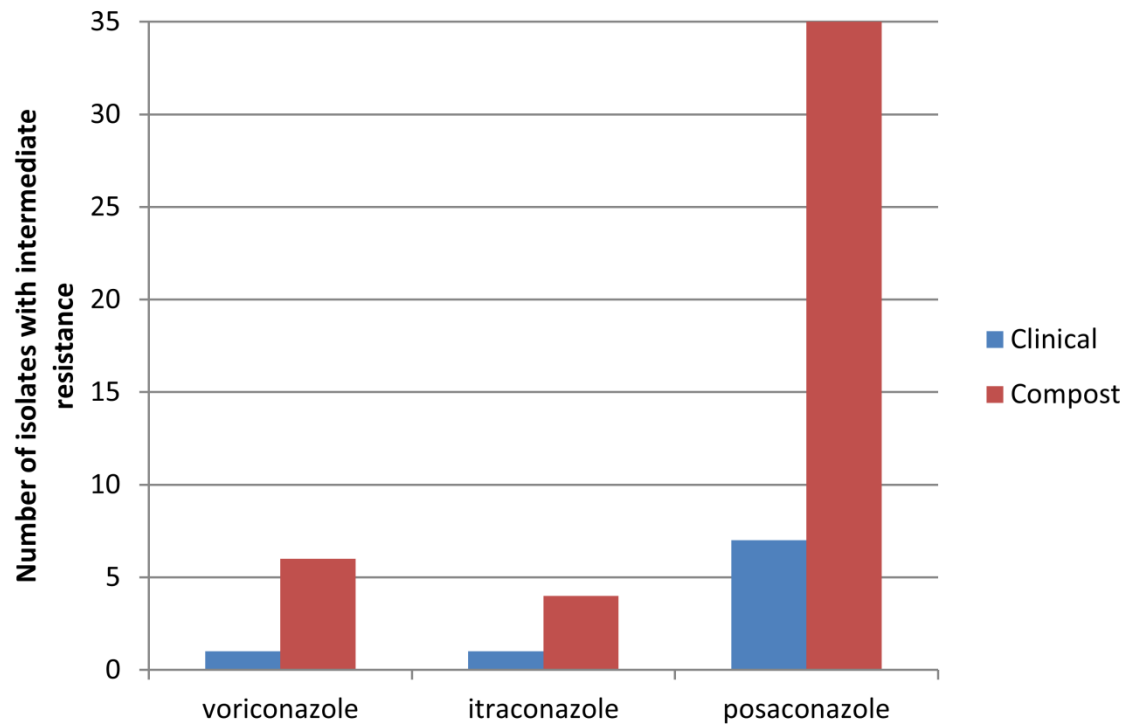
Figure 5-6: Isolates showing intermediate level of resistance to the three azole compounds. There were more compost-derived isolates than clinical isolates with intermediate resistance to all three compounds.
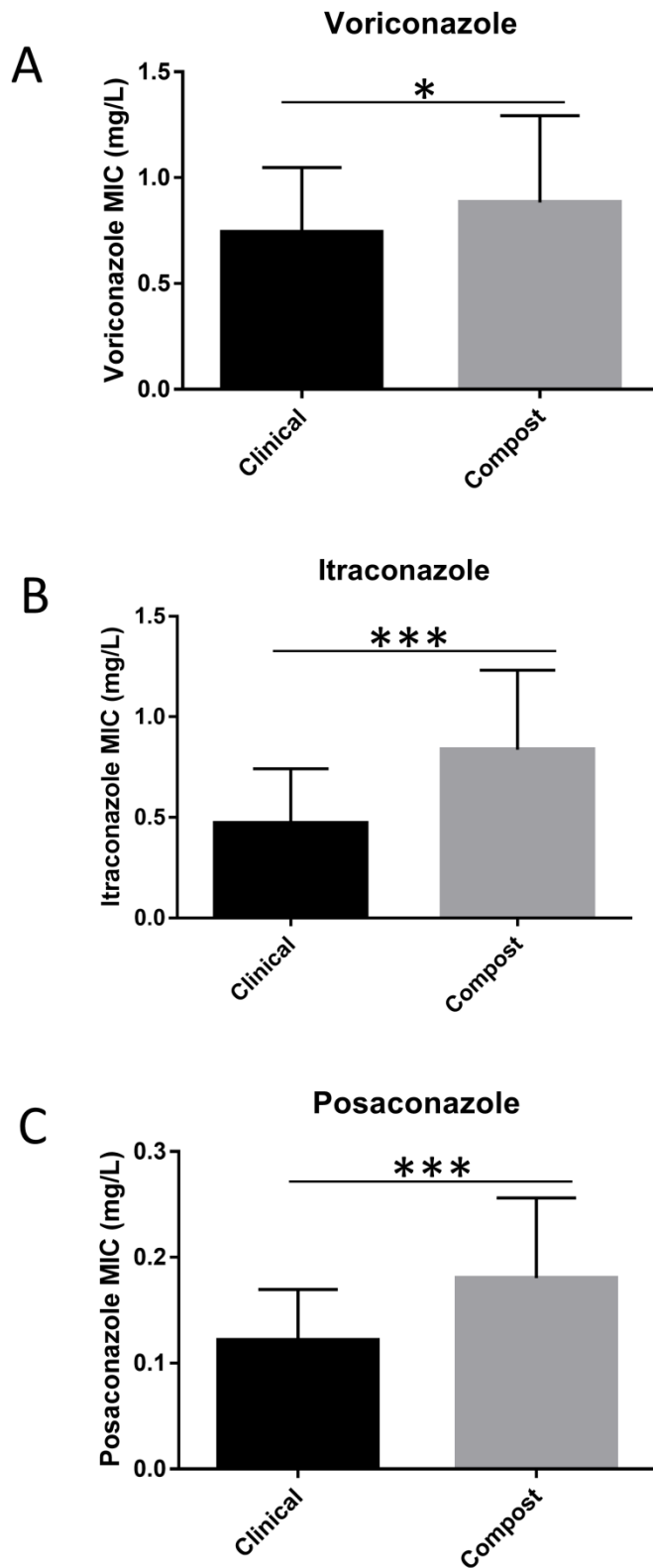
Figure 5-7: MIC values to the three azole compounds tested were significantly higher in the compost isolates than the clinical isolates for all three drugs. (Mann-Whitney test, *=p<0.05, ***=p<0.0001).

A
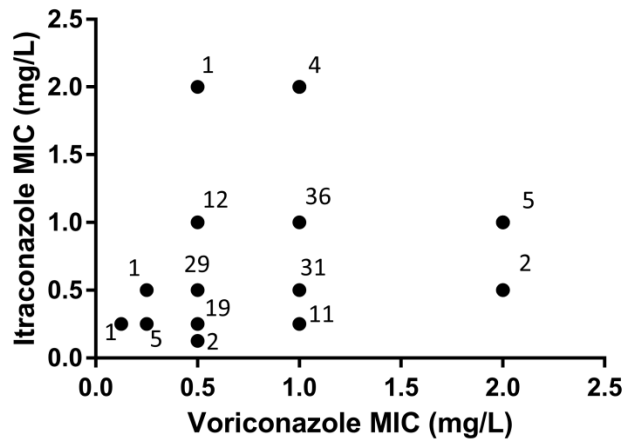
B
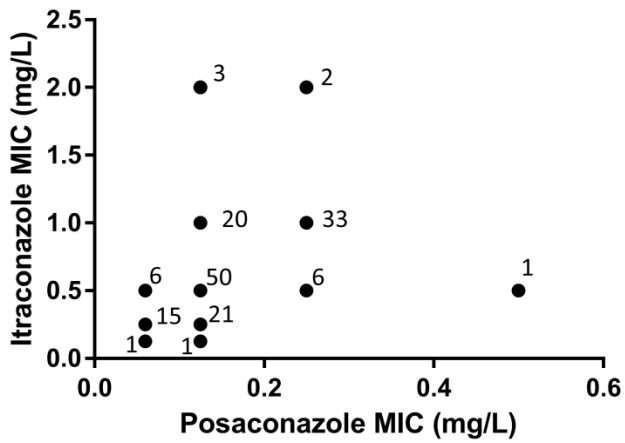
C
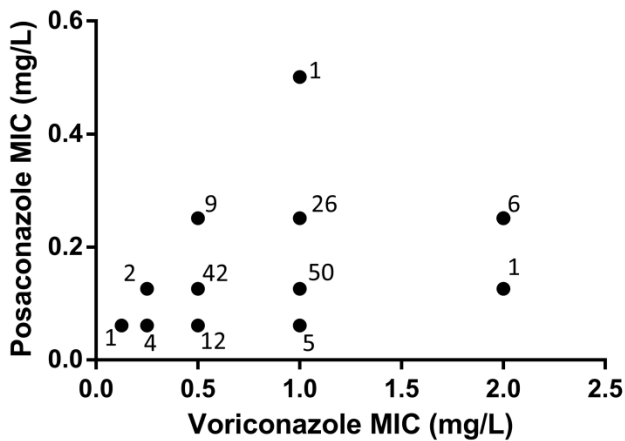
Figure 5-8: Correlation plots showing MIC values of the azole drugs correlated to one another. There was statistically significant correlation (p<0.0001) between MIC values for all three azole drugs. A) Spearman's rank correlation coefficient; r=0.4115; B) r= 0.6273, C) r=0.4049. Numbers show the number of isolates represented by each point.
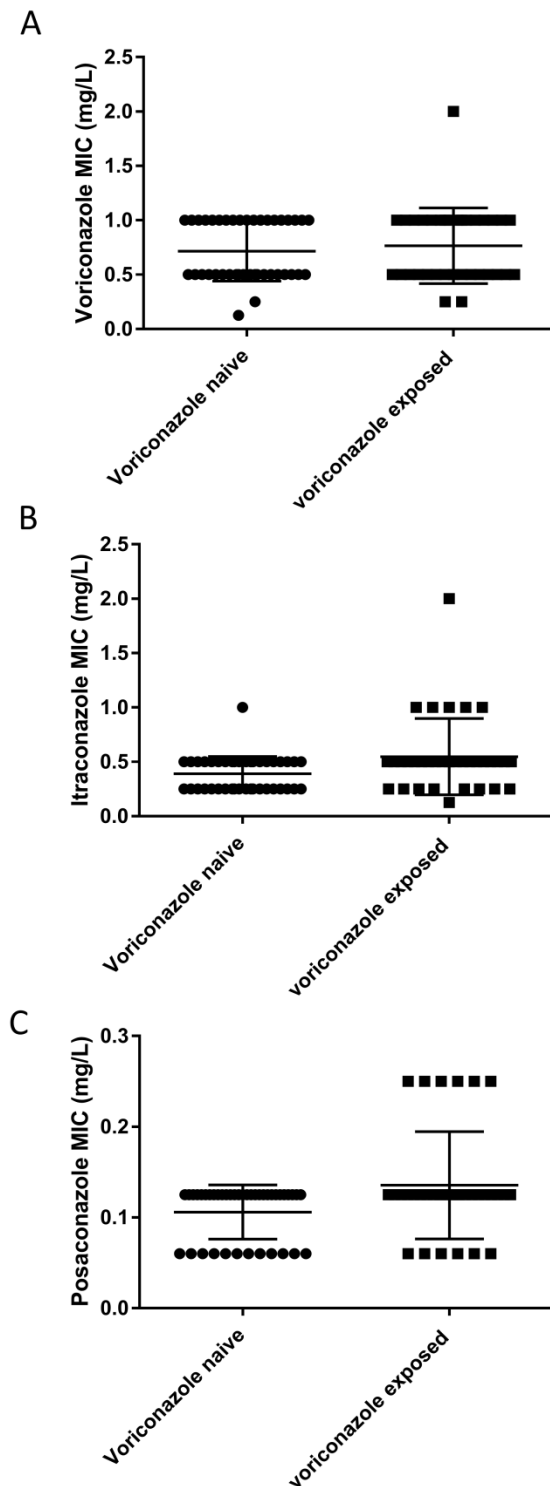
Figure 5-9: MIC values for the three azole compounds for isolates obtained from voriconazole naive and voriconazole treated patients. A) No significant difference in voriconazole MIC values (Mann-Whitney p=0.717). Significantly higher itraconazole (B) and posaconazole (C) MIC values in isolates from voriconazole treated patients (p=0.0194 and 0.0315, respectively).

5.3.2 cyp51 sequence

Eight isolates which gave intermediate resistance MIC values to more than one azole compound with EUCAST testing were chosen for cyp51A gene analysis. The isolates were; six isolates (five environmental, one clinical) which showed intermediate resistance to posaconazole and voriconazole and two isolates (both environmental) which showed intermediate resistance to posaconazole and itraconazole.

The coding sequence of PCR product A spans the first 507bp of the reference cyp51A sequence AD222068 and matches completely. There was no variation in this sequence in any of the 8 intermediate-resistant isolates tested.

The coding sequence of PCR product B spans from nucleotide 320-885 of the AF222068 sequence, again the sequences were identical for all eight samples tested, and matched the wild-type sequence fully over this region. This shows that there is no L98H mutation in these samples with intermediate resistance.

Figure 5-10 shows the relevant sequence data in the test samples (merged for simplicity as all eight were identical and shown as 'test samples') compared to the various reference sequences obtained from Genbank. The PCR amplified regions from each sample matched the Genbank wild-type cyp51A accession completely. The Genbank accession numbers of the sequences used for confirmation of genotype in the samples were; AF338659, EU807920, KJ210331 and KP270713. AF338659 is from a wild-type, non-resistant isolate and does not contain any of the mutations (Mellado *et al.*, 2001). EU807920 is from a multi-drug resistant isolate carrying the TR34 and L98H mutations (Although the promoter region is not included in the seq. deposited in Genbank so only L98H is seen in this sequence) (Howard *et al.*, 2009). KJ210331 is from an isolate resistant only to voriconazole which carries only the single SNP which causes the Y121F substitution. The combined sequence data shows that the PCR amplifications worked well and with complete fidelity and that none of the samples tested had any of the known resistance-associated mutations.
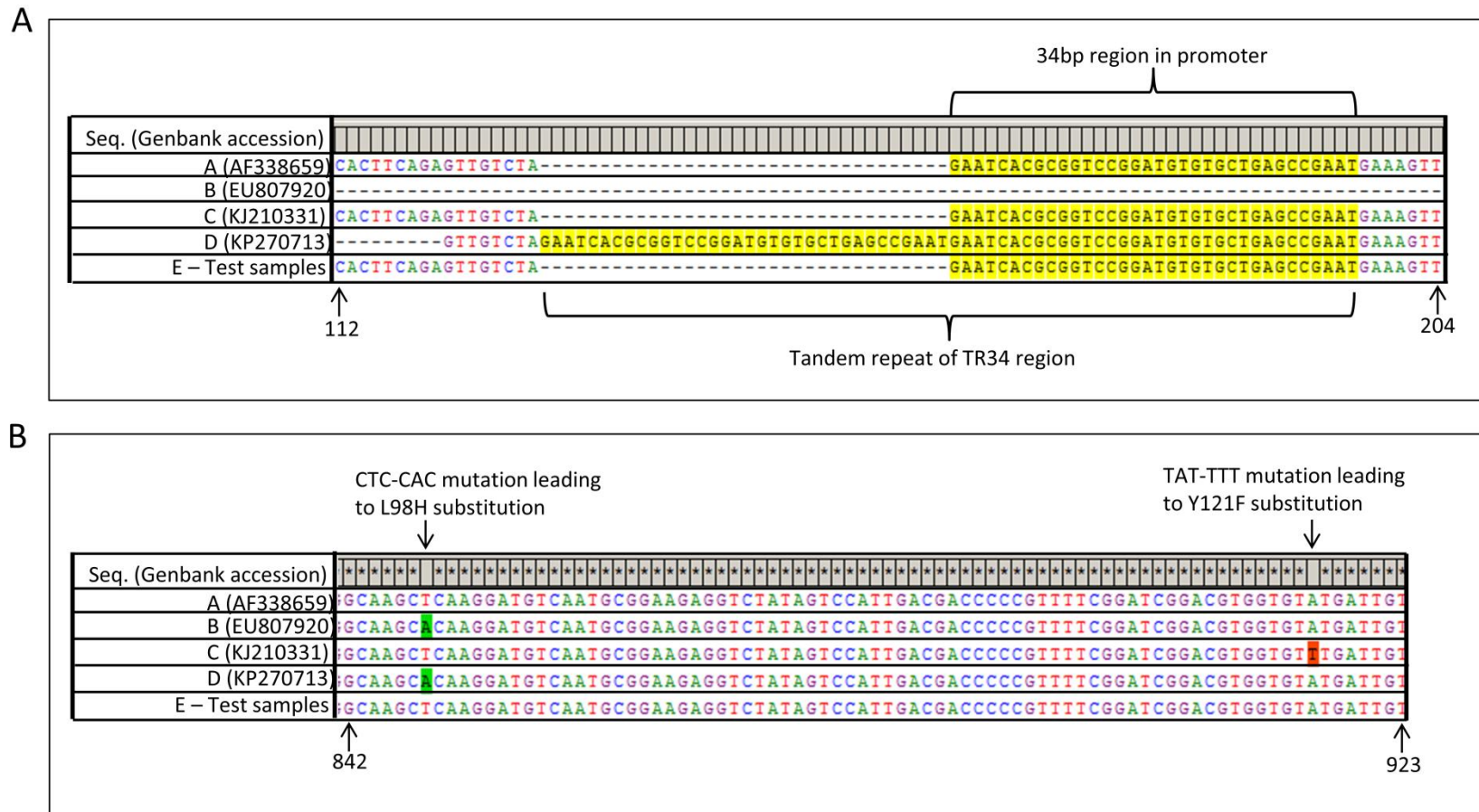
Figure 5-10: Merged sequence data for the test samples (row E) aligned against Genbank accession sequences (rows A-D). Neither TR34/L98H nor TR46/Y121F were present in the isolates tested. A) The region of the promoter in which the TR34 mutation occurs (yellow). B) The region within the coding sequence of the cyp51A gene in which the L98H and Y121F SNPs occur (shown in green and red, respectively).

5.3.3 Pathogenicity

Initial dose-response experiments showed that a dose-dependent effect on mortality was seen with spores of the *A. fumigatus* type strain NCPF7097, (Figure 5-11). Inoculation with 10,000 CFUs of this strain caused around 50% mortality between 4-5 days post inoculation and resulted in high mortality over 7 days. This dose was then tested a further 3 times in independent experiments, using different batches of *G. mellonella* and fresh spore stocks on each occasion. These repeats (Figure 5-12) show good reproducibility, with no significant difference between the Kaplan-Meier survival curves produced from these repeats (Log-rank (Mantel-Cox) test, p=0.9824).

When the survival data for all of the isolates tested are merged into compost and clinical groups, the survival curves appear similar in shape but the curves are significantly different (Log-rank (Mantel-Cox) test, p<0.0001). Overall, the compost isolates are more pathogenic, killing the *G. mellonella* quicker than the clinical isolates, Figure 5-13. When analysing the data in this way, the individual isolates lose their identity and are effectively considered as 'repeats' within the compost or clinical groups.

Two alternative ways of analysing this dataset is to calculate and compare the time taken to reach 50% mortality and the percent survival at 72 hours (Figure 5-14). These analyses support the Kaplan-Meier analysis demonstrating significantly higher mortality caused by the compost isolates than the clinical isolates (p <0.001 and p <0.01 for time to 50% mortality and %-survival at 72 hours respectively).

Grouping the clinical isolates by the status of voriconazole exposure of the patient from which the isolate was obtained, there is no significant association between voriconazole exposure and pathogenicity of the isolate obtained (Figure 5-15). There is also no significant correlation between the lung function measures of the patient from which the fungus originated and it's pathogenicity in *G. mellonella*. This is shown in terms of time to 50% death vs FEV1% pred. (post-bronchodilator) (Kruskal-Wallis, p=0.2087) in Figure 5-16.

There was no significant association between MIC values obtained in the EUCAST testing and pathogenicity in the *G. mellonella* model (Figure 5-17: voriconazole r= 0.04833, p=0.5465; itraconazole r=0.02510, p=0.7542, and posaconazole r=0.07459, p=0.3516).

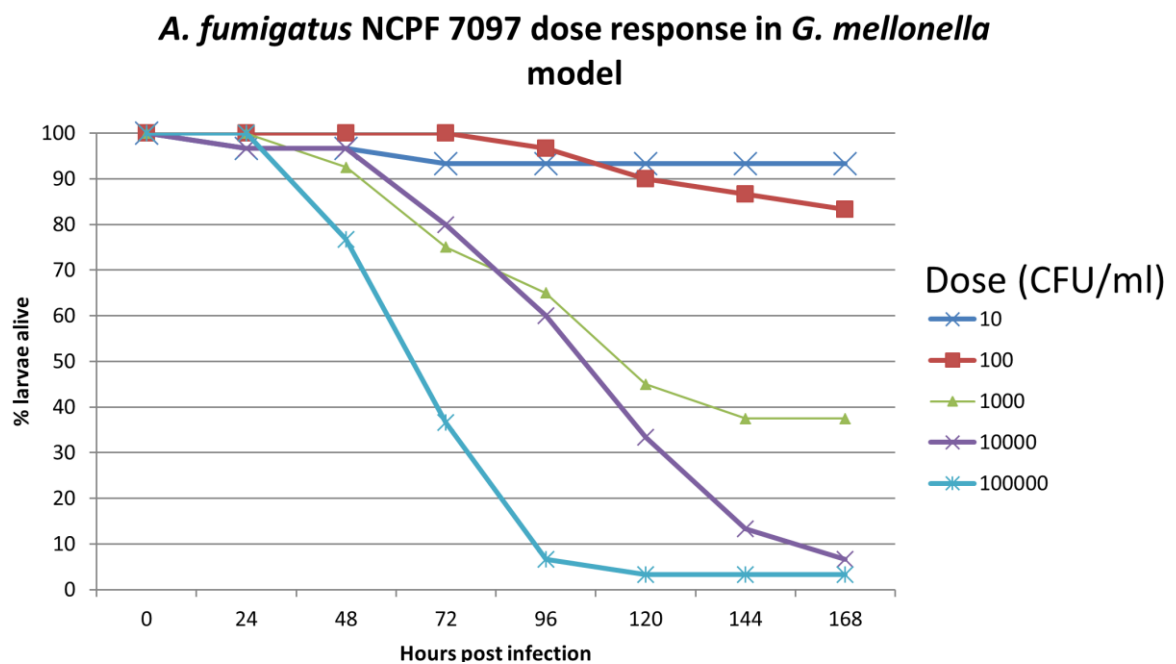**Figure 5-11: Dose response curves of infection of *G. mellonella* larvae with spore suspensions of *A. fumigatus* strain NCPF7097.**
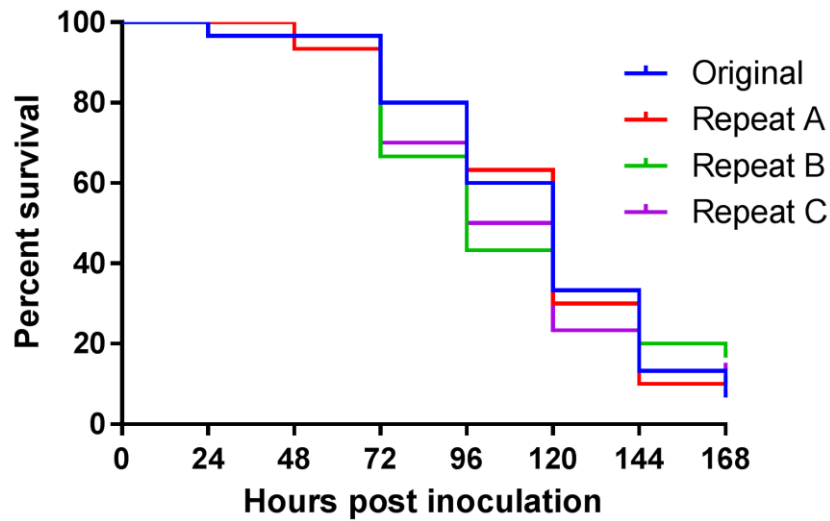
Figure 5-12: *G. mellonella* **Kaplan-Meier survival curves following infection with 10,000 CFUs of** *A. fumigatus* **strain NCPF7097.** No statistically significant difference between the curves produced from the original and three repeats (Log-rank (Mantel-Cox) test, p=0.9824).
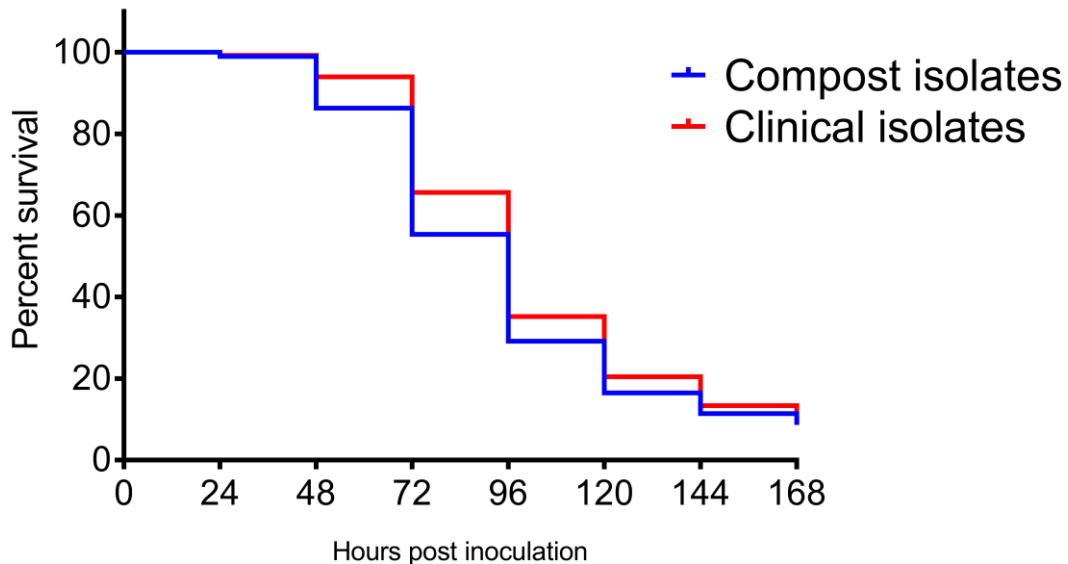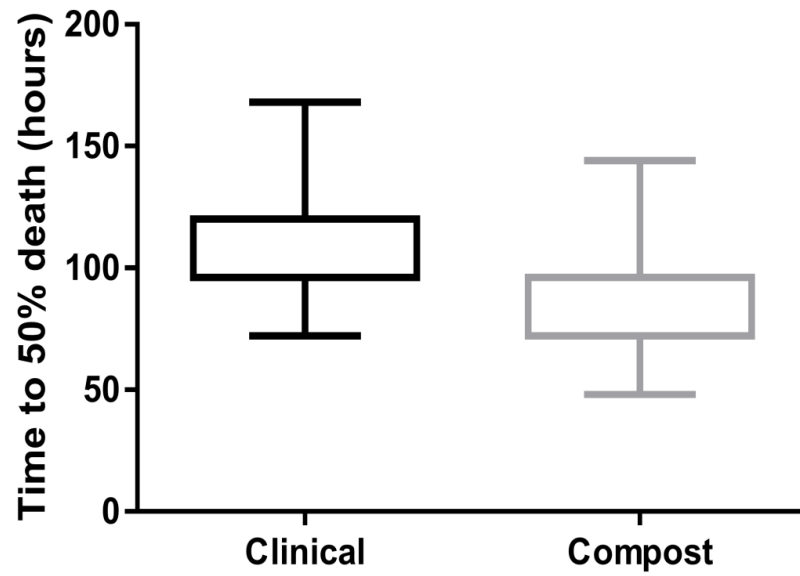

Figure 5-13: Kaplan-Meier survival curves plotted from the merged survival data of all 80 clinical isolates and 79 compost isolates. Compost isolates were more virulent, killing a higher proportion of the *G. mellonella* more quickly than the clinical isolates did (Log-rank (Mantel-Cox) test, p<0.0001).
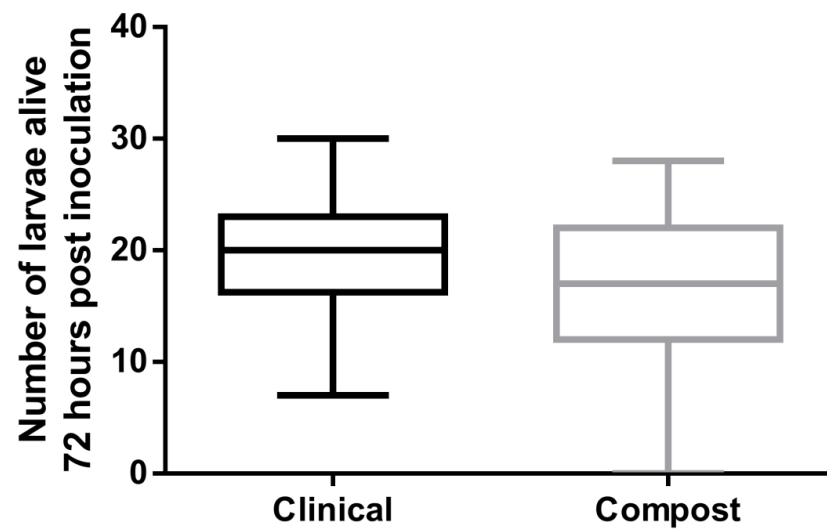
A

B

Figure 5-14: Larvae inoculated with compost-derived *A. fumigatus* isolates show significantly higher mortality rates than those infected with clinically-derived isolates. A) Time to 50% mortality (p=0.0007) and B) % survival 72 hours post-inoculation (p=0.0054). Mann-Whitney U-test.

Effect of patients' voriconazole exposure on virulence of *A. fumigatus* isolated
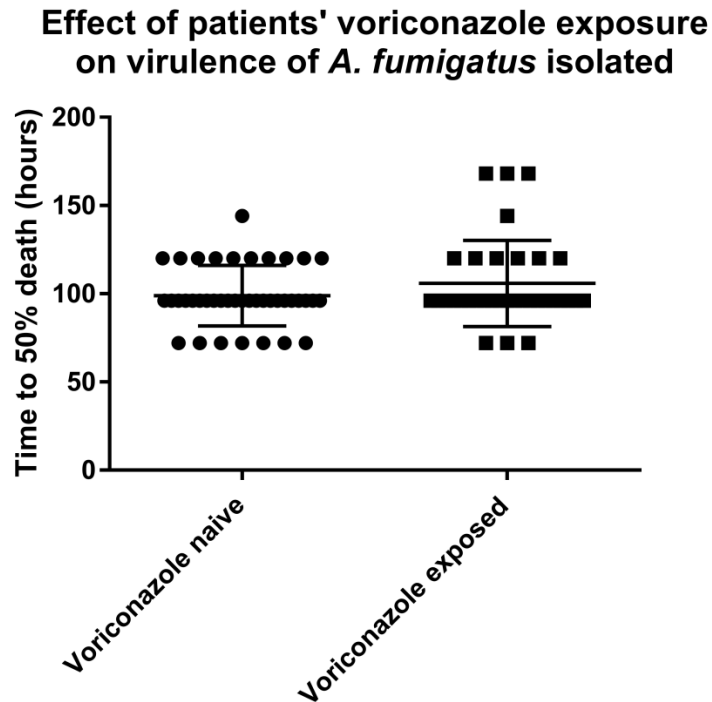

Figure 5-15: Status of voriconazole exposure of the patient from which the isolates were obtained has no significant effect on the time taken for the isolate to kill 50% of larvae in the *G. mellonella* model (Mann-Whitney p=0.3696).
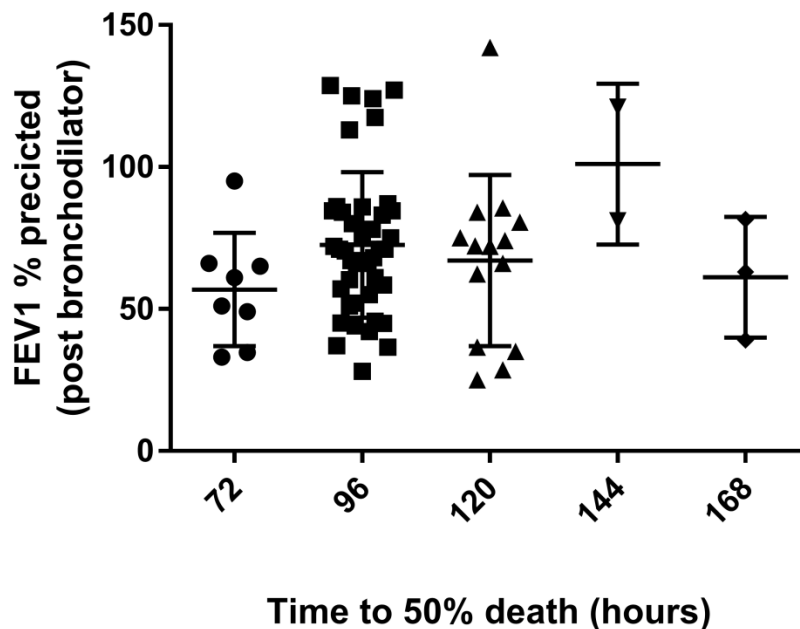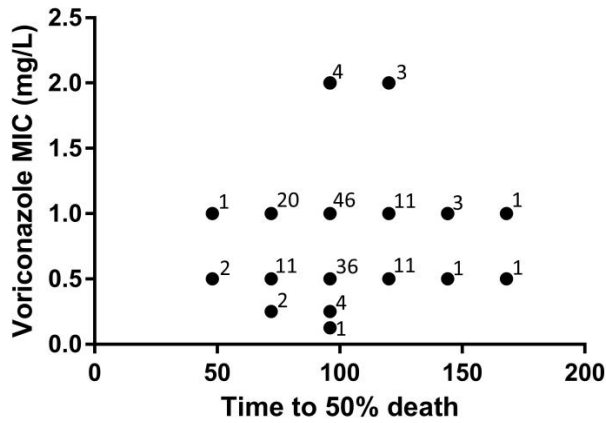

Figure 5-16: No significant difference between time taken to cause 50% mortality in *G. mellonella* and post-bronchodilator FEV$_1$ of the patient from which the isolate was obtained (Kruskal-Wallis p=0.2087).

155

A

B
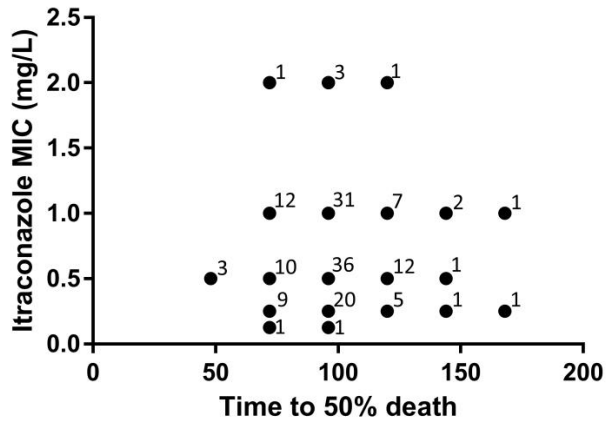
C

Figure 5-17: MIC – virulence correlation plots. No significant correlation between MIC values to any of the azole compounds and virulence in *G. mellonella*. A) Spearman's rank correlation coefficient; r= 0.04833, p=0.5465, B) r=0.02510, p=0.7542, C) r=0.07459, p=0.3516. Numbers show the number of isolates represented by each point.

5.3.4 Mating type
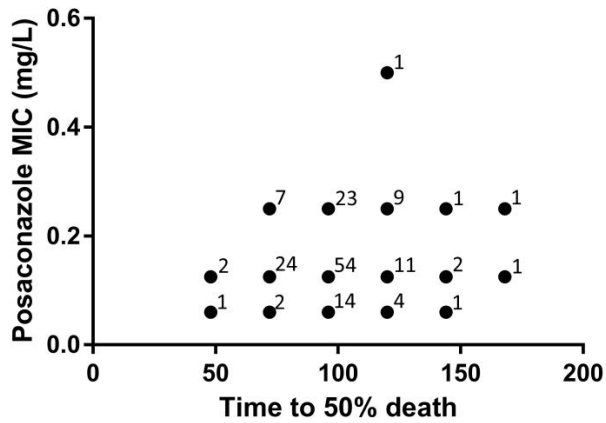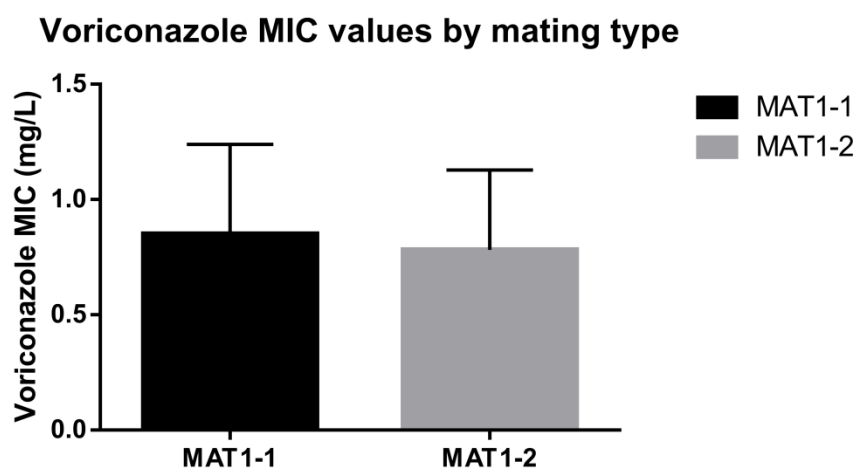
There was no significant difference between MIC values for any of the three drugs in the two different mating types MAT1-1 and MAT1-2. This is shown in Figure 5-18.

There was a difference in the proportion of each mating type found in the clinical and environmental collections of isolates. There was a very even split of MAT1-1 and MAT1-2 in the compost isolates, with 41 compost isolates (51.9%) being MAT1-1 and 38 isolates (48.1%) being MAT1-2. This is different to the uneven divide seen amongst the clinical isolates, in which a higher proportion were MAT1-2. Of the 80 clinical isolates, 30 (37.5%) were MAT1-1, and 50 (62.5%) were MAT1-2 (Figure 5-19), although this difference does not quite reach statistical significance (Fisher's exact test, $p=0.0801$).
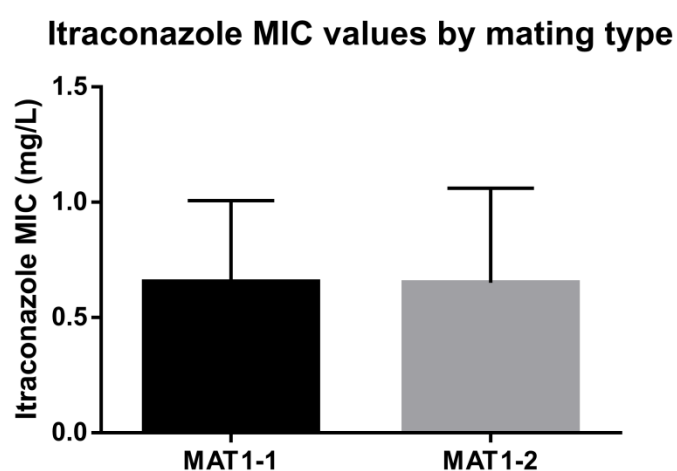
Figure 5-20 shows the difference in mating type prevalence in the clinical isolates when the azole exposure of the patients they were obtained from is taken into consideration. The pre-exposure isolate group consists of samples from patients assigned to the voriconazole-treatment group before the dosing visit and samples obtained from patients assigned to the placebo treatment group. The during-exposure isolates are the samples from patients in the voriconazole-treatment group obtained from visits during the voriconazole treatment phase of the study and the post-exposure isolates are those isolates obtained from visits after the final dosing of voriconazole. There appears to be a shift towards an increased prevalence of MAT1-2 associated with exposure to voriconazole, although this does not reach statistical significance (Fisher's exact test, $p=0.1632$).

There was no significant difference in lung function of the patients from whom the isolates were obtained with regards to the mating types of the fungi isolated (Figure 5-21). Also there was no significant association seen between mating type and virulence in the *G. mellonella* model regardless of method used to determine virulence (fFigure 5-22 and Figure 5-23).

A

Voriconazole MIC values by mating type

B

Itraconazole MIC values by mating type

C

Posaconazole MIC values by mating type
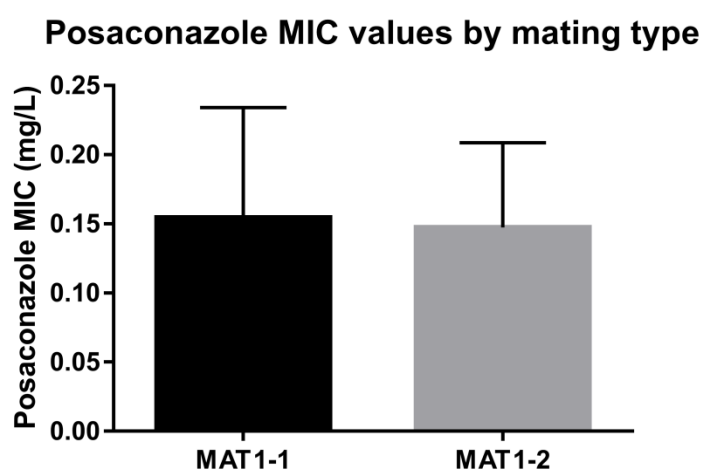
Figure 5-18: MIC values for the three azole compounds analysed by mating type.
There was no significant difference between MIC values for either mating type for any
of the drugs tested. A) Voriconazole (Mann-Whitney p=0.2265), B) Itraconazole
(Mann-Whitney p=0.6921), C) Posaconazole (Mann-Whitney p=0.8900)
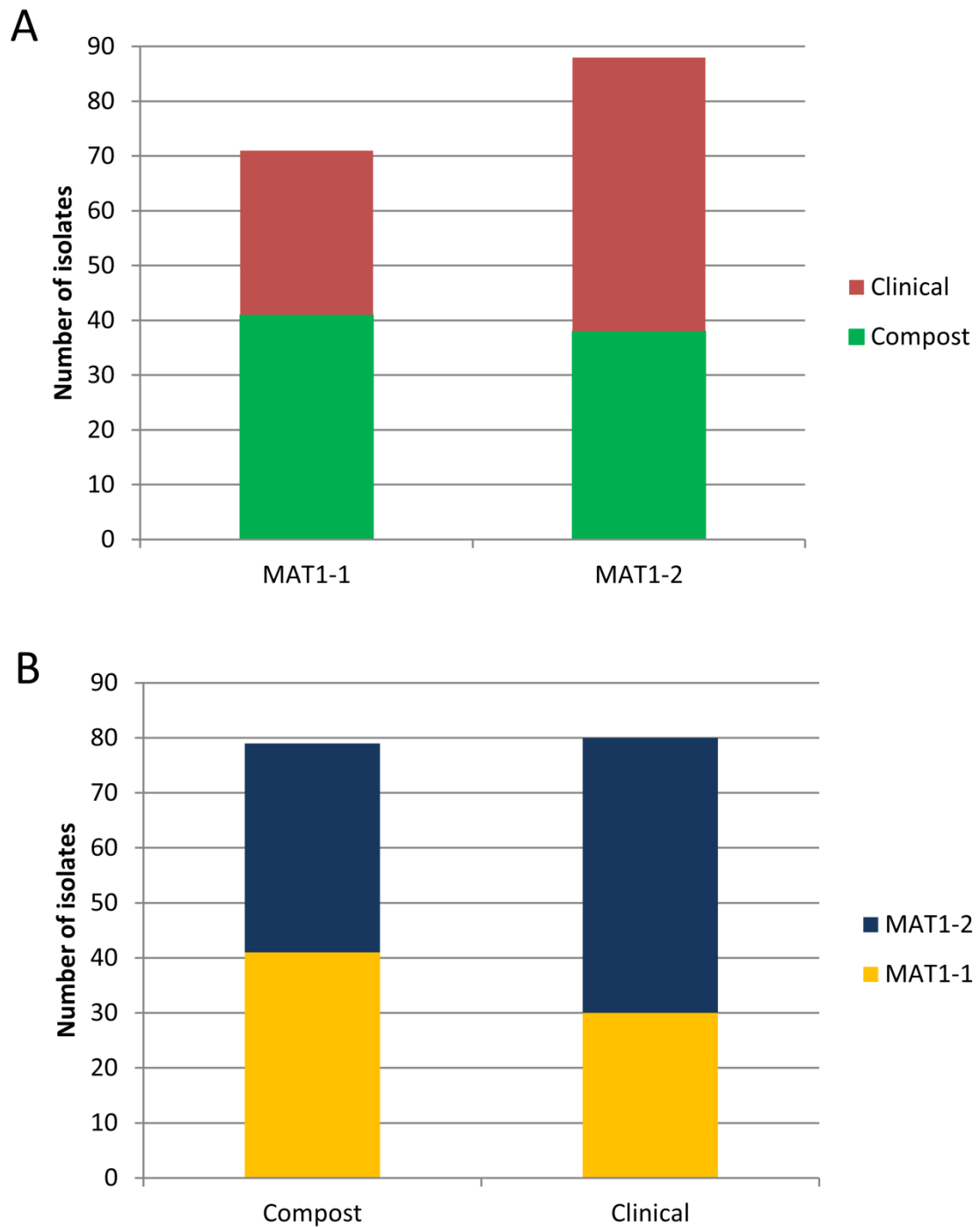
Figure 5-19: Prevalence of mating types amongst clinical and compost isolates.
A) Of the MAT1-1 isolates identified, 41 were compost-derived and 30 were clinical. Of the MAT1-2 isolates, 38 were compost-derived and 50 were clinical. B) 51.9% of the compost isolates were MAT1-1 and 37.5% of clinical isolates were MAT1-1. This difference is not statistically significant (Fisher's exact test, p=0.0801).
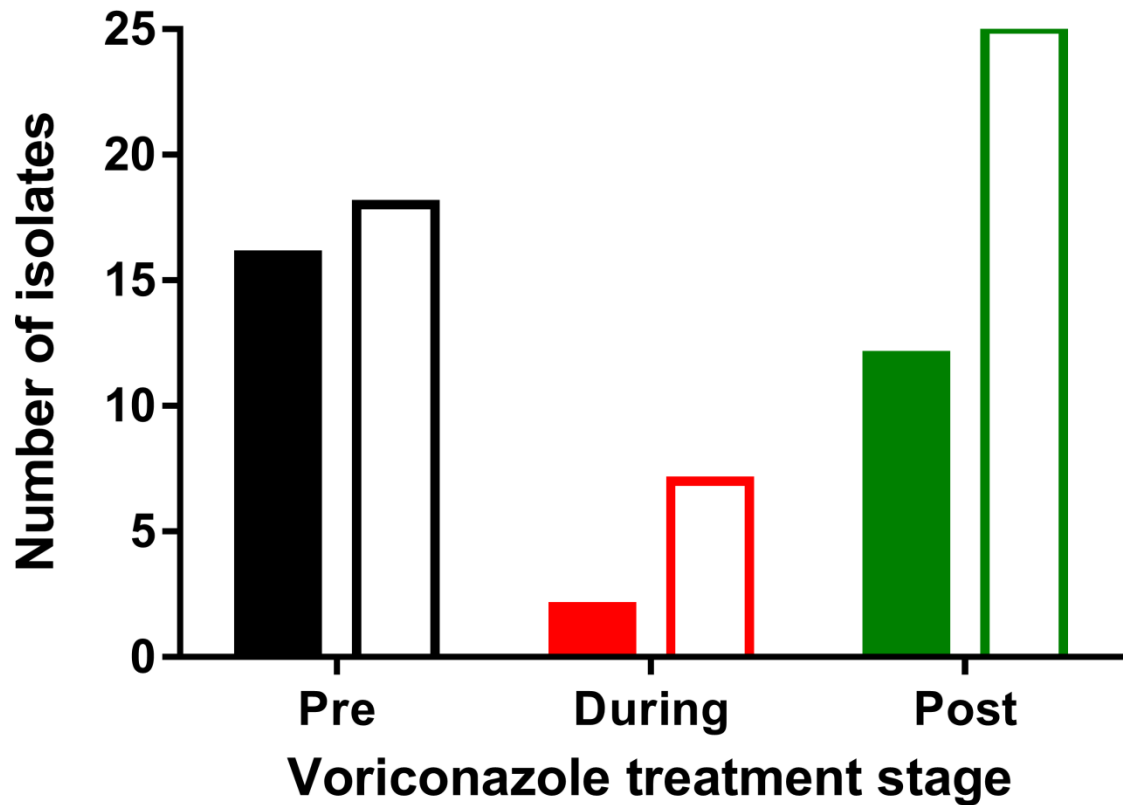
Figure 5-20: Mating type ratio by voriconazole treatment stage. The ratio of MAT1-2 to MAT1-1 *A. fumigatus* isolates is higher from patients during and post treatment with voriconazole compared to before-treatment, although this does not reach statistical significance (Fisher's exact test, p=0.1632). Open bars =MAT1-1, filled bars =MAT1-2.

A

MAT vs FEV1% predicted

B

MAT vs FEV1:FVC ratio (as %)
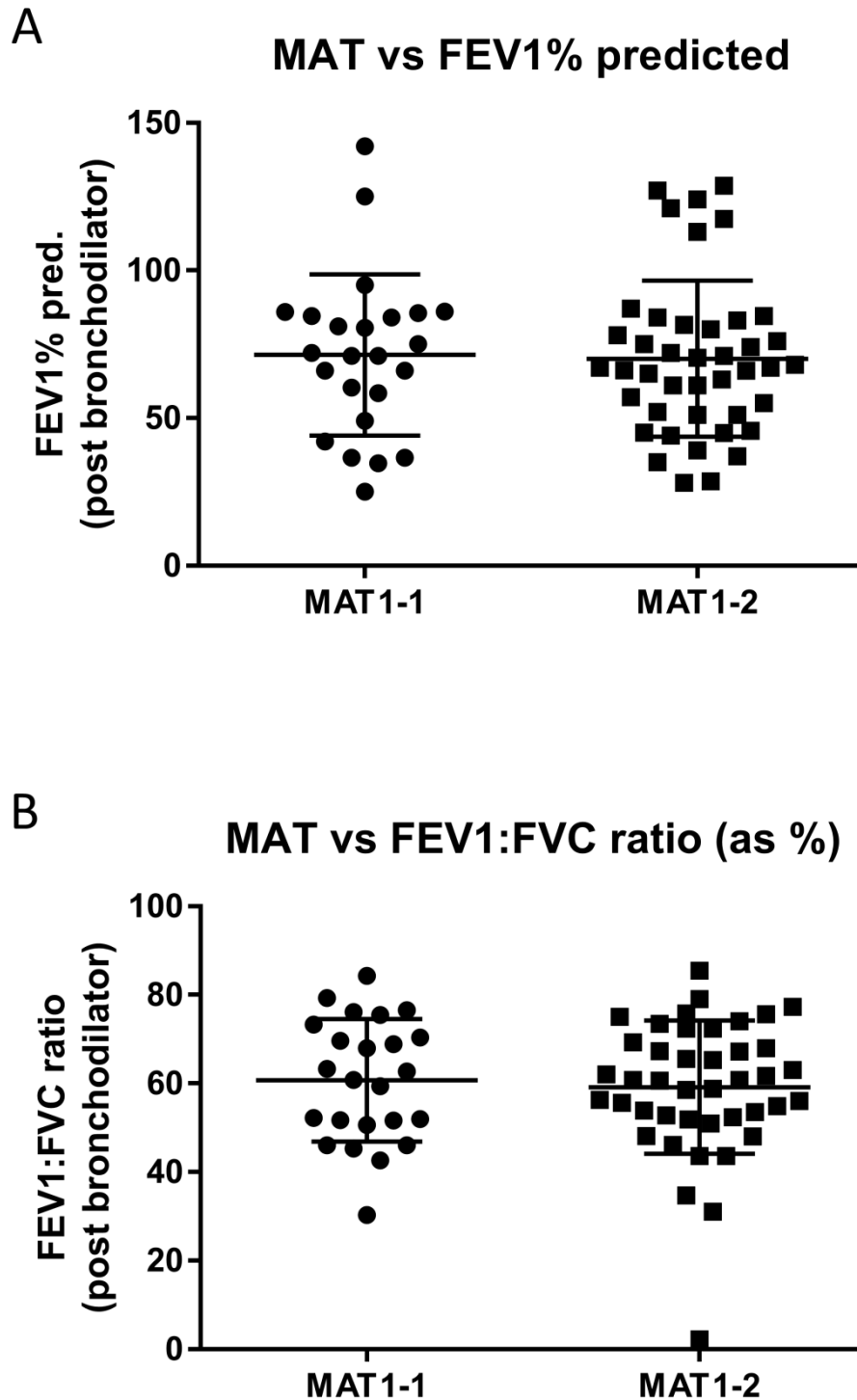
Figure 5-21: Mating type of isolates was not associated with differences in post bronchodilator lung function of the patient. A) FEV1% predicted grouped by mating type. Not significant, unpaired t test, p=0.8540.   B) FEV1:FVC ratio grouped by mating type. Not significant, p=0.6848.

Mating type and virulence
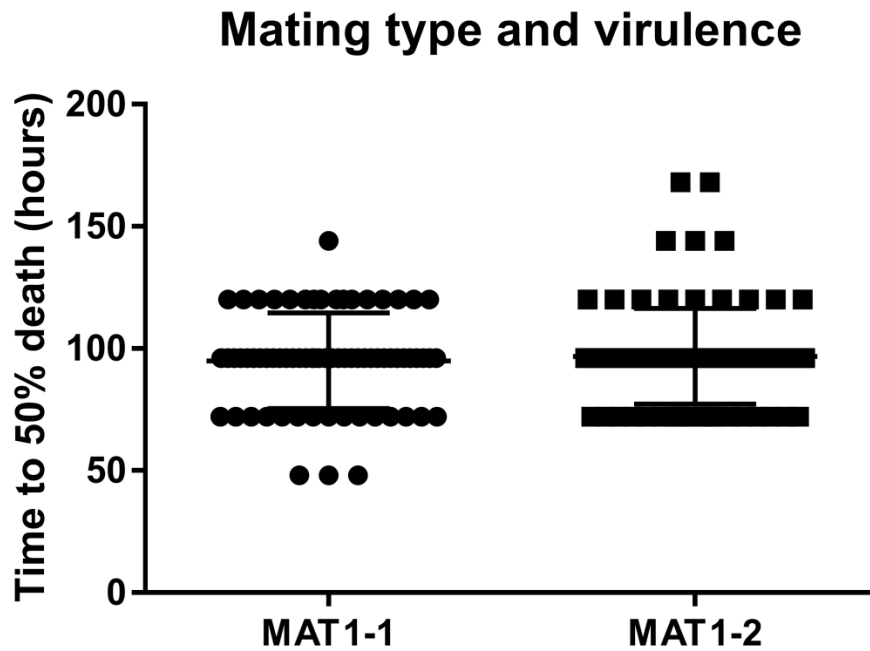
Figure 5-22: Time taken to cause 50% mortality in *G. mellonella* grouped by mating type. There is no significant difference in virulence between the two mating types (Mann-Whitney, p=0.9761).

Merged survival curves from isolates of different mating types
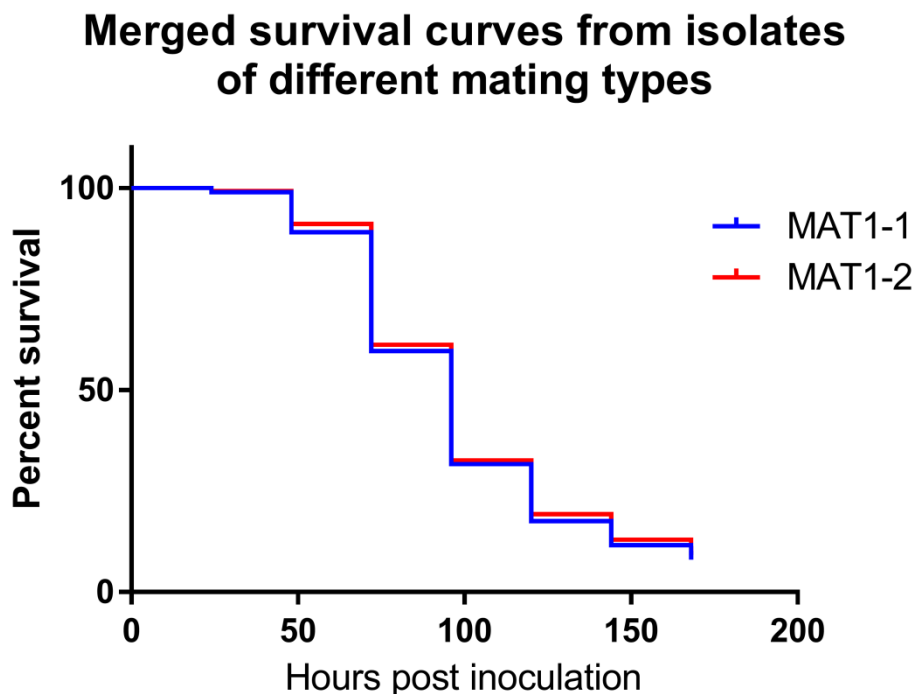
Figure 5-23: Kaplan-Meier survival curves plotted from merged survival data from the two mating types. There is no significant difference between the two survival curves (Log-rank (Mantel-Cox) p=0.0769).

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5.4 Discussion

5.4.1 EUCAST

One of the most striking findings from this part of the work is that there was very little azole resistance found in these isolates. The fact that none of the 159 samples were resistant to voriconazole or itraconazole and only one was resistant to posaconazole is surprising given the recent estimates of the prevalence of resistance being between around 6% and 17% (van der Linden *et al*., 2011 and Howard *et al*., 2009) and the fact that the isolates were all obtained from relatively recent samples (obtained within the last 6 years). Even if this collection of isolates was at the lower end of this expected frequency of resistance one would still expect some resistance to be detected in a study with this number of samples. The samples from voriconazole-treated patients did not produce MIC values above the threshold deemed to be the clinically significant breakpoint values. This suggests that either the voriconazole treatment was insufficient to get rid of all fungi from the patient's respiratory tract as opposed to the isolates remaining due to an inbuilt resistance to the drug, or that the isolate had not been in the lung long enough for the drug to be effective.

Although resistance in our collections was less prevalent than might be expected, the data did show significantly higher MIC values for all three azole drugs in the environmental isolates compared to the clinical isolates. This statistically significant difference is interesting as it would appear to fit in with the idea of environmental development of resistance, as proposed by Snelders *et al*., 2009. The hypothesis that compost-derived populations of *A. fumigatus* contain a higher prevalence of azole resistance is supported by these findings. Composting sites are inevitably fed with material which has previously been exposed to azole fungicides, and this background exposure to azoles (albeit possibly at low levels) may act as a selective pressure on *A. fumigatus*. With this azole exposure in mind, industrial scale composting sites could be an ideal environment for the selection and subsequent proliferation of azole-resistant strains. These composting sites have a constant and relatively high concentration of a wide array of different *A. fumigatus* strains being introduced (with each fresh batch of material, but with successful strains being able to spread from one windrow to another by airborne spore release). Previous work looking at non-compost-associated

environmental sources of *A. fumigatus* have also found resistant strains (e.g. Camps *et al*., 2012c), suggesting that resistance does already exist prior to the composting process and the composting process thus may act as a further selective process for these resistance isolates as well as the dense and heterogeneous mix of *A. fumigatus* within developing compost being a source of *de novo* development of resistance in some of these isolates. Interestingly, the azole resistant environmental isolates described by Camps *et al*. (2012c) did not have any mutations in the cyp51A gene, comparable with the intermediate resistant strains in this study.

Knowing the history of azole use in the material coming into the compost sites would be invaluable in helping to determine if this is the true method of resistance selection, but such analysis is beyond the scope of this project. The likelihood is that most of the sites will receive some material with a history of azole exposure.

Although not giving MIC values above the breakpoint thresholds for the azole drugs, there were a number of isolates whose MIC values are classed as intermediate resistance under the current EUCAST guidelines (**Error! Reference source not found.**) The intermediate resistant isolates are those giving a MIC value of 2 mg/l for voriconazole and itraconazole and those with a MIC value of 0.25 mg/l for posaconazole. The divide between clinical and environmental isolates with intermediate resistance concurs with the overall MIC data analysis; i.e. there were more compost-derived isolates with these high MIC values than there were clinically-derived isolates.

In some studies a high proportion of isolates found to be resistant to itraconazole are cross-resistant to other azole compounds; for example, in Manchester, UK, 65% of itraconazole resistant strains were cross-resistant to voriconazole and 74% to posaconazole (Howard *et al.*, 2009). This doesn't seem to hold true for the intermediate resistant isolates identified in this study. Of the 46 isolates reaching intermediate MIC values for one or more drug only 8 were also at the intermediate level for another drug, and none showed intermediate MIC values for all three drugs. There also was no cross-intermediate resistance between itraconazole and voriconazole, whereas itraconazole-voriconazole cross resistance is commonly reported amongst isolates showing resistance (Faria-Ramos *et al.*, 2014). Meneau and Sanglard (2005) however found no voriconazole-itraconazole cross resistance amongst their collection of isolates, and note

that the different shapes of these two azole drugs (Figure 5-24) and the fact that they bind to different residues on the cyp51A P450 enzyme is likely to explain this. Their conclusion is that SNPs altering the amino acid sequence of cyp51A are likely to confer specific resistance to single azole drugs, whereas mutations responsible for upregulation of resistance-associated genes would likely cause multi-drug resistance.

The isolates reaching the intermediate resistant threshold only account for 28.9% of the total isolates tested, and analysis of the entire MIC value dataset showed that generally higher MIC values for one drug do correlate with higher MIC values for both other azoles tested (Figure 5-8). This suggests that there may be a sub-set of isolates which, while not necessarily having mutations allowing full drug resistance, may feature higher levels of expression or action of a drug-resistance mechanism. Nascimento *et al.* (2003) looked at the expression of AfuMDR3 and AfuMDR4 multi drug resistance genes in *A. fumigatus* and showed either constitutively higher levels of expression or higher levels of expression when grown in the presence of an azole compound amongst their collection of resistant isolates. Similar increased expression of MDR genes has also has been shown to be true in some resistant isolates for AtrF and cyp51B genes (da Silva Ferreira *et al.*, 2004) and could explain the apparent association between high MIC values for one drug and high MIC values for the other drugs tested.

Unexpectedly voriconazole treatment had no significant effect on MIC values to voriconazole but there were significantly higher MIC values to both itraconazole and posaconazole in the isolates derived from samples obtained following voriconazole exposure relative to those obtained from voriconazole naïve samples. This finding does not fit in with the commonly accepted logic that voriconazole exposure is likely to lead to elevated voriconazole MICs and possibly elevated MICS to other drugs. These findings thus only partially support the hypothesis that isolates obtained from patients during and after antifungal therapy may show reduced susceptibility to antifungal drugs.
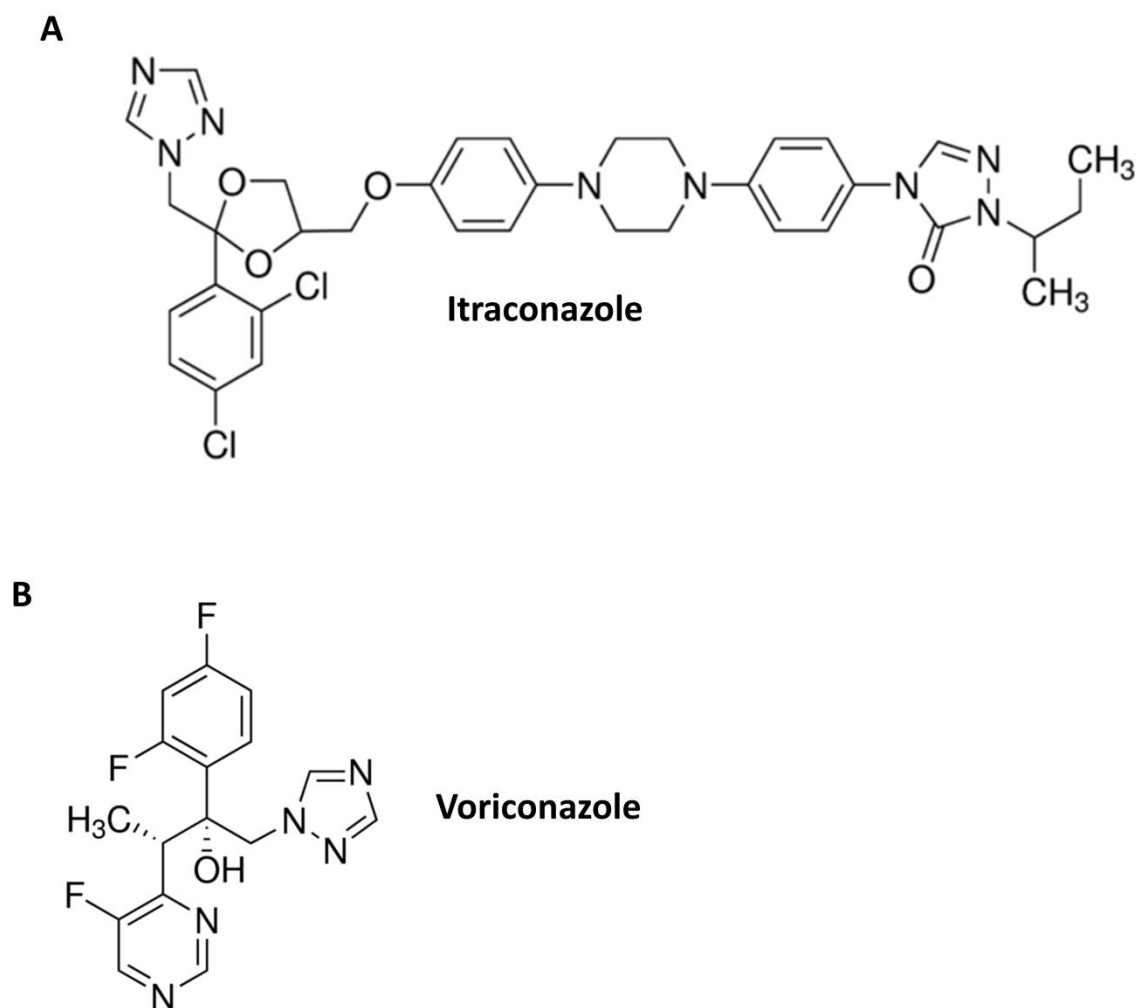
A

Itraconazole

B

Voriconazole

Figure 5-24: The structures of A) itraconazole and B) voriconazole showing the considerable difference in the shapes of these two azole compounds. (Compound diagrams from Sigma-Aldrich Ltd.)

5.4.2 cyp51A

The 8 isolates selected for cyp51A gene sequence analysis were those which gave intermediate resistance MIC values for more than one azole compound. The TR34/L98H and TR46/Y121F/T289A mutations are known to be associated with multi-azole resistance (e.g. Wiederhold & Patterson 2015) and so it is these samples which would be most likely to carry these mutations. Within the region sequenced two mutations in the protein-coding region of the gene would be detectable. These are the SNPs which cause a Leucine to Histidine substitution at residue 98 (L98H) and a Tyrosine to Phenylalanine substitution at residue 121 (Y121F). Either the 34 or 46 nucleotide tandem repeats within the promoter region of the cyp51A gene would also be detectable from the sequence data. Thus both of the common resistance genotypes TR34/L98H and TR46/Y121F/T289A are detectable from this analysis.

The fact that none of the resistance-associated mutations covered by the region sequenced were present shows that these most common resistance mechanisms are not responsible for the intermediate-level resistance seen in these samples, suggesting that there may be another mechanism responsible for this mild resistance phenotype. This may be due to mutations or increased expression of *A. fumigatus* efflux pump genes such as AfuMDR3 and AfuMDR4 and AtrF, as seen previously in non-cyp51A-associated resistance (Nascimento *et al.*, 2003; da Silva Ferreira *et al.*, 2004). Another possible explanation is that different cyp51 mutations in regions not covered by the PCRs used in this study may be the cause of the intermediate resistance. There are two cyp51 genes in *A. fumigatus*, cyp51A and cyp51B, both producing similar enzymes. Increased expression of cyp51B (as identified by Buied *et al*., 2013) could result in slightly elevated MIC values as seen in these samples. Generally it is thought that cyp51A is responsible for the most important 14-α sterol demethylase activity required for cellular growth (the conversion of lanosterol to ergosterol is a critical step in the genesis and maintenance of the fungal cell membrane). It has been postulated (Warrilow *et al.*, 2010) that cyp51B may be involved in a compensatory function, or become expressed under certain conditions. This compensatory function is backed up by the work of Hu *et al.* (2007), in a study which showed that a cyp51A knockout was

still able to produce viable cells, but that a cyp51A and cyp51B double knockout mutant was non-viable.

Ultimately, it isn't necessarily a surprise that the TR34/L98H mutations weren't identified in these samples, as in a recent study (Fraczek *et al.*, 2013) it is noted that over 50% of the azole resistant isolates in their clinical collection had no mutations in either the cyp51A gene or its promoter region. This further suggests that the mechanisms behind drug resistance are more numerous and varied than previously thought. The low-level resistance seen in the samples in the present study not being associated with these common cyp51A mutations does fit in with the idea proposed by Lescar *et al.* (2014) that resistance may develop in a step-wise fashion with accumulation of different mutations, each alone not able to confer full resistance but each adding to the overall fitness to survive in an azole-exposed environment.

Future work to clarify the importance and mechanisms behind this intermediate resistance would be to quantify cyp51A and B expression in these isolates relative to non-resistant isolates using RT-PCR. This would show if this intermediate resistance is associated with over expression of cyp51 or not. Slight overexpression of cyp51A independent of the TR34 or TR46 mutations in the promoter region has previously been shown (Arendrup *et al.*, 2010) and this could explain the mild resistance phenotype observed in these isolates. In addition to this, full cyp51A sequencing could be carried out on these samples in order to ascertain if there are any novel mutations in this gene responsible for the mild resistance phenotype observed, alongside expression levels or mutations of the efflux pump genes mentioned above. Other non cyp51-mediated resistance mechanisms have also been identified (e.g. Camps *et al.*, 2012a; Meneau *et al.*, 2016; Song *et al.*, 2016) and surveillance for these alternative resistance-associated genotypes should also be considered in future studies.

5.4.3 Pathogenicity

A reproducible and dose-dependent effect was seen in the preliminary tests of the infection procedure of *G. mellonella* larvae with *A. fumigatus* spore suspensions. This suggests that the dosing procedure and infecting dose used are appropriate for the intended study outcome (i.e. a direct comparison of survival rate following infection

with spore suspensions of the clinical and environmental isolates). Different groups have previously used different inoculum concentrations and volumes when infecting *Galleria* with fungal spores (Akhtar, 2014; Alshareef and Robson, 2014; Maurer *et al.*, 2015; Mcmillan *et al.*, 2015) as well as bacterial pathogens (e.g. Loh *et al*., 2013). For some pathogens it is possible to infect via the oral route rather than injecting into the haemocoel although this is not commonly used for fungal pathogens. Another variation in the protocols used is that some groups quantify the dose in terms of spores/ml whereas other studies use CFU/ml. It was decided for this work that CFU/ml would be used, as it had previously been noted that there is variation in the viability of fungal spores from different strains.

The data obtained from the testing of all 159 isolates (80 clinical, 79 compost) shows that the compost-derived isolates were more pathogenic than the clinical isolates and that this was statistically significant. Note that despite appearing to be similar in shape, the high number of repeats in the Kaplan-Meier analysis allows for relatively mild differences to be seen and confirmed as statistically significant. Due to the high number of these 'repeats', i.e. 80 clinical and 79 environmental isolates, with each isolate being inoculated into 30 individuals there is a total of 2400 individual subjects for the clinical group and 2370 subjects in the environmental group. This high number of individual infections for each group explains why even though the curves look similar, they are different enough to reach statistical significance. However, the lack of correlation between MIC and virulence suggest that the higher MIC values and higher virulence in the compost isolates are unrelated. The hypothesis that azole resistance is associated with altered pathogenicity would therefore appear not to be supported by the findings presented. It should be considered however that none of the isolates had MIC values above the resistance threshold values and so would not be classed as resistant.

Within the clinical isolates, pathogenicity did not correlate with lung function of the patient at the time of sample acquisition. This is perhaps not entirely surprising as the samples were not obtained from invasive aspergillosis patients and the *G. mellonella* model is essentially a model of invasive disease. Nonetheless, it is interesting that those isolates associated with increased virulence in the *G. mellonella* model were not

associated with worse lung function of the patients from which the samples were obtained.

The finding from this work that the clinical isolates were less pathogenic than the environmental isolates contradicts the findings of (Aufauvre-Brown *et al.*, 1998) who compared environmental and clinical isolates for virulence in a mouse model. It should be noted however that their study was based on a much smaller number of isolates (3 clinical and 4 environmental isolates) and also that the 'environmental' strains used in their study were obtained from the air in areas of the hospital from which the corresponding clinical isolates were derived. Although it is reasonable to class these as environmental isolates, this clearly is a very different source to the compost sites from which the environmental isolates in the present study were derived.

There was a greater range of pathogenicity in the environmental isolates than amongst the clinical isolates, which was also the case in the work of Alshareef *et al*., (2014). Contrary to their findings, however, was the fact that the clinical isolates in the present study showed less variation and slightly lower pathogenicity whereas their 10 clinical isolates showed less variation but higher pathogenicity. The conclusion drawn by Alshareef *et al*. is that there is high variation in pathogenic potential amongst environmental isolates and that the act of infection acts as a selective process, selecting for those more virulent strains. Clearly this does not explain the findings in the present study. A further interesting point which may prove to be an important factor is that in this paper it was also established that the environmental samples derived from Manchester, England were more pathogenic than the environmental samples from Dublin, Ireland. The possibility of geographical variation in pathogenicity profiles amongst environmental *A. fumigatus* populations could potentially explain the differences in the findings between their work and ours. However, similar to Aufauvre-Brown *et al*. (1998) the ten 'environmental' isolates from Dublin and Manchester were from the air of the respective University campuses which is likely to be very different to compost-derived isolates.

It should be considered that dead, non-viable and metabolically inactive spores of *A. fumigatus* as well as fragments of spores are able to cause allergic responses, and thus play a role in disease (Green, Sercombe and Tovey, 2005). By using CFU/ml rather

than spores/ml this cannot be taken into account, as the number of spores inoculated is inevitably greater than the number of CFUs and will vary from one strain to another (see section 2.2.). As this is primarily a model of invasive fungal infection, it was decided that the use of CFU/ml over spores/ml in this work would better ensure the consistency of the effective inoculum. It is also worth considering when viewing this data that mycotoxins may be produced by the growing fungi in culture which could then (irrespective of the growth and tissue invasion of the spores post-inoculation) have a negative impact on the health of the larvae. Again, for the purposes of this work it was not feasible to take this into account in the design of the experiments, however, standardising the number of viable spores between samples should standardise any effect of mycotoxins given that only viable spores will be producing mycotoxins.

It has been shown *in vitro* that *A. fumigatus* produces extracellular elastase only when cultured in the presence of elastin (Kothary *et al*., 1984). The lung linings are an elastin-rich environment and elastase secretion in some *A. fumigatus* strains has been shown to be associated with increased virulence, with elastase-producing strains causing 100% mortality in a mouse model compared to <50% mortality in low-elastase producing strains (Kothary *et al*., 1984). A future addition to the work in this chapter could be to measure elastase production or activity in these clinical and environmental isolates in order to identify the true significance of elastase as a virulence factor in respiratory infection with *A. fumigatus*. It would also be useful to test these isolates for virulence in an established mouse model of fungal pathogenicity to assist in further clarifying the usefulness and predictive power of the *G. mellonella* model in *A. fumigatus* human infections.

Generally in this work the compost isolates have been referred to as environmental isolates. It should be recognised however that not all environmental isolates are compost-derived. Indeed *A. fumigatus* is ubiquitous in the environment, regardless of proximity to composting sites. It may well be that the compost site environment harbours a distinct population of *A. fumigatus* with different characteristics to non-compost-derived environmental isolates. In the comparisons between clinical and environmental isolates throughout this work it has been assumed that the clinical isolates ultimately arise from the inhalation of spores of environmental isolates, but of

course many people may never be exposed closely to composting sites and thus if the samples collected on these sites do not represent the overall environmental *A. fumigatus* population then this would confound the results. This could explain the higher virulence in the compost isolates, despite previous findings (Alshareef *et al*., 2014, Aufauvre-Brown *et al*., 1998) suggesting that clinical isolates are more virulent than environmental isolates. Rather than thinking in terms of simply clinical *vs* environment it may be more accurate to further define the environmental populations in terms of proximity to composting sites. One explanation that could fit in with the earlier findings is that the clinical isolates may be more virulent than general environmental (non-compost) isolates whilst compost-derived isolates are more virulent than both non-compost environmental isolates and clinical isolates. As the environmental collection of *A. fumigatus* used in this study contained only compost-derived isolates it is not possible to address this question further.

The uncertainty about the importance of various putative virulence factors of *A. fumigatus* remains an ongoing problem in the development of new therapeutic compounds. Indeed some researchers have proposed that an alternative method to studying specific factors is to use genome comparison techniques to screen the *A. fumigatus* for possible essential genes and then use gene knockout models to identify genes essential to the growth of *A. fumigatus* in physiological conditions. Using this approach, Hu *et al*. (2007) identified the function and transcription of the ERG11 gene family as essential in order for *A. fumigatus* to cause successful infection in a mouse model. This demonstrates the use of novel techniques to identify potential targets for much-needed new therapeutic drugs in the future.

5.4.4 Mating type

Within the compost samples, there is a roughly even proportion of MAT1-1 to MAT1-2 isolates (~52-48%) but the clinical samples have a considerably higher prevalence of MAT1-2 (~63% being MAT1-2), particularly from isolates obtained during and after voriconazole treatment (~68% MAT1-2). This is interesting as Alvarez-Perez *et al*., (2010) had previously found the opposite to be true in their comparison of clinical and environmental isolates, showing that in their collections there was a higher proportion

of MAT1-1 in the clinical samples and a roughly even split in the environmental isolates. In their study, they also showed a striking association between MAT1-1 and samples derived from invasive aspergillosis patients (as opposed to other, non-invasive, *A. fumigatus* clinical isolates) and identified that higher elastase activity was associated with MAT1-1. This would appear to fit in with the idea of elastase-induced pathogenicity being an important potential factor in the instigation and perpetuation of an invasive *A. fumigatus* infection (Tomee and Kauffman, 2000), but does not explain the prevalence of MAT1-2 in the clinical isolates in the present study. Although it would appear from this data that infection in humans in the context of patients with asthma may select for the MAT1-2 mating type, there was no clear association with mating type and pathogenicity in the *G. mellonella* model (Figure 5-22 andFigure 5-23). Ideally this could be tested with a higher number of samples in a future study to ascertain the importance and reproducibility of this finding, alongside further characterisation of the expression profile of putative virulence factors in the two mating types.

The fact that there was no significant association with mating type and virulence in the *G. mellonella* model fits in with the observation that in the clinical isolates, lung function of the patient was not associated with mating type of the culture isolated (Figure 5-21). These findings together do suggest that, while the MAT1-2 mating type does seem to be associated with a clinical sample origin, this does not appear to determine clinical outcome. It should be considered however that all of the clinical isolates used in this study were obtained from asthma patients, none of which had invasive aspergillosis.

Generally, a roughly even divide between the two mating types has been observed globally (Paoletti *et al.*, 2005), as would be expected of a sexually reproducing fungus. This was indeed one of the key pieces of evidence for sexual reproduction in *A. fumigatus* before its sexual form was seen under laboratory conditions (O'Gorman, Fuller and Dyer, 2009). The even divide seen amongst the environmental isolates in the present study fits in with these findings and suggests that sexual reproduction is at least occasionally used in the compost population, as such an even split of mating types is unlikely to be maintained in a population reproducing purely by asexual means (Dyer,

2007). An extension of this study to confirm this hypothesis would be to use microsatellite markers, for example the Str*Af* primers of de Valk *et al.* (2005), to compare genetic diversity within and between the environmental and clinical isolates used in this study.

Mating type isn't only a marker by which *A. fumigatus* isolates can be categorised, it has real-world and widespread influence on the fundamental biology of the fungus. The mating type gene products alpha and HMG act as transcription factors controlling cascades of transcriptional regulation (Rydholm, Dyer and Lutzoni, 2007) and so while the effects of the differences induced by these different mating types isn't fully understood, it is likely that they have an impact on the fitness of *A. fumigatus* under different conditions. Such fitness advantages under different conditions could explain the apparent shift towards MAT1-2 within the clinical samples.

From the data comparing mating type in the clinical isolates before and after voriconazole exposure (Figure 5-20) it appears that human exposure to voriconazole may select for colonisation by MAT1-2 *A. fumigatus* strains. It should be noted however that these are relatively low numbers of isolates (40 isolates from azole naïve patients, 34 obtained from patients during or post-voriconazole treatment) and so a study specifically looking at the relationship between azole exposure in the human and mating type of colonising fungi would be useful to clarify this point. A further complication is that overall there was no association between increased MIC values and either mating type (although the MIC values didn't reflect voriconazole treatment either in this study). It would seem that this isn't a simple case of MAT1-2 conferring voriconazole resistance and thus fungi of this mating type being better at colonising people with asthma during voriconazole treatment. These data suggest that mating type alone is not associated with virulence, drug resistance or the disease state of the patient from which they are isolated. The significance of the apparent selection of MAT1-2 mating type in the clinical setting remains unknown in light of these findings, and is worthy of further study. Mating type appears to be associated with origin of isolate, in agreement with the original hypothesis, although the data suggest that mating type differences are not associated with altered pathogenicity.

Chapter 6 Summary and Future Directions

This project focused on three distinct but complementary areas of research involving different aspects of the biology of *A. fumigatus*; bioaerosol monitoring methods, mycoviruses and virulence and drug resistance differences between *A. fumigatus* populations. The purpose of the wind tunnel experiments was to compare commonly used sampling methods to determine which approaches may be best suited to the collection of air samples and quantification of bioaerosol load at compost sites and for measuring personal exposure to *A. fumigatus* bioaerosols. Ultimately this work was to test the hypothesis that some air sampling techniques are more efficient than others and may be more appropriate for sampling the high levels of fungal spores in the air around composting facilities.

Considering the increasing prevalence of drug resistance in *A. fumigatus* worldwide and the need for new treatment options for *A. fumigatus* infections the primary aim of the mycovirus work was to assess the validity of an enrichment technique for obtaining mycoviruses from environmental samples. This work was done with a view to determining if compost samples may be a potential source of *A. fumigatus* mycoviruses. The secondary aim of the mycovirus work was to further understand the basic biology of the mycovirus carried by the *A. fumigatus* type strain NCPF7367. The primary hypothesis behind the mycovirus work was that mycoviruses are present within environmental populations of *A. fumigatus* and *A. fumigatus* mycoviruses can be isolated from raw environmental samples.

The aim of the drug resistance work was to ascertain if clinical and compost-derived collections of *A. fumigatus* differed in terms of azole resistance and virulence, and to see if any differences seen were associated with mating type. The hypotheses being tested were that antifungal drug resistance is more prevalent in compost derived *A. fumigatus* isolates than in clinical isolates and this resistance is associated with altered pathogenicity in these two populations of the fungus. Isolates obtained from patients following antifungal drug treatment may also display higher antifungal drug resistance or greater pathogenicity. A further hypothesis was that mating type differences between *A. fumigatus* isolates are associated with altered pathogenicity and source.

6.1 Wind tunnel

The monitoring of bioaerosol load at and around composting sites is essential for understanding the risk of exposure for people working on sites and those who live and work nearby. It has been observed that working in environments with high bioaerosol load is associated with a range of health problems (e.g. Schlosser *et al.*, 2009). More definitive analysis of associations between health and exposure require robust exposure measures and there is a recognised lack of a "gold standard" for environmental exposure studies of fungal bioaerosols. A large variety of different sampling devices are available and some may be more suitable than others for sampling the air of this environment, as well as some being more suited to personal exposure monitoring than others.

The main conclusion to be drawn from the data collected in the wind tunnel experiments is that there were significant and highly variable differences in the collection efficiencies of the different samplers tested. The use of a combination of different sampling strategies would appear to be desirable in any in-depth study of compost site bioaerosol release, although clearly this increases the cost and complexity of any such study. The data collected suggest that the CIP-10M and the IOM filter samplers may be useful additions to a sampling protocol for both static and personal exposure sampling. The versatility of the CIP-10M sampler is particularly advantageous and yet this sampler is not routinely used in the majority of bioaerosol monitoring studies presently. This conclusion should be viewed bearing in mind that a sampling strategy that allows for molecular quantification and identification is a definite advantage. Recently the use of molecular quantification of bioaerosols has proven to be a useful approach (e.g. Madsen *et al.*, 2015; O'Brien *et al.*, 2016) and is likely to be increasingly important in future bioaerosol studies. In this regard, the CIP-10M liquid collection system may be more suitable than the IOM which collects onto filters, due to the inefficiencies associated with retrieving collected material from the filters for analysis.

Although further work is required in order to fully realise the potential of the wind tunnel experiments, the data shown support the hypothesis that some commonly used

sampling strategies are better suited than others for the monitoring of compost site bioaerosols.

6.2 Mycovirus

Viruses of microorganisms are ubiquitous and important in physiological and evolutionary processes of their host populations. They offer an alternative to antimicrobial drugs as well as being an invaluable tool for the study and manipulation of their hosts. The study of phages has set a precedent for an environmental source of mycoviruses which may be of use against fungal pathogens, as it is known that phages of environmental isolates of bacteria can be used in the treatment of clinical isolates. A recent example is that a cocktail of environmentally-sourced *A. baumanii* phages was shown to be efficient in clearing an *A. baumanii* infection in a mouse model (Regeimbal *et al.*, 2016).

The novel use of an enrichment procedure for obtaining mycoviruses from environmental samples appears promising with the nanosight, RNA extraction and TEM results providing good evidence of the presence of mycoviruses in a small proportion of the enrichments prepared. The findings strongly support the original hypothesis that *A. fumigatus* mycoviruses present in compost may be isolated from compost samples.

The nanosight proved to be a useful method by which to screen the large collection of enrichments for potential mycoviruses. This approach has not previously been used in the search for mycoviruses but the findings in the present study suggest that it may be a useful tool in such studies in the future. In addition the CSTEM protocol for fungal hyphal cells was a useful method, further helping to characterise the virus of *A. fumigatus* strain NCPF7367 as a non-capsid-forming mycovirus. The genome sequencing of the NCPF7367 mycovirus gave a fascinating insight into the biology of this virus and the observation that no capsid protein was encoded by the viral genome corroborates the earlier findings of the Bradford assay and CSTEM work on this fungal strain. The presence of an RNA-dependent RNA polymerase in the genome of this virus fits in with the current understanding of dsRNA viral replication strategies whilst the remaining genome segments encode a putative RNA capping enzyme and two other

putative products of unknown function. The genome segments showed significant homology to viruses of other fungi, demonstrating the conservation of certain motifs within apparently unrelated mycoviruses. The lack of a capsid protein in the genome of this virus also highlights the fact that a study based only on the identification of viral particles is likely to underestimate mycovirus prevalence. This is clearly an important consideration when using the nanosight system for the early screening of samples of interest and shows that a combination of approaches is needed in order to avoid such underestimation.

The use of mycoviruses either in the direct treatment of fungal infections or as a means to identify potential drug targets or simply to better understand the biology of *A. fumigatus* is clearly a worthwhile endeavour, especially in light of the emergence of A. *fumigatus* globally as a human pathogen and the increasing prevalence of drug resistance in this species.

6.3 Resistance

Azole resistance in *A. fumigatus* is an increasingly common and problematic phenomenon in clinical and environmental settings. In the present work however the prevalence of resistance in both the clinical and compost-derived culture collections tested was much lower than expected, with none of the over 150 isolates giving MIC values above the EUCAST resistance threshold values. This was a surprising finding in the context of recent studies' findings of high prevalence of azole resistance (e.g. van Ingen *et al.*, 2015). Some other studies however have also found a low frequency of resistance in their collections of *A. fumigatus* isolates. Alanio *et al.* (2016) conclude that there is low prevalence of azole resistance within the clinical isolate collections tested in their studies (obtained from IA patients from a region of France) and that the prevalence has not increased in recent years. This observation, when viewed alongside the findings in the present study suggests that geographical variation may be greater than has previously been recognised. Geographical variation in resistance rates is already established, and in a recent review of the epidemiology of resistant *A. fumigatus* in the Netherlands it was shown that hospitals with the highest prevalence of resistance

were all within the same geographic region. Those hospitals with the lowest rates were also geographically closest to each other (RIVM, 2015). It has also been highlighted that there is a large variation in clinical resistance prevalence in different patient groups (van der Linden *et al.*, 2016). As has been mentioned the clinical isolates used in the present study were all obtained from asthma patients, none of which were known to have invasive disease. The low prevalence of resistant isolates obtained from this group would concur with previous findings that resistance rates are highest amongst only the most high-risk groups of patients (Fuhren *et al.*, 2015; van der Linden *et al.*, 2016).

The significantly higher MIC values in the compost isolates supports the hypothesis that azole resistance is more prevalent amongst compost-derived isolates and would appear to fit in with the idea of an environmental origin of resistance. This finding suggests that compost sites may indeed be a source of such environmentally-acquired resistance. None of the intermediate resistance isolates tested had either of the common environmental resistance genotypes, suggesting that other mechanisms may be involved in the low-level intermediate resistance observed. Although the two most common resistance genotypes are seemingly continuing to spread globally and increase in prevalence (with TR34/L98H recently observed in Iran (Nabili *et al.*, 2016) and TR46/Y121F/T289A in Japan (Hagiwara *et al.*, 2015)) there are numerous other genetic mechanisms which are known to contribute to decreased azole sensitivity. The DAP family of damage resistance protein-encoding genes have been shown to be involved in the regulation of cyp51 activity, with the expression of these DAP proteins shown to be azole dependent (Song *et al.*, 2016). ABC transporter genes and major facilitator transporter genes can also mediate azole resistance independently of cyp51 and there is evidence that at least some such genes are overexpressed upon azole-treatment growth conditions (Meneau *et al.,*2016).

The requirement for more frequent and rigorous surveillance of drug resistance in *A. fumigatus* is clear and yet this still is not routinely performed in many clinical and environmental settings.

6.4 Pathogenicity

It is interesting that the compost isolates were shown to have higher virulence in the *G. mellonella* model than the clinical isolates but that this was not associated with the higher azole MIC values. This finding further confirms the distinct nature of these two populations and highlights the risks associated with compost-derived *A. fumigatus* bioaerosol exposure. This finding would however appear to be contrary to the conclusion drawn by Alshareef and Robson (2014) suggesting that the act of infection functions as a selective process, selecting (from the diverse environmental population) those more pathogenic fungi. The hypothesis that resistance is associated with altered pathogenicity does not appear to be supported by the correlation data although compost-derived isolates were, overall, more virulent than clinical isolates as well as showing increased MIC values to the azole drugs tested. The hypothesis that azole treatment of a patient may be associated with increased azole MIC values was only partially supported by the data obtained, with increased MIC values only to itraconazole and posaconazole following voriconazole treatment in the patient. The hypothesis that voriconazole treatment of a patient is associated with increased pathogenicity of isolated obtained is not supported by the data.

When viewed together the drug resistance, virulence and mating type data suggest that there are significant differences in the make-up of the two populations studied; that compost derived isolates are more pathogenic and less sensitive to azole drugs and that the clinical isolate collection has a higher prevalence of mating type MAT1-2. These differences at the phenotype and genetic level suggest that the distinction between the two populations has potential clinical and environmental relevance and is worthy of further study. These findings highlight the risk associated with inhaling fungi originating from compost sites and strengthen the need for a comprehensive exposure study of workers at these sites, which requires reliable means of measuring exposure.

6.5 Mating type

There was a higher prevalence of MAT1-2 within the clinical isolates tested, which is contrary to the findings of Alvarez-Perez *et al.* (2010) who identified MAT1-1 to be

more prevalent in their clinical collection of isolates. This data appears to support the hypothesis that mating type is associated with origin of isolate; however the difference in mating type prevalence was not statistically significant. There was no association between mating type and virulence in the *G. mellonella* model, which is contrary to the hypothesis that mating type differences are associated with altered pathogenicity. The data from the present study also appear to be contrary to the findings of a previous study (Cheema and Christians, 2011) although this study was based only on a small sample size (20 isolates in total).

There was also no association seen between mating type and lung function of the individual from which the fungus was obtained amongst the clinical isolates tested. Clearly virulence and ability to colonise is multifactorial and mating type is only one factor. The data does however suggest an association between MAT1-2 and clinical origin of *A. fumigatus* isolates. It is known that there are significant transcriptional differences between these two mating types (Rydholm *et al.,* 2007), and as the MAT locus proteins are transcription factors themselves it is likely that there are a number of yet unknown potential virulence factors whose expression is MAT-dependent.

6.6 Future work

As molecular techniques become more widely used a comparative analysis of different sampling techniques in the application of molecular identification and quantification of bioaerosols is needed, with particular emphasis on the monitoring of compost bioaerosols. Current monitoring guidelines still rely on culture-based methods (AfOR, 2009), and the devices currently used may not be the best suited for sequence-based analysis. Future bioaerosol monitoring guidelines should take this into account.

Further work is needed to fully verify the validity of the enrichment approach for the isolation of mycoviruses from environmental samples, and the storage of putative mycovirus suspensions particularly warrants further study in order to ensure the maintenance of viability of any mycoviruses obtained. It would also be beneficial to screen the clinical and environmental collections of *A. fumigatus* isolates for dsRNA to identify any differences in the rate of virus carriage between these two populations. In addition to this dsRNA screening of the compost virus enrichment collections would be

a logical next step for this work, if a suitably sensitive dsRNA extraction and detection method could be identified. The potential importance of mycoviruses with non-dsRNA genomes should also be considered in future work. ssRNA and dsDNA mycovirus genomes have also been previously described. Although these may be relatively rare compared to dsRNA mycoviruses, they may well be of importance and so future studies should also aim to incorporate strategies which would detect these viruses as well as dsRNA mycoviruses.

In addition, extending the range of environments studied in the search for *A. fumigatus* mycoviruses is likely to be the best way to ascertain the true diversity, function and prevalence of these viruses. Any environment in which *A. fumigatus* is present, such as degrading leaf litter, is a potential source of *A. fumigatus* mycoviruses and future studies should take advantage of this.

In order to more definitively characterise the intermediate resistance seen in the EUCAST testing, full cyp51A gene sequencing of all of the intermediate-resistance isolates would be useful to ascertain if specific mutations anywhere in this gene are responsible for the slightly reduced sensitivity to azoles observed. Both cyp51A and cyp51B expression could also be measured, as well as sequencing regions of other genes which may play a role in mediating azole resistance. Targets for such analysis could include the transporter genes identified by Meneau *et al,* (2016) to be associated with azole resistance or DAP genes which similarly appear to be overexpressed during azole exposure and contribute to the resistance phenotype (Song *et al.*, 2016).

One recently validated approach is the 'AsperGenius' multiplex PCR, a culture-independent method which can distinguish common *Aspergillus* species from one another as well as identifying the TR34, L98H, T289A, and Y121F mutations in the cyp51A gene (Chong *et al.*, 2015). This could be a useful tool for the rapid identification of the common resistance genotypes within the collections of *A. fumigatus*.

It would be useful to set the virulence results observed in the context of a wider variety of *A. fumigatus* isolates from more broad-ranging environments. A study of virulence differences between collections of other (non-compost) environmental collections and

compost-derived isolates from other geographical areas, as well as clinical isolates obtained from individuals with a known history of exposure to compost-related environments would be desirable. Such a study would allow more accurate comparisons to be drawn with compost-site *A. fumigatus* populations and so allow the risks associated with compost site bioaerosol exposure to be more fully appreciated.

6.7 Conclusion

This is the first time compost-derived isolates have been investigated for both azole resistance and pathogenicity. It is interesting that both resistance and pathogenicity were higher amongst the compost-derived isolates than clinical isolates, but that these two variables were not correlated with each other. This highlights the importance of compost *A. fumigatus* populations and, given the known health issues associated with compost-site workers, this is clearly a potentially important area which requires further work in order to gain a deeper understanding of the risks involved with exposure. The wind tunnel work demonstrated the differences in efficacy of different air samplers for bioaerosol monitoring. This should be taken into consideration in the experimental design of future studies as in-depth exposure studies require accurate, efficient and reproducible exposure measurements. This is also the first time the enrichment procedure has been used in the isolation of mycoviruses, and the first use of the nanosight system for the detection of putative mycovirus particles. The possibility of the isolation of mycoviruses from environmental samples and their efficient detection are promising steps forward in the study of these generally poorly-understood but potentially important viruses. The genome sequencing of the virus carried by *A. fumigatus* type strain NCPF7367 also reveals important new insights into the biology of this virus. Particularly relevant is the finding that the genome of this virus does not encode a capsid protein and thus viral particles are not formed. This highlights the complexity and variation amongst mycoviruses and the importance of using a combination of approaches for their isolation and further study.

Appendices

Appendix 1: Composition of media

Antibiotics

The antibiotics used in the preparation of the various media used in this project were prepared from stock powders as in **Error! Reference source not found.**. Once prepared, all antibiotics were stored at -20°C until use.

Table A-1: Details on the preparation of the antibiotics added to media used in this project

Antibiotic	Supplier (product number)	Stock concentration	Solvent
Chloramphenicol	Sigma-Aldrich (C0378)	34 mg/ml	Ethanol
Gentamicin	Sigma-Aldrich (G1264)	50 mg/ml	Deionised water
Fluconazole	Sigma-Aldrich (F8929)	5 mg/ml	Dimethyl sulfoxide (DMSO)
Penicillin G	Sigma-Aldrich (P3032)	100 mg/ml	Deionised water
Streptomycin sulphate	Sigma-Aldrich (S6501)	50 mg/ml	Deionised water

Glycerol nutrient broth (GNB)

9.45g Nutrient broth (Bioconnection, product number BC2014) and 75 ml glycerol (Sigma-Aldrich product number G6279) was added to 425 ml dH_2O, autoclaved at 121°C for 15 minutes and stored at 4°C.

Malt extract agar (MEA)

16.67 g Malt Extract Agar (Oxoid CM0059) and 5g Agar (Bacteriological agar No. 1, Oxoid LP0011) was added to 500 ml dH_2O. This was autoclaved at 121°C for 15

minutes and stored at 4°C. Before pouring agar plates, once the agar was molten but cooled to ~50°C, 60.5 µl Penicillin G [100 mg/ml] and 516 µl Streptomycin sulphate [50 mg/ml] was added. Agar plates were stored inverted in bags at 4°C and used within one month.

Phosphate buffered saline with 0.05% Tween 80 (PBST)

2 g NaCl (Sigma-Aldrich product number S7653) and 0.3025 g K_2HPO_4 (BDH product number 452331) were added to 0.085 g KH_2PO_4 (Sigma-Aldrich product number 22130) and made up to 250 ml with dH_2O. When fully dissolved, 0.125 ml Tween 80 (Merck product number 28830291) was added and stirred until dissolved. This was autoclaved at 121°C for 15 minutes and stored at 4°C.

Potato dextrose agar (PDA)

15.6 g Potato dextrose agar (Oxoid CM0139) was added to 400 ml dH_2O. This was autoclaved at 121°C for 15 minutes and stored at 4°C. Before pouring agar plates, once agar was molten but cooled to ~50°C, 188.2 µl chloramphenicol [34 mg/ml] and 32 µl gentamicin [50 mg/ml] was added for PGC agar and 40 µl fluconazole [5 mg/ml] was added in addition to chloramphenicol and gentamicin for PGCF agar. Agar plates were stored inverted in bags at 4°C and used within one month.

Double-strength RPMI-1640 (2%) glucose

20.8 g RPMI-1640 powder (Sigma-Aldrich R-6504), 69.06 g MOPS (Morpholinepropanesulfonic acid) (Sigma-Aldrich product number M1254) and 36 g D-Glucose (Sigma-Aldrich product number G7528) was added to 500 ml dH_2O and mixed until dissolved. The volume was then made up to 800 ml with dH_2O This was adjusted to pH7 with 10M Sodium Hydroxide (NaOH, Sigma Aldrich product number

S8045) and made up to 1 l with dH$_2$O. This was filter sterilised through 0.22 µm pore size filter unit and stored at 4°C.

Sabouraud dextrose agar (SDA)

26 g Sabouraud dextrose agar (Oxoid CM0041) was added to 400 ml dH$_2$O. This was autoclaved at 121°C for 15 minutes and stored at 4°C. Before pouring agar plates, once the agar was molten but cooled to ~50°C, 588.2 µl of chloramphenicol [34 mg/ml] was added and the plates poured. Agar plates were stored inverted in bags at 4°C and used within one month.

Sabouraud liquid medium (SLM)

15 g Sabouraud liquid medium (Oxoid CM0147) was added to 500 ml dH$_2$O. This was autoclaved at 121°C for 15 minutes and stored at 4°C.

Appendix 2: Andersen sampler correction table

i^A	ii^B	iii^C	i	ii	iii	i	ii	iii	i	ii	iii
1	1.0	0.0	51	58.8	3.1	101	140.6	7.9	151	281.3	18.1
2	2.0	0.1	52	60.2	3.1	102	142.7	8.0	152	285.4	18.5
3	3.0	0.1	53	61.6	3.2	103	144.8	8.2	153	289.6	18.8
4	4.0	0.2	54	63.0	3.3	104	146.8	8.3	154	294.0	19.2
5	5.1	0.2	55	64.3	3.4	105	148.8	8.4	155	298.4	19.6
6	6.1	0.3	56	65.7	3.4	106	151.0	8.6	156	302.8	20.0
7	7.1	0.3	57	67.1	3.5	107	153.2	8.7	157	307.4	20.4
8	8.2	0.4	58	68.5	3.6	108	155.3	8.8	158	312.2	20.8
9	9.2	0.4	59	69.9	3.7	109	157.5	9.0	159	317.0	21.2
10	10.2	0.5	60	71.3	3.8	110	159.7	9.1	160	321.9	21.6
11	11.3	0.5	61	72.8	3.8	111	162.0	9.3	161	327.0	22.1
12	12.4	0.6	62	74.2	3.9	112	164.2	9.4	162	332.2	22.6
13	13.4	0.7	63	75.6	4.0	113	166.4	9.6	163	337.5	23.1
14	14.6	0.7	64	77.2	4.1	114	168.8	9.7	164	343.0	23.6
15	15.6	0.8	65	78.6	4.2	115	171.2	9.9	165	348.6	24.1
16	16.6	0.8	66	80.0	4.2	116	173.5	10.1	166	354.4	24.7
17	17.8	0.9	67	81.6	4.3	117	175.9	10.2	167	360.4	25.2
18	18.8	0.9	68	83.1	4.4	118	178.3	10.4	168	366.6	25.8
19	20.0	1.0	69	84.6	4.5	119	180.8	10.5	169	372.9	26.5
20	21.0	1.0	70	86.2	4.6	120	183.2	10.7	170	379.4	27.1
21	22.2	1.1	71	87.7	4.7	121	185.8	10.9	171	386.2	27.8
22	23.3	1.2	72	89.2	4.8	122	188.3	11.1	172	393.2	28.5
23	24.4	1.2	73	90.8	4.9	123	190.9	11.2	173	400.5	29.3
24	25.6	1.3	74	92.4	5.0	124	193.5	11.4	174	408.0	30.1
25	26.7	1.3	75	94.0	5.1	125	196.2	11.6	175	415.9	30.9
26	27.8	1.4	76	95.6	5.1	126	198.8	11.8	176	424.0	31.8
27	29.0	1.5	77	97.2	5.2	127	201.6	12.0	177	432.6	32.8
28	30.2	1.5	78	98.8	5.3	128	204.3	12.2	178	441.4	33.8
29	31.3	1.6	79	100.5	5.4	129	207.1	12.4	179	450.8	34.8
30	32.5	1.6	80	102.2	5.5	130	210.0	12.6	180	460.6	36.0
31	33.7	1.7	81	103.8	5.6	131	212.8	12.8	181	470.8	37.2
32	34.9	1.8	82	105.6	5.7	132	215.8	13.0	182	481.6	38.5
33	36.1	1.8	83	107.2	5.8	133	218.7	13.2	183	493.1	40.0
34	37.3	1.9	84	109.0	5.9	134	221.8	13.5	184	505.2	41.5
35	38.5	2.0	85	110.6	6.0	135	224.8	13.7	185	518.2	43.2
36	39.7	2.0	86	112.4	6.1	136	227.9	13.9	186	532.0	45.1
37	40.9	2.1	87	114.2	6.3	137	231.0	14.2	187	546.8	47.1
38	42.2	2.2	88	116.0	6.4	138	234.2	14.4	188	562.8	49.4
39	43.4	2.2	89	117.8	6.5	139	237.5	14.6	189	580.2	52.0
40	44.6	2.3	90	119.6	6.6	140	240.8	14.9	190	599.3	54.9
41	45.9	2.4	91	121.4	6.7	141	244.2	15.2	191	620.4	58.3
42	47.2	2.4	92	123.2	6.8	142	247.6	15.4	192	644.0	62.2
43	48.4	2.5	93	125.1	6.9	143	251.0	15.7	193	670.8	66.9
44	49.7	2.6	94	127.0	7.0	144	254.6	16.0	194	701.8	72.5
45	51.0	2.6	95	128.8	7.2	145	258.2	16.3	195	738.4	79.6
46	52.2	2.7	96	130.8	7.3	146	261.8	16.6	196	783.4	88.9
47	53.6	2.8	97	132.7	7.4	147	265.6	16.8	197	841.8	101.7
48	54.8	2.8	98	134.7	7.5	148	269.4	17.2	198	925.1	121.3
49	56.2	2.9	99	136.6	7.6	149	273.3	17.5	199	1075.1	156.9
50	57.6	3.0	100	138.6	7.8	150	277.3	17.8	200	1175.6	253.8

Positive-hole correction table to adjust colony counts from a 200-hole impactor for the possibility of collecting multiple particles through a single hole. From Macher (1989). i^A= The observed number of colony-forming units (CFU). ii^B= The expected number of CFU, corrected for coincidence. iii^C= The standard deviation of ii^B.

Appendix 3: MiSeq sequencing platform

Purified dsRNA was DNase treated (Appendix 4) and the total RNA concentration confirmed by nanodrop. The cDNA library preparation and use of the MiSeq platform were performed at Fera Science Ltd. by Dr Ian Adams as outlined below.

Double-stranded cDNA was synthesised from the purified and DNase-treated RNA sample using the Scriptseq complete random hexamer-based cDNA synthesis kit (Invitrogen, UK) following the manufacturer's protocol. The cDNA was sequenced using the MiSeq sequencing-by-synthesis platform (Illumina Inc.), as per the manufacturer's instructions.

The raw sequence data was trimmed to include only sequences with a quality score of Q30 or higher (>99.9% base call accuracy) and exported for assembly of contiguous sequences using the Trinity software methodology (Grabherr *et al.*, 2013).

The resulting four genome segments were analysed using the MEGA (v6) and Mauve (v2.4.0) software packages and translated protein sequences analysed by BlastX (NCBI) for homology to other published genome sequences.

Appendix 4: Protocol for use of Promega DNase

RQ1 RNase-free DNase (Promega, UK) was used in the following protocol.

8 µl of RNA sample, 1 µl of RQ1 DNase 10x reaction buffer (Promega M198A) and RQ1 DNase (1 µl/µg RNA in sample) (Promega M610A) was mixed and PCR-grade H_2O added to make to 10 µl

This was incubated for 30 minutes at 37°C before 1 µl of RQ1 DNase stop solution (Promega M199A) was added and the solution incubated for 10 minutes at 65°C to stop the DNase activity. The products were then run on agarose gel electrophoresis to confirm the persistence of RNA in the original sample.

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