Evaluation of an in-house six-well screening plate as a means of detecting phenotypic triazole resistance in St. James's Hospital, Dublin with emphasis on *Aspergillus fumigatus*

A dissertation presented for the degree of M.Sc. in Clinical Microbiology in the department of Clinical Microbiology, Trinity College Dublin.

by
David Sheehan
2022

Department of Clinical Microbiology
Trinity College Dublin

Declaration

I David Sheehan declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish (EU GDPR May 2018).

David Sheehan

Abstract

Aspergillus fumigatus is a ubiquitous saprophytic mould fungus found in nature. This fungus is present in the air, in soil, in foliage in our food stock and on inanimate surfaces. For healthy individuals, *A. fumigatus* poses no threat to human health and lives in harmony with the general population. However, this is not always the case, as the fungus can also be responsible for causing a range of human diseases; the most deadly and serious of which is Angioinvasive aspergillosis which is seen in immunocompromised patients (Prasad *et al.,* 2016). There are a multitude of different forms of Aspergillosis including allergic pulmonary aspergillosis, aspergilloma and the serious condition invasive pulmonary aspergillosis. This is an extremely dangerous condition characterised by pneumonia which can prove life-threatening particularly in immunocompromised patients. This occurs mostly in people with impaired immune function as a consequence of immunosuppressive treatments due to a variety of reasons such as having treatment for acute leukaemia or having received a solid organ transplant, and more recently pulmonary aspergillosis has been described in patients with severe Coronavirus Disease-19 disease (COVID-19). It is noted by Mohamed *et al.,* 2020 that the cases of patients who had *Aspergillus* co infection with COVID-19 could be drastically under reported most likely due to the aggressive nature of the pandemic leading to clinical ambiguity. According to Wang *et al.,* 2003, Wang *et al.,* 2004 and Hwang *et al.,* 2004 there were only four documented cases of patients having severe acute respiratory syndrome caused by SARS-CoV-1 also developing invasive aspergillosis with none of the patients being immunocompromised prior to contracting SARS-CoV-1 although they did receive corticosteroids as part of their treatment. The aim of this project was to develop and validate a 6-well screening plate that is cost-effective and yields fast (48 hours) and effective results as part of the environmental surveillance of *Aspergillus* at St. James's Hospital, Dublin. The screening plates contain five different drugs currently licenced to treat a range of fungal infections (Itraconazole, Voriconazole, Posaconazole, Caspofungin, Terbinafine) and a growth control. Over the course of 6 months a variety of *A. fumigatus* isolates were gathered from both indoor and outdoor locations on the campus of St. James's Hospital in Dublin as well as from a variety of food samples originating from several different countries across the globe. The isolates were then screened and analysed for

triazole resistance yielding a total of 12 resistant out of over 287 *A. fumigatus* isolates (6 air isolates and 6 fruit & vegetable isolates).

Acknowledgements

I want to say a sincere thank you to Prof. Thomas Rogers for giving me the opportunity to undertake this project and for his continued guidance and support along the way.

I want to say a huge thanks also to Auveen Griffen and all of the staff in the diagnostic laboratory at St. James's Hospital, Dublin for your help and knowledge along the way.

A massive thank you to all of my family and friends for your continued support and encouragement along the way, especially in the year of COVID-19 and the craziness that came with it.

My biggest thank you goes to my Mam. Mam you are a constant source of inspiration and support to me. When times were tough you were always there and this degree is as much yours as it is mine because without you and all you have done for me I would not be where I am today. All of my opportunities are only possible because of the incredible job you have done as a mother and as person. I am so lucky to have you as a mother words can never describe and I look up to you in every way possible.

To my brother Gerard, you motivate me to work hard every day by being the inspiration that you are. You were always there to help, listen and make me laugh when times were stressful, you are a large part of the reason that I am where I am. The relationship I have with you is like no other, you are my brother and my father figure all in one and I cannot thank you enough for everything that you do for me and the positive impact that you have on my life.

To Chanice, you were always there for me to help me when times were tough. I would not be here without you and you made the tough times not so tough. I am eternally grateful.

To all of the friends I made during my time in St. James's, you all made my time there unforgettable. Peter, Naoise, Aileen, Aoife and Sinead, the Friday coffees and laughs are something I will always cherish. Thank you all so much for making me part of the gang.

Table of Contents

List of figures

List of tables

Chapter 1: Introduction

There have been several words employed in an attempt to capture the significance of *A. fumigatus* in our environment. These include “opportunistic”, “persistent”, “saprophytic”, “hardy” etc. It is difficult to truly convey the everlasting presence of *A. fumigatus* in the environment. *A. fumigatus* has been found in all corners of the world from the depths of Antarctica to the sands of the Sahara (Sewell *et. al.,* 2019; Gerginova *et. al.,* 2013), as well as being known to cause contamination on the International Space Station (Blachowicz *et al.,* 2016).

The genus *Aspergillus* comprises of a group of moulds that are extremely common in the environment, particularly in the autumn and winter months. Although possessing several important functions in our environment such as its saprophytic role in soil and decaying vegetation and a constituent of organic matter which is essential in the carbon and nitrogen cycle (Fang & Latgé 2018). *A. fumigatus* is mostly known for causing disease in both humans and animals. A person inhales approximately 300 *Aspergillus* spores on an average day with no adverse effects due to the easy handling of these propagules by the immune system (Bandres *et al.,* 2021). However, in the immunocompromised patient the inhalation of *A. fumigatus* spores can be life- threatening in some cases. The types of diseases caused by *A. fumigatus* are varied, ranging from an allergy-type disease to life-threatening pulmonary or disseminated infections. The severity of aspergillosis is determined by various factors but one of the most important is the competency of the immune status of the individual. Allergic bronchopulmonary aspergillosis and “fungal asthma” (SAFS) are conditions categorised by an allergic response to *Aspergillus* spores and is quite common in asthmatics and cystic fibrosis patients (Knutsen *et al.,* 2011).

A. fumigatus is perhaps the most important airborne fungal pathogen with the frequency of invasive infection caused by this strain increasing drastically during the last two decades (Margalit & Kavanagh 2015). In healthy individuals, *A. fumigatus* spores can be inhaled into the lung, but the establishment of disease is prevented by the host immune system whereas in the immunocompromised patient these spores can colonise within the lung upon inhalation and spores can germinate and produce hyphae in the lungs which are multicellular breaking off into the circulation to disseminate causing serious cases of invasive aspergillosis (Margalit & Kavanagh 2015).

Invasive aspergillosis is treated using prescription antifungal medication, usually voriconazole (Husain & Camargo 2019). Other treatment options include lipid amphotericin formulations, Posaconazole, Isavuconazole, Itraconazole, Caspofungin, and Micafungin (Fera *et al.,* 2014). Caspofungin is mainly utilised in combination with other azole antifungals and is effective due to its alternative target. It is also utilised because it is highly studied and regarded as safe by comparison to other antifungal drugs such as Amphotericin B which has toxic properties due to its nephrotoxicity (Heinz *et al.,* 2008). Although it is effective in some cases in combination therapy, the echinocandins are dramatically less effective for the treatment of invasive aspergillosis than the azole drugs (Heinz *et al.,* 2008).

A. fumigatus is a filamentous fungus which typically grows at a temperature of 37 °C or 99 °F (body temperature in humans) but can grow at temperatures of up to 50°C or 122 °F, with conidia surviving at 70 °C or 158 °F further consolidating its persistent nature (Bhabhra & Askew 2005). Originally it was believed that *A. fumigatus* reproduced only by asexual means as neither mating or meiosis had ever been witnessed up until the year of 2008 when *A. fumigatus* was found to undergo a full sexual reproductive cycle (O'Gorman *et al.,* 2009).

There are currently a wide variety of antifungal drugs used to combat *A. fumigatus* infections and its adverse effects on its host. Currently the main antifungal drugs in use are the Triazoles (predominantly Itraconazole and Voriconazole and Isavuconazole), Echinocandins (mainly Caspofungin, Micafungin and Anidulafungin) and Amphotericin B. These drugs have different modes of action, primarily targeting the fungal cell wall and membrane, but serve as the first line of therapy in fungal infection for several reasons such as their tolerability and their cost effectiveness (with the exception of Amphotericin B), as well as their quick infusion time and availability (Fera *et al.,* 2014).

The main mode of action of the triazoles is the inhibition of ergosterol synthesis, a vital component of the fungal cell membrane, by binding to the 14α-demethylase. However, resistance to these first-line drugs is on the rise. Triazole resistance can come about in a variety of ways such as by mutations in genes or efflux pumps (Chowdhary *et al.,* 2017*)* but it is usually caused by specific amino acid changes in the *Cyp*51A protein in combination with tandem repeats (TR) in the gene promoter such as TR_{34}/L98H and TR_{46}/Y121F/T289A for example (Mellado *et al.,* 2007). Isolates with environmental resistance seem to have overcome fitness competition having evolved alongside wild type isolates acquiring similar levels of fitness according to Verweij *et al.,* 2016*.* In some patients who suffer from invasive pulmonary aspergillosis mixed infections have been seen due to both triazole susceptible and triazole resistant strains (Kolwijck *et al.,* 2016).

Resistance is thought to have possibly come about due to the overuse of azoles in the agriculture setting. Agricultural azoles are highly efficient when it comes to deterring a wide range of plant diseases and infections including leaf spots, rust and powdery mildews, with a dramatic rise in the frequency of their use seen in the past 20 years (Kaur *et al.,* 2020). Despite their effectiveness in agriculture, their overuse can cause excess to contaminate water supplies leading to human consumption. Because these triazoles that are used in agriculture and floriculture share similar modes of action as those employed in the clinical setting, resistance is of huge concern and although it is not confirmed, it is suggested that this use of triazoles is a huge contributing factor to the rising incidence of triazole resistance in clinical *A. fumigatus* strains (Kaur *et al.,* 2020).

In some cases patients who have not had previous exposure to triazole therapy in any form have developed infection caused by triazole resistant *A. fumigatus*. There have been clinical cases where isolates of *A. fumigatus* that were once found to be susceptible later demonstrated resistance to triazoles during treatment due to the development of different resistance mechanisms. (Camps et. al., 2012; Buil et. al., 2019).

As referred to earlier, although we inhale several hundred spores of *A. fumigatus* on a daily basis, it is those who are immunocompromised that are at the most risk- this is a large proportion of patients who are being treated in St. James's Hospital, Dublin. This is because it is a major cancer treatment centre and has the national treatment centre for leukaemia. Such patients are at risk of aspergillosis because of building works which can disperse large numbers of *A. fumigatus* into the healthcare environment according to the Health Protection Surveillance Centre (HPSC) guidelines 2018 (Haiduven 2009). As can be seen in figure 1, construction work was the highest cause of nosocomial *Aspergillus* outbreaks in an environmental study undertaken by Weber *et al.,* 2009. Because of this there are a number of preventative measures hospitals employ in an attempt to minimize spore dispersal and the presence of *A. fumigatus* spores in the air which could otherwise prove to be potentially lethal to some patients particularly those receiving cancer treatments or critically ill in the Intensive Care Unit (ICU).

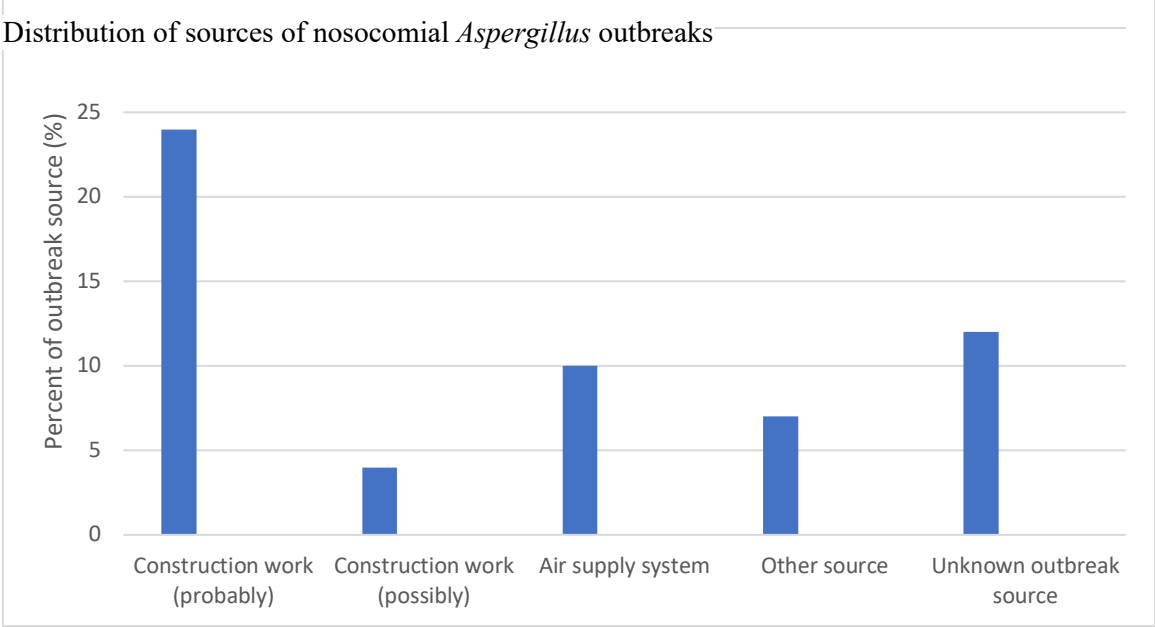


Figure 1. Distribution of sources of nosocomial *Aspergillus* outbreaks. Modified from Weber *et al*., 2009. Outbreaks were stratified by number and percent of outbreak source.

To give an overview of the effect *Aspergillus* infections have in the hospital setting, according to Singh *et al,* 2005 invasive *Aspergillus* infections have occurred in 2-26% of patients who receive hematopoietic stem cell transplants (HSCT) and 1-15% of those undergoing solid organ transplants with a mortality rate of between 74%-92%. Approximately 9.3-16.9% of the deaths that occur in transplant recipients in the first year after transplantation are caused by invasive aspergillosis. *A. fumigatus* was the main cause of infection in patients receiving HSCTs accounting for 43% of cases, and in those receiving solid organ transplants accounting for 57% making it the most common cause of infection in transplant procedures (Chiller *et al.,* 2008).

Triazole resistance is becoming more prominent across the globe according to Meis *et al.,* 2016. In a study undertaken in the Netherlands in 2007 by Verweij *et al*., 2009 multiple cases of triazole resistance were seen in patients with invasive aspergillosis in 2007. The countries that also had cases reported were Spain, Denmark, Greece, Turkey, Poland, Belgium, the UK and the Netherlands, with the Netherlands having the highest resistance frequencies (0.8%-9.4%) along with Turkey (10.2%) (Bueid *et al*., 2010; Snelders *et al*., 2008). *A. fumigatus* triazole resistance is a problem across the world and this is represented in figure 2 showing the distribution of countries where cases of triazole resistant *A. fumigatus* strains have been found.

Figure 2. Map showing the distribution of countries with reported cases of *A. fumigatus* triazole resistance highlighted in red. Countries in white are those with unknown resistance epidemiology (Sharpe *et al*., 2018).

There are a number of guidelines and recommendations in place that any given hospital should adhere to as proposed by the Health Protection Surveillance Centre which have been followed by St. James's Hospital in order to monitor and minimize the amount of *Aspergillus* isolates in the air which could be potentially deadly to immunocompromised patients, namely those individuals mentioned in table 2. These guidelines are outlined in the national guidelines for the prevention of nosocomial invasive aspergillosis during construction/renovation activities developed by a subcommittee of the scientific advisory committee of the national disease surveillance centre (www.hpsc.ie). These include dust control, ventilation of construction zone, debris removal and cleaning and traffic control. Ensuring contaminants from the construction zone are kept outside is essential. Another measure is surveillance by monitoring the counts of *A. fumigatus* as well as other fungal species in the air, particularly during times of construction, demolition, or renovation activities. Currently on St. James's Hospital campus there is major construction work to add a new national children's hospital. But due to this construction and the excavation, the building of foundations has the potential to cause a large amount of sediment and soil to be displaced in turn potentially causing an increase in the amount of *A. fumigatus* in the air which is of huge threat to the patient population on the grounds of St. James's Hospital (Kanamori *et al.,* 2015).

Table 1. Table adopted from Morris *et al*., 2000 summarising various occasions of importance when air sampling is a useful surveillance technique.

	Occasions when sampling for environmental *Aspergillus* spores may be useful
1.	To monitor levels of contamination prior to occupancy of special controlled environments e.g. to determine efficiency of HEPA filters in laminar flow facilities
2.	To identify potential sources of nosocomial aspergillosis when a case has been identified
3.	To predict environmental spore contamination from outside sources
4.	To identify defects/breakdown in hospital ventilation/filtration systems
5.	To correlate outbreaks of invasive aspergillosis with hospital construction or demolition work
6.	To monitor efficiency of procedures to contain hospital building wards where at-risk patients are managed

Table 2. Table showing the published incidence of invasive aspergillosis cases among immunocompromised patients from different studies.

Host group	**Incidence of invasive aspergillosis (%)**	**Reference**
Allogeneic bone marrow transplantation	5-10%	McWhinney *et al.,* 1993
Autologous bone marrow transplantation	0-5%	Iwen *et al.,* 1993
Peripheral blood stem cell transplantation	5%	Iwen *et al.,* 1993
Cytotoxic-therapy-induced granulocytopenia	Up to 70%	Schwartz *et al.,* 1984
Kidney Transplantation	0-3%	Toree-Cisneros *et al.,* 1993
Liver transplantation	1-15%	Collins *et al.,* 1994; Wajszczuk *et al.,* 1985; Kusne *et al.,* 1988
Heart/lung transplant	0-20%	Guillemain *et al.,* 1995 & Kramer *et al.,* 1993
Heart transplant	0-25%	Hofflin *et al.,* 1995

There are several standardised tests for confirming *Aspergillus* resistance with a number of options by which *Aspergillus* resistance is examined and assayed. In the diagnostic laboratory in St. James's Hospital Dublin, E-tests are routinely used by which the *Aspergillus* is streaked on a media plate and a MIC strip is placed in the centre. After incubation for 48h the growth of the *Aspergillus* can be inspected and inhibition can be read up to a certain breakpoint on the gradient strip. PCR and other commercial PCR kits such as the Aspergenius are also used to confirm resistance in possible *Aspergillus* isolates which is more time consuming and costly but is reliable. Antimicrobial susceptibility testing is routinely carried out according to EUCAST standard. This involves using antibiotic dilutions in a liquid growth medium and inoculating them with the *Aspergillus*. Following an incubation period of 48h the growth of the fungi can be examined and the MIC observed after inspection (Reller *et al.,* 2009).

Many patients that have undergone transplants which has rendered their immune system compromised (Table 2) are at high risk of developing invasive aspergillosis. In 2016 the first phase of construction to build the new national children's hospital began on the grounds of St. James's Hospital. With this in mind, there were several aims of this study with the main motivation being to provide information on *Aspergillus* prevalence in the environment as part of an overall strategy of protecting the patients of St. James's Hospital, Dublin and providing data that is crucial to the infection control strategy. These aims were:

1. The validation of the in-house six-well screening plate to provide a rapid method of diagnosing phenotypic triazole resistance.

2. The collection of air samples in the wards and outdoors on the campus of St. James's Hospital to establish the typical concentration of *A. fumigatus* in the air during major construction works on site.

3. The collection of *A. fumigatus* from fruit and vegetable samples originating from various locations to establish the presence or absence of *A. fumigatus* on Irish food items by comparison to abroad.

4. The screening of *A. fumigatus* isolates collected from air sampling and food isolates to identify the prevalence of resistance in an Irish setting and abroad.

Chapter 2: Materials and methods

2.1 Air Sampling

As part of an ongoing environmental surveillance study in St. James's Hospital Dublin samples from the air were taken to investigate the prevalence of different *Aspergillus* species both inside and outside of the hospital. This was largely influenced by the construction of the new children's hospital on campus to ensure the levels of microbes in the air are within safe levels for patients (Rooms and wards containing HEPA filters should have <1 CFU/m^3 and rooms or wards without HEPA filters should have <5 CFU/m^3 according to the national guidelines for the prevention of nosocomial aspergillosis 2008).

Table 3. Indoor air sampling locations

Plate identifier location	**Location**	**Air sampling point**
A	Oncology, haematology and radiation oncology ward	Centre of multi bedded bay C
B	Cardiac unit	HDU bay
C	Cardiac ICU	Open plan
D	ICU	Open plan at nurse's station
E	Respiratory ward	Corridor at nurse's station
F	Haematology & Hepatology ward	Neutral pressure room 15 or 16 within the patient's room
G	Haematological ward	Alternative each sample date to capture a room in red side, blue side & blue side 2
H	Bank of Ireland	Outside the entrance

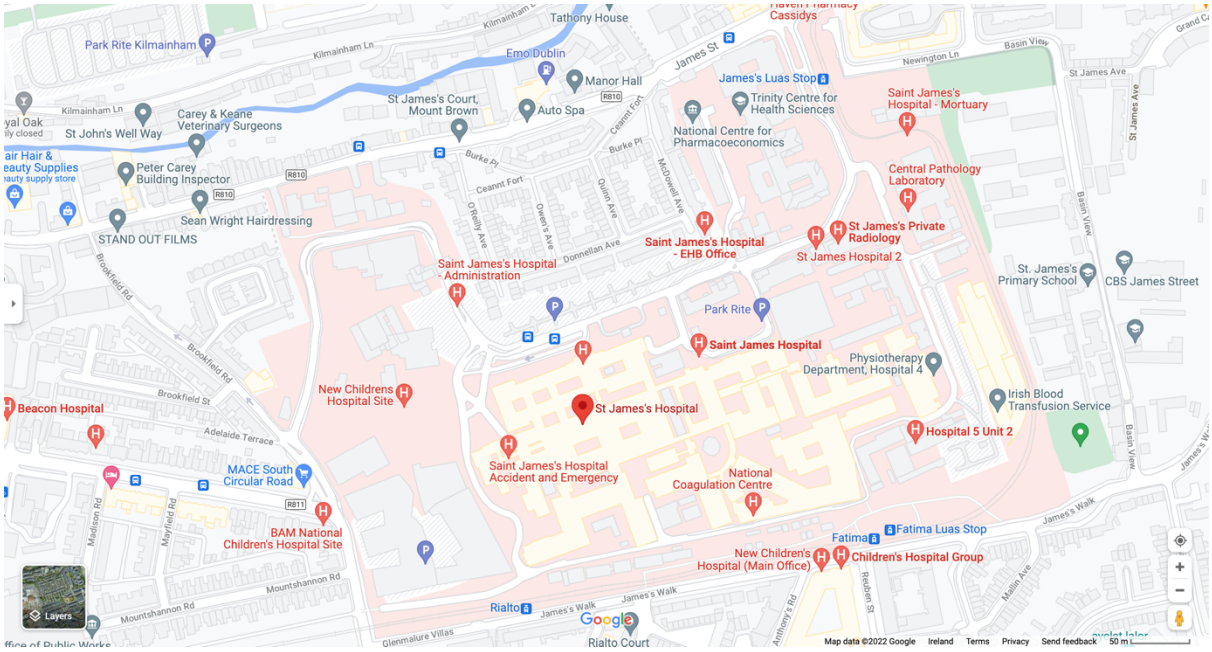


Figure 3. Google map showing the grounds of St. James's Hospital Dublin

Table 4. Outdoor air sampling locations

Plate identifier location	Location	Air sampling point
I	Out-patient department	Outside in car park
J	CEO building	Outside on ledge
K	DBW AHU	Access via H&H lift to go outside
L	Hospital 5	Outside foyer entrance
M	Central Pathology Laboratory	Outside main entrance on ledge

Sampling was undertaken in eight locations inside the hospital and five locations outside on the grounds of St. James's Hospital, Dublin. Before sampling took place, weather conditions for that day were noted using Accuweather and recorded in an Excel spreadsheet. The conditions of interest on the day of air sampling included temperature, humidity, precipitation in the last 24 hours (measured in millimetres), windspeed (kilometres/hour), wind direction, barometric pressure, and UV index. Air sampling was undertaken every two weeks on a day that there was no precipitation occurring. An SAS dual head Air Sampler was used for all sampling both inside and outside of the hospital. Before use both heads of the air sampler were autoclaved at 121°C for 20 min to ensure they were completely free from all micro-organisms. At each location a volume of $1m^3$ (1000L) of air was sampled per plate at a height of approximately 1 meter above the ground. Both heads contained a Sabouraud (SAB) agar plate supplemented with chloramphenicol which were used to measure the prevalence of *A. fumigatus* in the air by colony counts. Duplicate SAB plates were used to find the average colonies grown at a given location to give a better overall reading.

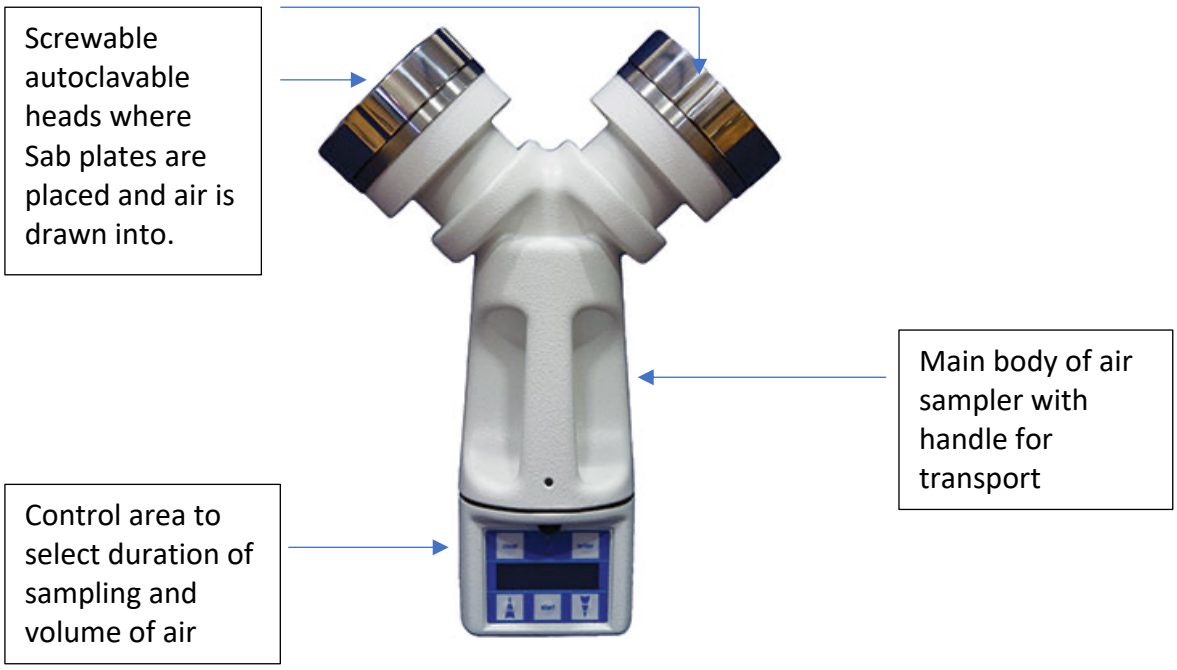


Figure 4. SAS dual head Air Sampler used to collect samples at various locations (PBI Duo SAS Super 360 Air Samplers, laftech).

In between each sampling location each head of the air sampler was thoroughly wiped down using a 70% v/v isopropyl alcohol wipe to disinfect the sampler before use at each new location (Sahiner *et al.,* 2019). Adequate time (approximately 10 minutes) was allowed at each point to ensure the alcohol had dried off before use as excess on the sampler could affect the fungal load gathered. After the required volume of air had been taken the plates were carefully removed from the air sampler by gloved hands and sealed with parafilm. The location, date and time was then recorded on the plate, and they were placed in the incubator at 37^{o}C for 24-48h. Plates were investigated at 24h and 48h and colonies of interest were sub-cultured separately and stored after 48h. All *A. fumigatus* were identified by macro and microscopic techniques using Lactophenol cotton blue.

Macroscopic identification involved note of the isolate's physical features such as shape, morphology and colour of the front and the beneath of the isolate. Microscopic identification was undertaken by gently pressing a small section of sellotape onto the colony to be identified being careful to avoid causing damage to the structural integrity of the fungal colony. A glass slide was then prepared with a droplet of lactophenol cotton blue, and the adherent side of the tape was carefully then placed on top to avoid air bubbles.

The *A. fumigatus* colonies were stored in sterile Phosphate-buffered saline (PBS) by taking a sterile moistened cotton swab and gently rolling it over the *Aspergillus* conidia and then whisking in a 1.5ml tube containing PBS. These tubes were then labelled and stored at 4^{o}C until further use, at which time they were regrown on SAB plates supplemented with chloramphenicol by incubating at 37^{o}C for 24-48h.

The collected isolates were screened for phenotypic evidence of triazole resistance by screening on the in-house 6-well plates and those that exhibited growth in any of the azole containing wells were inoculated onto VIP Plates. VIP Plates are four-well screening plates commercially available from Media Plates BV, Netherlands that are used to screen for evidence of phenotypic triazole resistance, validated by Arendrup *et al.,* 2017. These agar plates contain the triazole drug concentrations chosen for each well of the in-house 6-well

plate (Itraconazole 4mg/L, Voriconazole 2mg/L and Posaconazole 0.5mg/L). The plate layout can be seen in figure 5.

2.2 Screening for triazole resistance

All *A. fumigatus* isolates collected were screened for triazole resistance using the in-house 6-well agar screening plates. These plates contained 6-wells with five different drugs (Well 1: Itraconazole (4mg/L), well 2: Voriconazole (2mg/L), well 3: Posaconazole (0.5mg/L), well 4: Caspofungin (1mg/L), well 5: Terbinafine (1mg/L), well 6: growth control (agar only)). The plates were made in 1L batches using the ingredients in table 5.

Table 5. Table denoting the ingredients used, the supplier and reference number

Name of Chemical	**Supplier**	**Ref Number**
Itraconazole	Sigma	I6657
Voriconazole	Sigma	32483
Posaconazole	Sigma	32103
Caspofungin diacetate	Sigma	32343
Terbinafine	Sigma	T8826
Dimethyl sulfoxide	Sigma	D2650
RPMI-1640 Medium	Sigma	R7755
L-Glutamine solution	Sigma	G7513
D-(+)-Glucose	Sigma	G7021
Sodium hydroxide	Sigma	S8045
Agar	Sigma	A1296

3-(N-morpholino)propanesulfonic acid (MOPS)	Sigma	M3183
Phosphate Buffered Saline	Sigma	187672

The screening agar was made up in 1L batches using the following protocol: 35g of MOPS was dissolved in 100ml of sterile H2O and was then sterile filtered using 2nm pores. 900ml of H20 was added to a 1L glass duran with 10.4g of RPMI-1640 reagent, 18g of glucose and 14g of agar. The mixture was then autoclaved at 121°C for 15 minutes to sterilise and was then allowed to sit and cool until the temperature reached 56°C, which was then maintained by leaving the duran in a water bath set to 56°C. Once the agar had cooled to the desired temperature 0.3g of L-glutamine (500µl) and the sterile filtered MOPs was added. Sterile NaOH and pH strips were then used to pH the agar to 7.0-7.5. pH strips were utilised as agar could cause damage to the probe of a pH indicator.

The concentrations for the azole drugs were based on EUCAST breakpoints (Arendrup *et al.,* 2017) and those utilised in the commercially available and validated VIP Plates, however breakpoints for Caspofungin and Terbinafine are not readily available for *Aspergillus* and the data on this subject is minimal. To decide on a concentration of these drugs for the in-house 6-well plates, a series of experiments involving the production of dilution plates were run over the course of the project modelled on published literature (Arendrup *et al.,* 2008; Madureira *et al.,* 2007; Borman *et al.,* 2017; Effron *et al.,* 2004). Images of such plates can be referred to in section 3 figure 12.

This involved making up plates with each well containing doubling dilutions of each drug to obtain the minimum effective concentration (MEC) for Caspofungin and the minimum inhibitory concentration (MIC) for Terbinafine. Caspofungin plates were set up with the concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.016 and 8, 6, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.3 for Terbinafine with a growth control well containing agar only in each plate. The susceptible Af293 and resistant TR34 control strains were inoculated on the plates as a 0.5 McFarland along with a range of air sampling isolates for testing. The MEC of 1mg/L for Caspofungin and the MIC of 1mg/L for Terbinafine were decided upon and yielded consistent results showing either the cut off (Terbinafine) or alteration (Capofungin) of *Aspergillus* growth. For Terbinafine, susceptible strains were characterised as those that did not grow at the concentration of 1 and for Caspofungin colonies that exhibited a hard border as opposed to a "fluffy" border were exhibited as susceptible. The MEC for Caspofungin was defined as the lowest concentration causing aberrant growth.

To make the in-house 6-well plates, the entire contents of the antifungal drug powders were dissolved in DMSO in order to give the desired concentration (Itraconazole (4mg/L), Voriconazole (2mg/L), Posaconazole (0.5mg/L), Caspofungin (1mg/L), Terbinafine (1mg/L)). The reconstituted drugs were then aliquoted into separate labelled 1.5ml tubes and stored in the freezer until they were ready to be used. When making the screening plates, the drugs were added to the agar in 50ml batches. To a labelled falcon tube, 50ml of agar was added along with 50µl of Itraconazole, Voriconazole, Caspofungin, Terbinafine or 5µl of Posaconazole to give the desired concentrations. As the agar is the same colour when each drug is added particular care was given to labelling and workflow so as to avoid mixing up the drugs. The falcon was then inverted gently several times to adequately mix the drug in agar and ensure even distribution whilst avoiding air bubbles. 4mls of the drug/agar mixture was then added to each of the wells one at a time using an electronic pipette tube, taking note of which drug went into which well. Working quicky but accurately during the pouring phase was essential due to the quick solidification time of the agar. The control well contained agar only. The plates were then sealed in sample bags and stored at 4^{o}C until their time of use, with a maximum storage time of 4 weeks.

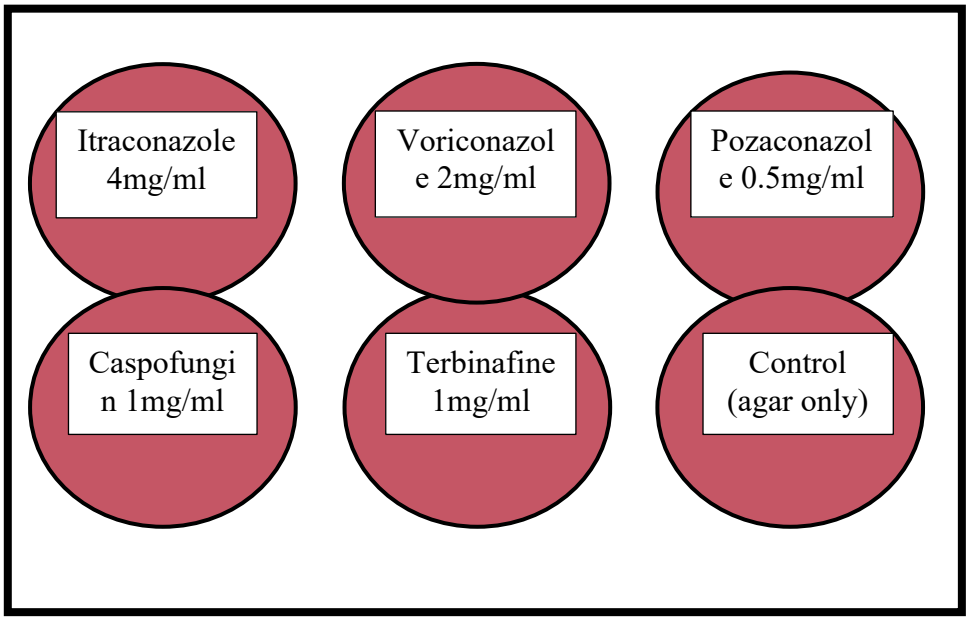


Figure 5. In-house 6-well plates displaying the layout of individual drugs in each well

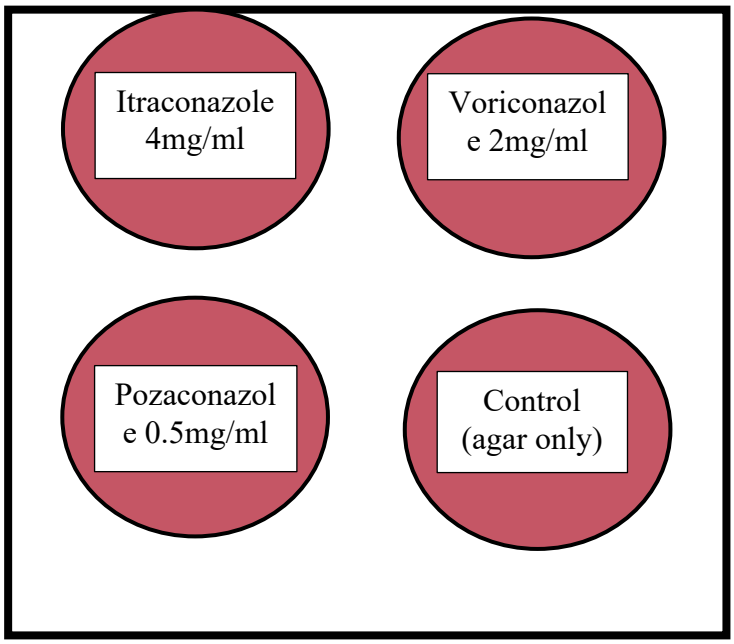


Figure 6. VIP Plates displaying the playout of individual drugs in each well

2.3 Inoculating the in-house six-well plates with *Aspergillus fumigatus*

There were three types of samples screened on the 6-well plates: environmental samples from the air both indoors and outdoors St. James's Hospital campus, fruit and vegetable samples from a range of locations globally and clinical samples from St. James's Hospital (anonymised).

After collection and storage previously described in this section, the samples were regrown by spreading the *Aspergillus*/ PBS suspension onto SAB plates supplemented with chloramphenicol and incubating for 48 hours at 37^{o}C. After the 48-hour incubation period the conidia were harvested from the SAB plate by gently rolling a sterile cotton swab moistened with sterile PBS over the sporulating colony in question and whisking in approximately 6mls of PBS. The concentration was adjusted by either adding more PBS (if concentration was too high) or more conidia (if concentration was too low) to a 0.5 McFarland using a densitometer for all samples tested. In the biological safety cabinet, a volume of 25µl of the 0.5 McFarland spore suspension was then inoculated onto each individual well of the azole agar plates. The plates were then left to sit in the cabinet in order to allow the droplet to dry and were then gently sealed using parafilm. Following this the plates were incubated at 37°C and the growth was assessed at 24 and 48 hours.

Initially several colonies of similarity were tested on the one plate. For example, 5 colonies from the same location the Respiratory Ward or five Colonies from the same vegetable sample such as a carrot were inoculated into the same well. Any colonies that were shown to grow in the drug containing wells were then isolated, sub-cultured and retested individually on their own in-house 6- well plate. If growth was observed again in any of the drug containing wells these samples were noted as "possibly resistant" and were kept for inoculation on VIP plates for comparison and further confirmation of phenotypic resistance. Isolates that only grew in the control well and exhibited no growth in the drug containing wells after 48 hours were categorised as azole susceptible.

2.4 Food sampling

As part of this study of *A. fumigatus* a variety of fruit and vegetable samples were investigated and screened for triazole resistance. The fruit and vegetables in question were chosen carefully based on several factors; whether it was a root plant or not, whether it was grown above or below soil, the country of origin, *A. fumigatus* prevalence and popularity.

Table 6. Food samples screened for *A. fumigatus* presence and triazole resistance with their country of origin.

	Food sample:	**Country of origin**
1.	Samphire	Morocco
2.	Onions	Netherlands
3.	Sweet potato	Egypt
4.	Avocado	Chile
5.	Fine beans	Kenya
6.	Potato	Ireland
7.	Parsnip	Ireland
8.	Ginger	Asia
9.	Tomato	Netherlands
10.	Pear	Netherlands
11.	Carrot	Ireland
12.	Strawberry	Ireland
13.	Potato Soil	Ireland
14.	Brown Onions	Ireland
15.	Cherry tomatoes	Ireland
16.	Beetroot	Ireland
17.	Turnip	Ireland
18.	Parsnip	Ireland
19.	Potato	Ireland

The food samples were freshly sourced from Supervalu in Maynooth, County Kildare, and the first task was to grow viable *A. fumigatus* for screening. Three methods were initially used to see which yielded the highest colony output.

Method one: Using a sterile cotton swab moistened with PBS the food sample was swabbed and then streaked onto a SAB plate supplemented with chloramphenicol, labelled and sealed with parafilm and incubated at 37^{o}C for 48 hours.

Method two: Using a sterile scalpel the food sample was cut into 2g sections under sterile conditions on the work bench. This 2g section of the food was then placed into a sterile 50ml falcon and suspended in 8ml of sterile PBS with 1% tween and vortexed thoroughly to ensure adequate mixing of the food particles in the solution. 1000µl of the food/PBS suspension was then plated onto a SAB plate supplemented with chloramphenicol using a pipette. The plate was then labelled, sealed with parafilm and placed in the incubator at 37^{o}C for 48 hours.

Method three: This involved crushing the food sample to be screened. The food sample was cut into 2g sections using a sterile scalpel under sterile conditions on the work bench. The 2g section of the food was then crushed using a sterile pestle and mortar and placed into a 50ml falcon and suspended in 8ml of sterile PBS with 1% tween. The falcon was then vortexed thoroughly to ensure adequate mixing of the food particles in the solution. 1000µl of the crushed food/PBS suspension was then plated onto a SAB plate supplemented with chloramphenicol using a pipette. Particular care was taken to ensure the correct volume was used as the food particles can become lodged in the pipette tip leading to a smaller volume being inoculated. The plate was then labelled and sealed with parafilm and placed in the incubator at 37^{o}C for 48 hours.

The third method utilising the crushed food in PBS yielded the best results and showed the highest output of *Aspergillus* colonies. After 48 hours, the colonies were inspected and identified both macro and microscopically and after confirmation of their identity up to five *A. fumigatus* colonies were selected and stored in sterile PBS for later screening. If more than five colonies of *A. fumigatus* grew on a particular plate five colonies were selected at random and stored. Macroscopic identification involved noting the isolate's physical features such as shape, morphology and colour of the front and the beneath of the isolate. Microscopic identification was undertaken by gently pressing a small section of

sellotape onto the colony to be identified being careful to avoid causing damage to the structural integrity of the fungal colony. A glass slide was then prepared with a droplet of lactophenol cotton blue, and the adherent side of the tape was carefully then placed on top to avoid air bubbles.

Chapter 3: Results

3.1 Air sampling

Over the course of this project air sampling took place from October 2019 until March 2020. This was cut short due to the disruption caused by the COVID-19 pandemic which prevented access to the hospital wards. During this phase two SAB plates supplemented with chloramphenicol were used in an SAS dual head air sampler for each location. A total of 13 locations were sampled on the grounds of St. James's Hospital Dublin (five outdoor locations and eight indoor). After incubation the average number of colony forming units (CFU) of the *A. fumigatus* isolates that grew on each of the two plates was taken and the results were recorded in an Excel spreadsheet. Over the course of the 6 months of the environmental sampling a total average of 130 *A. fumigatus* colonies from the duplicate SAB plates were gathered and screened for triazole resistance (263 *A. fumigatus* colonies collected and screened). From this sample set a total of six isolates were found to have phenotypic evidence of triazole resistance (2.28% resistant).

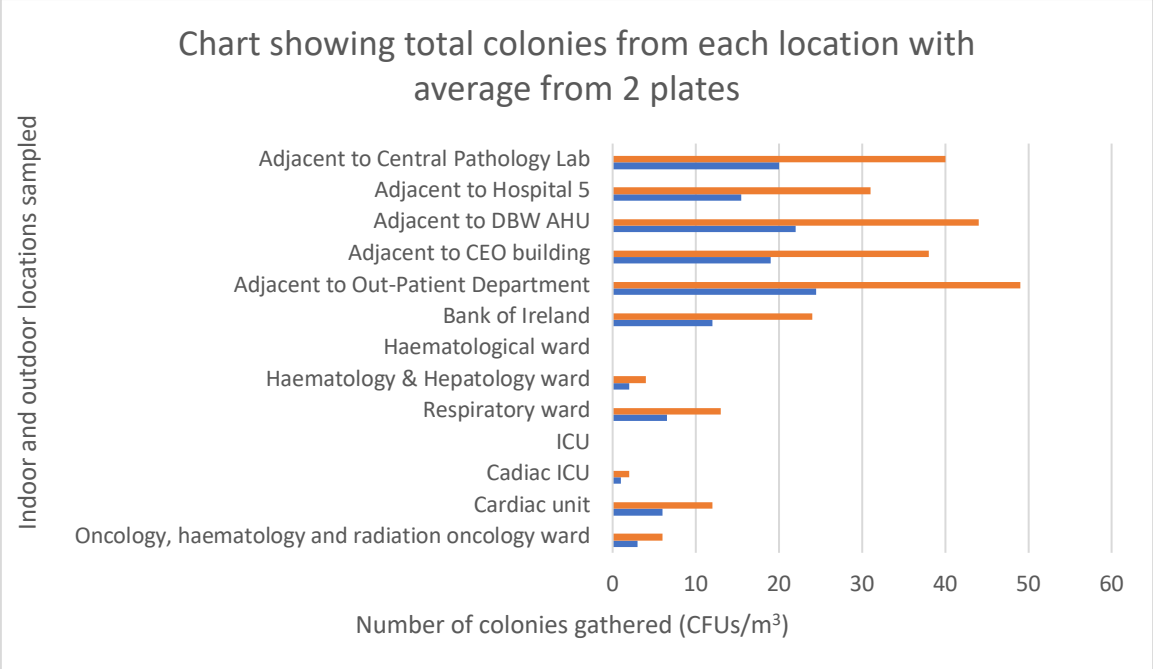


Figure 7. The total number of *A. fumigatus* colonies recovered from each location grown on Sabouraud agar incubated at 37oC for 48 hours from the beginning of February 2019 to the beginning of March 2020. Orange indicates the total number of colonies grown on SAB agar at 37°C for 48 hours from each location over the course of the air sampling. Blue indicates the average number from each sampling session from the duplicate SAB plates. Air samples were collected using an SAS Air Impact sampler. In total, 263 isolates of *A. fumigatus* were collected over the course of the air sampling. When duplicates were averaged the total was 130 isolates. Values are in CFU/m^3.

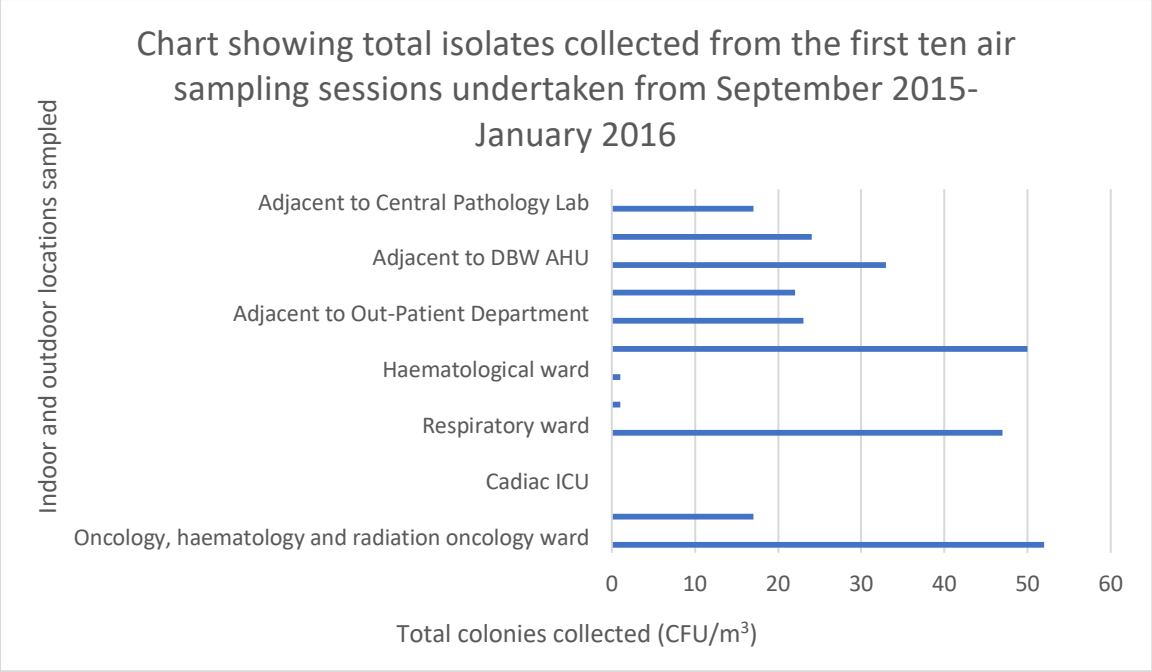
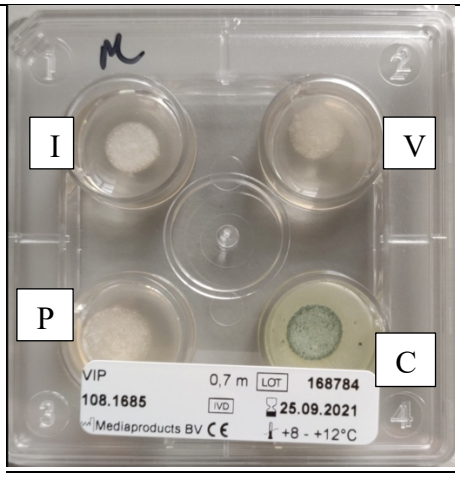
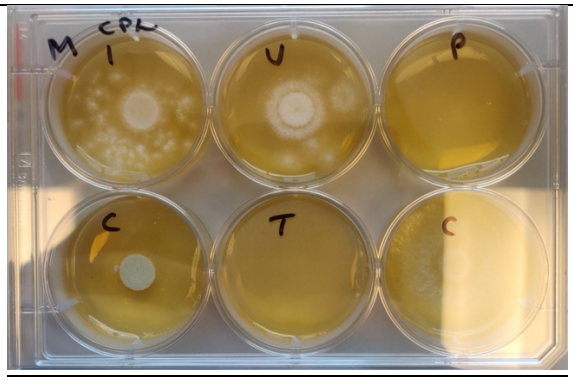
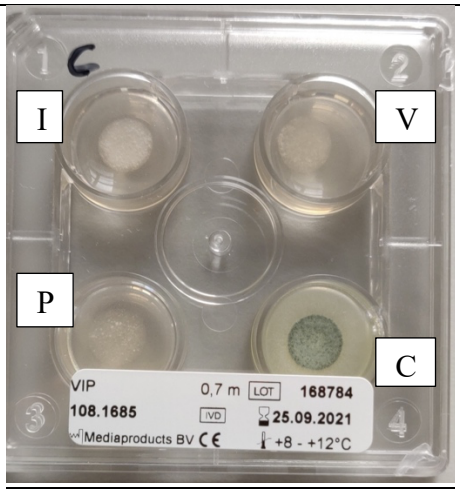
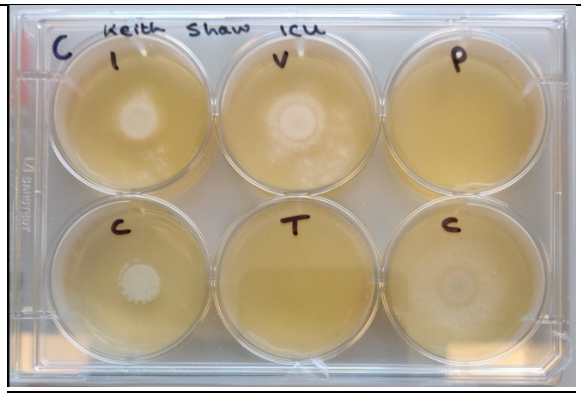
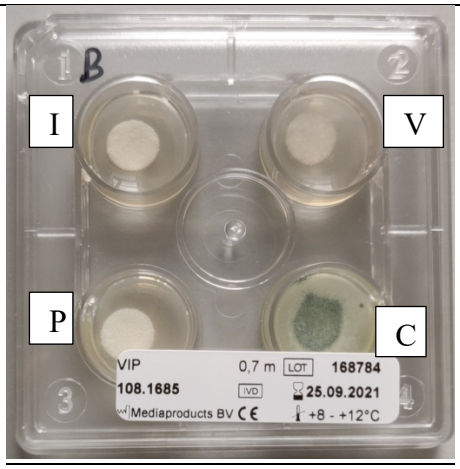
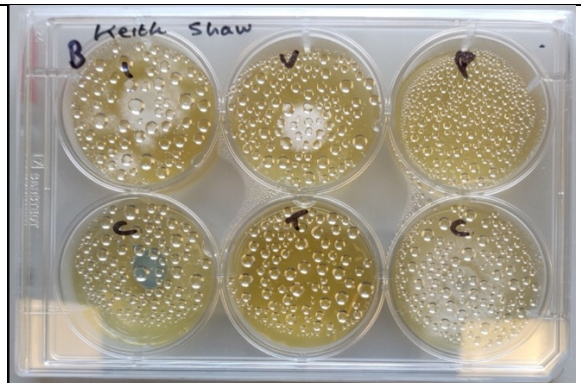


Figure 8. The total number of *A. fumigatus* colonies recovered from each location grown on Sabouraud agar incubated at 37°C for 48 hours from the beginning of September 2015 to the beginning of January 2016. Blue indicates the total number of colonies grown on SAB agar at 37°C for 48 hours from each location over the course of the 10 air sampling sessions. All samples were collected using an SAS Air Impact sampler. In total, 287 isolates of *A. fumigatus* were collected over the course of the air sampling from September 2015-January 2016. Values are in CFU/m^3.

Table 7. Tabulation of the results from the screening of the air isolates on the in-house 6-well plates and the VIP Plates for comparison and as a reference screening method. Six isolates in six different locations inside and outside of the hospital were found to have resistant phenotypes after screening on both plates, these isolates are highlighted in bold print. The remaining locations not highlighted yielded only triazole susceptible *Aspergillus* colonies. (I: Itraconazole, V: Voriconazole, P: Posaconazole, T: Terbinafine).

Location Code	**Location**	**In-house 6-well resistance**	**Drug resistance exhibited**	**VIP Plate resistance**	**Drug resistance exhibited**
A.	Oncology, haematology and radiation oncology ward.	✓	I, V	✓	I, V
B.	**Cardiac unit**	✓	**I, V**	✓	**I, V**
C.	**Cardiac unit ICU**	✓	**I, V**	✓	**I, V, P**
H.	**Bank of Ireland**	✓	**I, V, P, T**	✓	**I, V, P**
L.	**Adjacent to Hospital 5**	✓	**I, V**	✓	**I, V, P**
M.	**Adjacent to Central Pathology Laboratory**	✓	**I, V**	✓	**I, V, P**
D.	ICU	X	None	X	None
E.	Respiratory ward	X	None	X	None
F.	Haematology & Hepatology ward	X	None	X	None
G.	Haematological ward	X	None	X	None
I.	Adjacent to outpatient department	X	None	X	None
J.	Adjacent to CEO building	X	None	X	None
K.	DBW AHU	X	None	X	None

Location	VIP Plate	In-house 6-well plate
1. Outside Central Pathology Laboratory		
2. Cardiac unit ICU		
3. Cardiac Unit		

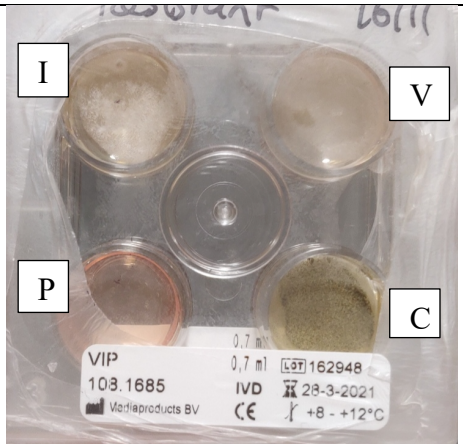
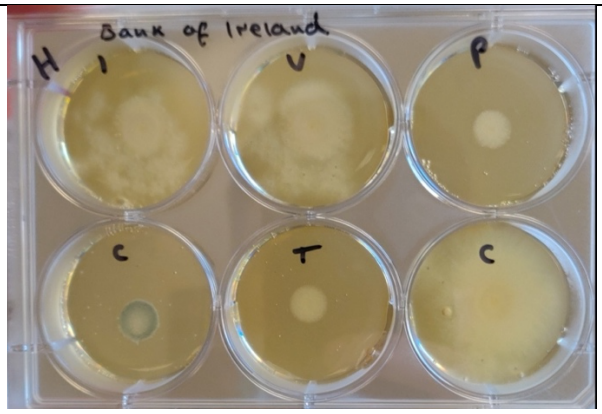
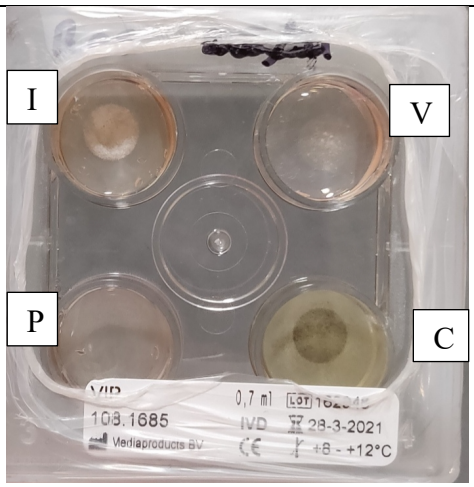
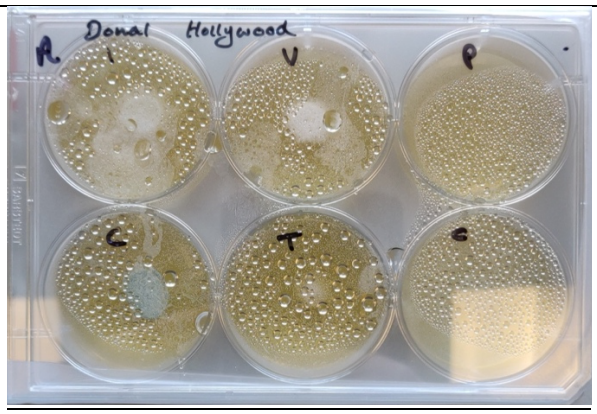
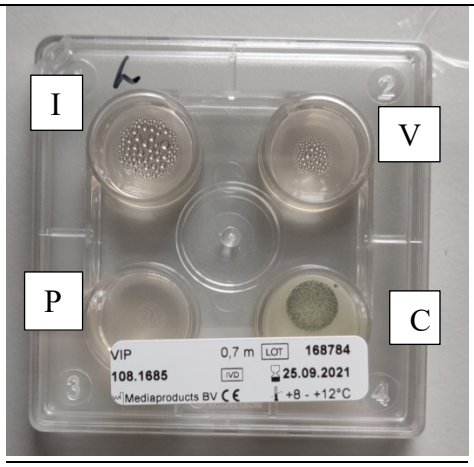
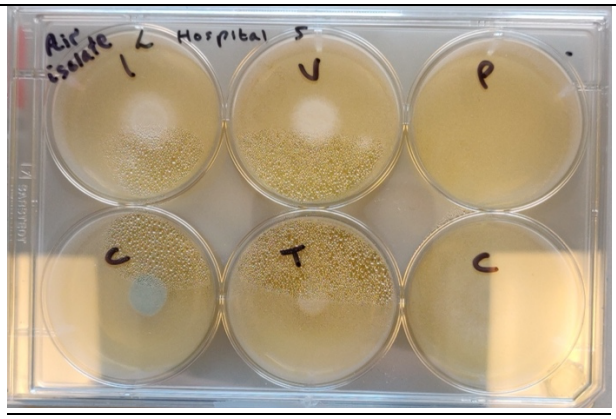
4. Outside Bank of Ireland		
5. Oncology, haematology and radiation oncology ward		
6. Adjacent to Hospital 5		

Figure 9. Images of plates inoculated with single colonies of A. fumigatus collected during air sampling showing direct comparison between the results obtained with the in-house 6-well plates and VIP Plates.

The isolates seen on the plates were originally screened using the multi colony inoculation method referenced in Materials and Methods. Having showed signs of possible resistance the individual colonies were then sub-cultured and re-inoculated (25μl of a 0.5 McFarland) as individual colonies onto these individual in-house 6-well plates and individual VIP Plates in figure 5 for confirmation and comparison. A grid for the drugs contained in each well of the in-house 6-well plates and the VIP Plates can be found in the Materials and Methods section.

All of the isolates in figure 9 are displaying phenotypic evidence of triazole resistance as indicated by the growth displayed in the azole containing wells. Isolates 1, 2, 3, 5 and 6 are showing resistance to Itraconazole and Voriconazole only. Isolate 4 is the only one of the air sampling isolates that exhibited resistance to all of the azole drugs (Itraconazole, voriconazole and Posaconazole) as well as to terbinafine. This isolate was collected from outside of the Bank of Ireland in the foyer of St. James's Hospital.

3.2 Food samples (fruit and vegetables)

Over the course of this study a total of 17 fruit and vegetable items were screened for triazole resistance. These fruit and vegetable items were selected based on a number of factors, one of particular interest was their country of origin. A proportionate amount from Ireland and across the globe were selected to investigate any potential links to triazole resistance in a particular location. The food samples were freshly bought in Supervalu in Maynooth, County Kildare.

Table 8. Tabulated results showing all fruit and vegetable samples that had *A. fumigatus* colonies grown and screened for triazole resistance with their original country of origin. The total number of food items sampled was 17 with six of these showing phenotypic triazole resistance highlighted in bold print. Two of these samples were grown in the Netherlands (Tomato and Pear) and the remaining four were grown in Ireland (Beetroot, Strawberry, Parsnip and Potato). The isolates recovered from these food samples tested positive for phenotypic triazole resistance on both the 6-well in-house plates and the VIP Plates ie. they exhibited growth in the azole containing wells.

	Food sample:	**Country of origin**	**Six-well resistance**	**VIP-Resistance**
1.	Samphire	Morocco	X	X
2.	Onions	Netherlands	X	X
3.	Sweet potato	Egypt	X	X
4.	Avocado	Chile	X	X
5.	Fine beans	Kenya	X	X
6.	Ginger	Asia	X	X
7.	**Tomato**	**Netherlands**	✓	✓
8.	**Pear**	**Netherlands**	✓	✓
9.	Carrot	Ireland	X	X
10.	Turnip	Ireland	X	X
11.	Potato Soil	Ireland	X	X
12.	Brown Onions	Ireland	X	X

13.	Cherry tomatoes	Ireland	X	X
14.	**Beetroot**	**Ireland**	✓	✓
15.	**Strawberry**	**Ireland**	✓	✓
16.	**Parsnip**	**Ireland**	✓	✓
17.	**Potato**	**Ireland**	✓	✓

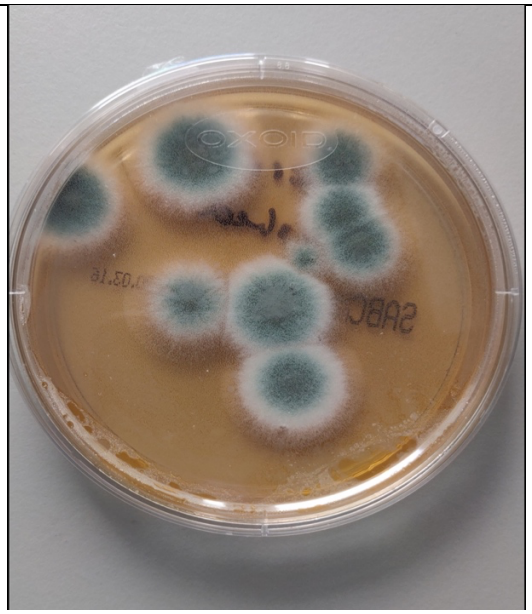
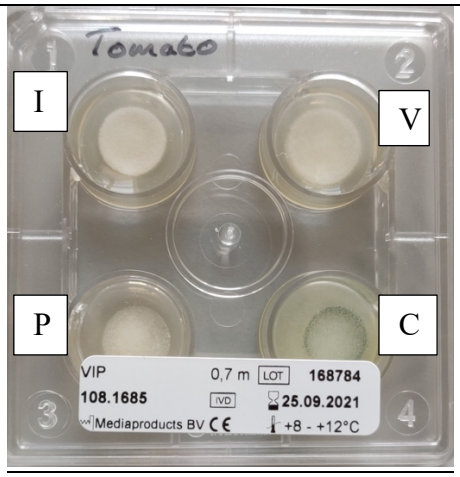
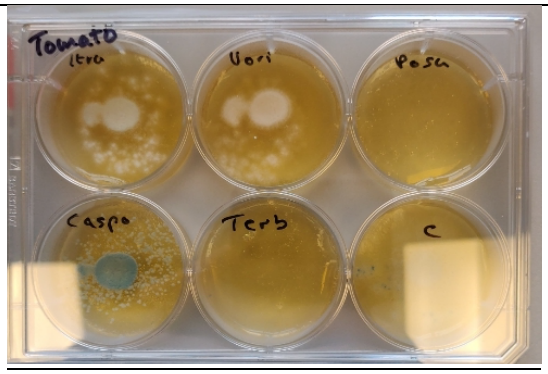
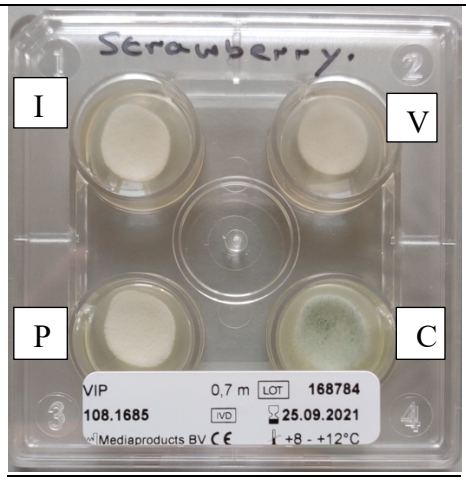
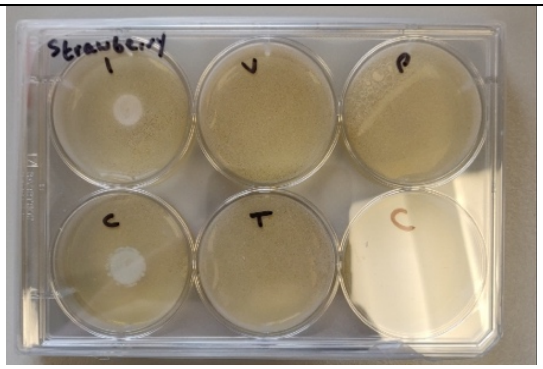
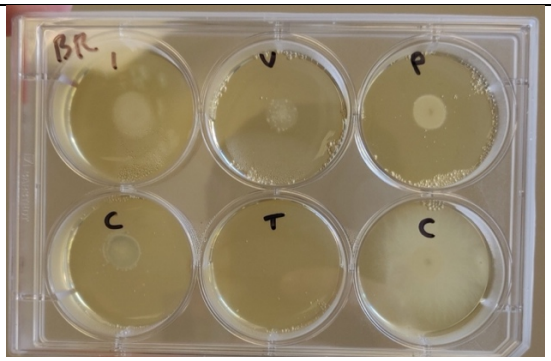
Technique used	Visual representation of growth	Results
1. Swabbing technique		Sab Plate showing no *A. fumigatus* colonies using swabbing technique referred to in Materials and Methods.
2. Crushing and inoculating technique		SAB plate showing multiple viable *A. fumigatus* colonies using crush and streak method referred to in Materials and Methods.

Figure 10. Illustration of the difference in viable colonies recovered from culture of fruit and vegetables. Plate 1 shows a SAB plate prepared using the swabbing technique on a beetroot sample referred to in the Materials and Methods section. After a 48-hour incubation no viable *A. fumigatus* colonies grew. Conversely, plate 2 shows a SAB plate prepared by utilising the crushing and inoculating technique on a beetroot sample referred to in materials and methods. After a 48-hour incubation several viable *A. fumigatus* colonies grew on plate 2 which were then sub-cultured and stored for later screening in batches for triazole resistance. This technique was used for all of the food samples.

Sample	VIP Plate	In-house 6-well plate
1. Tomato		
2. Strawberry		
3. Beetroot		

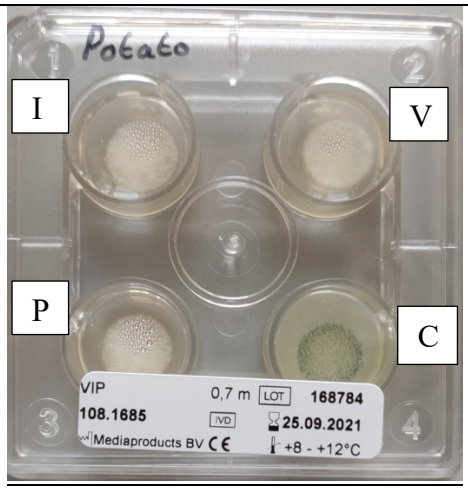
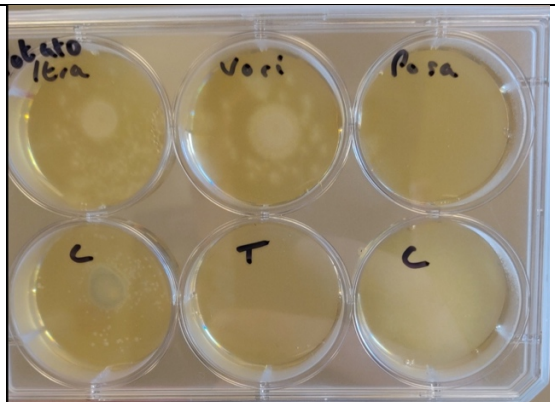
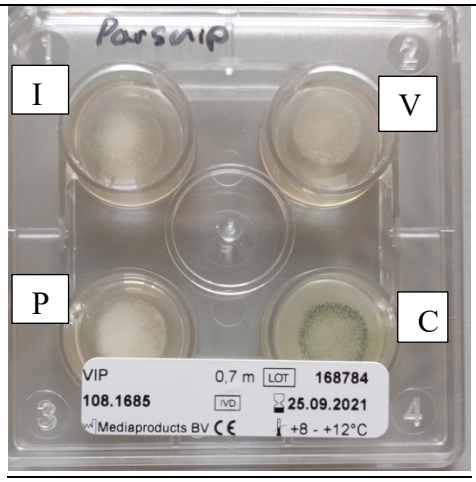
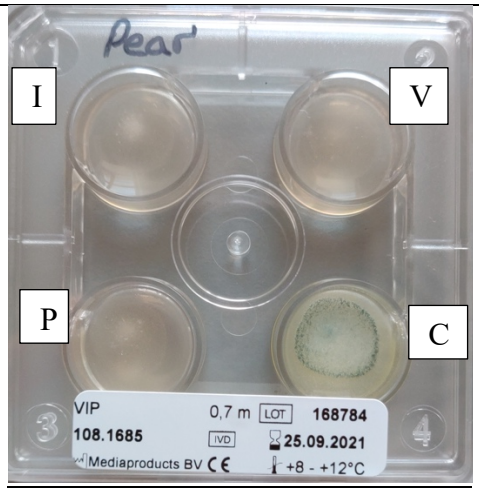
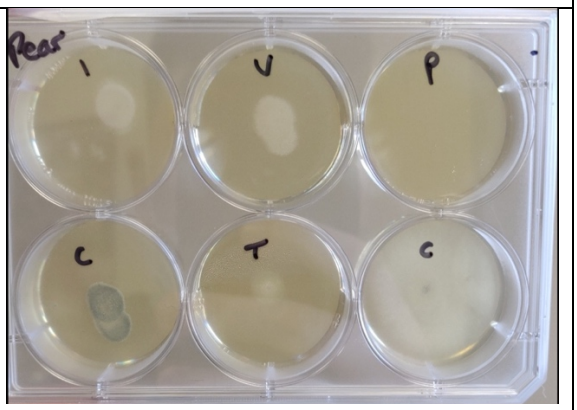
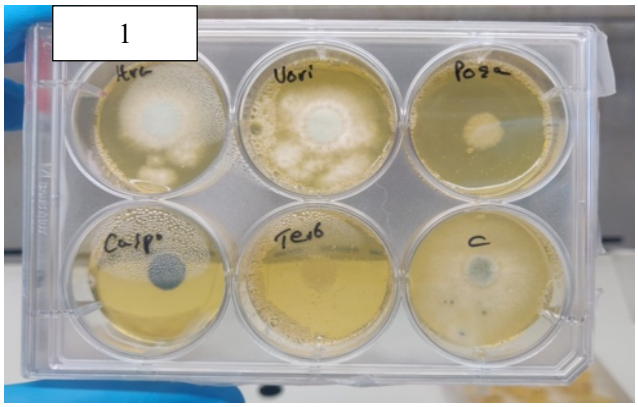
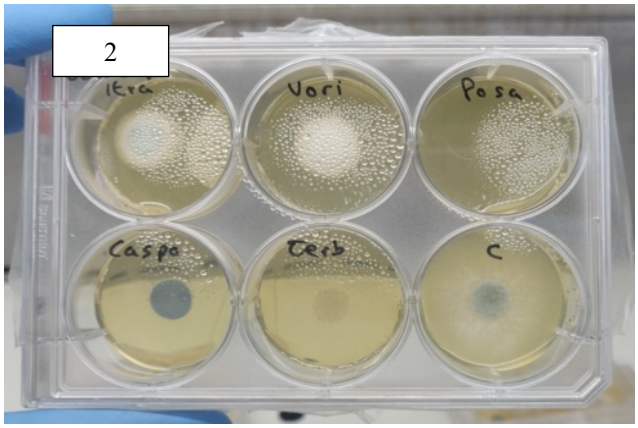
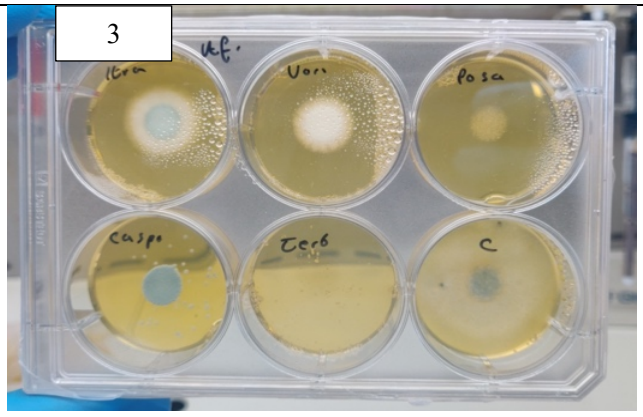
4. Potato		
5. Parsnip		
6. Pear		

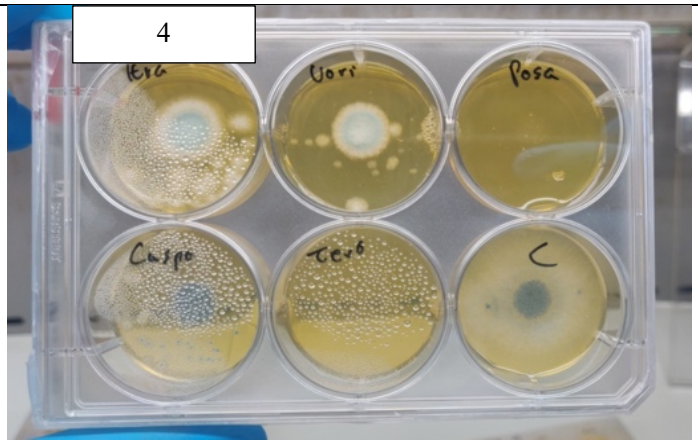
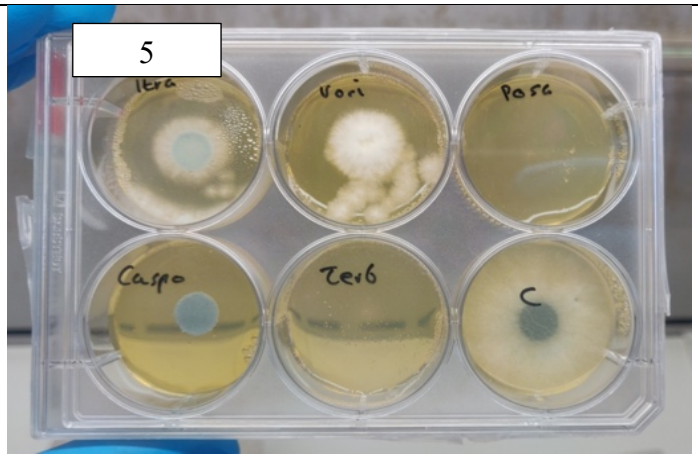
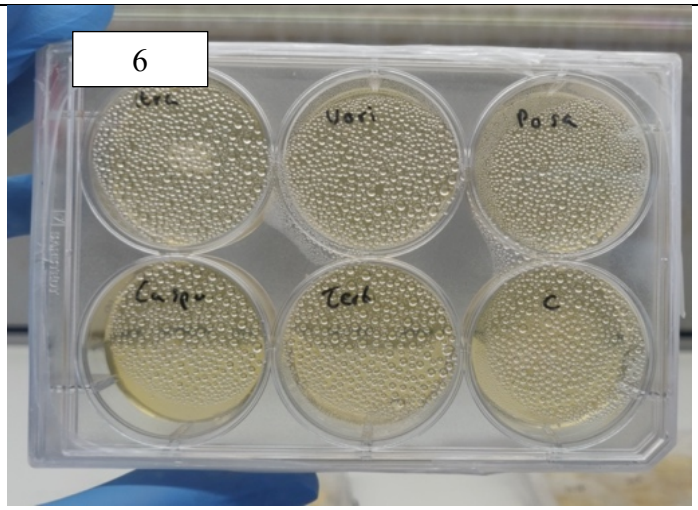
Figure 11. Direct comparison between the in-house six-well plates and VIP Plates when screening for triazole resistant *A. fumigatus* fruit and vegetable isolates. Images display isolates of *A. fumigatus* that were found to be possibly resistant during the initial screening and multi colony inoculation on the in-house six-well plates. The plates photographed show these isolates having been sub-cultured and re inoculated as individual colonies onto individual in-house 6-well plates and individual VIP Plates for confirmation and comparison. A grid for the drugs contained in each well can be found in the Materials and Methods section.

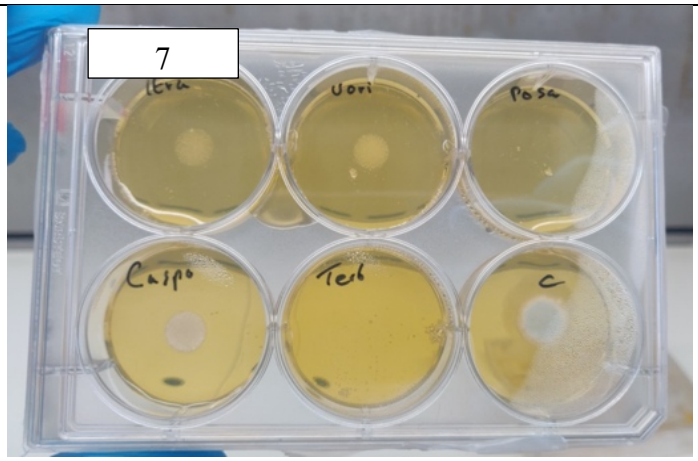
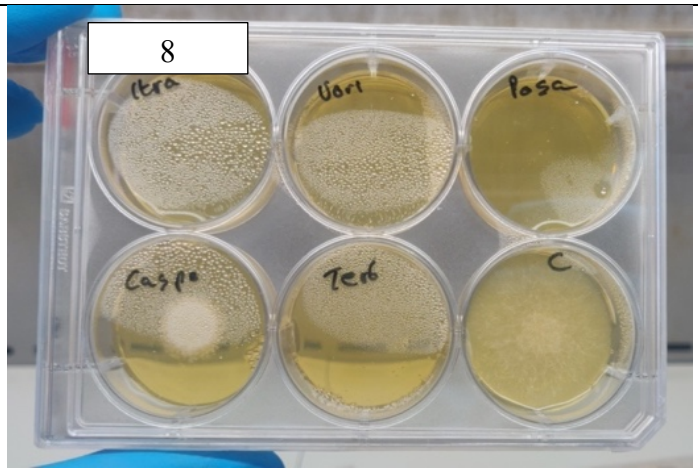
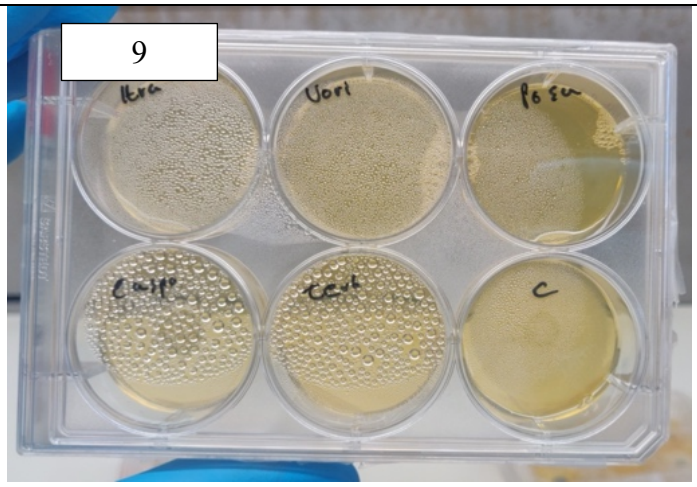
3.3 Clinical Isolates

Table 9. Tabulation of the results obtained from the screening of the clinical isolates on the 6-well in-house plates and the VIP Plates for comparison and confirmation. Isolates 8, 9 and 10 were susceptible *A. flavus* isolates, the remaining seven isolates were triazole resistant *A. fumigatus*. Af293 was included as a susceptible control and an *A. fumigatus* isolate with a known TR34 mutation was included as a resistant control. Isolates exhibiting triazole resistant phenotypes are highlighted in bold text.

Clinical isolates	**Source**	**6-well resistance**	**VIP resistance**
1	*A. fumigatus*	✓	✓
2	*A. fumigatus*	✓	✓
3	*A. fumigatus*	✓	✓
4	*A. fumigatus*	✓	✓
5	*A. fumigatus*	✓	✓
6	*A. fumigatus*	✓	✓
7	*A. fumigatus*	✓	✓
8	*A. flavus*	X	X
9	*A. flavus*	X	X
10	*A. flavus*	X	X
TR34 Control	*A. fumigatus*	✓	✓
Af293 Control	*A. fumigatus*	X	X

Sample ID	Visual representation of results on in-house 6-well plates	Sample type & results
1.		Clinical *A. fumigatus* isolate (resistant to Itraconazole, Voriconazole and Posaconazole)
2.		Clinical *A. fumigatus* isolate (resistant to Itraconazole and Posaconazole)
3.		Clinical *A. fumigatus* isolate (resistant to Itraconazole, Voriconazole and Pozaconazole)

4.		Clinical *A. fumigatus* isolate (resistant to Itraconazole and Voriconazole)
5.		Clinical *A. fumigatus* isolate (resistant to Itraconazole and Voriconazole)
6.		Clinical *A. fumigatus* isolate (resistant to Itraconazole and Voriconazole)

7.		Clinical *A. fumigatus* isolate (resistant Itraconazole and Voriconazole)
8.		Clinical *A. flavus* isolate (susceptible to all drugs)
9.		Clinical *A. flavus* isolate (susceptible to all drugs)

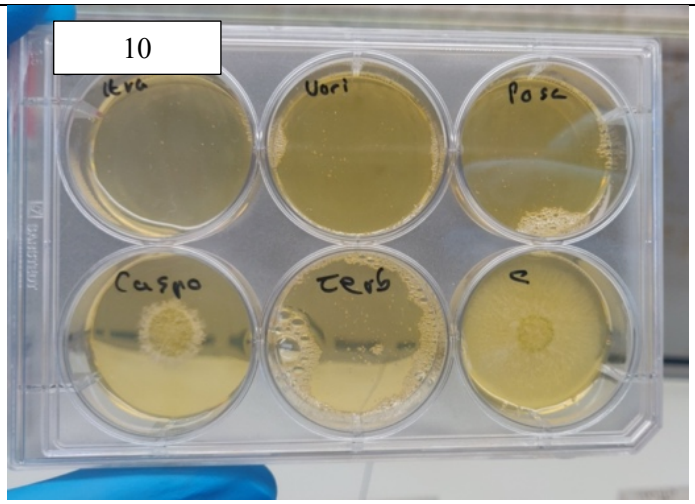
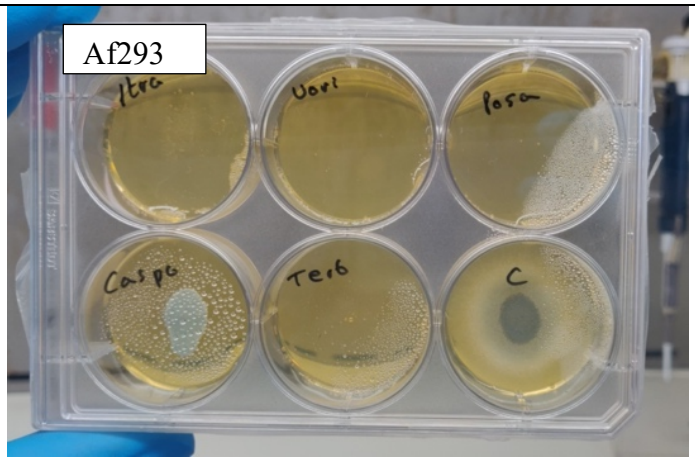
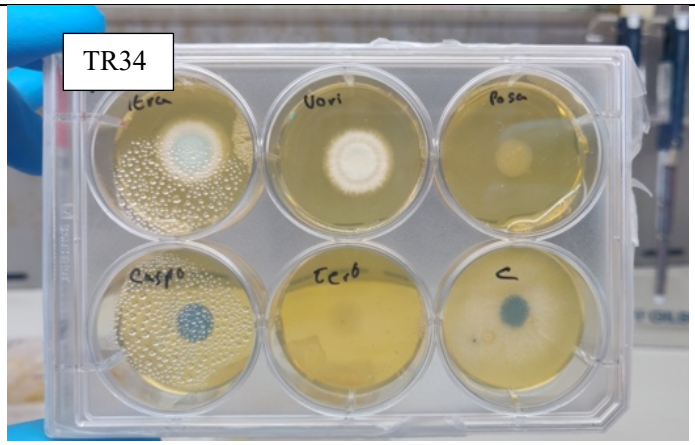
10.		Clinical *A. flavus* isolate (susceptible to all drugs)
11. Af293		Susceptible to all drugs
12. TR34		Resistant to Itraconazole, Voriconazole and Pozaconazole

Figure 12. Figure showing images of the results obtained during the screening of the clinical isolates on the in-house 6-well plates. Isolates 1-7 are clinical *A. fumigatus* isolates with known resistance as confirmed by the diagnostic laboratory in St. James's Hospital, Dublin by E- tests and molecular means. Isolates 8-10 are clinical *A. flavus* isolates exhibiting susceptibility as confirmed by the diagnostic laboratory also. Isolates 11 & 12 are susceptible and resistant controls Af293 and TR34/L98H respectively.

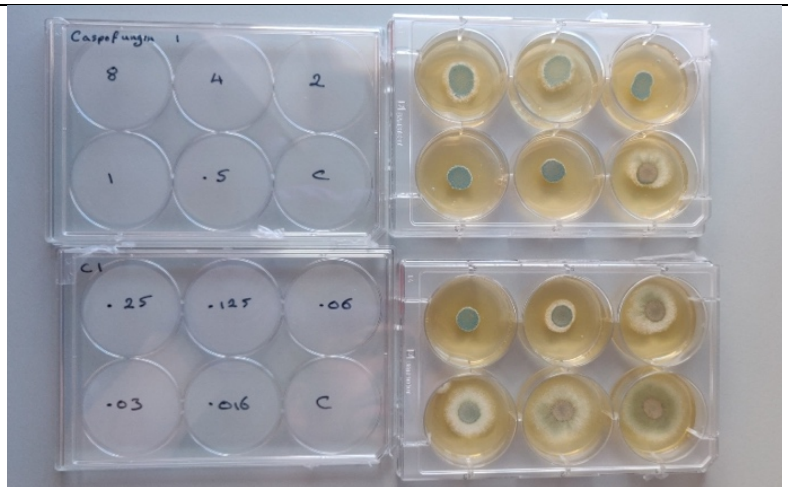
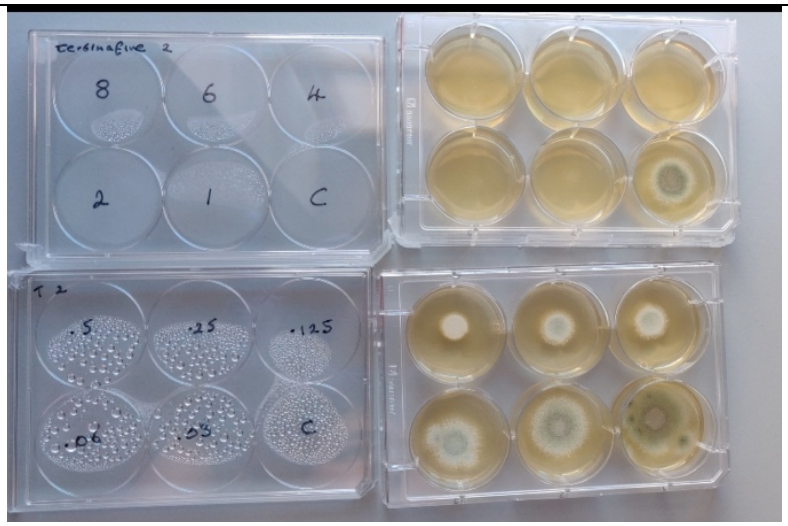
Plate ID	Visual representation of dilution plates	Key
1.		Dilution plate set up to find optimal concentration for Caspofungin.
2.		Dilution plate set up to find optimal concentration for Terbinafine.

Figure 13. Figure visualising the in-house 6-well dilution plates for Caspofungin and Terbinafine. Plates were set up in duplicate to find the MEC for Caspofungin and the MIC for Terbinafine. The dilution plates allowed for a visual representation of the effects of each drug on *A. fumigatus* over a range of concentrations. From these dilution plates and from the published literature (Arendrup *et al*., 2008; Madureira *et al*., 2007; Borman *et al*., 2017; Effron *et al*., 2004) an MEC of 1mg/L for Caspofungin and an MIC of 1mg/L for Terbinafine were decided upon.

For the Caspofungin dilution plate, growth can be seen in all wells of varying concentrations (from 8mg/L-0.016mg/L). As discussed in chapter 2, the MEC for Caspofungin was described as the concentration that elicited aberrant growth of the fungus. Originally this was thought to be at around a concentration of .25mg/L due to the change in colony morphology that can be seen between colonies in the .125mg/L well and the .25mg/L well. This halo growth that ceases at .25mg/L was initially the MEC of choice. Following a series of confirmation experiments where 30 air isolates of *A. fumigatus* were screened on the dilution plates and a review of the original dilution plates it was confirmed that the true MEC for Caspofungin was 1mg/L. At concentrations greater than 1mg/L more fuzzy and undefined borders are evident which is a better indicator of resistance, as can be seen in wells containing a concentration of 8mg/L, 4mg/L and 2mg/L. The defined border observed around the colony in the well of a concentration of 1mg/L is an indicator of *A. fumigatus* Caspofungin susceptibility.

For Terbinafine, the MIC was clearer to visualise as it is represented by the complete suppression of growth by comparison to the Caspofungin MEC categorised by aberrant growth. At a concentration of 1mg/L complete suppression of growth can be seen with gradual change in colony morphology in wells containing .125mg/L, .25mg/L and .5mg/L respectively, leading up to this.

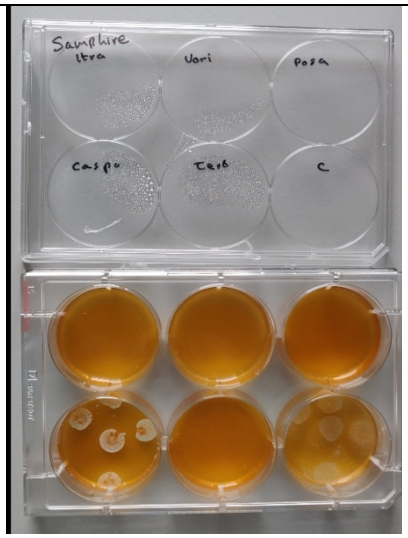
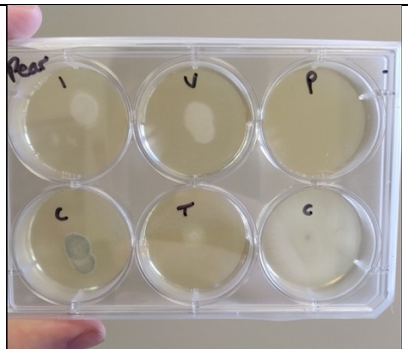
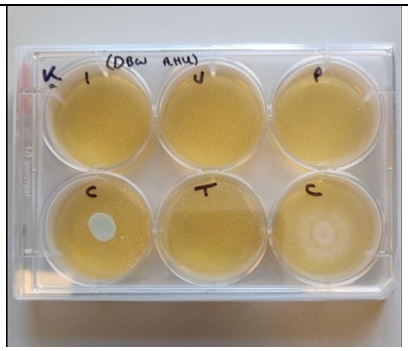
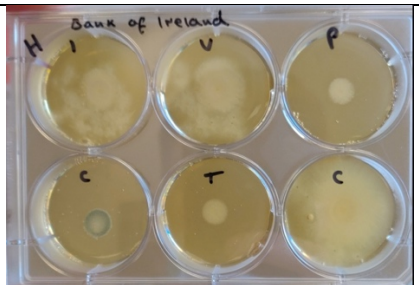
Plate ID	Visual representation of results	Sample type
1.		Food isolate from samphire screened using multi colony method showing negative results for resistance (susceptible).
2.		*Aspergillus fumigatus* isolate from a pear sample screened individually having previously showed resistance during multi colony screen (resistant).
3.		Air isolate obtained from an outdoor location screened individually having previously showed potential for resistance during multi colony screen showing to be negative after single colony inoculation (susceptible).
4.		Air isolate from outside the Bank of Ireland screened individually having previously showed resistance during multi colony screening showing to be positive for triazole resistance (resistant).

Figure 14. Figure showing different phenotypic representations of plate results. Example 1 illustrated a visual representation of results expected having inoculated the in-house 6-well plate with multiple colonies. No growth can be seen in the three azole containing wells or the Terbinafine well, but 5 individual colonies can be seen in the Caspofungin and Control well categorising this isolate as susceptible to all drugs. Example 2 illustrates a representation of results after a single resistant colony is inoculated onto the well, in this case it was an *A. fumigatus* colony isolated from a pear showing resistance to Itraconazole and Voriconazole. Example 3 illustrates expected results from a susceptible isolate after inoculating the plate with a single colony, in this case a susceptible air sample. Lastly example 4 shows a resistant phenotype against Itraconazole, Voriconazole, Posaconazole and Terbinafine from an air sampling isolate and is indicative of results expected after inoculating an in-house 6-well plate with a single resistant colony.

Chapter 4 Discussion

4.1 Air sampling

The environmental *Aspergillus* monitoring on the campus of St. James's Hospital, Dublin was initiated in 2015 in response to the ongoing major building work on-site. During this time there has been a large data set collected in relation to the colony counts of different *Aspergillus* species along with some other commonly found microbes in the air. Over the course of this long-term environmental surveillance project several circumstances have varied affecting the results such as the construction of the new national children's hospital on the campus and COVID-19 interrupting sampling sessions for a number of months leaving a period between March 2019 to August 2020 where no data was recorded for the first time since the study began back in 2015.

In previous years, work in this area had been undertaken by other researchers in the department (Dr. Katie Dunne & Dr. Patrick McCluskey). This current project aimed to further investigate and build upon their respective findings by continuing the environmental sampling at St. James's Hospital to further research the isolates in the area and screen for triazole resistance in *A. fumigatus*. Following on from earlier research, colonies from a range of different sources (environmental isolates, clinical isolates and fruit & vegetables) were also screened for triazole resistance as part of the continued validation of the in-house 6-well screening plate. A large proportion of the time over the course of the project involved gathering the isolates for screening which provided challenges in itself which will be discussed in this section along with the main findings of this project which will be broken down, analysed and compared with data previously gathered by researchers involved in this study.

The main isolate of interest during this project was *A. fumigatus*, and this was the only fungus stored if viable colonies were grown from the SAB plates, consistent with other studies assessing environmental *Aspergillus* levels (Mahieu *et al.,* 2000 & Guinea *et al.,* 2006). For the purpose of interest and surveillance, although other *Aspergillus* species were not stored, they were documented in an Excel spreadsheet along with other microbes

that were commonly observed during the sampling process. These included *Penicillium* spp, environmental "plate lifters" and a variety of other environmental moulds.

In comparison to previous air sampling results from the study undertaken by Dr. McCluskey in 2018, CFU/m^3 counts taken during this study were similar (263 over 10 sampling sessions in this study versus 511 over 19 sampling sessions in Dr. McClukey's study). Construction varied on the grounds of St. James's Hospital during this time with one limitation being that it was not known by researchers on which days excavation took place as this could have a significant impact on outdoor CFU counts. Interestingly, comparing the results from the first 10 air sampling sessions that took place from September 2015 to January 2016, colony counts were slightly higher during this phase as can be seen from figure 7 and figure 8 with a total of 287 colonies collected over 10 sessions between September 2015 to January 2016 and 263 collected between March 2019 and August 2020. With the construction project getting underway in 2015 and this section of the environmental sampling commencing in 2019, it was expected that CFU counts would be lower on average during this project as sediment displacement and aerosols would be at their highest levels in the earlier stages of construction leading to higher levels of spores in the environment, and this slight decrease was seen during this project.

Other factors that may play a significant role in CFU counts are the weather conditions (Panackal *et al.,* 2011). The main conditions of interest in this study were the temperature, humidity, precipitation in the last 24 hours (measured in millimetres), windspeed (kilometres/hour), wind direction, barometric pressure, and UV index. These conditions can have an effect on the CFU counts for several reasons; It was noticed over the course of the study that on sunnier days CFU counts were slightly higher; this could be due to the higher temperatures. This was also seen by Coleman *et al.,* 2019 when a study was undertaken to investigate the effects of climate change on *Aspergillus* load in the environment. It was concluded during this study that the amount of *Aspergillus* spores in the air was higher when temperatures were higher and lower when wind speeds were lower. Interestingly, in one study looking at *Aspergillus* abundance in the air in three renovation areas of a neonatal intensive care unit no association between outdoor temperature, air pressure, wind speed, humidity, rainfall, patient density and variance

in *Aspergillus* colony collection was detected (Mahieu *et al.,* 2000). In juxtaposition to this however, another study undertaken by Guinea *et al.,* 2006 found that variances observed in different atmospheric variables such as temperature, humidity, wind speed and season did have an influence on *Aspergillus* abundance in the air finding and that *A. fumigatus* was the most frequently recovered isolate (47%), followed by *A. niger* (20%) and *A. flavus* (8%). These mixed results show the variability in data that can be obtained and encourages further work to be done to develop more compounding links between environmental factors and *Aspergillus* spore counts in the air.

Date of sampling	Colony counts (CFUs)	Temperature (°C)	Humidity (%)	Rainfall (mm)	Wind speed (km/h)	Wind direction (North, South, East , West)	Pressure	UV Index
02/10/19	6	10 °C	87%	0mm	26 km/h	WNW	1022	0.8
30/10/19	12	9 °C	74%	0mm	26 km/h	ESE	1008	0.9
13/11/19	2	4 °C	84%	17mm	12 km/h	SSW	997	0
27/11/19	0	7 °C	97%	0.8mm	12 km/h	NE	984	0
11/12/19	13	4 °C	83%	0mm	22 km/h	WSW	998	0
07/01/20	4	13 °C	82%	0.3mm	26 km/h	NE	1009	0
23/01/20	0	8 °C	97%	0mm	6 km/h	W	987	0
06/02/20	24	7 °C	74%	0mm	26 km/h	SSE	992	0
19/02/20	49	8 °C	90%	0mm	26 km/h	SW	1010	1
04/03/20	38	8 °C	71%	0mm	10 km/h	ENE	1005	1

Table 10. Table showing recordings of weather conditions on air sampling days for outdoor locations on the grounds of St. James's Hospital Dublin.

Air sampling was undertaken on a twice monthly basis on a day when there was no precipitation during the air sampling period. It was noticed that CFU counts were lower on days where there was no precipitation during the time of air sampling but there had been earlier on in that day. This could be due to the wet surfaces preventing the spores from being dispersed. Generally, on days where the conditions were windy, CFU counts were also higher.

Over the duration of this project, a total of 263 *A. fumigatus* isolates were collected from the different sources. 202 of these were collected from outdoor locations accounting for 77% of all the isolates gathered. The remaining 61 *A. fumigatus* isolates came from indoor locations accounting for 23%. For the outdoor locations, the most colonies were collected at location OPD, near the carpark where a total of 49 *A. fumigatus* isolates were gathered. Out of all the outdoor sampling points, this location is nearest to the construction grounds and is also the most populated which could be indicative of the effects of the construction work. There were no outdoor sampling points where no *A. fumigatus* isolates were gathered, but the location yielding the fewest colonies was outside Hospital 5 with a total of 31 isolates.

For indoor locations, the sampling point where the most *A. fumigatus* isolates were collected was outside the Bank of Ireland in the hospital foyer (24 total). This result is unsurprising as it is located near the hospital entrance and the foyer, which does not have the same measures in place to reduce the number of *Aspergillus* spores in the air by comparison to the wards and ICUs. This area is also located near food halls that are usually densely populated by visitors, which could be another reason for the higher counts in this area. There were only two locations where no viable *A. fumigatus* isolates were gathered; these were the haemato-oncology ward Rm 2 (room vacant) and the ICU at the nurse's station. These clinical areas have HEPA filtration facilities.

This section of air sampling data began in October of 2019 and concluded in March of 2020 where COVID-19 interrupted sampling. One reason why counts obtained during this section of the study could be similar to previous data obtained in 2018 is due to the

sampling commencing in the autumn and being forced to stop in the winter. The windy and harsh conditions of these months is highly conducive with *Aspergillus* displacement and dispersal from the soil, trees, areas of construction and other sources. Despite the conditions, outdoor data was still in line with the hospital guidelines for CFU counts in the air according to the 2018 National Guidelines. In terms of indoor colony counts these were also in the normal range. A much higher CFU average was obtained in outdoor locations as expected. Overall, the results support the view that the protective infrastructure in place (HEPA filtration systems, antifungal paint, antimicrobial cleaning procedures) are working as expected.

Another factor which could have had an effect on CFU counts is the switch from using one SAB plate supplemented with chloramphenicol and one RPMI plate supplemented with Voriconazole to using two SAB plates with chloramphenicol. This switch was made towards the end of the previous block of air sampling which concluded in 2018. The change in methodology was made because in one instance it was seen that an isolate of *A. fumigatus* gathered during an air sampling run exhibited resistant phenotypes when investigated. This isolate grew on the SAB CHL plate after incubation at 37^{o}C for 48 hours but showed no growth on the RPMI plate supplemented with Voriconazole. However, when challenged with Itraconazole and Voriconazole the isolate exhibited growth indicating phenotypic resistance. This finding prompted a review of the data that had been collected during periods where this methodology had been utilised and it was concluded that it was likely that in the case of air sampling using a plate supplemented with Voriconazole for a single *A. fumigatus* spore, even if resistant it may be too challenging for it to grow and sporulate under these conditions meaning that some triazole *A. fumigatus* isolates could be missed. It can be deduced that in general from the overall study the utilisation of the two SAB plates for sample collection gives a better indication of CFU counts in a particular area. Although in some sampling batches there is some discrepancy between duplicate plates this usually ranges from approximately 1-3 CFU's and is negligible over the course of years. The overall average of the two SAB plates gives a better overall reading of CFUs as a whole. According to the national guidelines for the prevention of nosocomial aspergillosis 2008, rooms and wards containing HEPA filters should have <1 CFU/m^3 and rooms or wards without HEPA filters should have <5 CFU/m^3 and the results from this project were within these limits.

At some periods during the research undertaken prior to this project the incubation temperature was tried and tested at higher temperatures such as 40^{o}C which showed little to no difference in colony growth. *Aspergillus* is a fungus capable of growth at temperatures up to 70^{o}C (Kwon-Chung & Sugui 2013) and the temperature of 37^{o}C along with the incubation time of 48 hours gives a good indication of fungal growth and colonisation in the human body, coinciding with internal body temperature.

As mentioned, the environmental sampling on the grounds of St. James's began in 2015 and continues until present. It is a desirable process in the hospital when construction is not underway and essential when it is. The data which conveys the serious risks posed to patients consolidates this point and the surveillance data gathered over the course of the study's six years has proven to be very helpful in monitoring the fungal burden within the hospital and the potential risk posed to patients.

4.2 Screening for triazole resistance

Another aim of this project was to investigate the prevalence of triazole resistant strains in the air. These strains pose significant harm to patients as often they cannot be eradicated by the administration of traditional antifungal medication, mainly the first line of treatment that is the triazoles (Itraconazole, Voriconazole and Posaconazole). The development of an in-house 6-well agar plate to screen for phenotypic resistance has the potential to provide clinical microbiology laboratories with a cost effective and rapid means of screening. The in-house 6-well plate aimed to serve as an improvement on the VIP Plate with the addition of two new wells containing Caspofungin and Terbinafine respectively.

Although these latter 2 drugs are not typically used as first-line treatment for *Aspergillus* infection, they are occasionally used in combination with either a triazole or liposomal amphotericin B for difficult cases of aspergillosis, and traditionally are used in the treatment of Candida infections (Caspofungin) and dermatophyte infections (Terbinafine). In total 4 isolates were found to show resistant phenotypes to Terbinafine. These were 3 air sampling isolates from outside of the Bank of Ireland, an oncology Ward (two indoor locations) and outside Hospital 5 (one outdoor location) and one food isolate (pear from the Netherlands) that exhibited Terbinafine resistance as seen in figures 9 and 11. There were no isolates that exhibited Caspofungin resistance based on the criteria described in methods.

For validation of the in-house 6-well plates over 287 *A. fumigatus* isolates were screened with a total of 12 isolates (6 air isolates and 6 fruit & vegetable isolates) found to exhibit phenotypic azole resistance to multiple drugs as confirmed by direct comparison with VIP Plates (figures 9 and 11). The most successful method of validating the plates is by screening as many isolates as possible and confirming the results phenotypically (VIP Plates) and by molecular means (PCR). 10 clinical isolates, 7 *A. fumigatus* and 3 susceptible *A. flavus* were also tested yielding expected results that were previously confirmed by the diagnostic laboratory in St. James's ie. detecting the resistant and susceptible isolates as can be seen in figure 12.
Apart from the quantity of isolates tested the sample type is also important ie. air isolate, clinical isolate or food isolate. This study utilises a wide range of isolates for validation (environmental, food and clinical). The results display the competency of the plates as a phenotypic screening mechanism across a variety of sample types.

The in-house 6-well plates have many advantages: They are extremely cost efficient by comparison to their VIP Plate counterparts (€10.20 for 5 plates). The in-house plates cost just less than €1 and commercial PCR test kits cost upwards of €100. Although time consuming to produce, very large batches of the plates can be made with ease, and they can be stored for up to 4 weeks at 4^{o}C in the refrigerator. The plates have also been shown to produce fast and reliable results over a range of different isolates. The preparation of the sample and plate inoculation can be undertaken quickly and easily requiring minimal

training, and results are produced after only 48 hours. Furthermore, when results are obtained, they are easy to read and analyse. Results are depicted as growth in the drug containing wells indicating a resistant isolate and a lack of growth in the drug containing wells indicating a susceptible isolate. The only exception of this is the Caspofungin well which requires a slightly more trained eye as it is colony morphology and phenotype that determines the results as mentioned previously. Wild type isolates exhibit colonies with a hard defined border whereas non-wild type isolates exhibit colonies with a fluffy border.

The main purpose of the in-house 6-well plates was to act as a means of detecting phenotypic resistance and are not 100% accurate by comparison to molecular means of detection such as PCR. As the agar infused plates are man-made they are not as reliable as other diagnostic techniques. Given the fact that the drugs in question are dispersed within the agar, homogenisation of the antifungals is paramount in providing accurate results. If the antifungal drugs are not evenly distributed throughout the agar this can affect the growth of the fungal species in a particular well. Although this scenario is unlikely if the correct procedure is practised it could happen with the potential for false positives or worse, false negatives. Furthermore, although results are easy to read even by the untrained eye, they can be subjective in some cases. Growth versus no growth and fluffy border versus hard border are definitive result indicators, but in some cases it can be difficult to assess if little growth is enough growth to characterise the isolate as resistant meaning further testing would be necessary to provide results that are beyond reasonable doubt. This is not a major drawback of the in-house 6-well plates and is rarely an issue, but it is a point that requires consideration, but the objective of these plates is to be used only for screening and if a potentially resistant isolate is found followed by an E test plus/minus PCR confirmation.

4.3 Food sampling

Out of the 19 food items tested for triazole resistance, 35% gave rise to resistant *A. fumigatus* colonies. This number was surprising as it equals the number of resistant colonies but not the proportion obtained over the course of the air sampling, which took place over a 5-month period with 2 sampling sessions a month and yielded 263 isolates.

The fruit and vegetables were chosen based on a number of factors: Whether they were a root crop or not, if they were grown above or below soil and perhaps the most important was their country of origin. A collection of 9 fruit and vegetable samples (carrot, turnip, potato, potato soil, brown onions, cherry tomatoes, beetroot, strawberry and parsnip) were selected from Ireland and the remaining 8 from other locations across the world (samphire, onions, sweet potato, avocado, fine beans, ginger, tomato and pear).

The ratio between food items from Ireland and elsewhere across the globe is almost 50:50 with the majority of the resistant isolates coming from Ireland accounting for 4 out of the 6 total (66%). Interestingly, out of the remaining countries (Morocco, Egypt, Chile, Kenya Asia, Netherlands) the only other country that gave rise to resistant isolates was the Netherlands. This is interesting because of the literature describing a high incidence of azole resistant *A. fumigatus* isolates found in the Netherlands making it an environmental hotspot for azole resistant *Aspergillus* species. This point is well documented in the literature with Verweij *et al.,* 2012 noting that the prevalence of azole resistant *A. fumigatus* strains in Dutch hospitals was between 0.8% and 9.4%. Pieter *et al.,* 2020 also undertook a 6-year study to better understand the prevalence of *A. fumigatus* resistance in the Netherlands. Over the course of the 6 years, clinical *A. fumigatus* isolates were screened for the presence of *Cyp*51A mutations giving rise to clinical azole resistant isolates. Eleven percent out of the 4496 patients that were culture positive had an azole resistant isolate. The frequency of resistance also jumped from 7.6% in 2013 to 14.7% in 2018; a doubling in cases in just 5 years. Out of all of the *Cyp51A* gene mutations detected in these isolates, TR34/L98H accounted for 69% of these. The literature also suggests that a this could be due to the heavy use of industrial azoles for the protection of crops and in

the preservation in the Netherlands, in turn causing a higher incidence of triazole resistant *A. fumigatus* isolates. (Verweij *et al*., 2012).

Although only a relatively small number of food isolates from varied locations were sampled in this study it is difficult to conclude that this is in fact due to environmental factors in particular geographical areas. However, it is still noteworthy that the results obtained agree with other findings in the literature based on the geographical distribution of triazole resistant *A. fumigatus* isolates and the high incidence in the Netherlands (Verweij *et al*., 2012). A maximum of 3 individual food items for each food sample was tested, for example, 3 individual pieces from 3 different tomatoes from the same packaging. Furthermore, although 17 total food samples from a total of 7 different countries is not a very large sample size with a high degree of diversity, it is large for this study.

It is noteworthy that the two countries that delivered food samples exhibiting triazole resistant *A. fumigatus* strains were Ireland and the Netherlands. This is particularly concerning for the Netherlands, as samples from this location accounts for only about 18% of the total samples tested whereas Ireland accounts for over 50%. It is difficult to make a link and more work with a greater number of samples from a greater diversity of locations needs to be screened to compile a larger data set. Although food samples from the Netherlands accounted for just 18% of the total sample locations it was still the location with the second most samples tested, 3 in total, but out of these 3, two thirds tested positive for phenotypic triazole resistance. All other countries except for Ireland had only one sample per location (Morocco, Egypt, Chile, Kenya, Asia).

Prior to testing it was hypothesized that the Netherlands would contain a high number of resistant isolates and the fact that this was also shown by testing only a small number of samples shows the significance of this prevalence of triazole resistant *A. fumigatus* species. Ireland and the Netherlands have a multitude of similarities which could encourage *Aspergillus* growth and cause triazole resistant strains to thrive. The main similarity is the climate. Both Ireland and the Netherlands are relatively small countries with a marine west coast climate and are near or in the cool temperate moist forest biome. This moist environment is the perfect setting for *A. fumigatus* growth and colonisation. The mean

annual temperature is just 0.1^{o} cooler in Dublin and average temperatures over a month vary by approximately 5-6% with similar levels of humidity in both locations with a variation of around 5%. These similarities between the climates of Ireland and the Netherlands are a possible reason for the collection of resistant isolates. Another reason for this could be due to the heavy use of industrial azoles for the protection of crops and in the preservation of material in both countries. (Verweij *et al*., 2012). Out of all the countries that had food samples screened, Ireland and the Netherlands were the most similar. Morocco, Egypt, Chile, Kenya and Asia differ greatly in terms of the environmental conditions and geographical location by comparison to Ireland and the Netherlands. Interestingly during research undertaken on the air sampling in St. James's Hospital prior to this study (unpublished), it was found that *Aspergillus* counts were on average higher on sunnier days when the UV index were higher. By comparison to the Netherlands, Dublin gets approximately 65 fewer hours of sunlight also and evidence has been found to link higher *Aspergillus* spore counts in the air to higher temperatures and UV indexes by (Guinea *et al.,* 2006).

4.4 Clinical isolates

During the project a number of anonymised clinical *Aspergillus* isolates sourced from the diagnostic laboratory in St. James's Hospital, Dublin were screened on the in-house 6-well plates for validation purposes. Seven of these were *A. fumigatus* isolates exhibiting resistance as confirmed by the diagnostic laboratory, and three of these were *A. flavus* isolates exhibiting triazole susceptibility as confirmed by the diagnostic laboratory.

The results obtained from the screening were as expected as can be seen in table 9 and figure 12. The seven *A. fumigatus* isolates showed evidence of phenotypic triazole resistance and the three *A. flavus* isolates showed evidence of susceptibility to all the antifungal drugs on the screening plate. The findings of the diagnostic laboratory aligned with the results of the six well plates which again showed positive results and grounds to build on in the future.

4.5 Future work

As with most validation processes the more samples screened/tested on the product/process the better. Over the course of this project a large number of *A. fumigatus* isolates have been tested coming from a diverse group of locations and sources. This diversity of the sample group is important but can always be improved upon. The next step for further development of these plates would be to:

1. Test more sample groups of *A. fumigatus* (Screen a large number of isolates from different countries with known high and low incidences of triazole resistance in a clinical setting).
2. Test other fungal species (Dermatophytes)

It would be interesting to screen a larger batch of clinical isolates on the plates as patients are the main focus group for their development and tailoring. Out of all the isolates tested during this study the fewest number was from clinical isolates. Another point of note is that many of the environmental isolates from the food samples and air samples looked similar phenotypically in terms of colony morphology and sporulation. With the clinical isolates however, when sub-cultured onto SAB plates and the screening plates these colonies varied heavily in terms of appearance. Another point of note is that out of the small sample size of resistant *Aspergillus* isolates acquired from the diagnostic laboratory in St. James's Hospital, only 7 of these were *A. fumigatus.* It was noticeable that some of these isolates differed in terms of morphology. Some sporulated much less than others as can be seen in the difference between sample 1 and sample 7 meaning a slightly longer McFarland preparation as the fewer amount of spores being produced means they are less dense in PBS. But if this diversity can be seen among such a small group of *Aspergillus* samples it poses the question of how some strains that are very poor sporulators will grow on the plate, despite showing no issue with the clinical batch tested in this study, it is vital to confirm this for validation. This is an avenue that will be further explored in later research at the hospital when a larger batch of potentially resistant *A. fumigatus* can be screened.

Another area of exploration for the plates would be the screening of other fungal species, such as the dermatophyte species such as *T. rubrum* and *T. interdigitale*. Dermatophytic infections occur on the skin, hair and nails and are responsible for a large proportion of less serious fungal infections in the general population by direct contact from other individuals, animals and soil (Hainer 2003). Building on the earlier point of different fungi exhibiting different levels of sporulation, these dermatophytes (mainly *T. rubrum*) would be an excellent testing organism. They are very slow growing on agar and exhibit little sporulation which is why they are notorious for being difficult to grow. This could be challenging on drug infused agar plates, but it is definitely an interesting avenue of exploration. Unfortunately, due to the disruption caused by COVID-19, time restraints did not allow this project to investigate the dermatophytes despite hopes to do so but will be included in future research projects. The main motivation for exploration of the dermatophytes on the in-house 6-well plates would be to validate the Terbinafine well. Furthermore, Terbinafine is very occasionally used in combination therapy with the azoles for the treatment of aspergillosis. In research undertaken by Schiraldi *et al.,* 1996 it was found that terbinafine seemed to be extremely effective in the treatment of bronchopulmonary aspergillosis in immunocompetent patients, showing results to be as successful in treatment as Amphotericin B and Itraconazole. The six well plate containing the triazoles, an echinocandin and an allylamine which all show evidence of having antifungal effects on *A. fumigatus* isolates would be an excellent diagnostic tool for any mycology laboratory and show promise for the future.

Bibliography

1. Arendrup MC, Perkhofer S, Howard SJ, Garcia-Effron G, Vishukumar A, Perlin D, and Lass-Flörl C. (2008) Establishing In Vitro-In Vivo Correlations for *Aspergillus fumigatus*: the Challenge of Azoles versus Echinocandins. Antimicrob Agents Chemother. 52(10), pp. 3504–3511.

2. Arendrup MC, Verweij PE, Mouton JW, Lagrou K, Meletiadis J. (2017) Multicentre validation of 4-well azole agar plates as a screening method for detection of clinically relevant azole-resistant *Aspergillus fumigatus Journal of Antimicrobial Chemotherapy*, 72(12), pp. 3325–3333.

3. Bhabhra, R, Askew DS, (2005) Thermotolerance and virulence of *Aspergillus fumigatus:* role of the fungal nucleolus Med Mycol. 43(1), pp. 87-93.

4. Blachowicz, A, Knox, B. P, Romsdahl, J, Palmer, J. M, Huttenlocher, A, Wang, C. C, & Venkateswaran, K. (2016) Characterization of *Aspergillus fumigatus* isolated from air and surfaces of the International Space Station. In 13th European Conference on Fungal Genetics, pp. 3-6.

5. Borman AM, Fraser M, Palmer MD, Szekely A, Houldsworth M, Patterson Z, and Johnson EM (2017) MIC Distributions and Evaluation of Fungicidal Activity for Amphotericin B, Itraconazole, Voriconazole, Posaconazole and Caspofungin and 20 Species of Pathogenic Filamentous Fungi Determined Using the CLSI Broth Microdilution Method J Fungi (Basel). 3(2) pp. 27.

6. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW, (2010) Azole antifungal resistance in *Aspergillus fumigatus:* 2008 and 2009. J Antimicrob Chemother. (65) pp. 2116–2118.

7. Buil, J. B., Hare, R. K., Zwaan, B. J., Arendrup, M. C., Melchers, W. J., & Verweij, P. E. (2019) The fading boundaries between patient and environmental routes of triazole resistance selection in *Aspergillus fumigatus*. PLoS pathogens, 15(8).

8. Camps, S. M, Rijs, A. J, Klaassen, C. H, Meis, J. F, O'Gorman, C. M, Dyer, P. S, & Verweij, P. E. (2012) Molecular epidemiology of *Aspergillus fumigatus* isolates harboring the TR34/L98H azole resistance mechanism. Journal of clinical microbiology, 50(8) pp. 2674-2680.

9. Chiller TM, Marr K, Kontoyiannis DP, (2008) The epidemiology of invasive *aspergillus* (IA) infection among organ transplant recipients in the U.S 2001–2006: Review of TRANSNET. In Program and Abstracts, 3rd Advances Against *Aspergillus*, Miami, FL, abstract number 87, pp. 179.

10. Chowdhary A, Sharma C, Meis J.F. (2017) Azole-resistant aspergillosis: epidemiology, molecular mechanisms, and treatment. *J Infect Dis.* (216), pp.436-S444.

11. Coleman J, Jones A, Collier L, Richardson MD (2019), *Bright-T RJ* M1 Do climate changes influence environmental *Aspergillus fumigatus* load at the manchester university NHS foundation trust adult cystic fibrosis centre? (74), pp 235.

12. Collins LA, Samore MH, Roberts MS, Luzzati R, Jenkins L, Lewis WD, Karcher AW (1994) Risk factors for invasive fungal infections complicating orthotopic liver transplantation. *J Infect Dis*; (170) pp. 644-652.

13. Fang W & Latgé JP (2018) Microbe Profile: "*Aspergillus fumigatus*: a saprophytic and opportunistic pathogen" *Microbiology*. 164 (8), pp. 1009–1011.

14. Fera MT, Camera EL, & Sarro AD, (2014) New triazoles and echinocandins: mode of action, *in vitro* activity and mechanisms of resistance, pp 981-998.

15. Garcia-Effron G, Lopez AG, Mellado E, Monzon A, Rodriguez-Tudela JL, Cuenca-Estrella M., (2004) *In vitro* activity of terbinafine against medically important non-dermatophyte species of filamentous fungi (2004) *Journal of Antimicrobial Chemotherapy*. 53 (6), pp 1086–1089.

16. Gerginova, M, Manasiev, J, Yemendzhiev, H, Terziyska, A, Peneva, N & Alexieva, Z (2013). Biodegradation of phenol by Antarctic strains of *Aspergillus fumigatus*. Zeitschrift für Naturforschung C, 68(9-10), pp. 384-393.

17. Guillemain R, Lavarde V, Amrain C, Chevalier P, Guinvarc'h A, Glotz D (1995) Invasive aspergillosis after transplantation. (27), pp. 1307-1309.

18. Geuinea J, Peláez T, Alcalá L, Bouza E (2006) Outdoor environmental levels of *Aspergillus* spp. conidia over a wide geographical area *Medical Mycology*. 44 (4), pp. 349 356.

19. Haiduven D (2009) Nosocomial aspergillosis and building construction. Med Mycol; 47(1), pp 10-6.

20. Hainer BL (2003) Dermatophytic infections, Am Fam Physician. 67(1), pp. 101-8.

21. Hofflin JM, Potasman I, Baldwin JC, Oyer PE, Stinson EB, Remington JS (1987) Infectious complications in heart transplant recipients receiving cyclosporin and corticosteroids. *Ann Intern Med.* (106), pp. 209-216.

22. Husain S & Camargo JF (2019) Invasive Aspergillosis in solid-organ transplant recipients: Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice Clin Transplant; 33(9), e13544.

23. Hwang D.M, Chamberlain D.W, Poutanen S.M, E Low D, Asa S.L, Butany J (2004) Pulmonary pathology of severe acute respiratory syndrome in Toronto. *Mod. Pathol.* (18), pp. 1–10.

24. Iwen PC, Reed EC, Armitage JO, Bierman PJ, Kessinger A, Vose JM, Arneson MA, Winfield BA, Woods GL (1993). Nosocomial invasive aspergillosis in lymphoma patients treated with bone marrow or peripheral stem cell transplants. *Infect Control Hosp Epidemiol.* (14), pp. 131-139.

25. Kanamori H, Rutala WA, Sickbert-Bennett EE, Weber DJ (2015) Review of Fungal Outbreaks and Infection Prevention in Healthcare Settings During Construction and Renovation *Clinical Infectious Diseases*, 61(3), pp. 433–444.

26. Kaur H, Yadav A, Wadhwa K, Jain K, Ghorai S.M, (2020) Azoles Used in Agriculture as Possible Cause of Azole-Resistance in Clinical Candida Isolates. Biosci Biotech Res Asia;17(4).

27. Knutsen AP & Slavin RG (2011) Allergic Bronchopulmonary Aspergillosis in Asthma and Cystic Fibrosis Clin Dev Immunol. 843763.

28. Kolwijck E., Van der Hoeven H., de Sévaux R.G.L., ten Oever J., Rijstenberg L.L., van der Lee H.A.L. (2016) Voriconazole-susceptible and voriconazole-resistant *Aspergillus fumigatus* coinfection. *Am J Respir Crit Care Med.* (193), pp. 927-929.

29. Kramer MR, Marshal SE, Starnes VA, Gamberg P, Amitai Z, Theodore J (1993) Infectious complications in heart-lung transplantation. Analysis of 200 episodes. *Arch Intern Med*, pp. 153.

30. Kusne S, Dummer JS, Singh N, Iwatsuki S, Makowka L, Esquivel C, Tzakis AG, Starzl TE, Ho M (1988) Infections after liver transplantation. An analysis of 101 consecutive cases. *Medicine* (Baltimore). (67), pp. 132-143.

31. Kwon-Chung KJ & Sugui JA (2013)., *Aspergillus fumigatus*—What Makes the Species a Ubiquitous Human Fungal Pathogen? PLoS Pathog. (12), e1003743.

32. Madureira A, Bergeron A, Lacroix C, Robin M, Rocha V, Peffault de Latour R, Ferry C, Devergie A, Lapalu J, Gluckmana E, Socié G, Ghannoum M, Ribaud P (2007) Breakthrough invasive aspergillosis in allogeneic haematopoietic stem cell transplant recipients treated with caspofungin. Int J Antimicrob Agents2007, 30(6) pp. 551-4.

33. Mahieu LM, Dooy JJD, Van Laer FA, Jansens H, Leven MM, (2000) A prospective study on factors influencing *Aspergillus* spore load in the air during renovation works in a neonatal intensive care unit Journal of Hospital Infection, 45(3), pp. 191-197.

34. Margalit A & Kavanagh K (2015) The innate immune response to *Aspergillus fumigatus* at the alveolar surface FEMS Microbiol Rev, 39(5), pp. 670-87.

35. Maria V. Bandres; Pranav Modi; Sandeep Sharma (2021) Aspergillus Fumigatus Treasure Island (FL), StatPearls Publishing.

36. McWhinney PHM, Kibbler CC, Hamon MD, Smith OP, Gandhi L, Berger LA, Walesby RK, Hoffbrand AV (1993) Prentice HG Progress in the diagnosis and management of aspergillosis in bone marrow transplantation: 13 years' experience. *Clin Infect Dis*; (17), pp. 397-404.

37. Meis JF, Chowdhary A, Rhodes JL, Fisher MC, Verweij PE (2016) Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus.* Philos Trans R Soc B Biol Sci, (371).

38. Mellado E, Garcia-Effron G, Alcázar-Fuoli L, Melchers W.J.G, Verweij P.E, Cuenca-Estrella M, (2007) A new *Aspergillus fumigatus* resistance mechanism conferring *in vitro* cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrob Agents Chemother*; (51), pp. 1897-1904.

39. Mohamed A, Rogers TR, Talento AF (2020) COVID-19 Associated Invasive Pulmonary Aspergillosis: Diagnostic and Therapeutic Challenges J Fungi (Basel). 6(3), pp. 115.

40. Morris G, Kokki MH, Anderson K, Richardson M. (2020) Sampling of Aspergillosis spores in air. *J Hosp Infect*, (44), pp. 81-92.

41. O'Gorman CM, Fuller H, Dyer PD (2009) "Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*", 457 (7228), pp. 471-4.

42. Overberger PA, Waowsky RM, Schaper MM (1995) Evaluation of airborne particulates and fungi during hospital renovation. *Am Ind Hyg Assoc J*, (56), pp. 706-712.

43. Panackal AA, Li H Kontoyiannis DP, Mori M, Perego CA, Boeckh M and Marr KA (2011) Geoclimatic Influences on Invasive Aspergillosis after Hematopoietic Stem Cell Transplantation Clin Infect Dis, 50(12), pp. 1588–1597.

44. Pieter P.A. Lestrade, Jochem B. Buil, Martha T. van der Beek, Ed J. Kuijper, Karin van Dijk, Greetje A. Kampinga, Bart J.A. Rijnders, Alieke G. Vonk, Sabine C. de Greeff, Annelot F. Schoffelen, Jaap van Dissel, Jacques F. Meis, Willem J.G. Melchers, Paul E. Verweij (2020). Paradoxal Trends in Azole-Resistant *Aspergillus fumigatus* in a National Multicenter Surveillance Program, the Netherlands, 26 (7).

45. Prasad A, Agarwal K, Deepak D, and Singh Atwal S (2016) Pulmonary Aspergillosis: What CT can Offer Before it is too Late! J Clin Diagn Res., 10(4), pp. 01-05.

46. Reller LB, Weinstein M, Jorgensen JH, Ferraro MJ (2009) Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices *Clinical Infectious Diseases*, 49(11), pp. 1749–1755.

47. Sahiner A, Halat E, Algin Yapar E (2019) Comparison of bactericidal and fungicidal efficacy of antiseptic formulations according to EN 13727 and EN 13624 standards Turk J Med Sci. 49(5), pp. 1564–1567.

48. Schiraldi GF, Colombo MD, Harari S, Lo Cicero S, Ziglio G, Ferrarese M, Rossato D, Soresi E (1996) Terbinafine in the treatment of non-immunocompromised compassionate cases of bronchopulmonary aspergillosis Mycoses, 39(1-2) pp. 5-12

49. Schwartz RS, Mackintosh FR, Schrier SL, Greenberg PL. (1984) Multivariate analysis of factors associated with invasive fungal disease during remission induction therapy for acute myelogenous leukaemia, (53) pp. 411-419.
50. Sewell, T. R, Zhu, J, Rhodes, J, Hagen, F, Meis, J. F, Fisher, M. C, & Jombart, T. (2019). Nonrandom Distribution of Azole Resistance across the Global Population of *Aspergillus fumigatus*, MBio, 10(3).

51. Sharpe AR, Lagrou K, Meis JF, Chowdhary A, Lockhart SR, Verweij PE (2018) Triazole resistance surveillance in *Aspergillus fumigatus Medical Mycology*, (56), pp. S83–S92.

52. Snelders E, Van Der Lee HAL, Kuijpers J (2008) An Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism, PLoS Med(5) pp. 1629–1637.

53. Streifel AJ, Lauer, JL, Vesley D, Juni B, Rhame FS. (1983) *Apergillus fumigatus* and other thermotolerant fungi generated by hospital building demolition. *Appl Environ Microbiol*; (45), pp. 375-378.

54. Toree-Cisneros J, Lopez OL, Kusne S, Martinez AJ, Starzl TE, Simmons RL, Martin M (1993) CNS aspergillosis in organ transplantation: a clinicopathological study. *J Neurol Neurosurg Psychiatry*, (56), pp. 188-193.

55. Verweij PE, Howard SJ, Melchers WJG, Denning DW (2009) Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. Drug Resist Updat, (12) pp. 141–147.

56. Verweij P.E, Zhang J, Debets A.J.M, Meis J.F, van de Veerdonk F.L., Schoustra S.E (2016) In-host adaptation and acquired triazole resistance in *Aspergillus fumigatus*: a dilemma for clinical management. *Lancet Infect Dis,* (16), pp. 251-260.

57. Verweij PE, van de Sande-Bruisma N, Kema GH, Melchers WJ (2012) Azole resistance in *Aspergillus fumigatus in* the Netherlands--increase due to environmental fungicides? Ned Tijdschr Geneeskd, 156(25), A4458.

58. Wang H, Ding Y, Li X, Yang L, Zhang W, Kang W (2003) Fatal Aspergillosis in a Patient with SARS Who Was Treated with Corticosteroids. *N. Engl. J. Med,* (349) pp. 507–508.

59. Wang H.-J, Ding Y.-Q, Xu J, Li X, Li X.-F, Yang L, Zhang W, Geng J, Shen H, Cai J.-J, (2004) Death of a SARS case from secondary *aspergillus* infection. *Chin. Med. J* (117).

60. Wajszczuk CP, Dummer JS, Ho M, Van Thiel DH, Starzl TE, Iwatsuki S, Shaw Jr B (1985) Fungal infections in liver transplant recipients. *Transplantation*, (40) pp. 347-353.

61. Weber DJ, MD, Peppercorn A, Miller MB, Sickbert-Benett E, Rutala WA (2009) Preventing healthcare-associated *Aspergillus* infections: review of recent CDC/HICPAC recommendations, *Medical Mycology*. 47 (1) pp. 199–209.