Norwegian University of Life Sciences

Master's Thesis 2022 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Trouble in the air – Are pathogenic *Aspergillus* spp. in Norwegian barns azole resistant?

Samantha Kim Pettersen
Biotechnology

Figure 1: Illustration made for a poster presenting the "BARNS" project at the 31st Fungal Genetics Conference in Pacific Grove, California by Genetic Society of America (2022).

Acknowledgement

Science has always interested me from a very young age, and it is a path that I have long intended to pursue. Working on and finishing my Master's thesis has been a huge personal achievement in my career and education, and a goal that I have had since elementary school, but it always felt so far into the future. Now that I have finally reached this milestone, it feels very surreal. Throughout this project, I have not only learned a lot on an academic level, but also on a personal level. It has truly been an honour to be able to work on such an exciting topic alongside some great and wonderful people. It has really increased my appreciation for mycology and the research field in general. I cannot wait to see what endeavours awaits me next.

This thesis was written at the Norwegian University of Life Sciences (NMBU). All work was done at the Norwegian Veterinary Institute (NVI). A huge thanks goes to the Research Funding for Agriculture and the Food Industry (FFL/JA) for funding the "BARNS" project. I would also like to give a special thanks to the people at NVI for all their help and encouragement; especially when I had to unexpectedly put my work on hold for a while. Thank you for keeping up my work at the laboratory while I was away.

My deepest gratitude goes to my supervisor, Professor Ida Skaar, for her guidance, feedback and for introducing me to the wonderful and exciting world of mycology, and to my co-supervisor, Dr Hege Hvattum Divon, for all her guidance and valuable feedback on my work. I would like to thank Elin Rolén for teaching me all the laboratory techniques I required for my work, Ellen Christensen for helping and teaching me how to identify the different fungal colonies, and Lonny Margrethe Kløvfjell for her guidance and for her immense ability at tracking down the most elusive information I asked for. I would also like to extend my gratitude to the people at the media production department at NVI for providing me with all the media and solutions I used throughout this study.

Lastly, I want to thank my immediate and extended family for their endless support. Especially to my dad, Per Olav, for always being there for me and for patiently listening to my long rants about fungi and microbiology. To my sister, Kjersti Linn, for all her advice and motivation. And to my mum, Angela Lim, for every great and little thing she did to make me happy and comfortable. I love you all.

Abstract

Aspergillus fumigatus and *Aspergillus niger* are saprotrophic, ubiquitous fungi and some of the lead causes of aspergillosis; a human and animal fungal infection with high mortality rate. Azoles are one of the few efficient antifungal agents used to treat aspergillosis, however, a rise in azole resistant fungi have become increasingly widespread. It is thought that use of azoles in the environment, such as in agriculture, is the main driving force of this development. Little is known about the prevalence of azole resistant fungi in Norway. The current study is part of the research project "BARNS" where the aim is to map out the prevalence of azole resistant *A. fumigatus* and *A. niger* in Norwegian farm buildings.

Air samples from farms were gathered through a citizen science approach and cultivated on DG18 agar. Environmental isolates of *A. fumigatus* and *A. niger* were isolated and identified by morphology and *caM* and *bT2* sequencing. This resulted in 58 *A. fumigatus* isolates, whereas 37 *A. niger* isolates were molecularly corrected to *Aspergillus tubingensis* and *Aspergillus welwitschiae*. All fungal isolates were screened for resistance against three medical azoles; itraconazole, voriconazole and posaconazole, using VIPcheck™. Together with three clinical isolates from the Norwegian Veterinary Institute's strain collection, the environmental isolates with indications of resistance were further tested against the same azoles using E-test. Isolates that showed phenotypical azole resistance were screened for known environmental mutations in the *cyp51A* gene and promoter region (i.e. TR_{34}/L98H and TR_{46}/Y121F/T289A). The results were compared to a previously conducted sampling round within the same project.

None of the environmental isolates in this study showed phenotypic resistance on E-test to any of the azoles tested, and were therefore not sequenced. In comparison to the previous sampling, both the number of isolates and detection of resistance were much lower in this study. This was unexpected as seasonal temperature differences were believed to be a major factor in proliferation of the fungi. Other external determining factors for fungal proliferation may be the presence and activity around animals on the farms. The three clinical isolates all showed phenotypic resistance to all three azoles on E-test, and they all harboured the TR_{34}/L98H mutation in *cyp51A*. This is consistent with the hypothesis that environmental driving forces affect the development of resistance.

The low sampling number and the one-sided focus on Norwegian farms is not sufficient to draw any clear conclusions on how prevalent azole resistant *A. fumigatus* and *A. niger* are in Norway. However, the lack of findings might indicate that the prevalence is fairly low compared to other countries.

Sammendrag

Aspergillus fumigatus og *Aspergillus niger* er saprotrofiske sopp som finnes overalt og er blant de ledende årsakene til aspergillose; en soppinfeksjon med høy dødelighet hos dyr og mennesker. Azoler er et av de få effektive antimykotiske midlene som brukes til å behandle aspergillose, men azolresistente sopp har begynt å bli mer utbredt. Bruk av azoler i miljøet, som for eksempel i landbruk, er antatt å være den største drivkraften til denne utviklingen. Utbredelsen av azolresistens i Norge er ukjent. Denne studien er en del av "BARNS"-prosjektet, hvor målet er å kartlegge utbredelsen av azolresistente *A. fumigatus* og *A. niger* i norske gårdsbygninger.

Luftprøver fra gårder ble samlet gjennom folkeforskning og dyrket på DG18 agar. Miljøisolater av *A. fumigatus* og *A. niger* ble isolert og identifisert morfologisk og med *caM*- og *bT2*-sekvensering. Dette resulterte i 58 *A. fumigatus* isolater, mens 37 isolater tilhørende *A. niger*-gruppen ble molekylært identifisert som *Aspergillus tubingensis* og *Aspergillus welwitschiae*. Alle soppisolatene ble screenet for resistens mot tre medisinske azoler; itraconazol, voriconazol og posaconazol, ved hjelp av VIPcheck™. Sammen med tre kliniske isolater fra stammesamlingen til Veterinærinstituttet, ble miljøisolatene som indikerte resistens videre testet mot de samme azolene med E-test. Isolater som viste fenotypisk azolresistens ble så screenet for kjente miljørelaterte mutasjoner på *cyp51A*-genet og promotoren (TR34/L98H og TR46/Y121F/T289A). Resultatene ble sammenlignet med en tidligere prøvetakingsrunde i det samme prosjektet.

Ingen av miljøisolatene fra studien viste fenotypisk resistens mot noen av azolene på E-test og ble dermed ikke sekvensert. Sammenlignet med den tidligere prøvetakingsrunden var både antall isolater og resistente isolater lavere i denne studien. Dette var uventet ettersom det var antatt at sesongforskjeller i temperaturer ville være en sterk faktor for oppvekst og forekomst av soppene. Andre bestemmende, eksterne faktorer for forekomst av sopp i lufta kan være tilstedeværelsen og aktiviteten av dyr på gårdene. Alle de tre kliniske isolatene viste fenotypisk resistens mot alle tre azoler på E-test, og alle hadde TR_{34}/L98H-mutasjonen på *cyp51A*. Dette er samsvarende med hypotesen om at miljøpåvirkninger driver utviklingen av resistens.

En fullstendig konklusjon om utbredelsen av azolresistente *A. fumigatus* og *A. niger* i Norge kan ikke bli trukket på grunn av det lave antallet prøver som ble samlet og det ensidige fokuset på norske gårder. Likevel kan mangelen på funn være en indikasjon på at utbredelsen er ganske lav i forhold til andre land.

Abbreviations

°C	-	degree Celsius
µg	-	microgram
µl	-	microliter
µm	-	micrometer
µM	-	micromolar
ABC	-	ATP-binding cassette
ABPA	-	allergic bronchopulmonary aspergillosis
AE	-	elution buffer (DNA extraction)
AL	-	lysis buffer (DNA extraction)
AMR	-	antimicrobial resistance
ATCC	-	American Type Culture Collection
ATP	-	adenosine triphosphate
AW1	-	washing buffer 1 (DNA extraction)
AW2	-	washing buffer 2 (DNA extraction)
BLAST	-	Basic Local Alignment Searching Tool
bp	-	base pair
bT2	-	β-tubulin
caM	-	calmodulin gene
CCPA	-	chronic cavitary pulmonary aspergillosis
cdr1B	-	efflux transporter gene
CFPA	-	chronic fibrosing pulmonary aspergillosis
CFU	-	colony forming unit
CLSI	-	Clinical and Laboratory Standards Institute
cm	-	centimetre
CNPA	-	chronic necrotising pulmonary aspergillosis
CNS	-	central nervous system
COPD	-	chronic obstructive pulmonary disease
CPA	-	chronic pulmonary aspergillosis
Cyp51/Cyp51A	-	cytochrome P450 sterol 14α-demethylase protein
cyp51A	-	cytochrome P450 sterol 14α-demethylase gene
cyp51B	-	cytochrome P450 sterol 14α-demethylase gene
cyp51C	-	cytochrome P450 sterol 14α-demethylase gene
ddNTP	-	dideoxynucleotide triphosphate

DG18	-	dichloran glycerol agar
DMI	-	demethylation inhibitor
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleotide triphosphate
E-test	-	epsilometer test
EB	-	elution buffer (gel purification)
EBK	-	extraction blank control
ECOFF	-	epidemiological cut-off
EDTA	-	ethylenediaminetetraacetic acid
erg11A	-	cytochrome P450 sterol 14α-demethylase gene
erg11B	-	cytochrome P450 sterol 14α-demethylase gene
EtOH	-	ethanol
EUCAST	-	European committee on antimicrobial susceptibility testing
fwd	-	forward
g	-	gram
GRAS	-	generally recognised as safe
h	-	hours
H_2O	-	water
IA	-	invasive aspergillosis
ITS	-	internal transcribed spacer
kb	-	kilo base pairs
KCl	-	potassium chloride
KH_2PO_4	-	potassium dihydrogen phosphate
L	-	litres
mA	-	milliampere
MEA	-	malt extract agar
MF	-	McFarland
MFS	-	major facilitator superfamily
mg	-	milligram
MIC	-	minimum inhibitory concentration
min	-	minutes
ml	-	millilitres
MQ	-	Milli-Q water
N	-	nitrogen

Na_2HPO_4	-	disodium hydrogen phosphate
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
NCBI	-	National Center for Biotechnology Information
ng	-	nanogram
NMBU	-	Norwegian University of Life Sciences
NVI	-	Norwegian Veterinary Institute
PBS	-	phosphate buffered salin
PCR	-	polymerase chain reaction
PE	-	wash buffer (gel purification)
pH	-	pondus Hydrogenii (measurement for acidity)
QG	-	solubilising buffer (gel purification)
RNA	-	ribonucleic acid
RO	-	reverse osmosis
RPMI	-	Roswell Park Memorial Institute
SAB	-	Sabouraud dextrose agar
SAFS	-	severe asthma with fungal sensitisation
sec	-	seconds
sp.	-	specie
spp.	-	species
TBE	-	tris-borate-EDTA
T_m	-	melting temperature
TR	-	tandem repeat
TR_{34}/L98H	-	34 bp tandem repeats at the promoter region and bp substitution at codon no. 98 which shifts leucine into histidine
TR_{46}/Y121F/T289A	-	46 bp tandem repeats at the promoter region and bp substitution at codon no. 121 and 289 which shifts tyrosine into phenylalanine and threonine into alanine
U	-	units of activity
UV	-	ultraviolet
V	-	Volt
WT	-	wild type
x g	-	relative centrifugal force

Table of content

Acknowledgement ... III
Abstract ... IV
Sammendrag ... V
Abbreviations ... VI
Table of content ... IX
1. Introduction ... 1
2. Background ... 2
2.1 *Aspergillus* biology and taxonomy ... 2
2.1.1 *Aspergillus* section *Fumigati* ... 2
2.1.2 *Aspergillus* section *Nigri* ... 3
2.1.3 Genetic markers ... 4
2.2 *Aspergillus* as a pathogen ... 5
2.3 Azoles – an efficient antifungal agent ... 7
2.3.1 Azole definitions ... 8
2.3.2 Ergosterol biosynthesis ... 9
2.3.3 Mechanisms of azoles ... 11
2.4 Azole resistance – a result of mutation ... 11
2.4.1 Reducing azole and enzyme affinity ... 12
2.4.2 Overexpression of the target enzyme ... 12
2.4.3 Decreasing intracellular azole concentration through efflux pumps ... 13
2.4.4 Other known or proposed mechanisms ... 13
2.5 Causes and impact of azole use ... 14
2.6 The theory and principles behind the experimental methods ... 17
2.6.1 Citizen science ... 17
2.6.2 Resistance screening ... 18
2.6.3 Polymerase chain reaction ... 19
2.6.4 Sanger sequencing ... 20
3. Materials and methods ... 22
3.1 Experimental setup ... 22
3.1.1 Gathering participants ... 22
3.1.2 Sampling ... 22
3.1.3 Recurring lab techniques ... 23
3.1.4 Cultivation of the spore films and isolation of fungal colonies ... 25
3.1.5 Contamination test ... 26
3.1.6 Glycerol stock for storage ... 27
3.1.7 Resistance screening ... 27
3.2 Molecular ... 32
3.2.1 DNA extraction ... 32

3.2.2 PCR ... 33
3.2.3 Gel electrophoresis ... 36
3.2.4 Gel purification ... 38
3.3 Sequencing and sequence processing ... 39
3.3.1 Sanger sequencing ... 39
3.3.2 Bioinformatic ... 40
4. Results ... 43
4.1 Citizen science participant's information ... 43
4.2 Fungal cultivation and subsampling ... 45
4.2.1 Contamination test ... 45
4.2.2 Findings on primary plates ... 46
4.2.3 Morphological identification of *A. fumigatus* and *A. niger* on primary plates ... 48
4.3 Molecular identification of fungal isolates ... 53
4.3.1 *caM* primer optimisation and troubleshooting ... 53
4.3.2 Molecular identification of *A. fumigatus* isolates ... 56
4.3.3 Molecular identification of *A. niger* isolates ... 57
4.4 Resistance Screening ... 58
4.4.1 Primary resistance screening with VIPcheck™ ... 58
4.4.2 Secondary resistance screening with E-test ... 61
4.4.3 MF-value and spore count ... 62
4.5 Molecular characterization of the clinical isolates ... 64
5. Discussion ... 66
5.1 The sampling yield in relation to environmental conditions ... 66
5.1.1 Citizen science provided efficient sampling, but may have affected data quality ... 68
5.2 Species identification of the isolates revealed the importance of sequencing ... 69
5.3 *A. fumigatus* and *A. niger* require different approaches in analyses due to biological differences ... 70
5.4 The differences between phenotypic resistance screening methods ... 72
5.5 Mucorales contamination can be inconspicuous and persistent ... 73
5.6 Very few resistant *A. fumigatus* isolates have been detected in Norway ... 74
5.7 Azole resistance is a One Health challenge ... 76
5.8 Future approaches for azole resistance surveillance and management in Norway ... 77
5.9 Conclusion ... 78
6. References ... 79
7. Appendix ... 88
Appendix 1 – Instruction manual ... 88
Appendix 2 – Questionnaire ... 90
Appendix 3 – Instruments ... 92
Appendix 4 – Compounds ... 94
Appendix 5 – Software ... 99
Appendix 6 – Household sampling ... 100

Appendix 7 – Participant information 101
Appendix 8 – Contamination test photos 103
Appendix 9 – Findings on primary plates 105
Appendix 10 – Pale *A. fumigatus* 108
Appendix 11 – VIPcheck™ scores 109
Appendix 12 – VIPcheck™ photos 111
Appendix 13 – E-test results photos 112
Appendix 14 – Clinical sample information 115
Appendix 15 – Consensus of *cyp51A* references used mutation screening 116
Appendix 16 – Gel electrophoresis images of *caM, bT2* and *cyp51A* amplicons 119
Appendix 17 – Molecular sample numbers 123
Appendix 18 – Sequence information 124

1. Introduction

Fungi come in many forms and exist practically everywhere; each having developed their own niche in the ecosystem. *Aspergillus fumigatus* is a saprotrophic, filamentous fungus that exists almost everywhere in the environment (Samson 2019, p. 130). It is also a human and animal pathogen, and a lead cause of aspergillosis (Kwon-Chung and Sugui 2013). Invasive aspergillosis is an advanced stage of the infection and has been reported to have a mortality rate of up to 90% (Latgé 1999).

A class of antifungal agents called azoles inhibit the essential ergosterol biosynthesis in fungi, and is the primary drug used to treat aspergillosis (Latgé and Chamilos 2019). Azoles are also used to protect agriculture from fungal diseases to ensure food safety and high crop yield (Jørgensen and Heick 2021). Several studies have found evidence implying that azoles used in the environment are the main driving forces of azole resistance development. In fact, many clinical samples with environment mediated resistance mutations on the *cyp51A* gene, TR_{34}/L98H and TR_{46}/Y121F/T289A, have been uncovered (Verweij et al. 2016). The emergence of azole resistant *A. fumigatus* strains cause treatment failure and has over the last decade become an increasing problem worldwide (Verweij et al. 2020).

Azole resistance is a branch of antimicrobial resistance (AMR) that is often overlooked, but in recent years, there has been an increased effort to gain more knowledge about the prevalence of azole resistant *A. fumigatus* through studies and surveillance programs (Verweij et al. 2016, Lestrade et al. 2019). *A. fumigatus* with environment mediated resistance mutations has been uncovered in Norwegian clinical samples, but substantial data of prevalence is still lacking (Skaar et al. 2019). In order to gain a better understanding of where Norway lies in terms of azole resistance, the Norwegian Veterinary Institute (NVI) has launched a pilot project, "BARNS", in which *A. fumigatus* from farm buildings all over Norway are screened for azole resistance. The project was inspired by Shelton et al. (2020) and aims to map out potential *A. fumigatus* hotspots and the prevalence of azole resistance under the hypothesis that azole resistance arises from the environment/agriculture.

In the present study, it was hypothesised that the prevalence of azole resistance in Norway would be around the same or lower than other countries. Furthermore, it was hypothesised that *A. fumigatus* proliferation would be season dependent due to temperature differences, and because the optimum temperature of the fungus is relatively high (37 °C) (Samson 2019). It was also thought that barns could be a potential hotspot for *A. fumigatus* proliferation; NVI has previously experienced a high yield of this fungus in samples taken from the farm environment.

2. Background

2.1 *Aspergillus* biology and taxonomy

There are 339 different species within the genus *Aspergillus* (Samson et al. 2014); some species can be distinguished through studying phenotypic characteristics, such as morphology and metabolism of certain compounds. In later years, screening the genome with molecular methods has become a more prominent approach in taxonomy (Guarro et al. 1999, Samson et al. 2014). In fact, several new species have been defined after molecular approaches started to play a larger part in the field of microbiology (Luo et al. 2018). In this thesis, the main focus will be on two *Aspergillus* species; *Aspergillus fumigatus* and *Aspergillus niger*.

2.1.1 *Aspergillus* section *Fumigati*

A. fumigatus is a filamentous, saprotrophic Ascomycete that exists almost everywhere in the environment, but is commonly found in soils and compost, and thrives better in warmer climates (Samson 2019, p. 130). This fungus plays a niche role in decomposing organic materials in soils (Latgé and Chamilos 2019); namely dead plant matter (Tekaia and Latgé 2005). Some of the most common hotspots for *A. fumigatus* are in compost heaps (Kwon-Chung and Sugui 2013). This fungus mostly proliferate by creating conidia through asexual reproduction. Sexual reproduction has been observed in laboratories, but is yet to be discovered in nature. The sexual cycle is called the teleomorph stage, in which ascospores (sexual spores) are created through hyphal fusion and meiosis (Latgé and Chamilos 2019).

The colonies of *A. fumigatus* are typically smooth and round in shape with white mycelium and dark green to bluish green conidiophores (Samson 2019, p. 130). The fungus can also be recognised morphologically by the structure of the conidiophores. The conidia are formed in chains on the phialides (Latgé and Chamilos 2019), which are anchored to a round vesicle by metula. The conidia are globose to subglobose and 2-3 µm in diameter, and are dispersed into the atmosphere through air turbulence (Latgé 1999, Latgé and Chamilos 2019, Samson 2019, p. 130).

The reasons why this fungus is so ubiquitous is their ability to produce a high amount of conidiophores that propagate easily through currents in the air, but also their ability to adapt and survive environmental changes. (Kwon-Chung and Sugui 2013). The optimum temperature for *A. fumigatus* is somewhere between 25 °C and 37 °C, but it is also capable of germination in temperatures up to 50 °C (Samson 2019, p. 130). Furthermore, they struggle to grow in temperatures below 12 °C (Kwon-Chung and Sugui 2013).

Along with 63 other *Aspergillus* spp., *A. fumigatus* belongs to the complex called *Aspergillus* section *Fumigati*. These species share many common attributes to each other, but each possesses minor variations in the genome that declare them as their own species. Examples of fungi found in this

complex can be seen in Table 1 (Sugui et al. 2014, Frisvad and Larsen 2016). Several species within section *Fumigati* have been reported as potential pathogens for animals and humans (Sugui et al. 2014), however, the most important species in epidemiology within this complex is *A. fumigatus* (Latgé 1999, Lamoth 2016).

Table 1: Some species found within *Aspergillus* section *Fumigati*. The list is retrieved from, referenced and modified after Sugui et al. (2014) and Frisvad and Larsen (2016).

Species within section *Fumigati*	
A. brevipes	*A. parafelis*
A. caatingaensis	*A. pernambucoensis*
A. duricaulis	*A. pseudofelis*
A. felis	*A. pseudoviridinutans*
A. fischeri	*A. siamensis*
A. fumigatiaffinis	*A. spinosus*
A. fumigatus	*A. thermomutatus*
A. fumisynnematus	*A. turcosus*
A. hiratsukae	*A. udagawae*
A. huiyaniae	*A. unilateralis*
A. laciniosus	*A. viridinutans*
A. lentulus	*A. waksmanii*
A. marvanovae	*A. wyomingensis*
A. novofumigatus	

2.1.2 *Aspergillus* section *Nigri*

A. niger is, much like *A. fumigatus*, a filamentous, saprotrophic Ascomycete that decomposes organic matter. It is typically found on various vegetarian foods and soils from potted plants. The colonies are globose in shape with white to yellowish hyphae and dark brown to black conidiophores. It manages to grow under the same conditions as *A. fumigatus*, and has similar optimum temperatures at about 25 °C to 37 °C. Its conidia are globose and 3.5-4.5 µm in diameter. They are also dispersed through disturbance and air turbulence, and they propagate by airflow (Samson 2019, p. 146).

Since the development of molecular analysis methods, a total of 19 different black *Aspergillus* species have been defined (Gautier et al. 2016). Several of these species are equally ubiquitous as *A. niger* and share similar habitats and phenotypes, making them near impossible to distinguish without extensive analyses (Silva et al. 2011). Examples of other variants of black aspergilli can be seen in Table 2 (Cruz-Magalhães et al. 2019). As these strains are very similar to one another, they have been categorised under the *A. niger* complex called *Aspergillus* section *Nigri* (Gautier et al. 2016).

Table 2: Some species found within *Aspergillus* section *Nigri*. The list is retrieved from, referenced and modified after Cruz-Magalhães et al. (2019).

Species within section *Nigri*	
A. aculeatinus	*A. labruscus*
A. aculeatus	*A. luchuensis*
A. awamori	*A. neoniger*
A. brasiliensis	*A. niger*
A. brunneoviolaceus	*A. piperis*
A. carbonarius	*A. saccharolyticus*
A. costaricaensis	*A. sclerotiicarbonarius*
A. ellipticus	*A. sclerotioniger*
A. fijiensis	*A. trinidadensis*
A. floridensis	*A. tubingensis*
A. heteromorphus	*A. uvarum*
A. homomorphus	*A. vadensis*
A. ibericus	*A. welwitschiae*
A. japonicus	

Certain strains of black *Aspergillus* spp. are being used in the industry to produce a wide range of enzymes and other secondary metabolites for commercial and pharmaceutical use through fermentation (Li et al. 2020). *A. niger* has GRAS-status (Generally Recognised as Safe), meaning they are safe to use in the food industry (Vries and Visser 2001, Li et al. 2020). While some strains have value in the industry, others are also known to produce severe mycotoxins or cause infectious diseases, which makes it crucial to be able to distinguish between different strains.

2.1.3 Genetic markers

The advances in molecular methods have enabled mycologists to define fungal species down to a genetic level as well as mapping the phylogenetic and ecological aspects of the different species (Lindahl et al. 2013). Certain genes or DNA sequences have small sequence segments that vary between species or strains. Sequences like these, with a known location on the genome, are therefore established as genetic markers that can be used for screening as well as species and strain identification (Goutam et al. 2015). Some of the most used genetic markers for identification in the fungal kingdom are ribosomal DNA; this includes the genes for the large and small ribosomal subunits and the internal transcribed spacer region (ITS) (Lindahl et al. 2013, Lamoth 2016). The ITS region is used especially frequent as its primers are universal (Samson et al. 2014, Stielow et al. 2015). RNA polymerase II, mitochondrial ATP synthase and elongation factor 1α are also some other commonly used genetic markers in fungal phylogeny (Walker et al. 2012, Lindahl et al. 2013).

Among *Aspergillus* spp., ITS can be used to distinguish between some of the species. However, despite it being the most common genetic marker used for fungi, ITS usually does not yield a sufficiently high resolution to distinguish between all species of *Aspergillus*. This is why other

markers such as calmodulin (*caM*), β-tubulin (*bT2*) and RNA polymerase II second largest subunit (*RPB2*) are used instead or as a supplement to the ITS region. RNA polymerase II subunit is not easy to amplify, making it challenging to work with. Both *bT2* and *caM* are easy to amplify, and can distinguish between almost all *Aspergillus* spp. (Samson et al. 2014). Both *bT2* and *caM* are recommended to be used to distinguish between species of *Aspergillus* (Samson et al. 2014, Lamoth 2016), though *caM* yields a higher identity resolution for aspergilli than *bT2* (Alshehri and Palanisamy 2020). Additionally, an extensive database of *caM* sequences from nearly all accepted species has been established, making it a great tool in DNA barcoding (Samson et al. 2014).

2.2 *Aspergillus* as a pathogen

Superficial, cutaneous, subcutaneous and systemic infections caused by fungi are called mycoses (Walsh and Dixon 1996) and affect over a billion people worldwide (Bongomin et al. 2017). Furthermore, about 1.5 million people die from fungal infections annually, and there are more than 150 million people worldwide who suffer from serious diseases caused by fungi (Bongomin et al. 2017). One category of fungal infections is called aspergillosis, which is described as an infection caused by *Aspergillus* spp.; it can be local and chronic, or it can be acute and systemic (Latgé 1999). Both *A. fumigatus* and *A. niger*, along with *Aspergillus flavus*, *Aspergillus terreus* and *Aspergillus versicolor*, are some of the most common species to cause aspergillosis (Rudramurthy et al. 2019). In fact, *A. fumigatus* is the most common species to cause invasive aspergillosis (IA) (Kwon-Chung and Sugui 2013, Rudramurthy et al. 2019). This could be due to the wide proliferation of *A. fumigatus* and its high adaptability to new environments; this includes the airways of the host (Kwon-Chung and Sugui 2013, Verweij et al. 2016).

Aspergillosis starts by inhalation of airborne spores; every day, the average person breathes in hundreds of spores of *A. fumigatus* (Latgé 1999). Unlike other pulmonary infections, aspergilli were assumed to only infect people by spores dispersed from the environment, and not from person to person (Rudramurthy et al. 2019). However, a recent study shows that *A. fumigatus* can potentially also spread between people via aerosols (Engel et al. 2019). Due to the small size of the conidia, they can easily infiltrate deep into the airways where they can potentially proliferate and reproduce asexually (Bultman et al. 2017), or propagate to other areas of the body such as the central nervous system (CNS), liver, kidney, sinuses and spleen (Latgé and Chamilos 2019, Rudramurthy et al. 2019). The host environment also harbours the optimum temperature for *A. fumigatus* (37 °C) to germinate and grow (Kwon-Chung and Sugui 2013). In immunocompetent people, the conidia are eliminated by the host's immune system before they can germinate and cause infection (Segal 2009).

Pathogenic *Aspergillus* spp. are opportunistic pathogens, meaning infection takes place when the host's immune system is compromised (Latgé 1999, Foley et al. 2014). Aspergillosis therefore

mostly affects the immunocompromised, such as people with HIV/AIDS, people undergoing corticosteroid therapies, cancer patients and patients undergoing organ transplantation (Bongomin et al. 2017). The infection can also take place in people who are suffering from other pulmonary illnesses; this include patients who have tuberculosis, pneumonia, chronic obstructive pulmonary disease (COPD), sarcoidosis, bronchiectasis, asthma, influenza and cystic fibrosis (Bongomin et al. 2017, Schauwvlieghe et al. 2018, Zilberberg et al. 2018). Recent studies show that COVID-19 patients are also more susceptible to aspergillosis (Ismaiel et al. 2021, Szabo et al. 2021).

Aspergillosis can manifest in many ways, causing infections and conditions that varies in severity and persistence. They are generally divided into (i) allergic; in which the fungal antigens trigger an extensive immune response, (ii) non-invasive; in which the infection is contained within a location, and (iii) invasive; in which the mycelium invades the tissue and the infection can spread (Greene 2005, Latgé and Chamilos 2019).

ABPA (allergic bronchopulmonary aspergillosis) is a variant of aspergillosis in which the patient has an allergic response to the conidia of *Aspergillus* spp. (Latgé 1999, Riscili and Wood 2009). Conidia that are dead or uncultivable can also cause reactions (Samson et al. 2004, p. 304). ABPA mostly affects people with underlying respiratory conditions such as asthma and cystic fibrosis; almost all people with ABPA have a history of being asthmatic (Latgé and Chamilos 2019). The global burden of people who suffer from ABPA is at about 4.8 million (Bongomin et al. 2017). It is estimated that 1-3.5% of people with asthma develop ABPA, while 7-9% of people with cystic fibrosis are affected by this condition. Severe asthma with fungal sensitisation (SAFS) is another form of atopic aspergillosis, in which patients with severe asthma are sensitive to fungal allergens. What differs SAFS from ABPA is that it cannot be detected with the same diagnostic tools used to detect ABPA (Latgé and Chamilos 2019).

CPA (chronic pulmonary aspergillosis) is a non-invasive, persistent form of aspergillosis (Latgé and Chamilos 2019). The infection is progressive and slow; it destroys the lung tissue and cause symptoms that last for over three months (Alastruey-Izquierdo et al. 2018). There are several disease variants that are categorised as CPA, including chronic cavitary pulmonary aspergillosis (CCPA), chronic fibrosing pulmonary aspergillosis (CFPA), chronic necrotising pulmonary aspergillosis (CNPA) and aspergilloma (a fungal mass inside cavities of the body) (Denning et al. 2016). According to Bongomin et al. (2017), more than 3 million cases of CPA worldwide were reported in 2017. CPA is most commonly related to people who have or have had pulmonary tuberculosis; it is estimated that 20% of recovering cavitary tuberculosis patients develop aspergilloma over the course of three years (Latgé and Chamilos 2019). Other underlying lung conditions that increase susceptibility to CPA include sarcoidosis, COPD, mycobacterial lung infections and structural abnormalities in the lungs (Alastruey-Izquierdo et al. 2018, Latgé and Chamilos 2019).

IA (invasive aspergillosis) is an acute or chronic systemic infection caused by *Aspergillus* spp. This is the most serious form of aspergillosis in which the fungus spreads from the respiratory tracts to the CNS or other organs (Latgé and Chamilos 2019). The mortality rate of IA varies between sources and circumstances, but it is somewhere between 30-90% (Latgé 1999, Bongomin et al. 2017). There were more than 300 000 cases of IA reported worldwide in 2017 (Bongomin et al. 2017). IA mostly occurs in severely immunocompromised patients, such as HIV/AIDS patients, cancer patients and patients undergoing solid organ and stem cell transplantation (Latgé 1999, Latgé and Chamilos 2019). Lung diseases and other underlying health conditions have also been reported to increase the susceptibility to IA (Denning et al. 2016). Patients that have undergone liver-, lung-, heart- and small bowel transplantation have been reported to be especially susceptible to IA (Pappas et al. 2010, Neofytos et al. 2018). Among all invasive fungal infections in organ and stem cell transplant patients, IA is the most common type (up to 59%). Furthermore, it has been estimated that 15-20% of leukaemia patients die of *Aspergillus* spp. related fungal pneumonia (Latgé and Chamilos 2019).

The prevalence of fungal infections is not only a public health threat, but also an economical burden. A study done in the United States estimated that the economic burden caused by fungal diseases were more than $7.2 billion in 2017, with $4.5 billion going to hospitalisations. The cost of hospitalisation caused by aspergillosis was estimated to be at about $1.2 billion (Benedict et al. 2019). Over the last few decades, there has been an increase in number of people with fungal infections; some of this is due to the increased number of people at the risk group (Hagiwara et al. 2016). IA has generally been difficult to diagnose (Taccone et al. 2015), and there are no fully reliable serological methods that can be used to detect the infection. Often times, several diagnostic tools are utilised, but it is sometimes difficult to distinguish between invasive and non-invasive aspergillosis. The full extent of the epidemiology of aspergillosis is likely underestimated due to the lack of progress in diagnostics (Latgé and Chamilos 2019). Late or incorrect diagnosis can lead to lower success rate of treatments (Segal 2009, Hagiwara et al. 2016, Benedict et al. 2019). Today, the most common and effective therapeutic drugs used to treat aspergillosis are azoles (Latgé and Chamilos 2019).

2.3 Azoles – an efficient antifungal agent

A prerequisite for a good antifungal agent is that it targets essential properties unique to the fungus. Since fungi are eukaryotes, they have a lot in common with animal and plant cells. It can therefore be challenging to find chemical compounds that can efficiently eradicate the fungus, but also at the same time are low in toxicity for non-fungal eukaryotic cells.

There is a variety of antifungal groups used today to treat fungal diseases in humans, animals and plants. They target essential metabolic pathways in the fungi where the proteins and enzymes are unique in the fungal kingdom (Odds et al. 2003, Chen and Sorrell 2007). For aspergillosis, there are

four main classes of antifungal drugs used in treatment; polyene, pyrimidine, echinocandin and azoles (Hagiwara et al. 2016).

Azoles are perhaps the fungicide that are most widely used in the world (Chen and Sorrell 2007). The first azole was discovered in the 1940s, while the first azole was sold on the marked in the late 1950's to the 1960's (Sheehan et al. 1999, Bhagat et al. 2021). Since then, several varieties of azoles have been developed (Odds et al. 2003, Parker et al. 2014) and distributed as agents against fungal infections in both clinical and agricultural settings (Bhagat et al. 2021).

2.3.1 Azole definitions

Azoles are a family of artificially synthesised organic molecules that have a five ring heterocyclic functional group containing nitrogen (Kauffman and Carver 1997, Bhagat et al. 2021). These compounds inhibit a demethylation reaction step in the ergosterol biosynthesis in fungi so they lose their cell membrane integrity and dies (Bhagat et al. 2021); azoles are therefore classified as a demethylation inhibitor (DMI) fungicide (Garcia-Rubio et al. 2020). The inhibition only affects fungal cells, and the broad-spectrum activity and stability of the azoles make them efficient agents against several fungal species in multiple settings (Bhagat et al. 2021). Azoles are therefore used to both treat and prevent fungal infections in humans, animals, plants and food (Hof 2001, Bhagat et al. 2021, Jørgensen and Heick 2021). Depending on the concentration, azoles can act as both a fungistatic agent – where they do not kill the fungus, but inhibit its growth – and a fungicidal agent – where the fungus dies (Hagiwara et al. 2016). There are two main classes of azoles, and they are divided by the number of nitrogen atoms contained inside this azole ring. Imidazoles contain azole rings with two N-atoms, while triazoles have three N-atoms in their rings (Bhagat et al. 2021). The molecular structures of some commonly used azoles are shown in Figure 2.

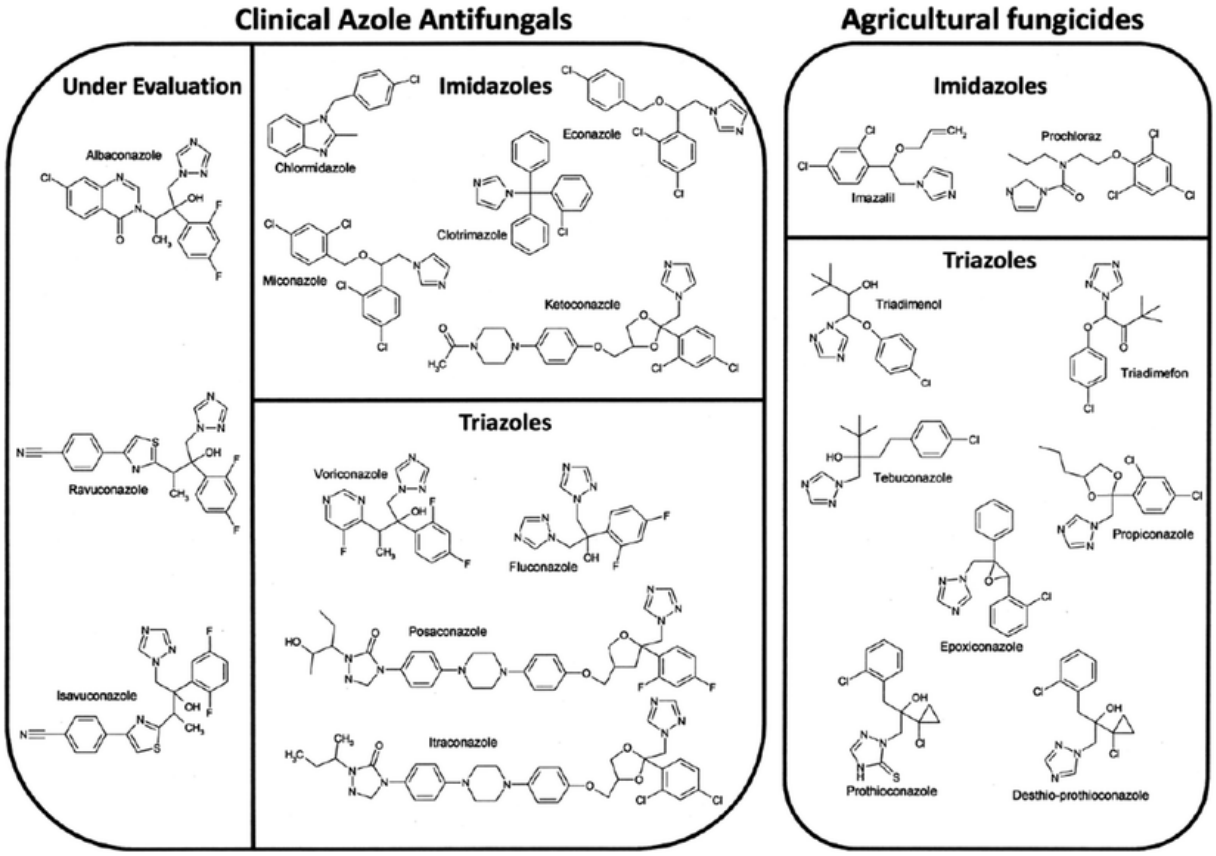


Figure 2: Some common azole drugs used in agriculture and clinically. Retrieved from Parker et al. (2014).

To better understand what makes azoles such successful antifungal agents, it is important to understand what their target site is and the mechanisms behind the inhibition.

2.3.2 Ergosterol biosynthesis

The presence of ergosterol in the cell membrane is a key difference between fungal cells and animal cells. While mammalian cell membranes contain the sterol cholesterol, fungi have a high percentage of the sterol ergosterol, in their cell membrane. The purpose of sterols in the cell membrane is to keep the cell structure rigid and control the distribution of integral proteins as well as the lipid membrane's fluidity, stability and permeability (Alcazar-Fuoli and Mellado 2013, Ermakova and Zuev 2017).

Ergosterol is synthesised in the cells' endoplasmic reticulum before being transported into the lipid membrane (Lepesheva and Waterman 2007, Jordá and Puig 2020). It is a complex metabolic pathway with about 20 enzymes involved (Alcazar-Fuoli et al. 2008). This pathway is best described in *Saccharomyces cerevisiae*, and is often used as reference for other eukaryotic organisms (Alcazar-Fuoli and Mellado 2013). The ergosterol biosynthesis pathway in *A. fumigatus* is less studied than that in *S. cerevisiae* (Alcazar-Fuoli et al. 2008), and research have shown that there are several genes in the *A. fumigatus* genome that encode for enzymes that carry out the same tasks (Mellado et al. 2001, Da Silva Ferreira et al. 2005, Dhingra and Cramer 2017).

The synthesis of ergosterol begins with two Acetyl CoA molecules, which are eventually turned into squalene through a few intermediate steps (Dupont et al. 2012). Figure 3 shows how squalene is then transformed into ergosterol through more reaction steps.

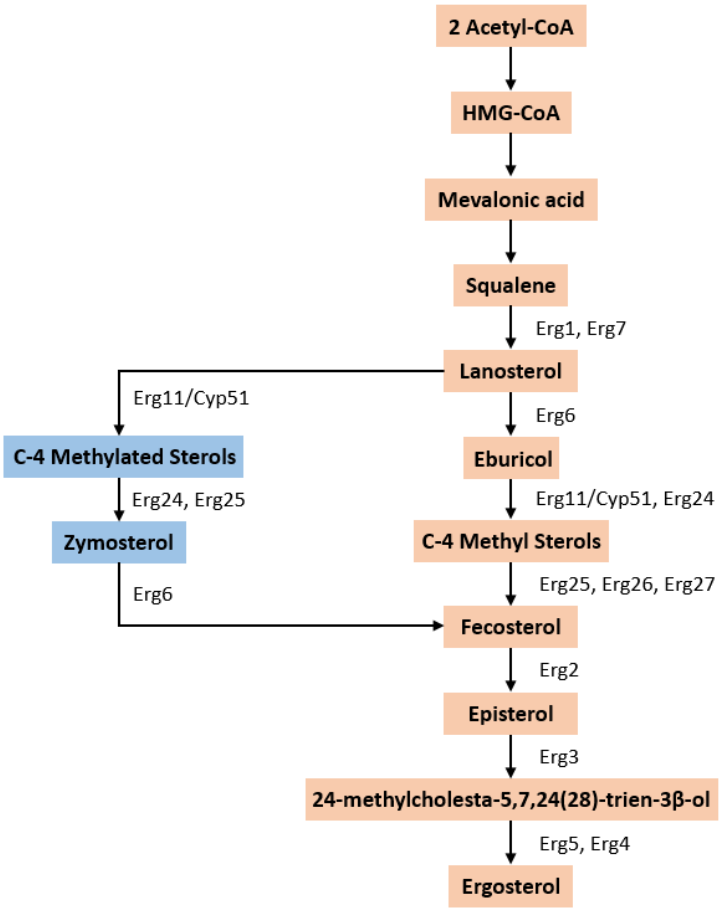


Figure 3: Schematic and simplified display of ergosterol biosynthesis. The pathway for *A. fumigatus* is described by the orange path, and the blue path describes *S. cerevisiae*. Enzymes involved are listed on the right side of the arrows. Referenced from Alcazar-Fuoli and Mellado (2013), and Dhingra and Cramer (2017).

One of the key enzymes in the biosynthesis of ergosterol, and the main target of azoles, is cytochrome P450 14α demethylase. It is part of a superfamily of proteins called cytochrome P450 (Mellado et al. 2001), which are found across several kingdoms of eukaryotes (Lepesheva and Waterman 2007). In *S. cerevisiae*, this protein converts lanosterol into other sterol intermediates by removing a methyl group. In *A. fumigatus*, the proposed pathway involves the protein converting eburicol into other sterol intermediates (See Figure 3) (Alcazar-Fuoli et al. 2008). There is a heme-iron prosthetic group at the active site of the enzyme (Lepesheva and Waterman 2007, Munro et al. 2018), which, when bound to oxygen, plays an important role in the enzyme's activity (Balding et al. 2008). There are several homologous genes that encode cytochrome P450 14α demethylase on the genome of *A. fumigatus* (Lepesheva and Waterman 2007), but the translation products most relevant for azole therapy stem from the *cyp51A*, *cyp51B*, *cyp51C*, *erg11A* and *erg11B* genes (Dhingra and Cramer 2017, Paul et al. 2018).

2.3.3 Mechanisms of azoles

Azoles inhibit the ergosterol synthesis by binding non-competitively to the cytochrome P450 14α demethylase, which are the Cyp51 proteins in *A. fumigatus* (Pérez-Cantero et al. 2020). They bind their azole nitrogen to the heme-iron, thereby blocking the oxygen out from the heme-iron and inhibiting the catalytic function of the enzyme (Balding et al. 2008). However, there are papers that suggest that some azoles bind competitively, where they bind to the enzyme's substrate binding site (Saad et al. 2006, Parker et al. 2011, Parker et al. 2014).

The inhibition of ergosterol biosynthesis leads to the cell collapsing due to loss of the membrane integrity. In addition, as a result of cytochrome P450 inhibition, lanosterol and other sterol intermediates accumulate and create a toxic environment inside the cell (Dupont et al. 2012, Zhu et al. 2021).

Parts of the gene sequence of cytochrome P450 14α demethylase are conserved across many species of fungi, which makes azoles a broad-spectrum drug (Becher and Wirsel 2012). On the other hand, cytochrome P450 sequences of non-fungal origin are significantly different, which enables the drug to target fungi while also remaining non-toxic towards mammalian and plant cells (Lepesheva and Waterman 2007). However, some individual fungal strains have managed to find ways to carry out the ergosterol biosynthesis despite the presence of azoles.

2.4 Azole resistance – a result of mutation

Fungi such as *A. fumigatus* proliferate massively through asexual reproduction. Spontaneous, random mutations happen regularly and create genome diversity among the organisms. Genome diversity is also achieved through sexual and parasexual reproduction. While most mutations do not amount to any phenotypic changes, these events can still lead to or pave the way for organisms to become more fit in their current environment and prevail. Azoles do not contribute to the mutation rate of fungi as they do not affect the genome directly. It is through natural selection that azole resistance rises, and the drug is its main driving force (Verweij et al. 2016). When azoles are introduced, they apply a selection pressure to the environment, and susceptible strains are eradicated. Strains that have achieved resistance through spontaneous alterations on the genome are left to fill the gene pool (Brauer et al. 2019). Additionally, it has been discovered that fungal strains that harbour common resistance mutations are genetically less diverse than their wild type (WT) counterpart. This is an indication of such selection pressure events (Camps et al. 2012, Chowdhary et al. 2012).

There is a list of known mechanisms fungi have developed in order to adapt to azole exposure. Several mutations behind these mechanisms have also been identified, but there are still some that are unknown or not yet fully characterised. A number of genes have however been reported or suggested

to be related to mechanisms behind azole resistance in *A. fumigatus* and other fungi. The most investigated mutations are found on the *cyp51A* gene, as it is the gene that encodes the target enzyme of the azoles (Pérez-Cantero et al. 2020). The next few subchapters will cover some of the most known azole resistance mechanisms; an illustration of these is shown in Figure 5.

2.4.1 Reducing azole and enzyme affinity

The target site for azoles are highly specific, however a change in the structure of Cyp51A can lead to azoles losing their affinity with the enzyme (Pérez-Cantero et al. 2020). This is due to point mutations on *cyp51A*, which are changes in single base pairs in the sequence. In some cases, this can lead to a shift in the amino acid sequence, which then can lead to changes in the three-dimensional structure of the enzyme. These structural changes can furthermore affect the enzyme's susceptibility to azoles (Berger et al. 2017, Chen et al. 2020, Pérez-Cantero et al. 2020). The structure of the enzyme is conserved in order to maintain its highly specific function (Becher and Wirsel 2012), so a structural change that diminishes its affinity with azoles is quite delicate. Sometimes, a structural change can affect the stability and activity of the enzyme as well (Pérez-Cantero et al. 2020).

Some point mutations in *cyp51A* that have been reported to reduce the susceptibility to azoles in *A. fumigatus* are G54R (-W, -E, -K), Y121, G138C, P216L, F219I, M220V (-K, -T, -I), A284T, Y431C, G432S, G434C and G448S (Pérez-Cantero et al. 2020). In *A. niger*, the point mutations in the *cyp51A* homolog are V104I, H382R and 1337V/S507I/L511M, while in *A. tubingensis*, they are L21F and A9V/L21F/A140V/P413S/D505E (Hashimoto et al. 2017). Resistance acquired from point mutations are often associated in patients who receive long term azole treatments (Verweij et al. 2016). Some of the most common azole resistance point mutations that are not coupled with tandem repeats (TRs) are G448, M220, G138 and G54 (Sharma et al. 2015). A schematic illustration of some of these point mutations can be seen in Figure 4.

2.4.2 Overexpression of the target enzyme

The concentration of effector drugs inside the cell in relation to the concentration of the target enzyme is the factor that determines the cell's survival. The essential ergosterol biosynthesis can still be carried out despite the presence of azoles if the Cyp51 enzyme concentration is substantial enough to override the enzyme inhibition. (Pérez-Cantero et al. 2020). The fungus can achieve this by creating enough Cyp51 through overexpression of the gene encoding the protein (Berger et al. 2017).

Mutations that have been observed in relation to this resistance mechanism are tandem repeats (TRs) in the promoter region of *cyp51A*, coupled with point mutations in the sequence. TRs are copies of a short nucleotide sequence that are repeated in tandem (Brauer et al. 2019). These mutations include TR_{34}/L98H and TR_{46}/Y121F/T289A, and are found in *A. fumigatus* (Pérez-Cantero et al. 2020). A strain with the TR_{34}/L98H mutation contains 34 bp TRs in the promoter region of *cyp51A* and a base substitution at codon 98 of the sequence (See Figure 4), which replaces leucine with

histidine (Mellado et al. 2007). This mutation type often leads to resistance against itraconazole, voriconazole and posaconazole (Chowdhary et al. 2012, Hagiwara et al. 2016). Furthermore, strains that have obtained the TR_{46}/Y121F/T289A mutation have been reported to become highly resistant to voriconazole (van der Linden et al. 2013, Brauer et al. 2019). Both TR_{34}/L98H and TR_{46}/Y121F/T289A are common in strains that have acquired resistance through the environmental route (Latgé and Chamilos 2019).

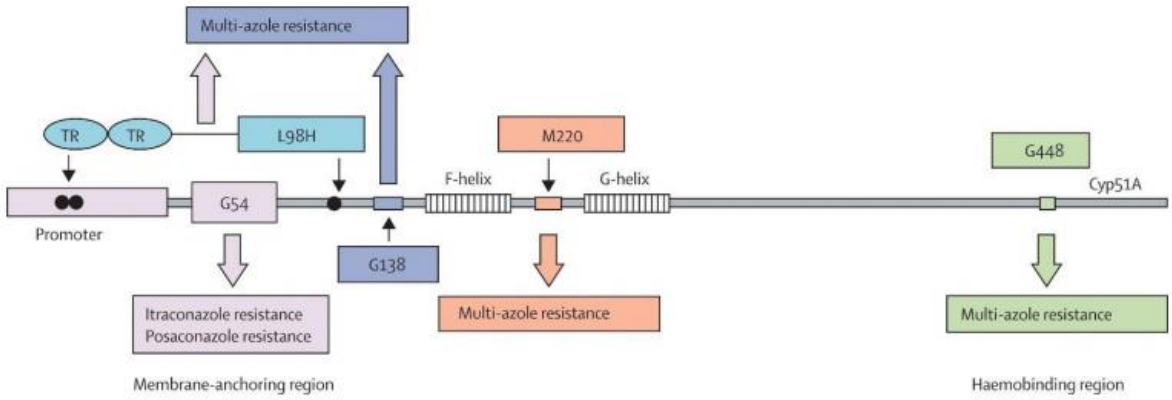


Figure 4: Schematic illustration over some known mutations on *cyp51A* that lead to azole resistance. Image was retrieved from Verweij et al. (2009).

2.4.3 Decreasing intracellular azole concentration through efflux pumps

Ergosterol biosynthesis takes place inside the fungal cell, so the azole drug needs to penetrate the cell in order to have an effect. With a high enough concentration of the drug inside the cell, it will bind to the target protein and inhibit its activity (Pérez-Cantero et al. 2020).

Cells can discard intracellular toxins out of the cell through transmembrane proteins called efflux pumps, such as ATP-binding cassette (ABC) transporters, or major facilitator superfamily (MFS) transporters. (Chen et al. 2020, Pérez-Cantero et al. 2020). If the cell has an overexpression of genes encoding efflux pumps, thus increasing the number of membrane transporters, it can lower the concentration of azoles inside the cells sufficiently for the cells' survival (Berger et al. 2017, Pérez-Cantero et al. 2020). The gene, *cdr1B*, have been reported to lead to azole resistance in *A. fumigatus* when upregulated (Fraczek et al. 2013, Paul et al. 2013).

2.4.4 Other known or proposed mechanisms

Inhibition from azoles can also be bypassed through using an entirely different enzymatic pathway in the ergosterol biosynthesis (Hagiwara et al. 2016). Alternatively, using a different sterol composition in the cell membrane could potentially be a resistance mechanism in fungi (Hitchcock et al. 1987). The affinity between the azole and cytochrome P450 is no longer relevant as the protein structure of the effector enzyme is something else entirely. The mutations behind this resistance mechanism are still unknown (Hagiwara et al. 2016).

Another mechanism that has been proposed is the degradation of azoles inside or outside the cell (Hagiwara et al. 2016). Few aspects of this pathway have been characterised, and the possible mutations related to it is unknown (Hagiwara et al. 2016, Pérez-Cantero et al. 2020).

Biofilm formation is a way for many microorganisms to avoid the immune system, and this can also be a relevant defence mechanism for fungi in a clinical setting. Biofilms may also make fungi less susceptible to azoles (Delattin et al. 2014). Pathogenic *A. fumigatus* has been reported to create biofilms as well (Ramage et al. 2012).

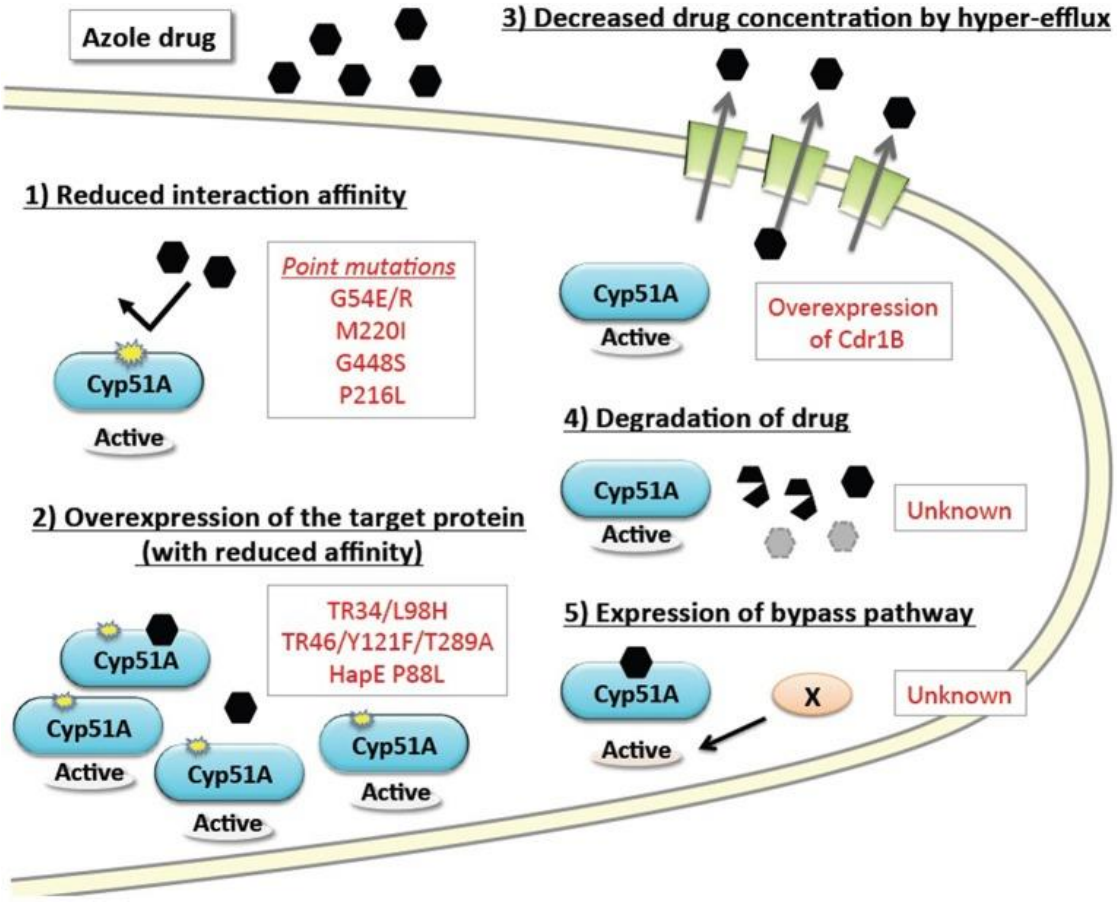


Figure 5: Schematic view of some known mechanisms of azole resistance in fungi. Retrieved from Hagiwara et al. (2016).

2.5 Causes and impact of azole use

There are two main routes in which *Aspergillus* spp. can develop azole resistance through exposure; in the environment where fungicides are used, and in patients receiving prolonged azole therapy (Hagiwara et al. 2016, Brauer et al. 2019). A concerning attribute is that an increasing amount of resistant strains found in patients harbour the TR_{34}/L98H and TR_{46}/Y121F/T289A mutations on *cyp51A*, which is a mutation found in most resistant *A. fumigatus* from the environment (Latgé and Chamilos 2019). This implies that people are getting infected by mutant strains originating from the

environment. Furthermore, it also suggests that the selection pressure in agriculture affects the health of humans and animals (Figure 7) (Brauer et al. 2019). Despite agriculture and medicine not using the exact same types of azoles, the structural similarities between some of the azoles could predispose the development of cross resistance when resistance against one compound is acquired (Snelders et al. 2012).

Azoles are one of the few efficient antifungal agents used to treat IA today, and it is because of this drug that fewer people die from aspergillosis compared to before it was first distributed commercially (Verweij et al. 2016). In recent years, azole resistant *A. fumigatus* has been found in an increasing number of human aspergillosis cases, which has become an issue in terms of effective treatment for patients with fungal infections. Resistant strains can be developed in patients with chronic aspergillosis and who are receiving long term azole treatments. Several types of point mutations on *cyp51A* in patients receiving therapeutic azole treatments have been recognised (Chowdhary et al. 2017).

The other route in which azoles are driving resistance is in the environment. The use of azoles in agriculture has become a major contributing factor that facilitates the rise of resistant fungal strains (Verweij et al. 2009). A correlation between azole fungicide use and prevalence of resistant *A. fumigatus* strains found has been reported (Cao et al. 2021). Agriculture uses azoles to secure safe food production with high yield by protecting plants from pathogens and preventing post-harvest spoilage (Jørgensen and Heick 2021). Additionally, azoles are also used in horticulture and wood industries (Skaar et al. 2019). The global use of pesticides in agriculture has increased significantly over the last decades (Brauer et al. 2019). DMI fungicides make up for 16% of the total amount of fungicides distributed today, and have doubled in the market for the last 25 years. Furthermore, over two thirds of the global amount of azoles are being distributed to Asia and Europe (Jørgensen and Heick 2021). A study in the U.S. showed that the amount of triazoles used has increased by 4-fold over the last couple of decades (Toda et al. 2021). The amount of triazoles sold in The Netherlands has doubled between 1995 and 2007 (Verweij et al. 2009). The total use of azoles in human systemic infections and agriculture in Norway between 2013 and 2017 can be seen in Figure 6 (Skaar et al. 2019). A study report done in The Netherlands identified that the three most prominent environmental hotspots for the development of azole resistant *A. fumigatus* were wood chippings, green compost and compost from flower bulb waste (Verweij 2017).

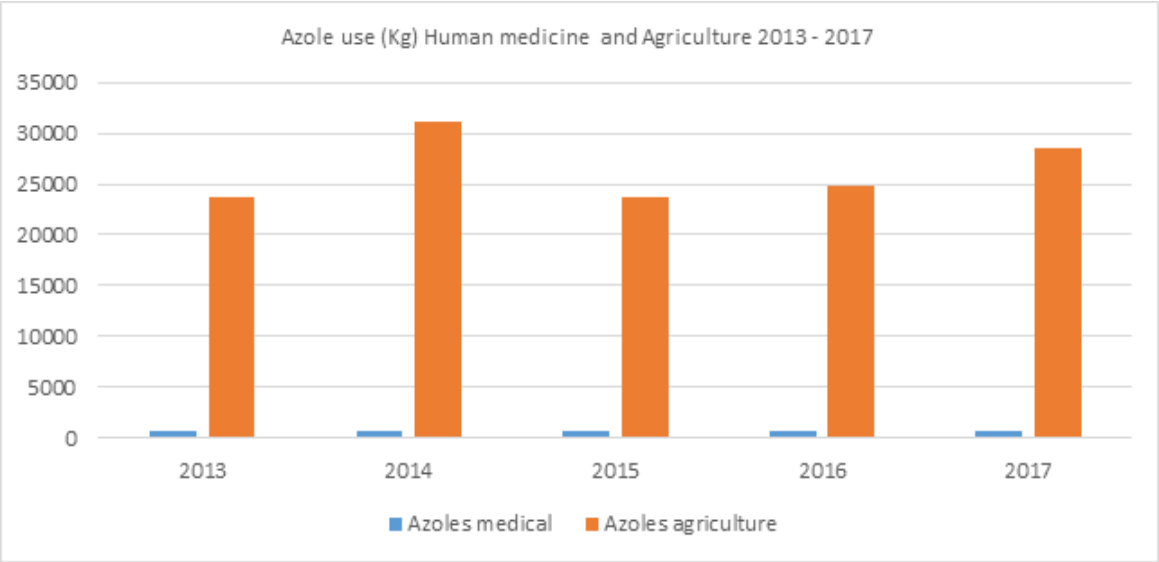


Figure 6: The total use of azoles for systemic infections (human medicine) and agriculture in Norway between 2013 and 2017. Retrieved from Skaar et al. (2019).

The first azole resistant *A. fumigatus* isolate was described in 1997 (Denning et al. 1997), and in recent years, there has been reported an increase in resistance in both clinical and environmental isolates (Pérez-Cantero et al. 2020). Significant levels of azole resistant *A. fumigatus* strains with environmental mutations have been detected in several surveillance studies in Europe, Asia and Africa (Verweij et al. 2015). A study in the UK found that 14% of their isolates taken from soil samples across the country were resistant to tebuconazole, which is a common fungicide used in British agriculture, and is often associated with cross resistance to medical azoles (Shelton et al. 2022). The study also found that a large majority of the tebuconazole resistant samples possessed the TR_{34}/L98H or TR_{46}/Y121F/T289A mutation in *cyp51A* and promoter region. In The Netherlands, azole resistance was found in about 7% of the *A. fumigatus* samples taken from patients between 2012 and 2016 (Buil et al. 2019). The *cyp51A* mutations, TR_{34}/L98H and TR_{46}/Y121F/T289A, have been found in 70% to over 90% of resistant isolates gathered from patients in the Netherlands, with over 70% of them not having had any history with azole therapy (Snelders et al. 2008, Verweij et al. 2016, Buil et al. 2019). In Denmark, they found resistance in 4-6% of their samples, and over 50% of the clinical samples had the TR_{34}/L98H mutation (Jensen et al. 2016). There was also a study in Vietnam found that over 50% of their samples were azole resistant, and most of them harboured the TR_{34}/L98H mutation (Duong et al. 2021).

New resistance mechanisms and mutations will likely appear along our current azole consumption, which will then limit their efficiency in clinical therapy. Unless new drugs are developed, the mortality rate of fungal infections is likely to rise (Verweij et al. 2016). Additionally, with temperatures and humidity rising with climate changes, *A. fumigatus* is expected to spread even more and increase its prevalence (Skaar et al. 2019). This concern has encouraged many countries to do surveillance studies in order to find the prevalence of resistant pathogenic fungi (Lestrade et al.

2019). Management of AMR requires a global effort. It is therefore important to monitor the resistance pathways and development in both the agricultural and clinical setting as well as spread awareness so authorities can take steps that will help keep azoles effective.

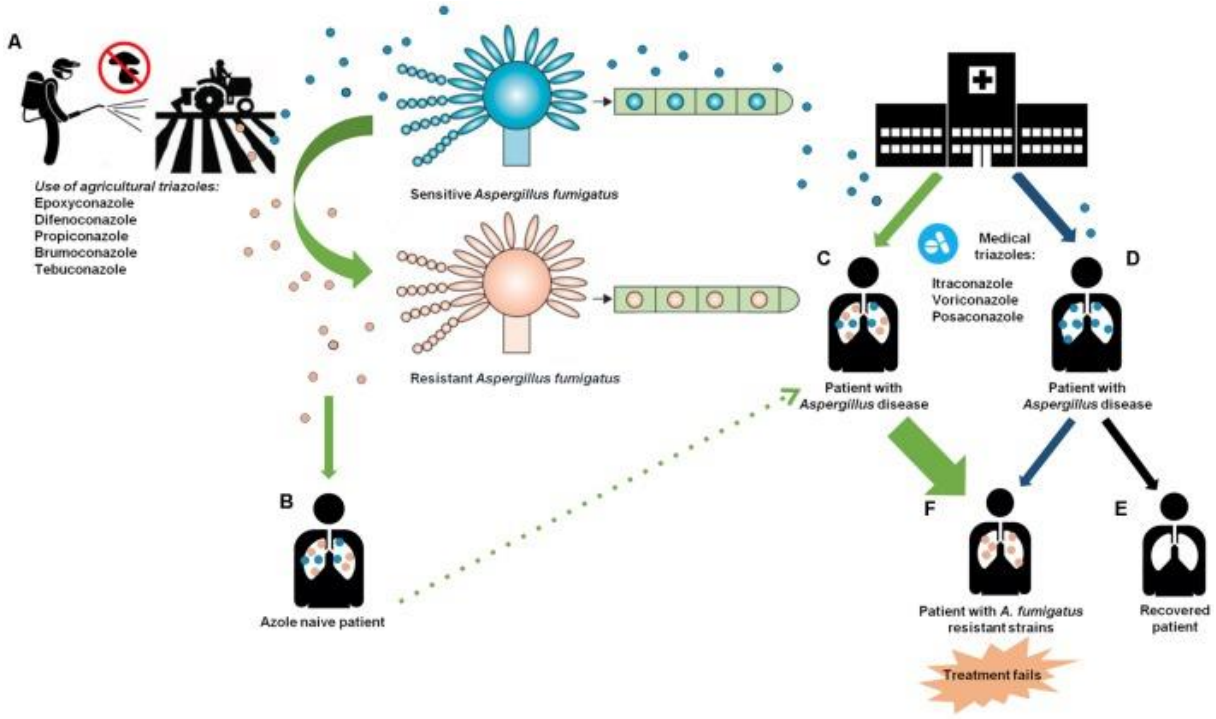


Figure 7: Schematic model of how azole resistance is developed. **A:** azole use in agriculture creates selection pressure in the environment, in which resistant strains are developed and propagate. **B:** People who get aspergillosis from *A. fumigatus* that has been subjected to selection pressure in the environment are **C:** admitted to the hospital for azole treatment. **D:** Other patients infected with WT aspergilli are also getting the same treatment. **E:** They either make a full recovery, **F:** or the treatment is ineffective due to azole resistant infection in the patient. Patients who were initially not infected with resistant strains can develop resistance due to selection pressure created from long-term treatments. Retrieved from Berger et al. (2017).

2.6 The theory and principles behind the experimental methods

2.6.1 Citizen science

Gathering samples in a wide field offers a variety of challenges; most noteworthy, time and expenses. Citizen science is an approach that has existed in over a century, but has in later years become a term used to describe the collaboration between scientists and the public community. The idea is to let volunteering non-researchers get involved in research by taking and sending samples to the laboratory for analysis, and/or process data. The citizen science approach is becoming more used in later years (Silvertown 2009).

The advantages of citizen science is that sampling becomes cheaper and less time consuming than if the samples were to be gathered manually. This has allowed scientists to study a wider field and larger numbers of samples (Silvertown 2009). Additionally, citizen science allows sampling over a wide area happen at the approximately same time, which contributes to a more representable model

(Shelton et al. 2020). Furthermore, citizen science has also been reported to be a good way to convey and spread knowledge and information about the subject of the research and raise awareness among people. (Bonney et al. 2016, Hecker et al. 2018).

The challenges of using citizen science for sampling is that it can affect the quality of the data as there are many different people involved in the sampling. According to Isaac et al. (2014), there are four main violations of fundamental principles making up for a good experimental design; firstly, the intensity of recording getting uneven over time. The second violation is an uneven coverage spaciously. Samples taken by the general public tend to correlate with population density, leading to geographical areas with few inhabitants being neglected (Mair and Ruete 2016). Depending on the aim of the study, a less than perfectly covered area can still provide valid data (Geldmann et al. 2016). If an evenly covered geographical area is necessary, the solution can be to gather samples of less populated areas manually, or to deem these spots negligible (Mair and Ruete 2016). The third violation is the uneven effort made by the participants (Isaac et al. 2014). There is little control over how the samples are taken, and there is a high probability that the effort varies among the participants. Equipment not being handled properly due to the lack of knowledge is also another matter of concern that can affect the quality of the data (Hecker et al. 2018). The last violation is that each volunteer has different levels of knowledge and experience (Isaac et al. 2014). With so many variables that can affect the quality of the data, it is recommended to use simple, well designed and standardised sampling methods with clear instructions, follow up and give feedback to volunteers and take the variables into consideration (Silvertown 2009).

2.6.2 Resistance screening

There are several laboratory techniques that are used to test a strain's susceptibility to antimicrobial substances; both culture based and molecularly. One of the commercially available azole resistance screening methods is the VIPcheck™, developed by Professor Paul Verweij, which is used to test both environmental and clinical fungal samples (Guinea et al. 2019). This four-well agar plate contains one well with 4 mg/L itraconazole, one with 2 mg/L voriconazole and one with 0.5 mg/L posaconazole (Buil et al. 2017). The fourth well is a control and contains no azoles. Phenotypic resistance can then be detected by inoculating the plates and observe where there is growth (Guinea et al. 2019).

A more quantitative culture based screening method is the E-test (epsilometer test), which reveals the minimum inhibitory concentration (MIC) of an antimicrobial agent on a microorganism. This test is used on clinical samples in order to plan the best treatment for the patient with the right antibiotic at the lowest efficient dose to restrict development of AMR (bioMérieux n.d.). The setup consists of an agar plate inoculated with a lawn of the isolate strain, which is then incubated with a plastic strip that has a concentration gradient of an antimicrobial agent (Joyce et al. 1992, bioMérieux

n.d.). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is a committee that works as a guideline on how to interpret breakpoints when testing strains for antimicrobial susceptibility. It is the MIC-value that indicates whether a strain can be considered resistant or a wild type (WT). The breakpoints for resistance is decided by a specific MIC-value from EUCAST's susceptibility tables, which is unique for each species at each antimicrobial agent (EUCAST 2022). What can be considered a WT in terms of resistance is decided by the epidemiological cut-off value (ECOFF). This value is based on breakpoints found in a species of microorganisms that do not display any phenotypic resistance across numerous studies. This distribution draws the upper breakpoint value for the WT criteria; a strain that has a MIC-value lower than ECOFF is likely a WT. However, this value cannot be used to evaluate drugs for medical treatment (Morrissey et al. 2014, Espinel-Ingroff and Turnidge 2016).

Molecular screening methods are used to confirm the resistance mechanism of an isolate. PCR and sequencing of the *cyp51A* gene is the most common method as this gene is often associated with azole resistance in *A. fumigatus* (Guinea et al. 2019). The sequence of the isolate strain is aligned and compared with other reference strains with bioinformatic softwares to detect mutations (Pontes et al. 2020).

2.6.3 Polymerase chain reaction

Molecular tools and methods have become more available over the last decade (Luo et al. 2018). These methods give highly specific information about the subject of analysis, which can be useful to identify species, strains and metabolic pathways (Houpikian and Raoult 2002).

Polymerase chain reaction (PCR) is an *in vitro* DNA replication reaction in which a selected nucleic acid sequence is being amplified. This cyclic reaction is controlled by adjusting the temperature of the reaction mix in specific time intervals. The exact temperatures used depend on the sequence that is being amplified and what components are being used. The reaction mix consists of a DNA template, which contains the nucleic acid sequence. There is also, nucleotides (dNTPs), a buffer, DNA-polymerase that withstands high temperatures and short oligonucleotide chains called primers, which are complementary to flanks of the sequence that will be amplified (Lorenz 2012).

The reaction starts by first denaturing the double-stranded DNA template into single-strand DNA; this is done by heating the reaction mix to 94-98 °C. Once the double-stranded structure denatures, primers can anneal to their complementary sequence on each of the template strand flanks. This happens when lowering the temperature to below the primers' melting temperature (T_m), which is often at 52-58 °C; this is called the annealing phase. The next phase is the elongation phase, where the temperature is risen to the optimal temperature for the DNA-polymerase. The primers are elongated in 5'→3' direction when complementary dNTPs bind to the chain along the template strand. Eventually, the newly synthesised chain is detached from the template when temperatures rise again, and the cycle

is repeated. The newly synthesised strand can also act as a template. This cycle is then repeated multiple times, and the concentration of the PCR product grows exponentially in the reaction mix. Once the cycles are complete, a longer elongation step is initiated, where incomplete amplicons can be synthesised. The PCR reaction is terminated when the temperature is lowered to around 4 °C indefinitely (Lorenz 2012). See Figure 8 for a schematic illustration of how PCR runs its course.

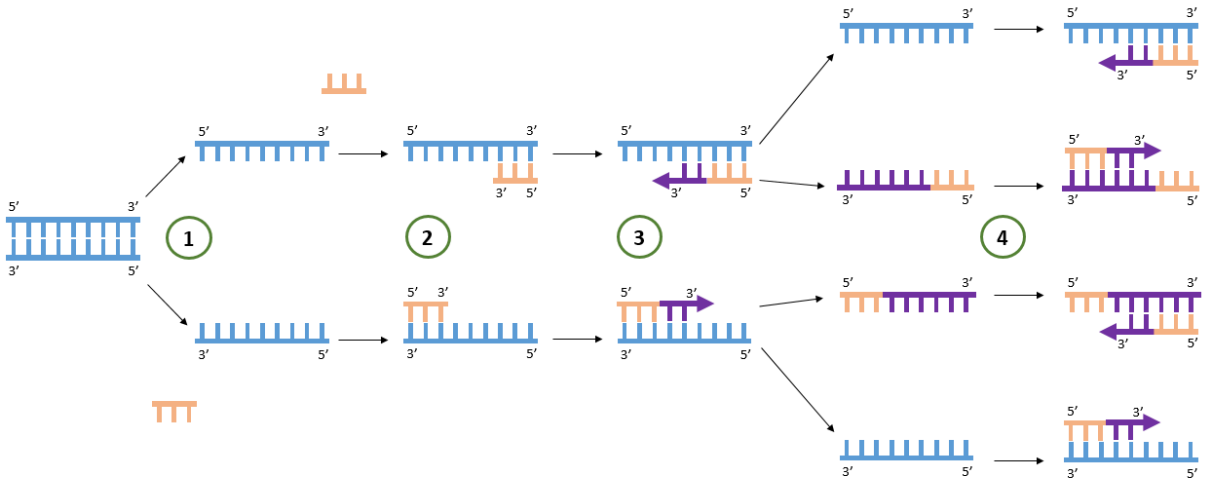


Figure 8: Schematic illustration of PCR. **1**; Template strand is denatured. **2**; Primers anneal to a specific site of each template strand. **3**; Chain synthesis by 5'→3' elongation of the primer on the template. **4**; The newly synthesised chain detaches from the template, making room for a new chain to be synthesised. The new chains can also act as a template.

2.6.4 Sanger sequencing

Sanger sequencing is a sequencing method that was developed by Frederick Sanger in 1977, and is still being used till this day (Totomoch-Serra et al. 2017). Other sequencing methods have also been developed since then, such as Illumina sequencing and Nanopore (Heather and Chain 2016).

The principle behind Sanger sequencing is to synthesise nucleotide chains, tag them with a fluorescent molecule, sort the chains by size and read the order of the fluorescent colour. Sequencing starts with a DNA template, e.g. the target gene that has been amplified by PCR. A primer complementary to the sequence flank is used to initiate the reaction. A DNA polymerase adds nucleotides to the chain like in PCR and DNA replication in cells, until a dideoxynucleotide (ddNTP) is added. The ddNTP is fluorescently tagged and terminates the polymerase chain reaction because it is unable to bond with the next dNTP. Each of the four nucleotides that are tagged is assigned a specific fluorescence colour so they can be recognised (Valencia et al. 2013, Heather and Chain 2016).

At the end of the reaction, several strands that differ in size by one nucleotide are present in the reaction mix; all of them are tagged with a fluorescent colour that indicates which nucleotide is at the end. All the strands are sorted by size through a form of gel electrophoresis called capillary based electrophoresis. A detector reads the order of strand size by fluorescing colour, which correlates to the order of nucleotides in the template strand. A chromatogram based on the reads is created, and thus, a

sequence can be determined (Valencia et al. 2013, Heather and Chain 2016). Sequencing the sense and antisense strands separately and aligning them bioinformatically afterwards is a common practice to restrict any ambiguous reads and achieve a longer consensus; especially towards the ends where the quality read tends to drop (Crossley et al. 2020).

Sanger sequencing is still a reliable sequencing tool today (Totomoch-Serra et al. 2017); it has an accuracy of up to 99,999% (Shendure and Ji 2008), and is most efficient to use for sequences that are up to 800-1000 bp long (Crossley et al. 2020). Longer strands can be sequenced with for example shotgun sequencing, in which short, random segments of the template are cloned and sequenced. The overlapping sequences can then be assembled with bioinformatic softwares (Heather and Chain 2016).

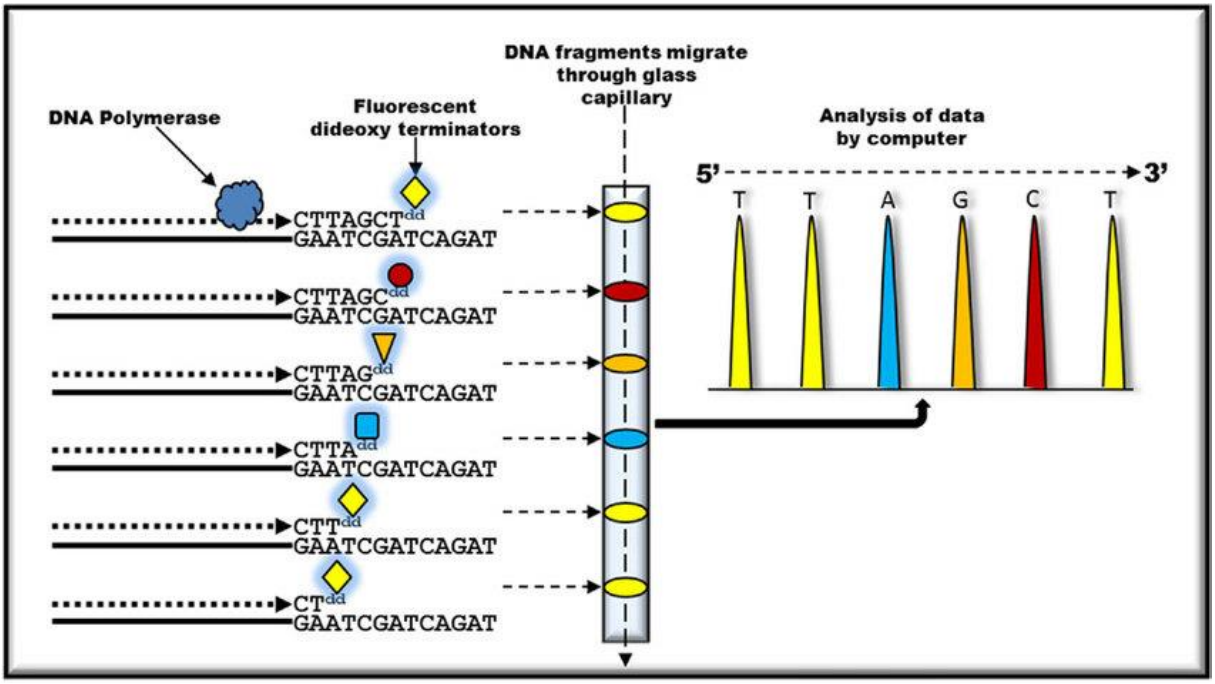


Figure 9: Simple schematic illustration of Sanger sequencing. Retrieved from Wangler and Bellen (2017).

3. Materials and methods

3.1 Experimental setup

3.1.1 Gathering participants

In this study, samples were gathered through a citizen science method approach; the volunteering participants were farmers from all over Norway. They were reached out to, asked to take air samples of their storage rooms and animal housing rooms and send them to our laboratory for analysis. Usually, participants in citizen science methods are reached out to through campaigns, advertisements, networks and social media. However, as this was the second sampling round of the "BARNS" research project, the same participants from the first round were contacted again through Email. During the first sampling round by Henriksen (2021), they had been reached out to through an advert in the magazine "*Bondebladet*" and the website "*Norges Bondelag*". While the first sampling round took place in January-March of 2021, the second round issued sampling during October-November of the same year. Using the same participants in both rounds would grant a more accurate representation upon comparing results between different seasons.

The first Email invited the old participants for the new round and was sent early September 2021. Once they confirmed they were interested, equipment was prepared and sent out using the same addresses gathered from the first sampling round; equipment was sent late September. An Email confirming the equipment had been mailed was sent soon after the consignment. Samples began coming in at the beginning of October and continued trickling in until early November. Participants that did not respond within two weeks after the first Email had been sent were issued a reminder. If participants had not sent their samples back within a month, they were sent another Email as a reminder. If the animal housing rooms were empty by the time the study was initiated, the participants were inquired to take samples two weeks after they had brought their livestock inside.

3.1.2 Sampling

The means and tools that are used to collect spores from the air are versatile. In this study, MicroAmp® Clear Adhesive Films by Thermo Fisher Scientific were used as spore traps; these films are normally used to cover 96-well PCR plates. They have a surface area of 8 cm x 14 cm, with a sticky surface area of 8 cm x 11.5 cm. They were also used for air sampling during the first sampling round of this study, and are efficient at catching any stray fungal spores that drift around in the air.

The participants received two adhesive PCR films each to sample the air from their farms with. The films were sent by mail along with a questionnaire to fill in (See Appendix 2), an instruction manual (See Appendix 1), a zip-lock bag, a return envelope and a return label. One of the short ends of the adhesive films had been taped shut to act as a hinge and make sampling easier to handle (See Figure 1 in the instruction manual in Appendix 1).

One of the two films was to be placed in the middle of an animal housing room if the participant had livestock, and the other was to be placed in a storage room selected by the participant themselves. The films were left with the sticky side up for 6 hours in these rooms to gather airborne spores. The participants were encouraged to take samples before the end of autumn so that samples were collected within the time frame of the warmer season. Additionally, a small, random selection of households was sampled for comparison. They were given the same sampling set and instructions as the farmers, and were inquired about the type of room sampled, the temperature of the room, number of residents and pets (See Appendix 6).

Once the sampling was complete, the films were sealed and put into the zip-lock bag before they were sent back to the laboratory together with the questionnaire. The samples were then ready to be cultivated and analysed.

3.1.3 Recurring lab techniques

Many laboratory techniques were repeated across several of the steps in the analysis process. These techniques will therefore be described in detail here and referenced in later text.

3.1.3.1 Sterile techniques

Working with microorganisms that propagate by air can be challenging in terms of avoiding cross contamination. Evaluation and test procedures on the cross contamination is written more in detail under section 3.1.5. In order to reduce the risk of cross contamination as much as possible, various sterile techniques were performed.

All work related to cultivated fungal colonies was done in a Labmodul Greenlife PRO airflow cabinet. Slow and gentle movements inside the cabinet while working was also done to reduce air turbulence. Anything that carried spores and was exposed to air, such as agar plates without their lids, was moved around minimally. Working with several samples at the same time was also avoided. The usage of gloves mostly applied during the molecular analyses.

The work space was wiped with 70% EtOH and a paper tissue before and after use. Wiping the work space was also done between handling every pure sample (for primary plates, this was only the case if they originated from different farms); normally, the work bench was wiped after working on one sample, then after at least 10 minutes, the bench was wiped again before moving onto the next sample. Hands were also disinfected with 70% EtOH between every sample.

Sterilising tools such as scissors and tweezers was done by dipping them in 96% EtOH and holding them under the gas flame until they stopped burning. For loops, the end was held under the flame until it was glowing red. The tools were cooled down in the air for approximately 30-60 seconds before use, or cooled down on clear agar. Sterile one-time-use tools such as plastic L-rods, plastic loops, 2 ml pipettes and swabs were also utilised during several occasions.

3.1.3.2 Plating techniques

During the study, three different techniques were used to plate spores on agar plates for cultivation or isolation.

The three point method involved using a sterilised loop, dipping it into the chosen colony or stock solution and tapping the end on three different points in a triangular formation onto the agar plate. This method was the most used plating method.

The streaking method was done using a loop and streaking the spores of a colony into a Z-formation. This method was done in cases where target colonies on primary plates or contaminated secondary plates were entangled in other colonies such as Mucorales or other species of *Aspergillus*. The streaking method drags the spores over a wider area, increasing the likelihood to achieve sections with only the target colony on the plate, and thus making it easier to isolate. The streaking method was also done when a substantial amount of fresh spores was needed for the analysis.

The drizzle method is a technique that was used whenever target colonies were too cluttered by other genera or species, such as Mucorales. A sterile loop was dipped into the target colony and held a couple of centimetres over a new agar plate while tapping a finger on the loop handle in order to drizzle spores over the plate; spores from *A. fumigatus* are much drier than Mucorales, so the principle was that the spores of Mucorales would remain on the loop, while *A. fumigatus* would fall onto the plate. The plates were also placed into 50 °C cabinets for incubation to further make sure only *A. fumigatus* would grow, as most other fungi are unable to grow in such high temperatures.

A variety of media was used throughout the study to cultivate the fungi, and each had properties that were more suited for their intentions. Dichloran-glycerol agar (DG18) contains less water than many other agars and is designed to select the growth of fungi with lower water activity and prevent bacteria from growing. It also discourages Mucorales from spreading as well as restricting colonies from growing too fast due to the dichloran component in the agar. The agar gives organisms of different requirements a more similar competition starting point, which allows more elusive organisms the opportunity to grow without being trampled by more competitive genera and species (Corry et al. 1995, p. 300-302). Media such as Sabouraud (SAB) and malt extract agar (MEA) are both excellent for cultivating fungi. SAB also contains antibiotics that inhibit bacterial growth and is often used to cultivate yeast (E. Christensen and E. Rolén, Personal Communication, 10.06.2022). The composition of each agar is listed in Table 25 in Appendix 4.

Most plates were packed into porous plastic bags that were either folded shut or shut tight with a rubber band before incubation. The only inoculated plates that were packed differently prior to incubation were the plates used in the E-test (See section 3.1.7.2).

3.1.3.3 Making spore suspensions

Suspending spores in solutions was done repeatedly throughout the study. While the solution in which the spores were suspended in varied, the principle in making the suspensions was the same.

In order to make the suspension, a sterile cotton swab was used to gather the spores from the agar plates. The cotton end was first soaked into the solution to restrict spores from spreading into the air too much while disturbing the colonies. In cases where the amount of spores produced by the fungus was minimal, i.e. very young colonies, it was better to quickly dip the swab into the solution instead of soaking it. Excess solution was removed from the cotton tip by pressing it against the walls of the tube with the solution.

The cotton tip was then gently rolled on the surface of the fungal colonies on the agar plate to gather spores. The spores were then suspended into the solution by rubbing the cotton tip at the bottom and walls of the tube. The suspension could be diluted by adding more of the base solution if the spore concentration was too high. The amount of spores was monitored using a Grant bio DEN-1B McFarland Densitometer if a specific spore concentration was required. The instrument measures light absorbance of a solution in McFarland (MF), which is proportionate to the cell/spore concentration. In a test tube, 0.00 MF is considered a blank solution.

3.1.4 Cultivation of the spore films and isolation of fungal colonies

Each spore film sample was placed on a square (11.5 cm x 11.5 cm) DG18 agar plate after their non-sticky ends had been snipped off using a pair of sterilised scissors (see section 3.1.3.1) to properly fit it into the petri dishes. Tweezers were used to place the films onto the plate, and a sterile, plastic L-rod was used to press the film properly down into the agar to get rid of any air bubbles. The plates were then left to incubate at 37 ± 1 °C. The spore films were removed from the plates with a pair of tweezers after 24 h and discarded, and the plates were left to further incubate for another 2-5 days at 37 ± 1 °C.

Fungal growth was inspected, described and photographed once they had become visible on the primary plates. All colonies were identified to group level and counted, and sometimes they had to be further examined under a microscope (Olympus BX-50) or stereo microscope (Nikon Stereo Microscope SMZ1270i) in order to determine their species or genera.

Microscope slides were prepared by adding a drop of lactofuchsin onto a glass slide, and a few drops of 70% EtOH on a separate slide. A piece of Sellotape Crystal Clear ultra transparent premium quality tape was gently pressed at the edge of the colony with the sticky side down. Excess spores were washed off in the EtOH drops, and afterwards, the piece of tape was pressed onto the slide with lactofuchsin. Using WypAll® X60 paper, the surface of the slide was properly pressed into place and wiped off of excess liquids. The slides could then be examined under the microscope using the settings as described in Table 3.

Table 3: Microscopy settings.

Objective	Phase	Immersion oil
10x	Ph1	No
20x	Ph1	No
40x	Ph2	No
100x	Ph3	Yes

Up to three individual colonies each of *Aspergillus fumigatus* and *Aspergillus niger* from each primary plate were selected and placed on round MEA or DG18 plates (9 cm in diameter) and left for incubation at 37 ± 1 °C for 2-5 days. The main plating technique that was used for secondary plates was the three point method (See section 3.1.3.2). It was attempted to select colonies that differed visually from each other in terms of shape and colour whenever they were distinguishable. If the target colonies were cluttered by other fungal colonies, either the Z-streaking method or the drizzle method were used to separate the target colonies from contaminating fungi (See section 3.1.3.2). There were a few secondary plates where Mucorales or other *Aspergillus* spp. persisted, and it was in these cases where the isolation methods had to be repeated in order to obtain pure samples.

All secondary plates were marked according to which farm they were sampled from (Farm number G1, G2, G3 etc., which were distributed to designated farms during the first sampling round of the study), and what room they originated from (L2 for storage room, and H2 for animal housing room). The secondary isolates were also marked S1, S2 and S3 for *A. fumigatus*, and A1, A2 and A3 for *A. niger*. A total of 58 *A. fumigatus* and 37 *A. niger* isolates were collected from this round of the study.

The square primary plates were stored in sealable box containers in room temperature in case the secondary plates needed to be redone. Once spores had been produced on the secondary plates, the isolates could be further processed and analysed.

3.1.5 Contamination test

As spores from *Aspergillus* spp., especially *A. fumigatus*, spread easily through airflow, cross contamination was a very possible issue during this study. Some of this issue was resolved by using an airflow cabinet, using sterile techniques and avoiding working with several samples at the same time. To better determine the risk of cross contamination, a separate troubleshooting experiment was conducted; based on a similar experiment that was done by Henriksen (2021).

Eight MEA plates were used to monitor the contamination risk over the first 8 minutes after a random *A. fumigatus* plate had been processed, and the airflow cabinet had been wiped with 70% EtOH. Four of the plates were placed right at the centre where the secondary plate had been processed

(M), and the other four were placed about 30-40 cm to the side of the field of work (S). One M- and S-plate were immediately placed to their designated spots in the airflow cabinet after spore transfer of the secondary plate and EtOH wiping were finished. Their lids were kept open for 2 minutes before they were replaced by the next pair in line. This was monitored using a stop watch and continued until all plates had been exposed for about 2 minutes each. All the plates were put into incubation in 37 ± 1 °C for 3-7 days.

Table 4: Overview of plate labels and time of placement. Each plate was left inside the airflow cabinet without their lids for 2 minutes.

Time of placement	Middle	Side
0 min	M0	S0
2 min	M2	S2
4 min	M4	S4
6 min	M6	S6

The same procedure was repeated one more time the exact same way, and another time with DG18 plates where the airflow cabinet had been exposed to *A. niger*.

3.1.6 Glycerol stock for storage

A spore stock solution was made from each pure secondary plate for long term storage. Spores from the secondary plates were suspended into two individual 1 ml, 10% glycerol tubes, using a sterile swab (see section 3.1.3.3). Only one swab was used to make both spore stock solutions for each isolate.

One tube was stored in 5 ± 1 °C and was used as a stock solution for other analyses further down the line such as the E-test. The second tube was stored in -75 ± 15 °C for long term storage. If the tests showed that the strains expressed azole resistance, the tubes were included in NVI's strain collection.

3.1.7 Resistance screening

3.1.7.1 VIPcheck™

A VIPcheck™ (Mediaproducts BV, Groningen, The Netherlands) is a tool used to screen for azole resistance in fungal isolates. The method is simple and relatively quick to execute, which is suitable for screening. The VIPcheck™ plate is a four well agar plate imbued with medical azoles; one well is with 4 mg/L itraconazole, one with 2 mg/L voriconazole, one with 0.5 mg/L posaconazole, and one is a control without azoles. Figure 10 displays the schematic setup of a VIPcheck™ plate. To make sure the same amount of spores is inoculated into each well, a spore suspension is made in advance. The MF-value to use in VIPcheck™ screening is 0.50-2.0 MF. The protocol used to set up VIPcheck™ is retrieved from VIPcheckTM (2015).

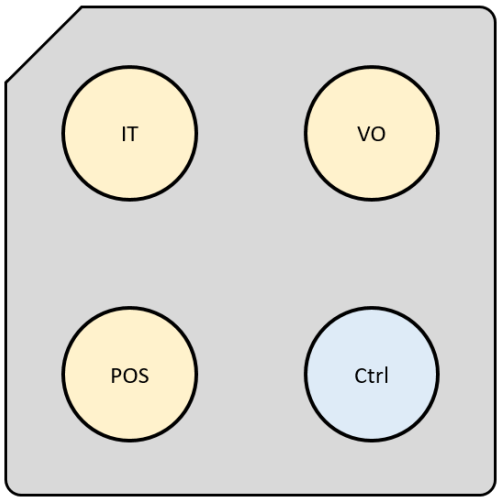


Figure 10: Schematic view of the set-up of the VIPcheck™ plate. IT = Itraconazole (4 mg/L), VO = voriconazole (2 mg/L), POS = posaconazole (0.5 mg/L), Ctrl = Control.

The VIPcheck™ was done accordingly for each *A. fumigatus* and *A. niger* isolate: spores from a secondary plate were suspended in an autoclaved test tube with 5 ml – 10 ml distilled water using a sterile swab to gather the spores from the plate (see section 3.1.3.3). It was aimed to achieve a MF-value that was similar across all spore suspensions so all the VIPcheck™ analyses had a similar starting point; around 1.1 MF. After the spore suspension had been made, a drop was immediately placed into each of the four wells on the VIPcheck™ plate using a sterile plastic pipette.

The VIPcheck™ plates were then placed into 37 ± 1 °C for incubation. The plates were analysed 24 h and 48 h after inoculation, and rated -, (+), +, ++ and +++ according to observations of growth.

3.1.7.2 E-test

The E-test is an *in vitro* phenotypic resistance test that is more quantitative than VIPcheck™, and are commonly used to determine the minimum inhibitory concentration (the MIC-value) of an antimicrobial agent against a microorganism. Here, Itraconazole, voriconazole and posaconazole ETEST® strips (bioMérieux, Marcy-l'Étoile, France) were used for the E-test; there is a concentration gradient of antifungals of 0.002 µg/ml – 32 µg/ml along these plastic strips. RPMI (Roswell Park Memorial Institute) agar plates are used to inoculate a lawn of the isolates to conduct the E-test on. This medium is used to cultivate cells and promotes healthy cell growth as it contains a wide variety of vitamins and amino acids (Incyte Diagnostics 2018, Thermo Fisher Scientific n.d.). The ETEST® strips are placed on top of the inoculated agar plate and are read after incubation. The edge of the inhibitory zone defines the MIC-value, which determines the susceptibility of the fungal isolate. Whether an isolate is resistant or sensitive to a drug is decided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These MIC-values are listed in a regularly updated table, which is used as reference in drug susceptibility testing (EUCAST 2022). The protocol for the E-test was retrieved from (bioMérieux 2016).

Isolates that showed signs of resistance on either of the azoles in the VIPcheck™ (i.e. a score of ++ and/or +++) were further screened with the E-test to confirm the results from the VIPcheck™. Four isolates of *A. fumigatus* and yeasts (*Candida parapsilosis*, *Candida albicans* and *Candida krusei*) with known MIC-values were used as reference strains for the E-test (See Table 5). Three additional resistant *A. fumigatus* strains from clinical isolates stored in NVI's strain collection were also screened with the E-test (See Table 34 in Appendix 14).

Table 5: Overview of the reference strains used for the E-test and the control MIC-values. Retrieved from bioMérieux (2018) and ATCC (2020).

Sample ID	Species	Control MIC after 48 h (µg/ml)		
		Itraconazole	Voriconazole	Posaconazole
ATCC MYA 4609	*A. fumigatus*	0.12	0.5	0.06
ATCC 22019	*C. parapsilosis*	0.064-0.25	0.016-0.064	0.032-0.25
CCUG 32723*	*C. albicans*	0.064-0.25	0.004-0.016	0.032-0.125
CCUG 35869*	*C. krusei*	0.25-1	0.25-1	0.125-0.5

*The MIC-values of these strains were listed from ATCC 6258 (*A. krusei*) and ATCC 90028 (*A. albicans*) strains in the tables of bioMérieux (2018).

All samples that were going to be tested were cultivated on DG18 agar plates, using the three point method from the glycerol stock solution stored in 5 ± 1 °C, or streaked on SAB plates before they were left to incubate in 30 ± 1 °C (see section 3.1.3.2). The E-test required spores from fresh plates, so the tests were therefore done two-three days after inoculation for *Aspergillus*, and one day after for yeasts.

A. fumigatus and *A. niger* were plated on RPMI plates by first making a spore suspension. First, a saturated spore suspension was made in a test tube with 4.5 ml physiological saltwater (see section 3.1.3.3), and left to rest on the bench for 10-15 minutes until unsuspended spores had either sunken to the bottom or floated to the top. The suspension was then diluted further into another test tube of 4.5 ml saline water, using a pipette, until the dilution had an MF-value of 0.45-0.55. In this set-up, the tubes' MF-value varied between 0.50 and 0.56 MF (See Table 19). The entire content was poured into the RPMI dish and spread around evenly by tilting the plate. Excess water was removed with a pipette. The plates were set aside to dry for 10-20 minutes with the lid slightly open in the airflow cabinet.

For yeast cultures, the colonies were transferred directly into a 4.5 ml test tube of saline water with a sterile swab and suspended until the solution had reached a MF-value of 0.50. Using a new

swab, the cotton tip was soaked into the suspension; excess liquid was removed by pressing the tip against the walls of the test tube. The swab was then used to streak evenly over the entire surface of the RPMI plate in three layers; each layer having streaks in different directions. The swab was soaked into the suspension one more time, and the same procedure was repeated. The plates were then left to dry for approximately 10 minutes in the airflow cabinet.

Once dry, the ETEST® strips were placed at the centre of the RPMI plate using a pair of sterile tweezers. The plates could hold up to two strips each, and in these instances, the strips were placed parallel from each other with some distance between them and the gradient going in opposite directions. A damp tissue paper was placed at the bottom of a zip-lock bag before the plates with the ETEST® strips applied were put into the bag. The bags were then partly sealed (the plates needed to incubate aerobically) before they were incubated at 37 ± 1 °C for approximately 24 h for *Aspergillus* samples, and 48 h for yeast samples.

After 20 h, the MIC breaking point for each azole strip was recorded and compared with EUCAST's tables (EUCAST 2022). Samples with a MIC-value indicating resistance would be further characterised molecularly by sequencing the *cyp51A* gene and promoter region. The MIC-values of the reference strains were used to evaluate the accuracy of the E-test, using Table 5.

3.1.7.3 MF-value and spore count

The Grant bio DEN-1B McFarland Densitometer was used in both the VIPcheck™ and the E-test during a few important steps of the procedures. As stated in section 3.1.3.3, this instrument measures absorption of light, which is proportionate to cell/spore concentration. As *A. niger* has much darker pigmented spores than *A. fumigatus*, it was anticipated that this would influence the MF-values; in other words, the spore concentration could be significantly different between the two species at the same MF-value. A simple experiment was therefore conducted to correlate MF-values and spore concentration for *A. niger* and *A. fumigatus*.

A spore suspension of each *Aspergillus* sp. was made in 10 ml of PBS (phosphate-buffered saline), 0.1% Tween® 20 solution (see section 3.1.3.3) by using randomly selected secondary samples. The buffer creates better conditions for the spores and prevents them from rupturing, and Tween 20 is an emulsifying agent to prevent the spores from clumping together. The spore suspension rested on the bench for 10 minutes before it was further diluted into another test tube with 5 ml PBS, 0.1% Tween 20 until it had obtained an MF-value of 0.50.

The following steps are based on LO-Laboroptik (n.d.). A haemocytometer (Marienfeld, Berlin, Germany) was prepared using a sterile cotton swab dipped in water to wet the mounting supports. The cover glass was carefully slid on top of the haemocytometer, and the clamps were gently pressed into place until interference rings were visible on the mounting support (Newton rings); that indicated that the cover glass had the correct distance from the haemocytometer. The haemocytometer

had two separate Bürker-Türk grids, so both spore suspensions could be inserted on the same slide at the same time (See Figure 11). Each grid contained four counting chambers, which were divided into 16 squares.

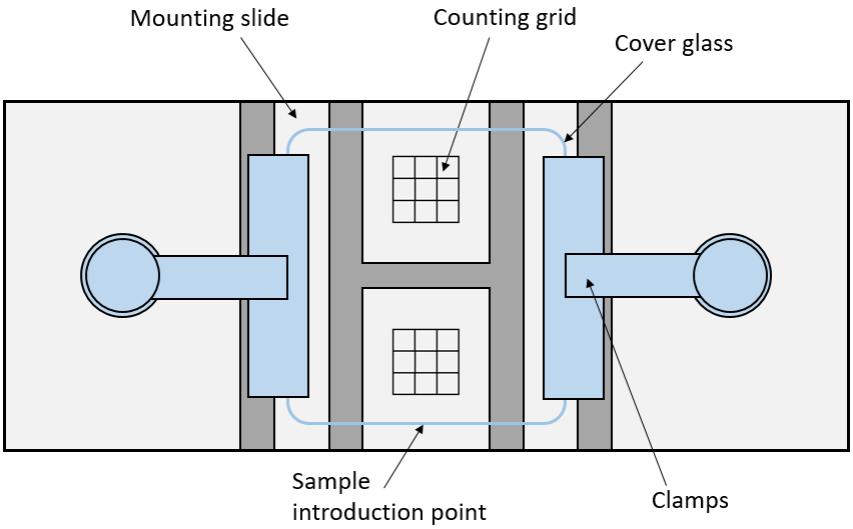


Figure 11: Schematic view of a haemocytometer with two counting grids.

A drop of the spore suspension was placed on a plain glass slide with a plastic pipette for easier access. Using an automat pipette, 7-8 µl of the spore suspensions was gathered. The tip of the pipette was placed at the sample introduction point (See Figure 11) in an angle of approximately 45° to 60° and inserted until the grid was covered in the suspension; driven by capillary forces.

Using 10x objective on the microscope, all four 16-squares chambers in the grid were counted for spores on each Bürker-Türk grid. See Figure 12 to see how the spore placements were interpreted in relation to the lines of the grid. The following formula was used to calculate the number of colony forming units (CFU) per ml:

$$\bar{n} = \frac{n_1 + n_2 + n_3 + n_4}{4}$$

$$[Spores] = \bar{n} \cdot 10^4 = CFU/ml$$

$n_1 =$ number of spores in chamber 1

$n_2 =$ number of spores in chamber 2

$n_3 =$ number of spores in chamber 3

$n_4 =$ number of spores in chamber 4

$\bar{n} =$ average number of spores per chamber

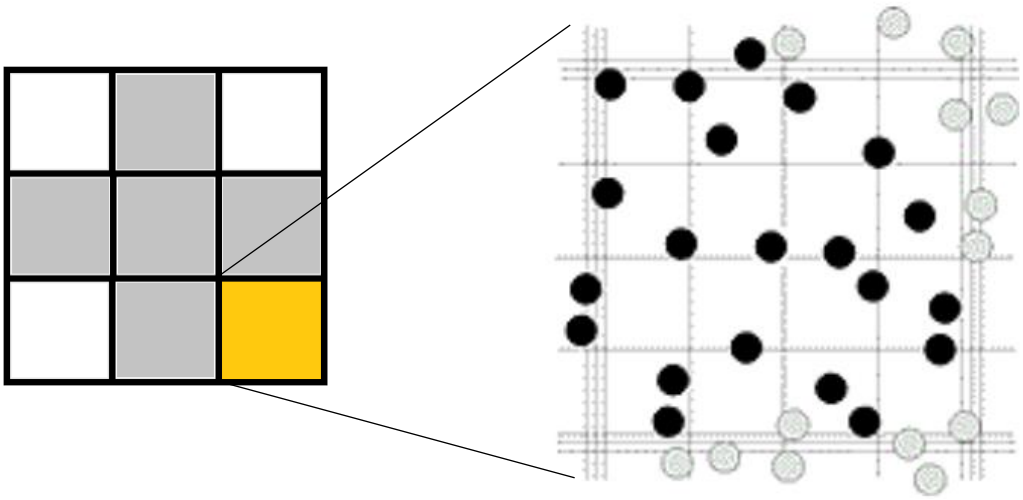


Figure 12: Rules applied for the counting grid. Spores that are coloured black were counted, while the grey were left uncounted. Adapted from LO-Laboroptik (n.d.).

The experiment was repeated twice with fresh 0.50 MF spore suspensions for each replicate.

3.2 Molecular

3.2.1 DNA extraction

The process of extracting DNA started with a spore suspension in a microtube. A sterile steel bead of 4 mm in diameter was placed into a 2 ml microtube together with 350 µl lysis buffer (AL-buffer from QIAGEN®, Vienna, Austria). Spores from a secondary plate were then suspended into the microtube (see section 3.1.3.3) until the suspension had turned dark and murky. Each tube was marked with a designated number.

The tubes were sealed and homogenised using a RETSCH® mixer mill for 3 minutes at 25 Hz. The samples were then spun down in a centrifuge for a few seconds to compress some of the foam created during the homogenisation. After that, 10 µl of 20 mg/ml Proteinase K (Sigma-Aldrich, St. Louis, Missouri) was added in each tube, mixed on a vortex for a couple of seconds and placed on a Thermomixer heating block at 56 °C, shaking on 550 rpm for 30 minutes.

Once the mixing had ended, the tubes were centrifuged for 5 minutes at 12 000 x g, and 200 µl of the supernatant was transferred into new 2 ml microtubes. All the samples were stored in -20 ± 1 °C until further extraction with QIAcube Connect by QIAGEN®. For every 11th sample, an extraction blank control (EBK) was also made using the exact same methods as the samples, except no spores nor substitutes were added.

DNA extraction was performed on a QIAcube machine, using the QIAamp® DNA Mini Kit (Vienna, Austria). With each run containing 11 samples and 1 EBK. The setting used on QIAcube for

the DNA extraction was DNA > QIAamp DNA Mini Kit > Tissue > Standard. The reagents inserted into the machine for DNA extraction were absolute EtOH, lysis buffer AL, wash buffers AW1 and AW2, and elution buffer, AE; these reagents, along with the QIAamp spin column, came from the kit.

The extracted DNA samples were stored in -20 ± 1 °C. DNA concentration was also measured in a few of the DNA samples using NanoDrop™ One to get an approximate measurement among all the samples; this was to see how successful the DNA extraction was, and to estimate if the amount would be sufficient for PCR.

3.2.2 PCR

PCR was used to amplify gene fragments prior to sequencing. To confirm the species of the fungi, either parts of the calmodulin gene (*caM*) or β-tubulin (*bT2*) were amplified and sequenced. For this set of isolates, *caM* was the main marker used for species identification; the protocol used for this PCR was based on Hong et al. (2005). The *bT2* gene was used for *A. niger* and a few *A. fumigatus* isolates whenever *caM* PCR was insufficient; the protocol used for *bT2* PCR was based on Glass and Donaldson (1995). The *cyp51A* gene and promoter region were sequenced to search for mutations that indicate azole resistance in the fungi. The protocol for the *cyp51A* PCR was based on the article from Mellado et al. (2001) and Mortensen et al. (2011).

Table 6: Forward and reverse primers of the genes that were amplified with PCR in this study.

Gene	Primers	Direction	Sequence (5' → 3')
caM	CMD5	Fwd	CCGAGTACAAGGAGGCCTTC
	CMD6	Rev	CCGATAGAGGTCATAACGTGG
bT2	BT2A	Fwd	GGTAACCAAATCGGTGCTGCTTTC
	BT2B	Rev	ACCCTCAGTGTAGTGACCCTTGGC
cyp51A	P-A07	Fwd	TCATATGTTGCTCAGCGG
	cyp51A_4R	Rev	CCTATTCCGATCACACCAAA

3.2.2.1 Species identification – caM and bT2 PCR

Since there were several samples that had to be sequenced, a master mix was made prior to the PCR; this is a mix of all components needed for the reaction except for the DNA template. The concentration and volumes of all the components in the master mix were pre-calculated and dependent on the number of samples. For PCR of *caM* and *bT2*, the master mix contained only Milli-Q water and primers. Volumes of each component used per sample reaction can be viewed in Table 7 for *caM* PCR and Table 8 for *bT2* PCR. For each master mix made, a negative control was also prepared where the template was replaced with Milli-Q water.

In order to make the master mix, the primer solutions were first defrosted and stirred with a vortex. In *caM* PCR, forward primer CMD5 and reverse primer CMD6 were used, while *bT2* PCR used forward primer Bt2A and reverse primer Bt2B (See Table 6). The correct amount of each primer was measured according to the number of samples and added into a 1.5 ml microtube. The Milli-Q water was then added to complete the master mix. The master mix was mixed with a vortex and spun down with a centrifuge to collect droplets along the walls of the tube.

The PCR-kit used for species identification of the isolates was GE Healthcare 27955702 illustra™ PuReTaq Ready-To-Go™ PCR beads (Buckinghamshire, UK). These dry beads contain all components needed for PCR except the primers and the template.

Table 7: Added volumes of each component for *caM* PCR. Master mix contained CMD5, CMD6 and MQ.

Component	Volume (µl)
CMD5 (5 µM)	1.5
CMD6 (5 µM)	1.5
MQ	18
PuReTaq™ Ready-To-Go™ PCR bead	-
DNA template	4
Total volume	25

Table 8: Added volumes of each component for *bT2* PCR. Master mix contained Bt2A, Bt2B and MQ.

Component	Volume (µl)
Bt2A (5 µM)	3
Bt2B (5 µM)	3
MQ	17
PuReTaq™ Ready-To-Go™ PCR	-
DNA template	2
Total volume	25

The end volume of each reaction mix for both *caM* and *bT2* PCR was 25 µl. In the *caM* PCR, 21 µl of the master mix was added into a PCR tube with a bead from the kit along with 4 µl template made in section 3.2.1. For *bT2* PCR, 23 µl of the master mix and 2 µl of the template were added into a PCR tube with a bead. The DNA templates were all defrosted and mixed with a vortex before use. Once everything was added into the PCR tube, the tubes were sealed shut with lids and gently tapped against the table surface a few times to mix. The tubes were then spun down with a centrifuge to collect all droplets to the bottom and get rid of any air bubbles.

The tubes were then placed into a Bio-Rad 96-well T100™ Thermal Cycler PCR machine, or a Thermo Fisher Scientific Veriti 96 Well Thermal Cycler PCR machine. The program used can be seen in Table 9 for *caM*, and Table 10 for *bT2*. All the PCR products were stored in -20 ± 1 °C for further analyses.

Table 9: PCR program for *caM*.

Temperature	Time	
95 °C	4 min	
95 °C	1 min	x 35
55 °C	1 min	
72 °C	2 min	
72 °C	8 min	
12 °C	∞	

Table 10: PCR program for *bT2*.

Temperature	Time	
95 °C	5 min	
95 °C	30 sec	x 35
58 °C	20 sec	
72 °C	30 sec	
72 °C	5 min	
8 °C	∞	

3.2.2.2 PCR on cyp51A

The isolates that showed azole resistance during the E-test in section 3.1.7.2 were selected to have their *cyp51A* and promoter region sequenced for mutation. Dissimilar to the PCR for species identification, *cyp51A* PCR used components from Thermo Fisher Scientific (Waltham, Massachusetts) for the master mix; it consisted of 10x Dream Taq buffer, dNTP and Dream Taq DNA polymerase along with the primers and Milli-Q water (See Table 11). The forward primer used in this PCR was P-A07, while the reverse primer was cyp51A-4R (See Table 6). The volumes of each component in the master mix was calculated according to the number of samples, mixed together in a 1.5 ml microtube by pipetting the mix up and down a few times and spun down in a centrifuge to collect droplets.

Table 11: Added volumes of each component for *cyp51A* PCR.

Component	Volume (µl)
10x Dream Taq buffer	5
dNTP (10 mM)	1
P-A07 (10 µM)	1
cyp51A-4R (10 µM)	1
Dream Taq (5 U/µl)	0.25
MQ	37.75
DNA template	4
Total volume	50

In each PCR tube, 46 µl of the master mix and 4 µl of vortexed DNA template were mixed together, sealed and spun down in a centrifuge to collect all the droplets and get rid of the air bubbles. The tubes were then placed into the PCR machine from BIO-RAD or Thermo Fisher Scientific (See Table 22 in Appendix 3) with the program in Table 12 running. The PCR products were stored in -20 ± 1 °C afterwards.

Table 12: PCR program for *cyp51A*.

Temperature	Time	
95 °C	2 min	
95 °C	30 sec	x 35
58 °C	30 sec	
72 °C	1 min, 30 sec	
72 °C	7 min	
8 °C	∞	

3.2.3 Gel electrophoresis

All PCR products that were made in this study were run on gel electrophoresis after the reaction to verify whether the amplification was successful. This process separates DNA fragments by size; this is done by running the amplicons through a gel with an electrical current. The TBE (Tris-borate-EDTA) buffer used in gel electrophoresis creates conditions that enable DNA to obtain maximum negative charge so they can travel with the current. The gel matrix creates resistance so the speed of the DNA fragments is proportional with their size; shorter fragments will then be able to travel further than longer fragments. The gel is also stained with GelRed™ dye that creates fluorescent light under ultraviolet (UV) light when bound to nucleic acids; this makes the samples visible to the eye (Yılmaz et al. 2012).

For *caM* and *bT2* PCR product, a 1.5% agarose gel was made for the electrophoresis; firstly, 1.50 g peqGOLD agarose universal powder (VWR, Spain) was weighed. After adding the agarose into a flask, 100 ml 1:10 dilution TBE buffer was measured and added in as well. To solve the agarose into the buffer, the mix was put into the microwave on medium high effect. Once all the agarose was fully dissolved, 10 µl GelRed™ (Sigma-Aldrich, St. Louis, Missouri) was added into the content of the flask. Later in the study, 7 µl for 100 ml agarose mix was used as that was sufficient as well. GelRed™ was homogenised into the agarose mix by swirling the content in the flask around before it was poured into the mould with the appropriate comb (or combs) for the wells. For *cyp51A* PCR products, 1.0% agarose gel was prepared instead. See Table 13 for an overview of the different gels and measurements used throughout the procedure.

Table 13: Measurements used for different gels.

Gel	Agarose (g)	1:10 dilution TBE (ml)	GelRed™ (µl)
1.5% agarose (100 ml)	1.50	100	7
1.5% agarose (50 ml)	0.75	50	3.5
1.0% agarose gel (50 ml)	0.50	50	3.5
1.0% agarose gel (120 ml)	1.20	120	8

The comb was carefully extracted from the gel once it had settled. The gel was then taken out from the mould and submerged into an electrophoresis box with 1:10 dilution TBE buffer. More buffer was added if the gel was not fully submerged.

The PCR products were prepared by mixing 5 µl of the product with 1 µl 6X DNA Loading Dye (Thermo Scientific™, Vilnius, Lithuania), by pipetting up and down 5-7 times. The dye would increase the density and visibility of the samples so they would sink into the wells and become easier to monitor during electrophoresis. The entire mix (6 µl) was carefully loaded into each well in the gel. Along with the *caM*- and *bT2*-amplicons, 3.5 µl GeneRuler 1 kb DNA Ladder (Thermo Scientific™, Lithuania), was added into the first well of the gel. In instances where there were more than 15 samples, 3.5 µl of the ladder was also loaded at the other end of the row. In gel electrophoresis on *cyp51A*, 3.0 µl ladder was used instead. The ladder was usually added as the last step before the electrophoresis started. Figure 13 displays the lengths of each band of the ladder and was used as reference for interpreting the chain size of the PCR products.

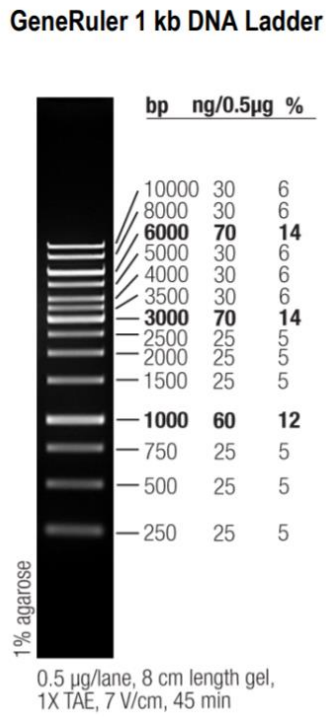


Figure 13: Reference of the GeneRuler 1 kb DNA Ladder by Thermo Scientific™, (Vilnius, Lithuania). Retrieved from Thermo Fisher Scientific (2019).

Once all the samples were loaded, an electric current (90 V and ~80 mA) was applied to the instrument for 40 minutes to 1 hour. For the *cyp51A* PCR product with 1.0% agarose gel, the electrophoresis was run on 100 V and ~90 mA for 50 minutes to 1 hour. Once the electrophoresis was complete, pictures of the gel were taken; UV302, 5 seconds exposure time with an Azure™ c150 geldoc instrument.

3.2.4 Gel purification

The results of the gel electrophoresis on *cyp51A* PCR product revealed some impurities, which seemed to be persistent. The impurities were contained in a different band size, and it was decided that gel purification was necessary for some of the samples.

A 120 ml, 1.0% agarose gel was made and moulded with 8 µl GelRed™ (See Table 13). All the PCR products were loaded with loading dye (1 µl for every 5 µl sample) and inserted into the wells as full as possible; the aim was to use as few wells as possible. In a separate well, 3 µl GeneRuler 1 kb DNA Ladder was added. The gel was running on 80 V and ~80 mA for an hour, and then on 60 V for an additional hour.

An image was taken under the geldoc (UV302, 5 sec exposure time) to check for bands. Working under UV326, the bands were cut out from the gel with a scalpel and collected into a 2 ml microtube for each sample.

For the proceeding steps of the gel purification, the QIAquick® Gel Extraction Kit from QIAGEN® (Vienna, Austria) was utilised; in this protocol, some of the volumes were stated in relation to the amount of gel collected. Three times the volume of buffer QG was added to the samples. The tubes were then placed on a 50 °C heat block and vortexed every 2-3 minute for 10 minutes until all the gel had fully dissolved. One volume of isopropanol was then added and vortexed. The start amount of each sample and component used before the first run through of the column are shown in Table 14.

Table 14: Sample size at the start of gel purification, and volumes of components used before the first run through the column.

	Sample B	Sample C
Gel collected (g)	0.39	0.28
Amount of start sample (µl)	390	280
QG added (µl)	1170	840
Isopropanol added (µl)	390	280

The samples were then transferred into a QIAquick® spin column with a 2 ml tube that came with the purification kit and centrifuged at 17 900 x g for 1 minute. The flow-through was discarded afterwards. Since there was a large volume of each sample, this step was repeated until all the sample had gone through the spin column, using 500 µl at the time. After all sample had passed through the spin column, an additional 500 µl QG buffer was added into the column and centrifuged for 1 minute; the flow-through was discarded.

For the next step, 750 µl PE buffer was added into the spin column. The tubes were then left on the bench for 2-5 minutes to rest before they were centrifuged for 1 minute, and the flow-through was discarded. The tubes were centrifuged once more to dry off the column extensively.

The spin columns were transferred into clean 1.5 ml microtubes, and 30 µl EB buffer was inserted at the centre of the column filter. The tubes rested for 1-4 minutes on the bench before they were centrifuged for 1 minute. The flow-through collected was then sent to sequencing.

3.3 Sequencing and sequence processing

3.3.1 Sanger sequencing

The sequencing of each sample was done by Eurofins Genomics (Ebersberg, Germany). 15 µl of every *caM* and *bT2*, and 20 µl *cyp51A* PCR product was packed into 1.5 ml microtubes and labelled with barcodes before they were sent off to be sequenced with Sanger sequencing methods. The primers that were used for sequencing can be seen in Table 15.

Table 15: Primers and their sequences used during Sanger sequencing for each gene written below.

Gene	Primers	Direction	Sequence (5' → 3')
caM	CMD5	Fwd	CCGAGTACAAGGAGGCCTTC
	CMD6	Rev	CCGATAGAGGTCATAACGTGG
bT2	BT2A	Fwd	GGTAACCAAATCGGTGCTGCTTTC
	BT2B	Rev	ACCCTCAGTGTAGTGACCCTTGGC
cyp51A	P-A07	Fwd	TCATATGTTGCTCAGCGG
	P-A04	Fwd	CAGACATGATATGGAACC
	cyp51A_2F	Fwd	CGGCAATCTTGCTCAATG
	cyp51A_1R	Rev	CATTGAGCAAGATTGCCG
	cyp51A_2R	Rev	GGTGAATCGCGCAGATAGT
	cyp51A_3R	Rev	GTCAAGATCCTTGTACTGGAGC
	P450.2	Rev	CTGTCTCACTTGGATGTG

3.3.2 Bioinformatic

3.3.2.1 Sample identification - Assembly and BLAST

After receiving the *caM* and *bT2* sequences, they had to be aligned and edited. The program used for this task was Geneious Prime (Biomatters, New Zealand). Every sample had a sense and antisense sequence, as a forward and reverse primer was utilised for the DNA sequencing (Table 15). By aligning these two sequences with each other, a higher quality read and longer contig could be achieved.

De Novo Assembly was used to align the sequences pairwise. The settings were adjusted to high sensitivity, and variants that had coverage of over 6 were not to be merged. Samples where one strand had a high quality read, and the other was of poor quality, were not aligned; only the strand of high quality was further processed.

After obtaining a consensus, ends with bad sequence quality were cropped. Base pairs where the read was too ambiguous were further looked into and edited based on comparing the chromatogram of the sense and antisense sequences. The ambiguous base pair reads were left unedited if the chromatogram was too unclear.

Once alignment and editing were complete, all the consensuses were put through the Basic Local Alignment Search Tool (BLAST), developed by the National Center for Biotechnology Information (NCBI), and was a feature in Geneious Prime. The BLAST was searching through the nucleotide collection database using Megablast. See Figure 14 for a detailed view of the settings used.

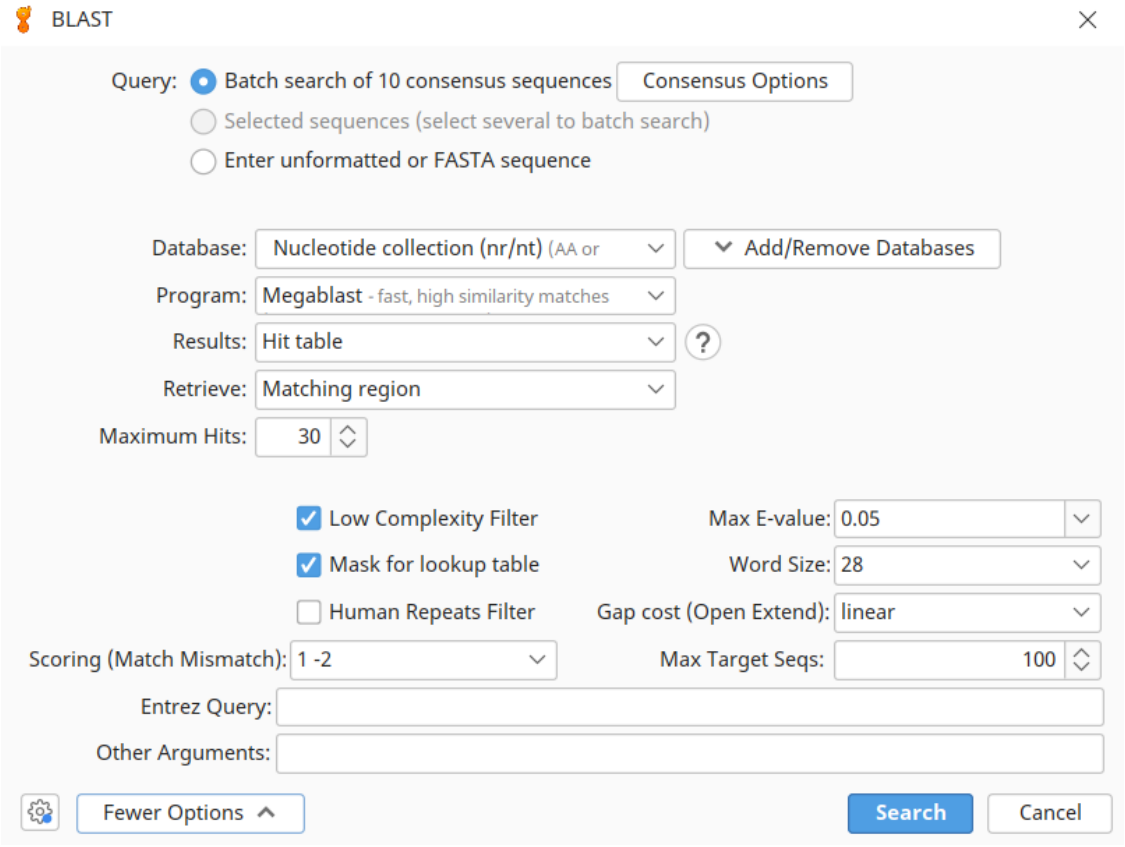


Figure 14: Snippet of the settings used in the BLAST feature in Geneious Prime.

3.3.2.2 Screening for mutations on cyp51A

Using De Novo Assembly with the same settings used for *caM* and *bT2*, all seven sequences from the *cyp51A* sequencing were aligned into one longer sequence. Low quality reads at the ends were cropped, and ambiguous base pairs were evaluated by the chromatogram and edited accordingly.

The consensuses of the samples were copied and pasted into their own file and aligned with three *cyp51A* and promoter region reference sequences with De Novo Assembly. The reference sequences used were a wild type (WT) sequence, a TR_{34}/L98H mutation sequence, and a TR_{46}/Y121F/T289A mutation sequence; these are depicted schematically in Figure 15, and the consensus of each reference sequence can be found in Appendix 15. The software marked where the sequences differed, and by comparing this, the mutation and its identity could be spotted.

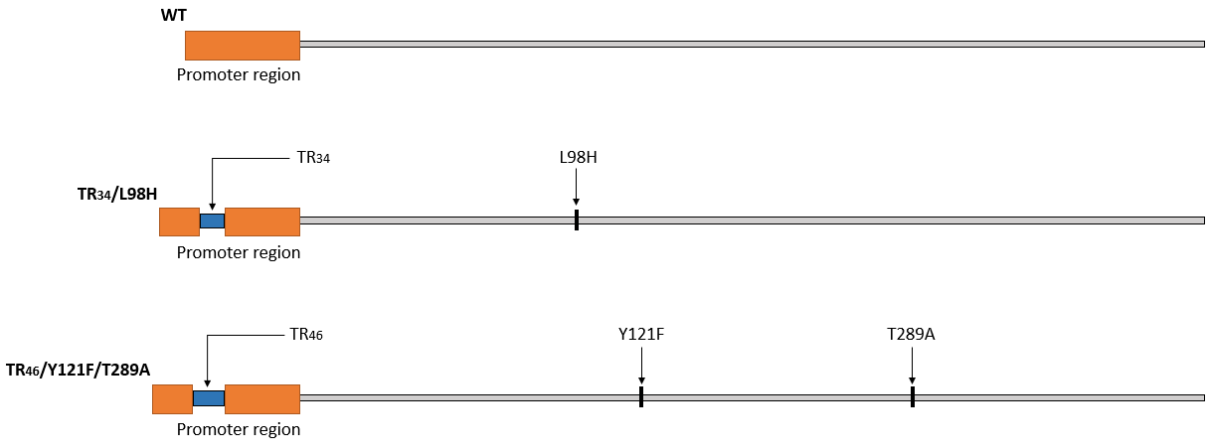


Figure 15: Schematic view over the reference sequences of *cyp51A* and its promoter region.

4. Results

4.1 Citizen science participant's information

Air samples were collected from the participants along with the information they provided in the form. In total, 43 participants were contacted to participate in the second round of this research project, due to their participation in the first round. There were in total 41 people who showed interest, which amounted to a response rate of 95.3%. Out of the 41 participants who responded, 37 actually sent their samples back; resulting in a response rate of 90.2%. This was 9 participants fewer compared to the first round of sampling, where 46 participants had sent their samples back. During the second round, 72 films were received in total; 35 from animal housing rooms, and 37 from storage rooms. Figure 16 shows a map over all the areas in Norway the samples were taken.

Figure 16: Map over air sampling locations. Map base was retrieved from Kartverket (n.d).

Information about the farms based on the responses given in the questionnaires that were sent to them (Appendix 2) was structured and sorted. Out of the 37 farms that were sampled, 35 of them housed animals. In total, 26 farms housed dairy cows and/or meat cattle; dairy cows were the most common livestock among the farms. The number of farms that housed each animal type is listed in Table 16. The type of bedding used for the animals was also inquired in the questionnaire; the most common type of bedding was wood shaving, which was used as the only bedding material in 20 of the farms, and in a mix in 9 of the farms. Furthermore, the questionnaire also inquired about plots; 5 of the 37 farms owned plots that they used to grow crops and hay.

Table 16: Number of farms housing each animal type. There were 37 farms in total. Take note that some farms housed more than one type of animal.

Animal	Number of farms (n = 37)
Dairy cow	21
Meat cattle	9
Sheep	8
Goat	1
Pig	1
Horse	5
Chicken	3
Others	3

A total of 6 of the 37 participants stated they ran an organic farm, according to the questionnaire. Two farms noted that they used pesticides on their plot; one farm stated they used all three categories of pesticides (herbicides, fungicides and insecticides) inquired in the form, but fungicides were used on only one of their two plots (wheat). The other farm used fungicides and herbicides on their plot.

The participants also noted what was kept inside their storage rooms. Some of the items listed in the questionnaire by the participants were silage, hay, other types of animal feed, straw, wood shaving, tools, equipment, vitamins, vegetables and fertilisers (some of the farmers actually kept their animals in their storage room). The most common items kept in were hay (18 farms), silage (13 farms) and other types of animal feed (14 farms).

Because temperature is one of the key factors that affect fungal growth, the temperatures of the sampling rooms were measured. The temperature measured in the animal housing rooms was between 7 °C and 21 °C, with a median temperature of 15 °C (± 3.5 °C). Next, the temperatures of the storage rooms were between 6 °C and 21 °C, with a median temperature of 10 °C (± 3.1 °C). Figure 17 shows the distribution of temperatures measured at the different farms.

Figure 17 also shows the differences in temperatures measured during the first and second sampling rounds in this study. The data from the first round are gathered from Henriksen (2021). In general, the first round had a lower median temperature with a higher standard deviation than the second round. The highest and lowest temperatures measured in the animal housing rooms were 22 °C and 3 °C, and in the storage rooms, 22 °C and -15 °C. The median temperature of the first round was 10 °C (± 4.9 °C) for the animal housing rooms, and 2.5 °C (± 6.7 °C) for the storage rooms.

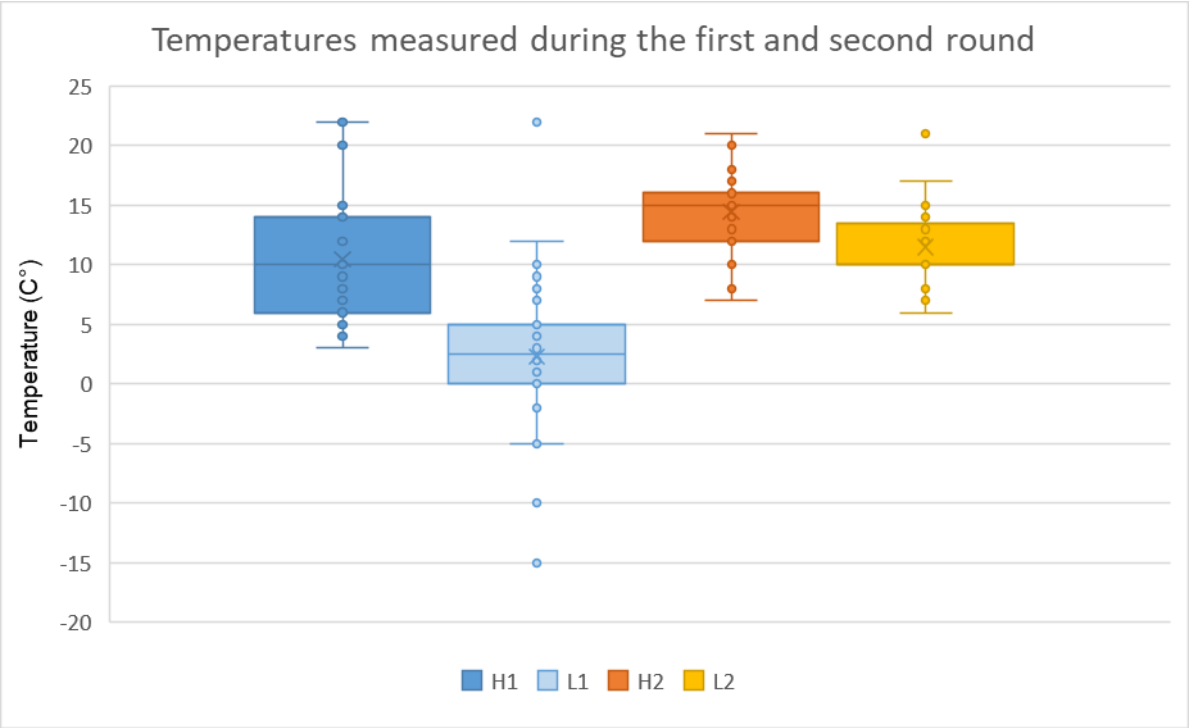


Figure 17: Comparison of temperatures measured during the first round (blues) and the second round (red and yellow). The box diagram displays the temperatures measured in the animal housing rooms (H1 and H2) and the storage rooms (L1 and L2) respectively. There are 34 points in H1, 36 in L1, 31 in H2 and 33 in L2. The first round had generally lower temperatures and higher standard deviation than the second round.

It is important to note that not all the questionnaires that were sent back with the samples were filled in completely; this has led to some data getting lost or becoming more challenging to sort. See Table 30 in Appendix 7 for a more detailed description of the information the participants granted.

4.2 Fungal cultivation and subsampling

4.2.1 Contamination test

Cross contamination was a constant concern while extracting, cultivating and processing the fungal isolates. A small test was therefore done parallel to the main processes, where agar plates were placed within and next to the field of work after processing the isolates to check for airborne spores.

The results of the contamination test are displayed in Table 17. Photos of the agar plates and setup of the test can be found in Appendix 8. The first round of MEA plates used for testing *Aspergillus fumigatus* contamination risk displayed no growth. The second round of MEA plates had *A. fumigatus* growth on the middle plate placed in the airflow cabinet after 0 minutes, and on the side plates after 0 minutes and 6 minutes. The DG18 plates used for testing *Aspergillus niger* contamination risk had growth only on the side plate placed in the airflow cabinet after 2 minutes.

Table 17: Results of the contamination test that shows growth (+) or no growth (-) on the agar plates. Plates marked with M were placed at the centre of the work area, while plates marked with S were placed 30-40 cm to the side of the work area.

Plate number	Time of placement	*A. fumigatus* – MEA (1) Growth (-/+)	*A. fumigatus* – MEA (2) Growth (-/+)	*A. niger* – DG18 Growth (-/+)
M0	0 min	-	+	-
M2	2 min	-	-	-
M4	4 min	-	-	-
M6	6 min	-	-	-
S0	0 min	-	+	-
S2	2 min	-	-	+
S4	4 min	-	-	-
S6	6 min	-	+	-

There did not seem to be any coherent patterns in terms of cross contamination according to this test. According to the similar test done by Henriksen (2021), cross contamination was persistent at the centre where isolates had been disturbed, while 30 cm to the side had no growth. Because of that, switching between workbenches was imperative to avoid cross contamination. For the remainder of this study, however, the measures done to prevent cross contamination was to work on one side of the airflow cabinet, wipe with 70% EtOH, wait 10 minutes, wipe with EtOH again, and work on the other side of the airflow cabinet.

4.2.2 Findings on primary plates

A total of 72 primary plates were made from the films received. About 11 different genera and/or species of fungi were identified morphologically on the primary plates; this included *A. fumigatus*, *A. niger*, *Aspergillus glaucus*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus tritici*, *Penicillium* spp., *Scopulariopsis* spp., *Paecilomyces* spp., yeasts, and Mucorales. Little or no distinction was made between the different species of yeasts and Mucorales due to morphologic similarities, and because it had little relevance to the study. Hence, these species were usually categorised within the same group. In cases where it was apparent that there were more than one species of yeast or Mucorales on the same plate, the observation was noted as such. Species within *Penicillium*, *Scopulariopsis* and *Peacilomyces* were usually grouped together respectively as well. See Table 31 in Appendix 9 for a more detailed description of the different findings on each primary plate. Figure 18 shows an example of how a primary plate could look like after incubation.

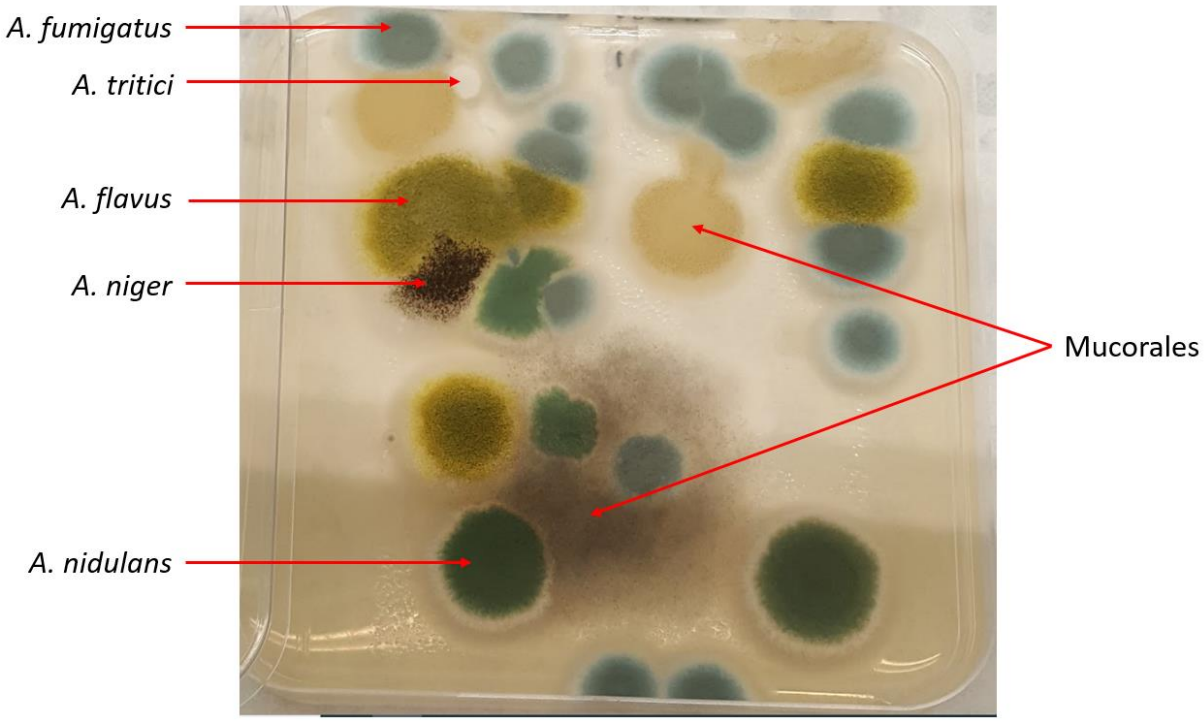


Figure 18: Primary plate (G23L2) with growth of various fungal species on DG18 after incubation at 37 °C for 48 h without the film. The plate displays colonies of *A. fumigatus*, *A. niger*, *A nidulans*, *A. flavus*, *A. tritici* and two types of Mucorales.

In order to create an overview of the diversity of species observed on the primary plates, each genus and *Aspergillus* spp. were scored as present or absent on each plate. Figure 19 shows the percentage of primary plates from animal housing rooms (H2) and storage rooms (L2) respectively that had each genus and *Aspergillus* species appearing. The figure also shows the percentage of plates with unidentified colonies. Yeasts appeared most often and were found on 47 of the 72 primary plates (~65%). The genus that appeared the rarest was *Paecilomyces*, which appeared on only two primary plates (~3%). Three primary plates (~4%) had no fungal growth after 2-5 days of incubation, and 14 of the 72 plates (~19%) had unidentified colonies.

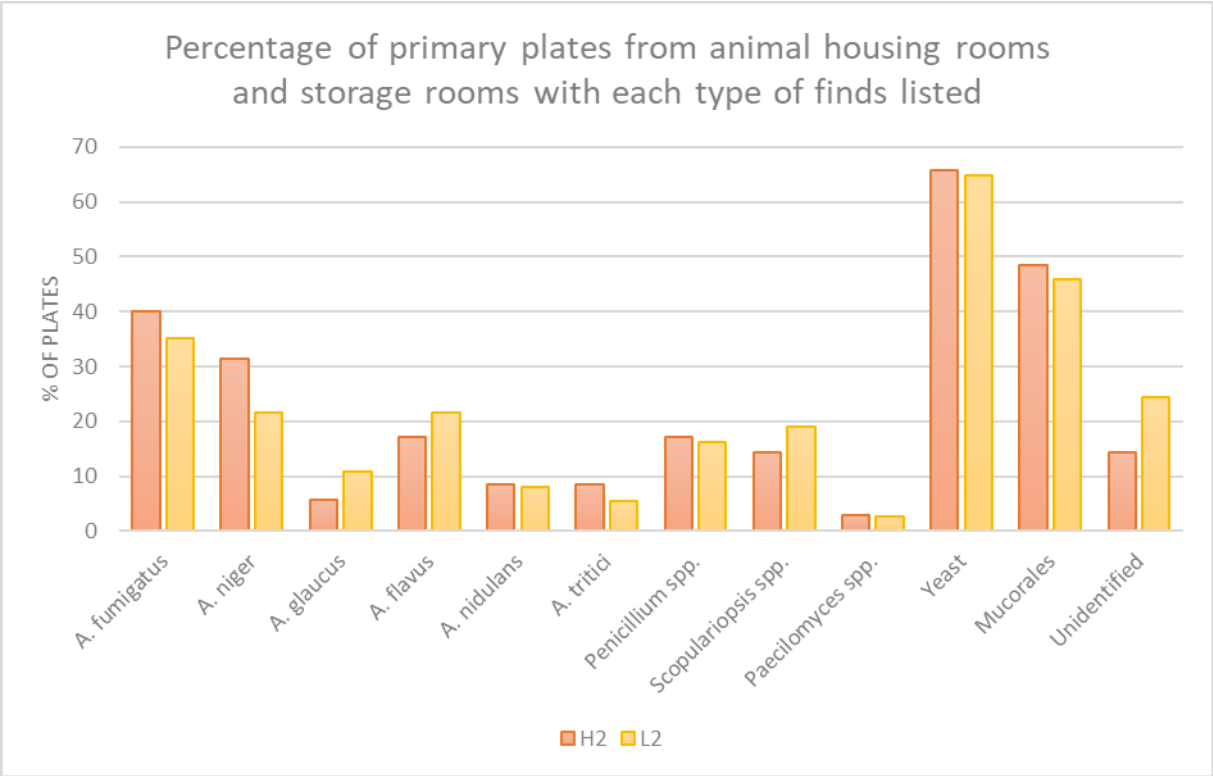
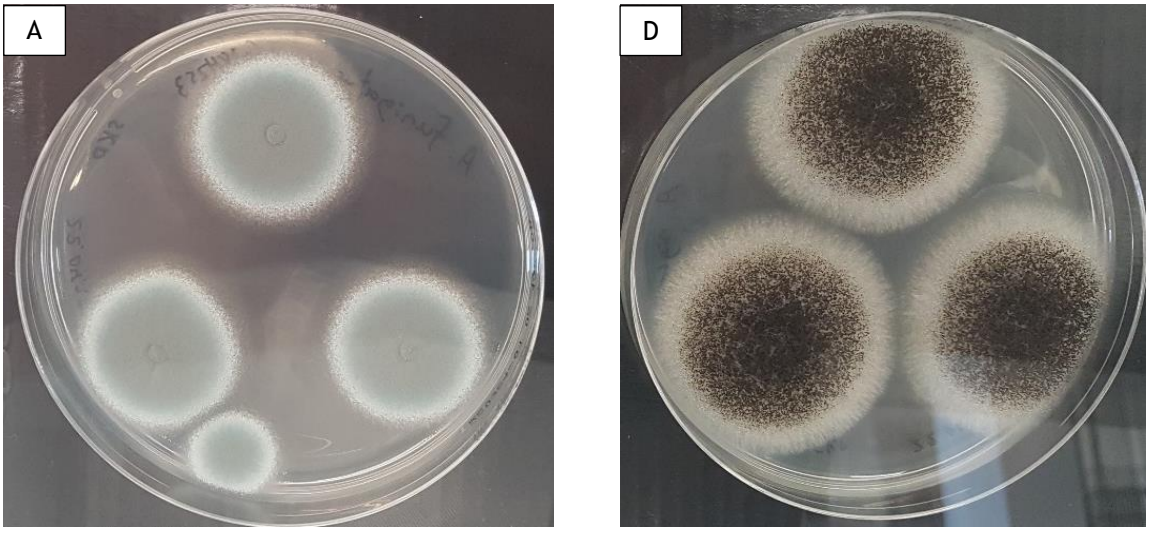


Figure 19: The percentage of primary plates from animal housing rooms (H2) and storage rooms (L2) that had growth of each type of finding listed. There were 35 H2 plates, and 37 L2 plates.

4.2.3 Morphological identification of *A. fumigatus* and *A. niger* on primary plates

Colonies of *A. fumigatus* and *A. niger* were isolated onto secondary plates from the primary plates. Aspergilli of both section *Nigri* and section *Fumigati* were recognisable through colony inspection and microscopy. Figure 20 shows the morphological characteristics of each of the two fungal species.



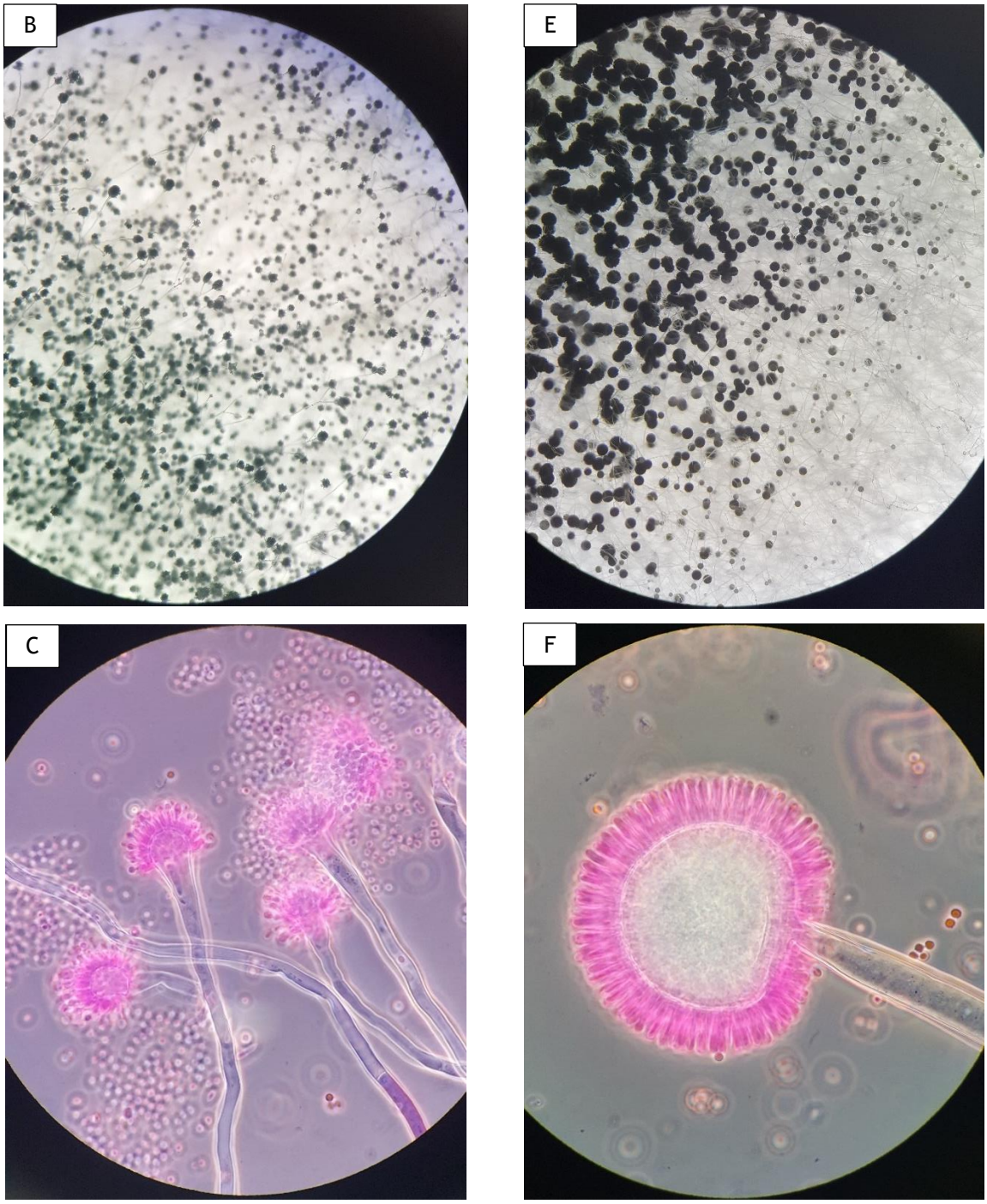


Figure 20: Morphological characteristics of *A. fumigatus* (**A-C**) and *A. niger* (**D-F**). The colonies of the two *Aspergillus* spp. on DG18 after incubation at 37 °C for three days can be seen in **A** and **D**. The conidiophores under the stereo microscope at 4x zoom are seen in **B** and **E**. Microscope images of the conidiophores 100x objective are shown in **C** and **F**.

Of the 72 films that were plated, 27 produced at least one colony of *A. fumigatus*, and 19 plates had at least one colony of *A. niger* (this includes plates with growth of both *Aspergillus* spp.). The number of plates that had both *A. fumigatus* and *A. niger* was 13. In total, there were 58 *A. fumigatus* (31 from animal housing, and 27 from storage rooms) and 37 *A. niger* isolates (22 from animal housing, and 15 from storage rooms) collected from all the primary plates (Figure 21). The

number of animal housing and storage rooms that gave one, two or three isolates of each *Aspergillus* spp. is shown in Figure 22.

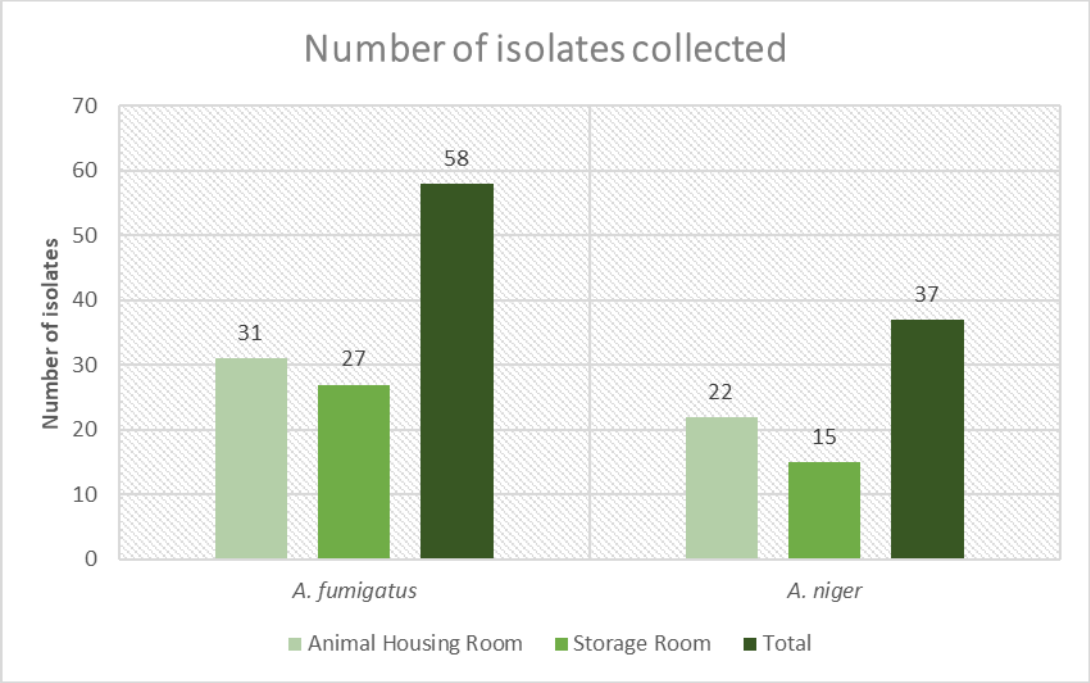


Figure 21: Number of *A. fumigatus* and *A. niger* isolates gathered from animal housing rooms, storage rooms and in total.

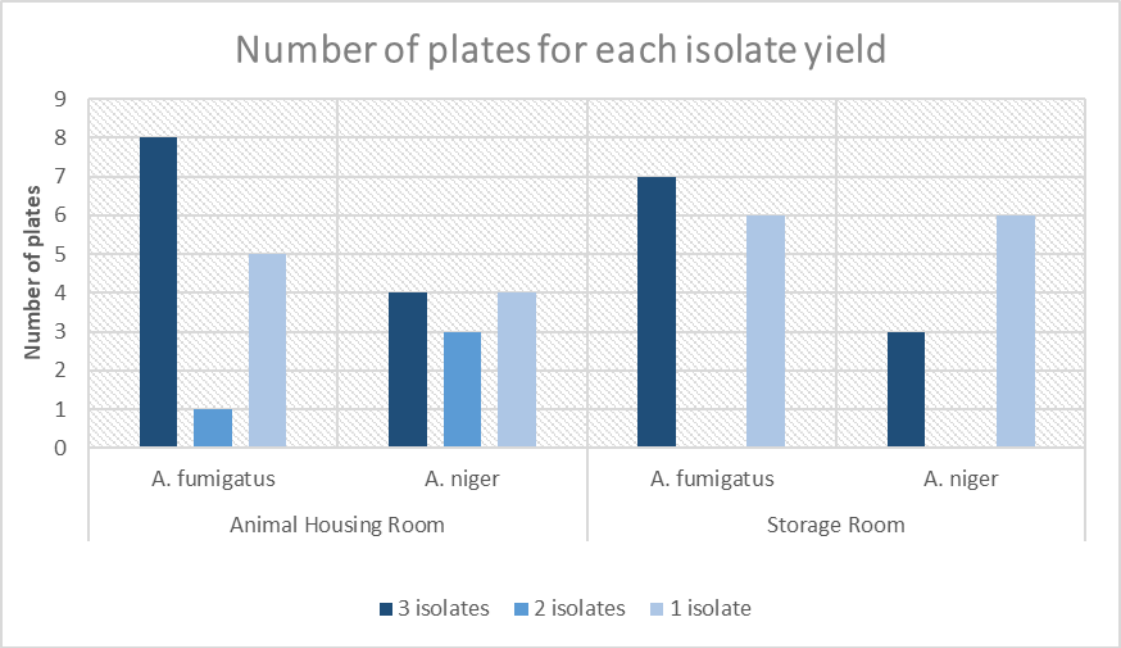


Figure 22: Number of primary plates that provided one, two or three isolates of *A. fumigatus* and *A. niger* from animal housing and storage rooms.

The map in Figure 23 shows where in Norway the *A. fumigatus* and *A. niger* isolates came from. Very few isolates were found in the more northern parts of the country, while clusters are located at the South-West and eastern parts.

Figure 23: Map over locations where the *A. fumigatus* and *A. niger* isolates originated. Map base was retrieved from Kartverket (n.d).

The comparison of how often *A. fumigatus* was found on the primary plates between the first (January-March) and second (October-November) sampling rounds can be seen in Figure 24. This was to see if different seasons affected proliferation of *A. fumigatus*. Data from the first sampling are retrieved from Henriksen (2021). It is clear that *A. fumigatus* appeared more frequently during the first sampling than in the second sampling, which is also reflected in the total number of isolates collected between the two rounds (Figure 25). There was a substantially higher number of *A. fumigatus* isolates gathered from the first sampling, which was almost double of what was collected during the second sampling.

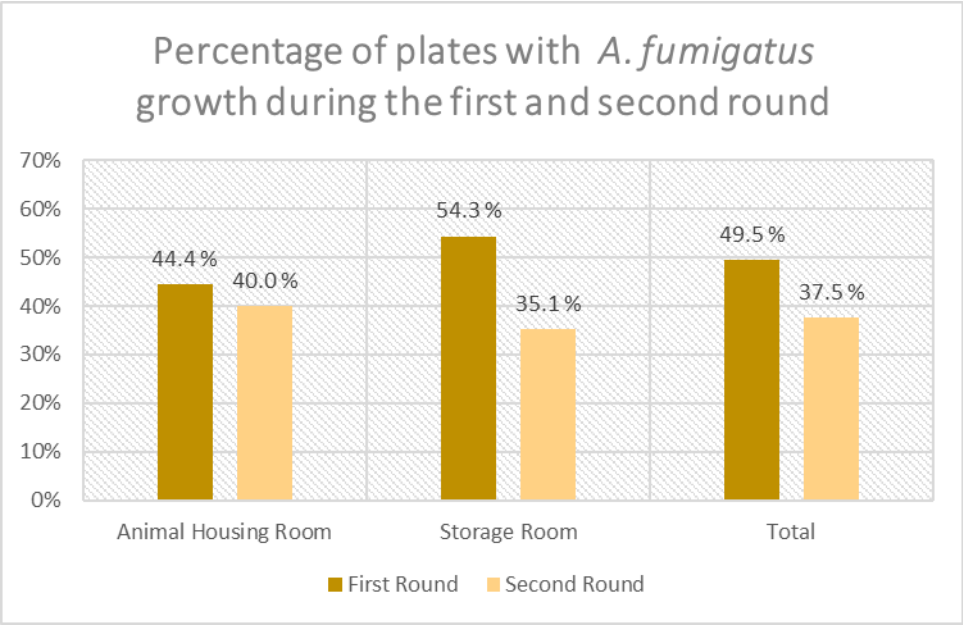


Figure 24: Comparison between percentage of primary plates with *A. fumigatus* in the first and second sampling.

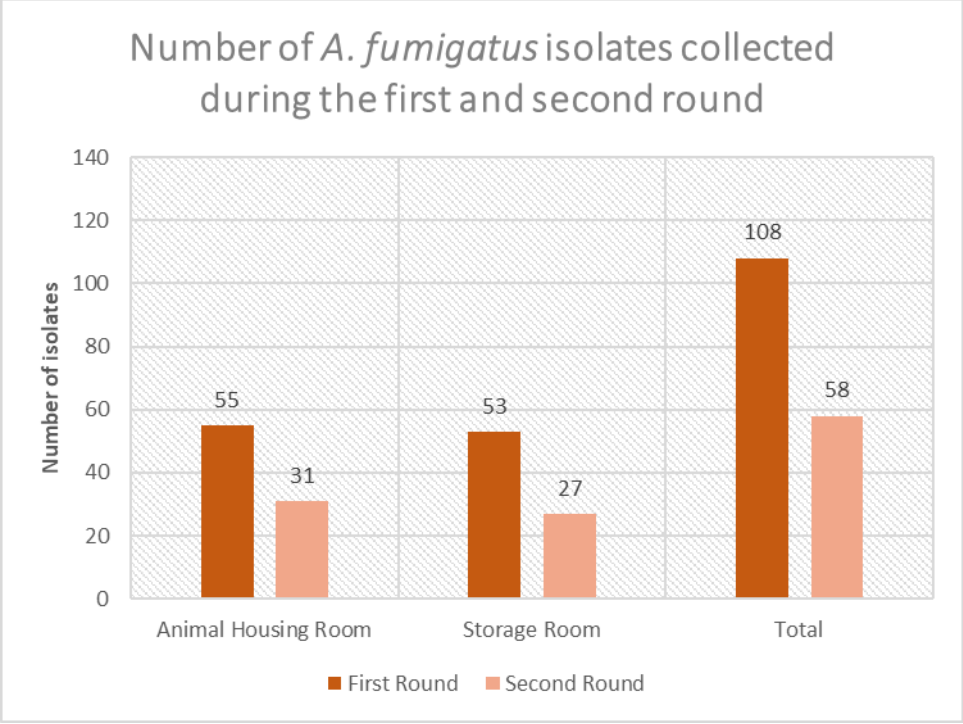


Figure 25: Comparison of number of *A. fumigatus* isolates collected during the first and second round.

4.3 Molecular identification of fungal isolates

4.3.1 *caM* primer optimisation and troubleshooting

Fungal isolates were identified molecularly by amplification and sequencing of appropriate genes. The *caM* gene gives the best resolution for *A. fumigatus* and was the primary choice as genetic marker. However, based on experience, the *caM* PCR can vary in quality, and a small test was conducted to find out what primer concentration and what template volume would optimise the *caM* PCR reaction. Combinations of two different primer concentrations (one with 0.6 µM, and one with 0.3 µM of forward primer CMD5 and reverse primer CMD6), with three different amounts of template were tested on representative isolates of *A. fumigatus* and *A. niger*.

The isolates of *A. fumigatus* and *A. niger* had the same DNA concentration (2.1 ng/µl), and the template amount was adjusted by changing the volume of the template (i.e.1 µl, 2 µl and 4 µl). See Figure 26 for the schematic view of the PCR setup.

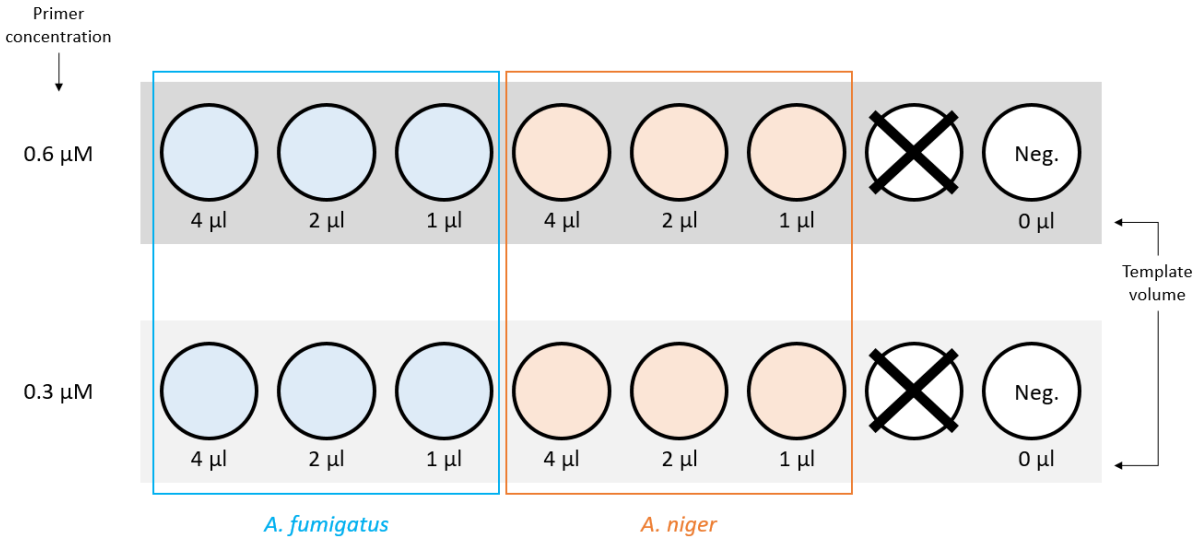


Figure 26: Schematic view of the set-up used for the troubleshooting of *caM* PCR. There were 25 µl in each tube.

After gel electrophoresis (Figure 27), the PCR products were visible in every well where the primer concentration was 0.3 µM. In the wells where the primer concentration was 0.6 µM, only the samples with 4 µl *A. fumigatus* DNA template and 2 µl *A. niger* DNA template had visible PCR products; the rest were obscure or not visible on the gel. One clear band with the expected length of the *caM* amplicon is visible wherever the PCR was successful. It also appears that the wells containing *A. niger* isolates created several other PCR products of different sizes during the reaction; this was especially prominent in the samples with 4 µl and 1 µl *A. niger* DNA template and 0.3 µM primer concentration. The negative controls were all blank, so this was unlikely due to a contamination. Regardless, based on the results of this test, the PCR protocol for *caM* was adjusted to 4 µl template volume and 0.3 µM primer concentration.

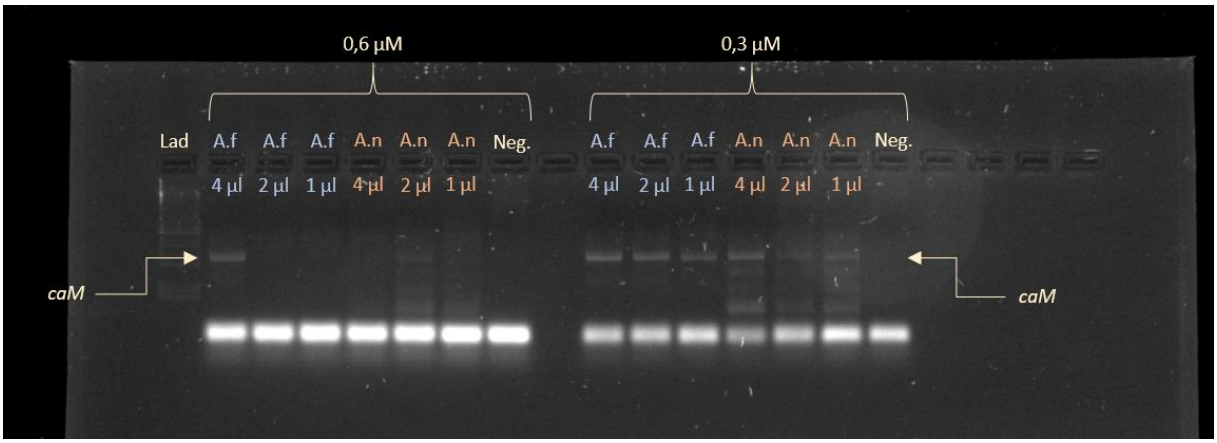


Figure 27: Gel image of *caM* PCR optimisation. The wells are marked with sample, template volume and primer concentration. A.f is for *A. fumigatus*, A.n is for *A. niger*. Neg. is negative sample and Lad is ladder. The band containing the PCR product of *caM* is marked with an arrow. All negative samples were blank.

The phenomenon of multiple bands seemed to only appear in *A. niger* and not *A. fumigatus*. The number and intensity of these bands also varied slightly from sample to sample. In order to figure out a possible explanation for why this was the outcome for *caM* PCR on *A. niger*, a few random *caM* sequences from *A. fumigatus*, *A. niger* and *Aspergillus tubingensis* were downloaded from the NCBI's nucleotide database and inspected. After performing multiple alignment of the sequences in Geneious Prime (New Zealand), a search for the primer sequences, CMD5 and CMD6, was conducted (See Figure 28). The results show that CMD5 was only found on the *A. fumigatus* sequences. Upon further inspection, there were some base pair variation on one of the *A. niger* sequences. One of the *A. tubingensis* sequences had a segment similar to the CMD5 sequence, but lacks a couple of base pairs at the end to draw a complete conclusion. The CMD6 sequence was found on one *A. fumigatus* and one *A. tubingensis* strain. Most of the other reference sequences had a few base pair variations in the primer region. This shows a possible explanation as to why the *caM* PCR did not work well on the black *Aspergillus* isolates; the base pair variations in the primer region would disable the primers from annealing perfectly. This could in turn have made the primers favour other loci on the genome with similar sequences; creating multiple bands during PCR. It is not very clear as to why CMD6 was not found on both *A. fumigatus* reference strands. Furthermore, it was also unexpected that the one primer sequence that was found on the black *Aspergillus* references was CMD6, as it was the forward strand (CMD5) that had a higher success rate during sequencing. This has demonstrated that molecular techniques do not always yield the expected outcome every time. Using different primers better suited to the black *Aspergillus* isolates could have increased the quality of the amplicon.

Figure 28: Primer sequences marked on random *caM* sequences of *A. fumigatus*, *A. niger* and *A. tubingensis* from NCBI's nucleotide library. The grey area in the upper image marks the CMD5 (forward primer) sequence, while the grey area in the bottom image marks the CMD6 (reverse primer) sequence.

The multiple bands *A. niger* created during *caM* PCR would interfere with sample sequencing. In order to try and solve this issue, the annealing temperature was increased from 55 °C to 56 °C, and the number of cycles was changed from 35 to 37 on a few selected *A. niger* isolates; both numbers of cycles were tested parallel to each other. As seen in Figure 29, the multiple bands still persisted after the adjustments. Changing the number of cycles from 35 to 37 seemed to have noticeably increased the intensity of the bands. Given the problems with *caM*-amplification on *A. niger*, the genetic marker used for *A. niger* identification was switched from *caM* to *bT2*.

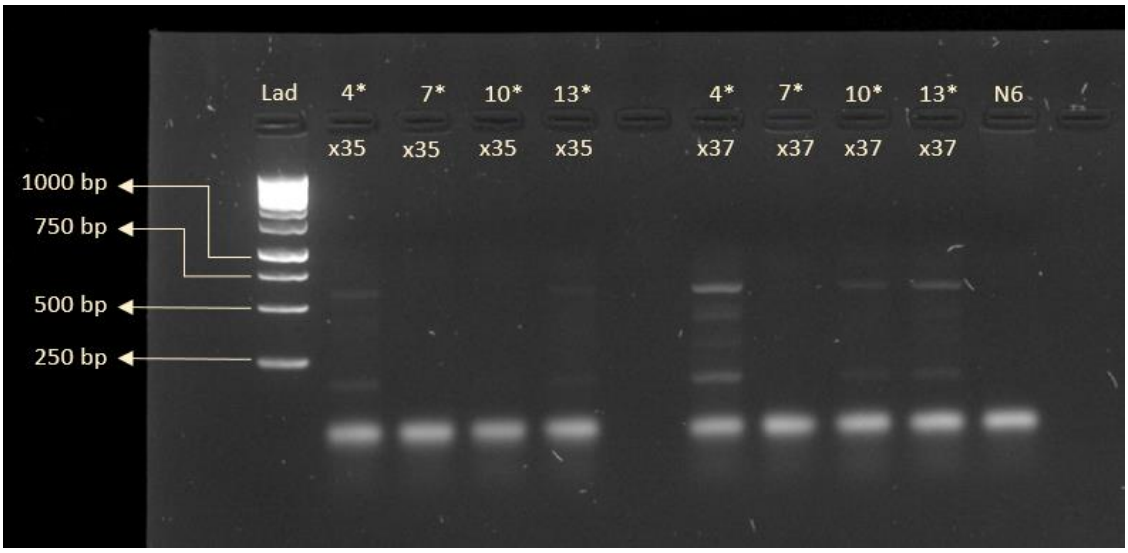


Figure 29: Gel image of *A. niger* isolates after an adjusted *caM* PCR; the product is between 500 bp and 750 bp. The annealing temperature had been increased to 56 °C. The number of cycles was also changed; 35 cycles to the left, and 37 cycles to the right. The negative control (N6) was blank. Band sizes are marked on the ladder (Lad). Sample numbers can be seen in Table 35 in Appendix 17.

4.3.2 Molecular identification of *A. fumigatus* isolates

PCR on *caM* was successful for most of the *A. fumigatus* isolates as they acquired a band somewhere between 500 bp and 750 bp; *caM* has a band size of 580 bp. The quality of the product varied moderately from sample to sample; some samples had a very clear, singular band at correct band length, while others were very faint or not visible. All EBK samples and negative controls turned out blank. A representative gel electrophoresis image of *caM*-amplified *A. fumigatus* can be seen in Figure 30. Gel images of all of the *caM* PCR products are shown in Figure 50 in Appendix 16, while the sample numbers are listed in Table 35 in Appendix 17.

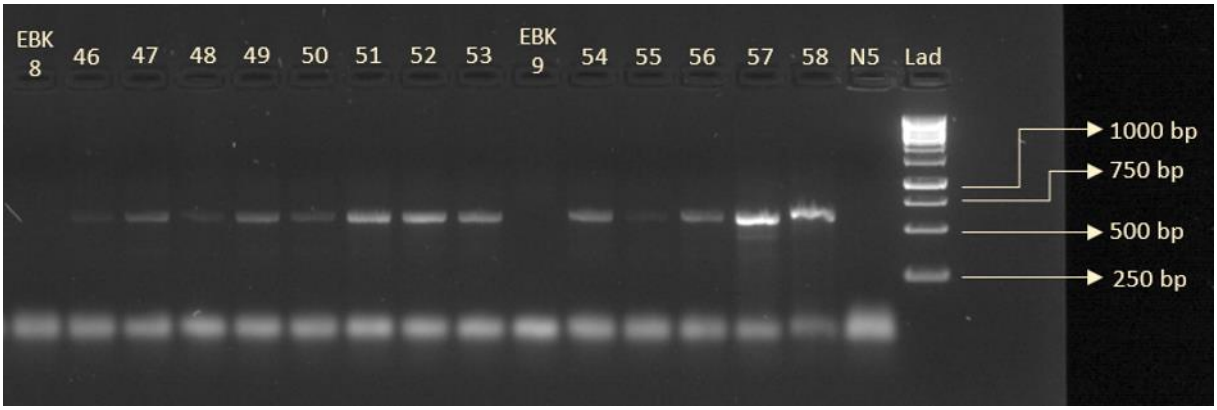


Figure 30: Gel image of *caM* PCR product for some of the *A. fumigatus* isolates (between 500 bp and 750 bp). EBKs are extraction blank control, and N5 is negative control, which were all blank. Band sizes are marked on the ladders (Lad). The description of what samples are in which wells is listed in Table 35 in Appendix 17.

There were a few *A. fumigatus* isolates that did not obtain a good enough sequence due to poor PCR-amplification. In order to increase the amount of PCR product, an additional amplification was set up, increasing the number of cycles to 36 (Figure 31). Five samples were sent to sequencing. Next, it was decided that three other samples had a high enough read quality on one of their two strands from the initial sequencing round and were not molecularly processed any further. The remaining four samples were put through another round of *caM* PCR where the number of cycles were increased to 37, and the template volume used was increased to 6 µl. This did not seem to have had an impact on the quality of the PCR products, so these four *A. fumigatus* isolates were identified using *bT2* instead (Figure 51-C, Appendix 16). The results of the last modified *caM* PCR round can be seen in Figure 51-A in Appendix 16.

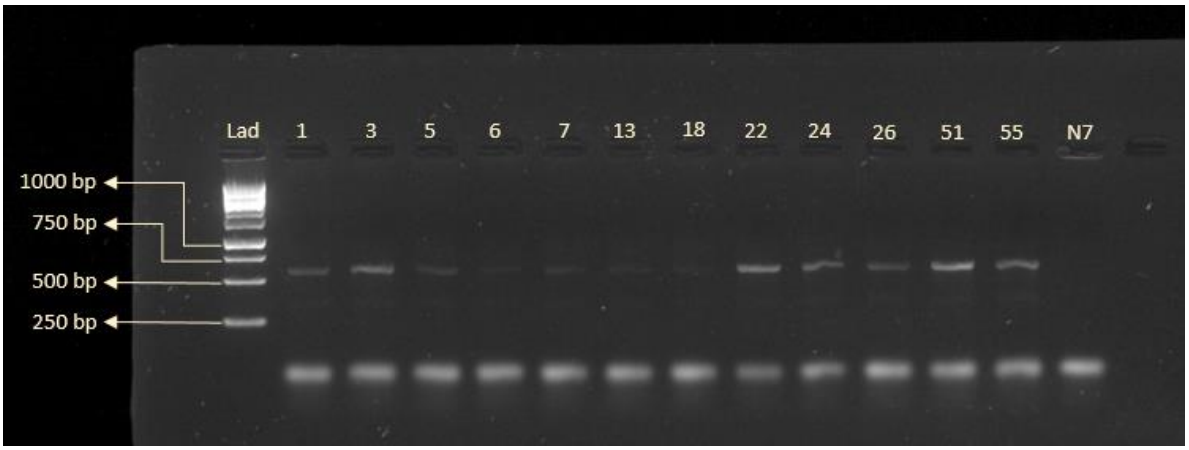


Figure 31: Gel image of *A. fumigatus* isolates where *caM* PCR had been modified to 36 cycles (between 500 bp and 750 bp). Sample 3, 22, 24, 26 and 55 were sent to get sequenced. Sample 1, 5 and 51 had a sufficient quality read on one of their strands in the initial sequencing round. Another modified PCR reaction was done on sample 6, 7, 13 and 18. Negative control (N7) was blank. Band sizes are marked on the ladder (Lad). Sample numbers are listed in Table 35 in Appendix 17.

The BLAST results of all the *caM* sequences from the *A. fumigatus* isolates confirmed that they were all indeed *A. fumigatus*, with a 99.5-100% similarity with the top hits. The *bT2* sequences of the *A. fumigatus* isolates also confirmed that they were *A. fumigatus* with a 100% similarity to the top hits. Details about the sequences and the BLAST hit results of the environmental *A. fumigatus* isolates are listed in Table 36 in Appendix 18.

4.3.3 Molecular identification of *A. niger* isolates

As all *A. niger* isolates had multiple bands in *caM* PCR, it was decided to use *bT2* as the molecular marker instead. The *bT2* PCR was successful for all samples. A clear band at somewhere around 500 bp was visible in all samples; this fits the description of *bT2*, which is 400-600 bp. Negative controls displayed no bands. Some of the samples had some additional bands of other sizes (Figure 32), but none of them harboured the same intensity as the main PCR product and would most likely not affect the results during sequencing. Gel images of the rest of the *bT2* amplicons of the *A. niger* isolates can be seen in Figure 51 in Appendix 16.

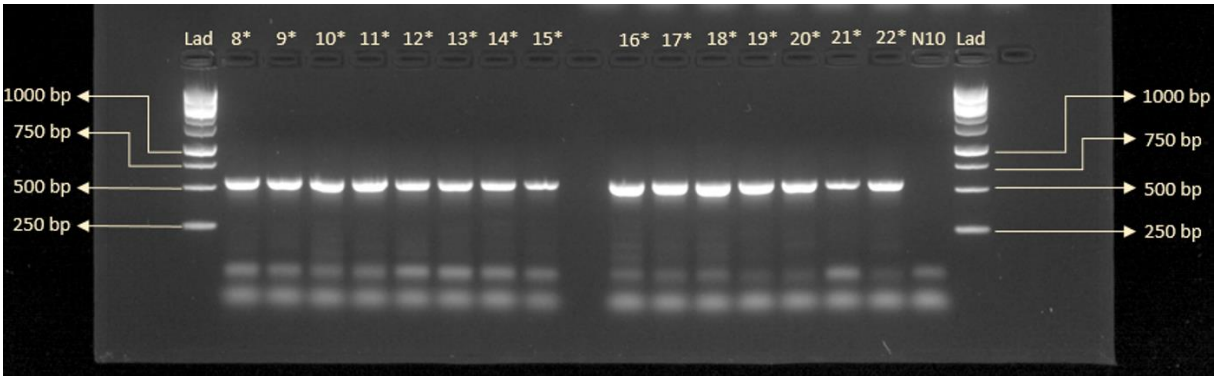


Figure 32: Gel image of some of the *A. niger bT2* PCR products (at around 500 bp). All *bT2* products were very clear, and negative control (N10) was blank. Band sizes are marked on the ladders (Lad). Sample numbers are listed in Table 35 in Appendix 17.

The BLAST results of the *bT2* sequences from the *A. niger* isolates revealed that they were all *A. tubingensis*; except for one that was *A. welwitschiae* (G53H2A1). All *bT2* sequences had a 100% similarity with the top hits of the search. The *bT2* amplicons seemed to also have a smaller variety in BLAST hits than the *caM* amplicons. Details of the sequences and the BLAST hit results of the environmental black *Aspergillus* spp. isolates are listed in Table 37 in Appendix 18.

4.4 Resistance Screening

4.4.1 Primary resistance screening with VIPcheck™

A total of 95 fungal isolates, 58 *A. fumigatus* and 37 *A. niger*, were tested for resistance against voriconazole, itraconazole and posaconazole using VIPcheck™ test plates. The scoring of potential resistance was determined by the mycelial growth, number and size of the colonies in the wells with azoles. The scoring system was divided into -, +, ++ and +++, and the inoculums were ranked according to these criteria. A control well without azoles served as a positive control for the spore inoculum. Figure 33 displays examples of VIPcheck™ plates that were granted each of the four scores. All VIPcheck™ plates that were granted a high score can be seen under Appendix 12.

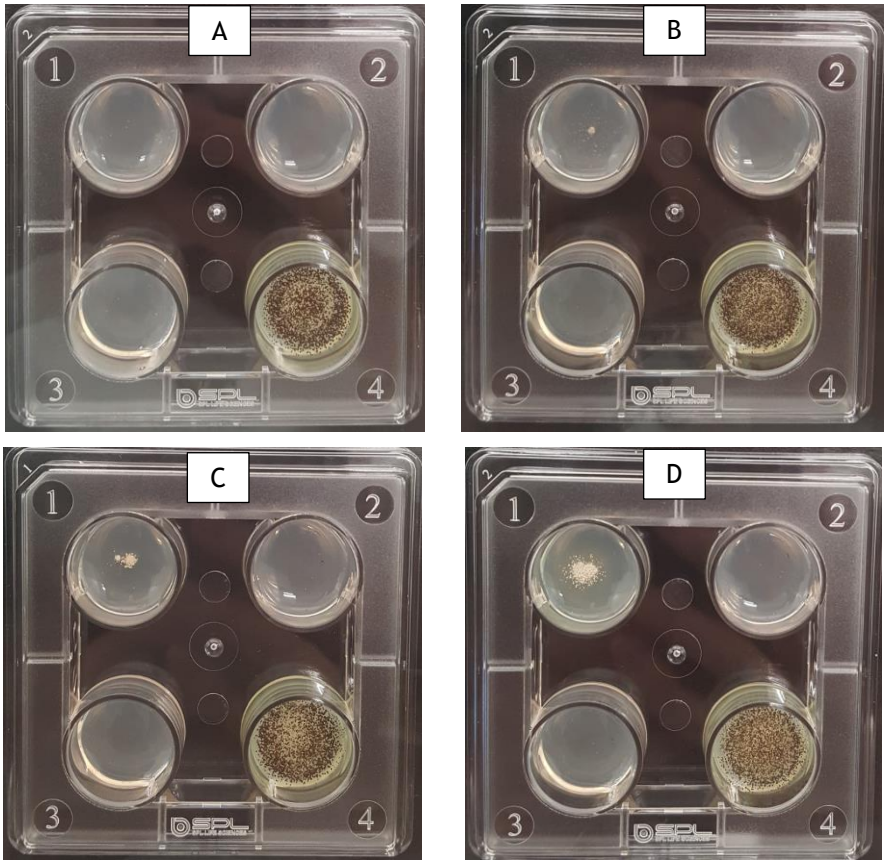


Figure 33: VIPcheck™ plates of *A. niger* after 48 h incubation. The images show isolates that were granted with different scores for itraconazole resistance (well 1). Well 4 is the control. **A**; G22H2A3 (-), **B**; G38L2A2 (+), **C**; G23L2A1 (++), **D**; G38H2A1 (+++).

Among the *A. fumigatus* isolates, only two showed significant growth after 48 hours in at least one of the wells containing azoles. Among the *A. niger* isolates, however, there was a significantly higher frequency in growth on azole containing wells, with eight isolates showing some degree of resistance to one or more of the three azoles. The conidia of all *A. niger* isolates that managed to grow on the azole wells were less pigmented than those of the control well, which could be a stress response. Table 18 displays the number of *A. fumigatus* and *A. niger* isolates that were granted scores of -, +, ++ or +++ after 48 hours incubation (there was too little growth after 24 h). The tables in Appendix 11 displays the scores granted to every VIPcheck™ plate after 24 h and 48 h incubation.

Table 18: VIPcheck™ scores for all *A. fumigatus* (n = 58) and *A. niger* (n = 37) isolates after 48 h incubation at 37 °C. Scores of the control wells are excluded from this table.

	Score	**Itraconazole**	**Voriconazole**	**Posaconazole**
A. fumigatus	-	50	55	55
	+	7	2	3
	++	1	0	0
	+++	0	1	0
A. niger	-	19	37	23
	+	11	0	12
	++	6	0	1
	+++	1	0	1

In order to see if seasonal differences affect the frequency of azole resistant strains appearing in the environment, a comparison on the VIPcheck™ results between the first sampling (January-March) and second sampling (October-November) was made. Data from the first round were retrieved from Henriksen (2021). Figure 34 summarises the percentage of *A. fumigatus* isolates with different scores for the various azoles from the first and second round. In general, the first round had a higher percentage of isolates with scores indicating growth (+, ++, +++) than the second round.

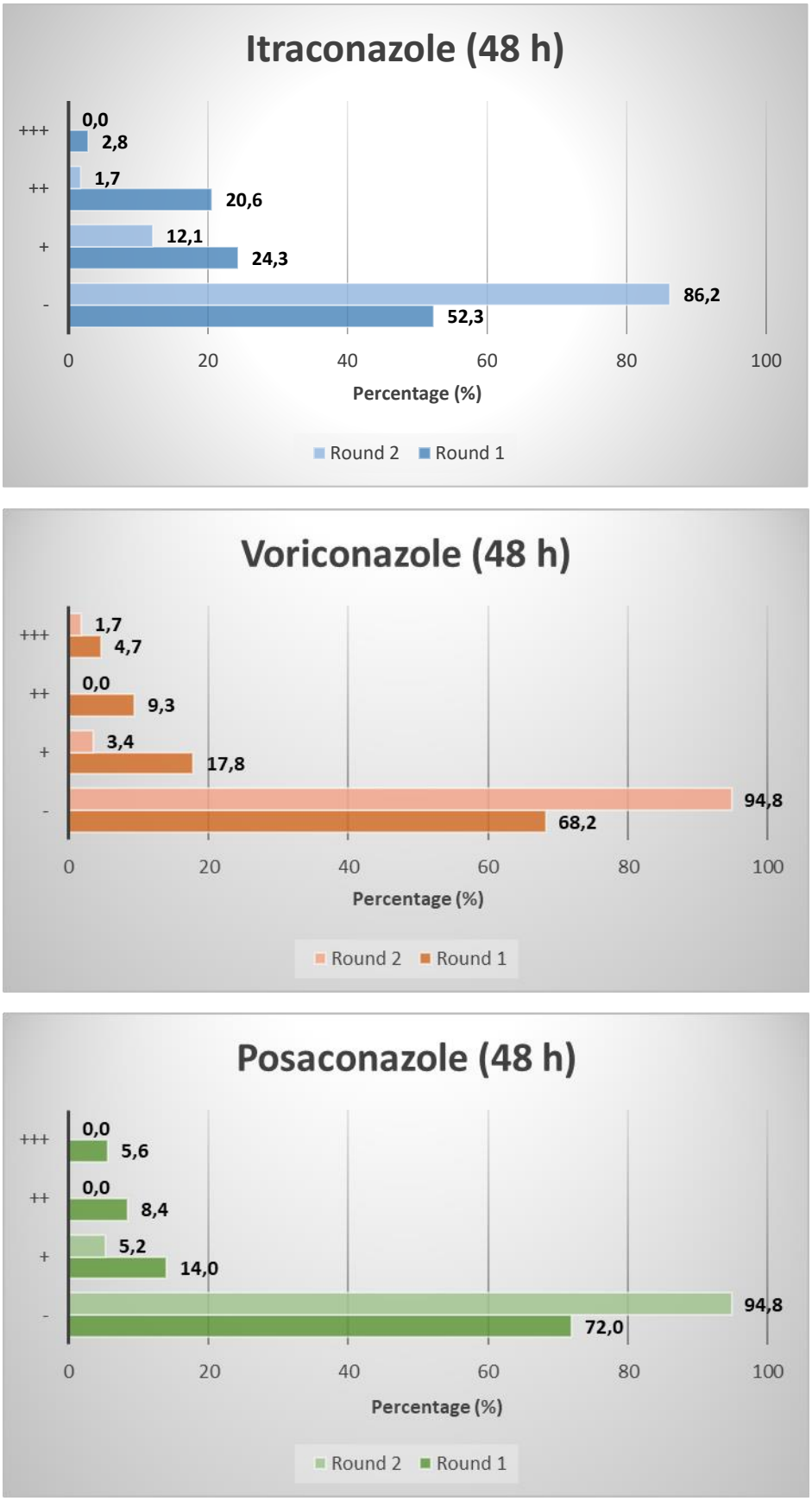


Figure 34: VIPcheck™ results comparison of *A. fumigatus* between the first and second sampling. Percentage of *A. fumigatus* isolates that were granted a score of -, +, ++ or +++ on each azole type after 48 h in 37 °C. **Top**: Itraconazole. **Middle**: Voriconazole. **Bottom**: Posaconazole.

4.4.2 Secondary resistance screening with E-test

Eleven fungal isolates from the VIPcheck™ were further screened with the E-test. Additionally, 3 resistant clinical *A. fumigatus* isolates from NVI's strain collection were included, and 4 commercially available isolates serving as references. The MIC-values for all these isolates can be found in Table 19. Photos of the E-test results of the resistant isolates as well as an environmental *A. fumigatus* and *A. niger* isolate can be viewed under Appendix 13.

Table 19: MIC-value for each isolate screened with E-test. MIC on *Aspergillus* spp. were read 24 h after inoculation, while yeast isolates (*Candida* spp.) were read after 48 h. G38H2A1 was read 48 h after inoculation for voriconazole and posaconazole due to slow growth. The isolates marked in orange had their *cyp51A* gene and promoter region sequenced.

Sample no.	Species	Itraconazole		Voriconazole		Posaconazole	
		MF[1]	MIC (µg/ml)	MF[1]	MIC (µg/ml)	MF[1]	MIC (µg/ml)
G18H2A1	*A. niger*	0.54	1.0	0.50	0.19	0.52	0.047
G23H2A1	*A. niger*	0.52	0.75	0.50	0.125	0.52	0.125
G23L2A1	*A. niger*	0.53	1.0	0.51	0.25	0.51	0.064
G27H2A1	*A. niger*	0.50	0.5	0.50	0.19	0.50	0.064
G27L2A1	*A. niger*	0.52	0.5	0.52	0.125	0.52	0.064
G33L2A1	*A. niger*	0.51	0.5	0.50	0.38	0.51	0.064
G38H2A1	*A. niger*	0.52	0.75	0.50	0.5	0.50	0.38
G42H2A2	*A. niger*	0.53	1.0	0.50	0.19	0.50	0.047
G22H2S2	*A. fumigatus*	0.50	0.064	0.50	0.125	0.50	0.047
G23H2S2	*A. fumigatus*	0.50	0.5	0.51	0.25	0.50	0.094
G38L2S1	*A. fumigatus*	0.50	0.5	0.50	0.25	0.50	0.19
VI06245[2]	*A. fumigatus*	0.51	4.0	0.51	1.5	0.51	0.75
VI06584[2]	*A. fumigatus*	0.50	3.0	0.50	1.0	0.50	0.38
VI06658[2]	*A. fumigatus*	0.50	4.0	0.50	1.5	0.50	0.75
ATCC MYA 4609[3]	*A. fumigatus*	0.50	0.38	0.50	0.5	0.50	0.19
ATCC 22019[3]	*C. parapsilosis*	0.56	0.19	0.56	0.032	0.56	0.032
CCUG 32723[4]	*C. albicans*	0.51	0.064	0.51	0.016	0.51	0.032
CCUG 35869[4]	*C. krusei*	0.50	0.38	0.50	0.25	0.50	0.19

1. E-tests with different azoles were done on separate occasions due to shortage of equipment at the time; hence the different MF-value across the same isolate as multiple spore suspensions were made.

2. Resistant clinical strains from the NVI strain collection.
3. Reference strains from American Type Culture Collection.
4. Reference strains from Culture Collection University of Gothenburg.

All the yeast reference strains displayed MIC-values similar to those indicated by bioMérieux (2018). The *A. fumigatus* reference strain, ATCC MYA 4609, had MIC-values that were higher than what was listed by ATCC (2020). The breakpoints in this table were determined with a microdilution series instead of an E-test, and it was stated that the spore concentrations used as inoculums were a thousand times more diluted than what was used on the E-test. These differences could potentially explain the high reads on the *A. fumigatus* strain, as the yeast cultures got MIC-values within the intervals given.

The MIC-value seemed quite consistent for each azole type between each environmental isolate; the MIC for itraconazole was somewhere between 0.064 µg/ml and 1.0 µg/ml, and half the environmental isolates had a MIC of 0.5 µg/ml for this azole type. The MIC-value for voriconazole ranged between 0.125 µg/ml and 0.5 µg/ml, while isolates tested on posaconazole had a MIC-value between 0.047 µg/ml and 0.38 µg/ml. According to EUCAST (2022), all the environmental isolates were below the ECOFF-value, and none of the *A. fumigatus* isolates exhibited phenotypic resistance. It is a bit more unclear for the *A. niger* isolates as the EUCAST table does not contain any resistance breakpoints for this species. However, since none of them exceeded the ECOFF-value, it is unlikely to assume they harboured any resistance.

One of the *A. fumigatus* isolates (G22H2S2) possessed a different morphology than the rest of the isolates. This isolate had less pigmented phialides and a lower spore production. Additionally, the edges of the colonies seemed to fuse into each other when they met (Figure 42 in Appendix 10). Since it had not shown resistance on the VIPcheck™, it was tested with E-test to see if it was exceptionally susceptible. No significant sensitivity towards posaconazole and voriconazole was detected on the E-test, but it had a significantly lower MIC-value on itraconazole (0.064 µg/ml) compared to the other *Aspergillus* isolates. No further analyses were done on this isolate.

The clinical isolates, VI06584, VI06658 and VI06245, had MIC-values higher than the rest of the isolates tested. The MIC-values also exceeded the threshold for the resistance definition stated by EUCAST (2022) on all azoles. These three strains were therefore the only isolates for which the *cyp51A* gene and promoter region was characterised.

4.4.3 MF-value and spore count

A few of the steps in the resistance screening were reliant on absorbance as a measurement for spore concentration. Because *A. niger* is much more pigmented than *A. fumigatus*, a test was conducted to see if the protocols, which were designed for *A. fumigatus*, were applicable for both

Aspergillus spp. A comparison in spore count of suspensions with the same MF-value between both species was done on a haemocytometer.

The spore count of a 0.50 MF *A. fumigatus* and *A. niger* spore suspension were done in three replicates, which are listed in Table 20. The spore concentration was calculated using the formula in section 3.1.7.3.

Table 20: Number of spores counted in all replicates on the haemocytometer, the mean spore count and the spore concentration of *A. fumigatus* and *A. niger* at 0.50 MF.

	Replicate 1	**Replicate 2**	**Replicate 3**
Spore count– *A. fumigatus*	152	157	143
	133	145	155
	143	186	178
	159	161	128
Mean spore count	146.75	162.25	151
Spore concentration (CFU/ml)	**1 467 500**	**1 622 500**	**1 510 000**
Spore count– *A. niger*	44	65	82
	40	70	68
	47	69	46
	33	56	52
Mean spore count	41	65	62
Spore concentration (CFU/ml)	**410 000**	**650 000**	**620 000**

The calculations show that the mean spore concentration of a 0.50 MF *A. fumigatus* suspension was 1 533 300 CFU/ml. The mean spore concentration was at 560 000 CFU/ml for *A. niger*. The results show that the spore concentration for *A. fumigatus* is two-three times higher than that of *A. niger* when the MF-value of the spore suspension is 0.50. Whether this trend is the same at other MF-values remains unknown. The analyses were not adjusted accordingly to the results of this experiment, but they have been noted for future references.

4.5 Molecular characterization of the clinical isolates

The resistant clinical strains from NVI's strain collection were also put through *bT2* PCR as the success rate was higher than that of *caM* PCR. The gel image is displayed in Figure 35, and the isolates are listed in Table 21. More details about each isolate are listed in Table 34 in Appendix 14.

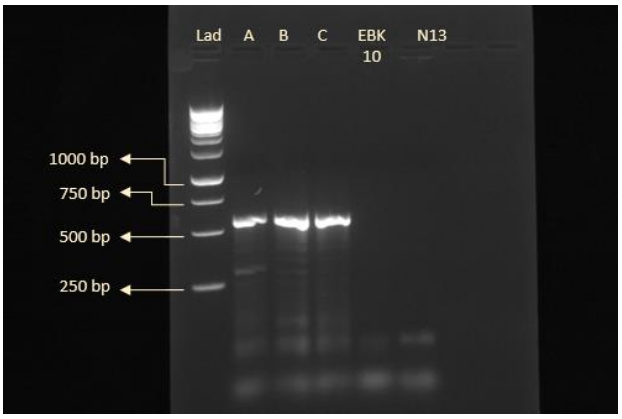


Figure 35: Gel image of *bT2* PCR products from the resistant strain (around 500 bp). The negative control (N13) and EBK10 were blank. Band sizes are marked on the ladder (Lad). Samples are listed in Table 21.

Table 21: Clinical, resistant *A. fumigatus* isolates.

Well	Sample no.
A	VI06245
B	VI06584
C	VI06658

The PCR product of *cyp51A* and promoter region from the three resistant *A. fumigatus* strains can be seen in Figure 36. A band appeared somewhere between 2000 bp and 2500 bp in the gel, which seems correct as the expected size of the PCR product is 2130 bp. The negative control appeared to be blank.

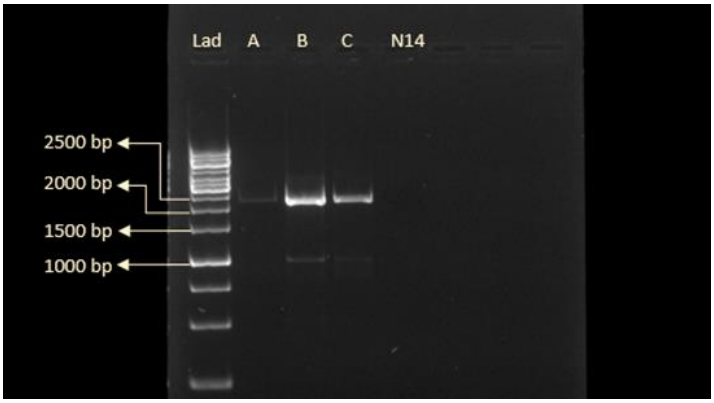


Figure 36: Gel image of *cyp51A* and promoter region PCR products from the resistant *A. fumigatus* strains (between 2000 bp and 2500 bp). Sample A was a little faint, and an extra band at around 1000 bp appeared in all of them. Negative control (N14) was blank. Band sizes are marked on the ladder (Lad). Samples are listed in Table 21.

In addition to the main PCR product, there was also a second, fainter band at 1000 bp that appeared in the gel. The PCR was repeated twice in order to gain a purer product; three parallels were run at the same time in one of these attempts. The product came out with poor quality, and the extra band still persisted (See Figure 52 and Figure 53 in Appendix 16). However, a sufficient PCR product without the extra band was achieved in one of the parallels of strain VI06245, so it was sent to sequencing without any additional processing. Because the extra band persisted in the other two samples, gel purification was executed on them before sequencing to avoid any potential noise.

After aligning the *cyp51A* and promoter region sequence with the reference strains, it appeared all three strains harboured the TR_{34}/L98H mutation. As displayed in Figure 37, all three sample sequences were identical to the TR_{34}/L98H reference strain.

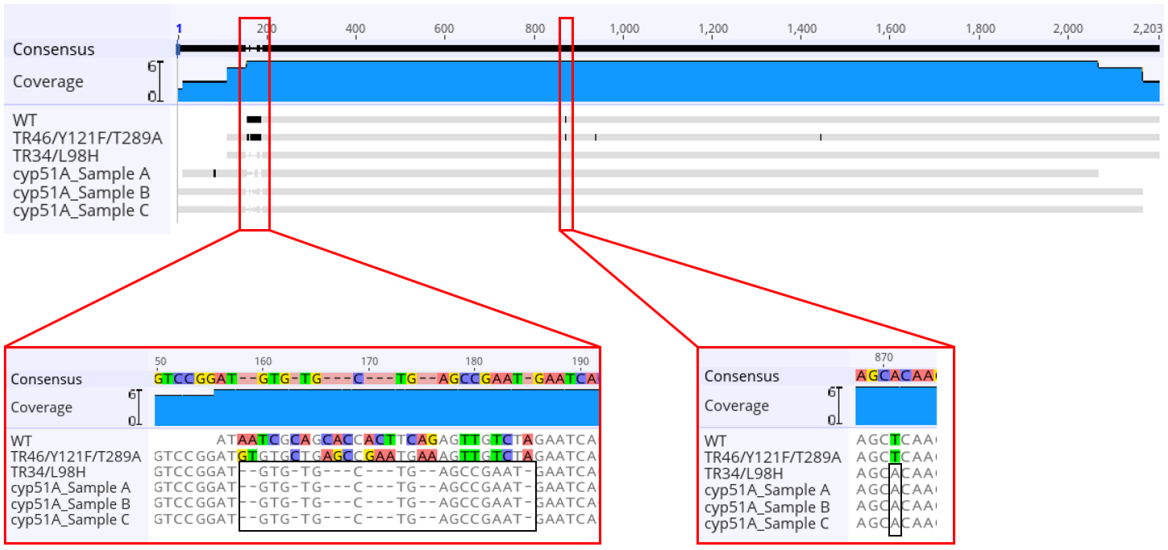


Figure 37: The *cyp51A* gene and promoter region from the three NVI resistant clinical strains aligned with the reference strains. Tandem repeats in the promoter region are marked to the left of the sequences, and the L98H point mutation is marked to the right. All isolates seem to have the same sequence as the TR_{34}/L98H mutation strain. Snippet from Geneious Prime (New Zealand).

5. Discussion

None of the 58 *Aspergillus fumigatus* or 37 *Aspergillus niger* isolates collected during this sampling displayed phenotypical azole resistance on the E-test. However, the three clinical isolates showed resistance against all three azoles (itraconazole, voriconazole and posaconazole) on the E-test, and had the *cyp51A* gene and promoter region screened for mutation. It was revealed that they all harboured the environment mediated mutation, TR_{34}/L98H.

5.1 The sampling yield in relation to environmental conditions

A total of 37 farms participated in the second sampling round of this research project, which resulted in 72 air samples; from these air samples, 58 *A. fumigatus* and 37 *A. niger* isolates were collected. Yeast, Mucorales and *Aspergillus* spp. were the most common fungi to appear on the primary plates. *A. fumigatus* was the most common *Aspergillus* sp. observed; this confirms how ubiquitous this species is in the environment, in accordance with previous reports (Latgé 1999). Generally, more isolates were collected from animal housing rooms than from the storage rooms, though the difference between the two was not very significant in terms of *A. fumigatus* isolates collected (31 from animal housing rooms, and 27 from storage rooms). In terms of *A. niger* isolates, however, the difference in number was a little wider (22 isolates from animal housing rooms and 15 from storage rooms). These trends could imply that animal housing rooms generally harbour more growth of *A. fumigatus* and *A. niger* compared to storage rooms. This does not seem to have always been the case for other fungal species that were found. In fact, some fungi were found more often in the storage rooms than the animal housing rooms. The exact distribution of species cannot be determined with this dataset as there were few samples, and several of the species were compiled into one group when the data were sorted.

In addition to the main sampling, a different set of samples from random households in Norway were taken in order to compare prevalence of fungi between farms and a different setting (See Appendix 6). Out of the 19 air samples, only two had *A. fumigatus* growth, and no *A. niger* appeared. Generally, there was significantly less fungal growth from households compared to barns (See Table 29 in Appendix 6). This was not unexpected despite households generally harbouring more stable conditions and warmer temperatures. The results might imply that farm buildings are hotspots for *A. fumigatus* proliferation, but it cannot be concluded with certainty as the sampling size from both settings were small.

During the first sampling (Henriksen 2021), the frequency ratio of *A. fumigatus* appearing between animal housing and storage rooms was similar to that of the second sampling. When comparing the number of isolates collected and the total fraction of plates with *A. fumigatus*, it is clear that the first sampling had a higher yield (108 isolates from 91 films in the first round, and 58 isolates from 72 films in the second round). One of the initial hypotheses was that proliferation of *A. fumigatus*

is controlled by seasonal differences as its optimum temperature (37 °C) is higher than compared to most other fungi (Kwon-Chung and Sugui 2013, Samson 2019, p. 130). However, the results were unexpected because more isolates were gained during the first sampling when the temperatures were generally lower. The median temperatures during the first sampling were 10 °C and 2.5 °C in animal housing and storage rooms respectively, while they were 15 °C and 10 °C in the second sampling (See Figure 17). Kwon-Chung and Sugui (2013) discloses that *A. fumigatus* does not grow when the temperature reaches below 12 °C; by that admission, the fungus should have been especially prominent in animal housing rooms during the second sampling compared to the first sampling, though that did not seem to be the case. According to Rhodes (2006), the germination rate for *A. fumigatus* does not vary significantly when the temperatures are below 30 °C; it is more prominent once the optimum temperature is reached.

One possible explanation to why the first sampling yielded more *A. fumigatus* growth is that livestock were kept inside the animal housing rooms during the winter, and the activity from the animals could have caused more *A. fumigatus* spores to disperse into the air. Animals are released out on the fields during spring and stay outside until autumn, leaving the rooms mostly vacant during this period. The differences in activity between the two sampling rounds could therefore have affected the number of *A. fumigatus* appearing. It is not clear how many of the participants took samples when there were no animals inside, but activity might still have been a contributing factor in isolate number from the animal housing rooms. However, this does not explain the differences in the storage rooms. Even though some of the participants mentioned they had kept animals in the storage rooms during sampling, these rooms were thought to remain vacant of activity most of the time during all seasons. According to Tekaia and Latgé (2005), *A. fumigatus* degrades dead organic materials such as decaying vegetation. This means that over time, they will produce more spores when conditions are stable. Items such as hay and bedding are stored over winter, while during the rest of the year, these items are replenished. This could possibly explain why more isolate were collected in the storage rooms during the first sampling compared to the second sampling. It could also be coincidental, given how small the sampling sizes were; especially during the second sampling. The temperatures were also fairly low for optimal growing conditions in the first sampling (Kwon-Chung and Sugui 2013). It is unspecified how close the storage rooms were to the animal housing rooms at each farm, or if the activity differed with the seasons. Seasonal differences, as well as climatic differences, may still be of interest to investigate further with larger samplings in order to determine the proliferation patterns of *A. fumigatus*.

Other factors that could have affected the isolate yield were considered. The participants were handed a questionnaire with various relevant questions to answer and fill in (Appendix 2). Based on the data provided, there does not seem to be any clear correlation between how often *A. fumigatus* and *A. niger* appeared and the type of animal, bedding, storage items or pesticides used during the second round (See Table 30 in Appendix 7 with Table 31 in Appendix 9). The results may imply that *A.*

fumigatus appears more frequently in the farm environment, suggesting that farm buildings harbour potential hotspots for proliferation of this fungus, which was one of the initial hypotheses. However, because of the small sampling size and the one-sided focus on Norwegian farms specifically, it is difficult to draw any final conclusions. More investigation is necessary in order to determine what factors affect fungal proliferation, and if they favour any species specifically.

5.1.1 Citizen science provided efficient sampling, but may have affected data quality

The information data and the samples were all provided by citizen scientists. Samples were acquired much quicker and cheaper than if they were to be retrieved manually; it cut down on both travelling and manual labour. During the first sampling, over fifty farms were interested in participating, and 85.2% sent their samples back (Henriksen 2021). In total, 95.3% of the same participants that were contacted showed interest in joining the second round. During the second round, 90.2% of the participants who responded sent in their samples. It is expected that engagement drops a little over time (Eveleigh et al. 2014), but there was a high percentage of participants that showed interest for the second round, and the engagement seemed good overall.

One of the four violations of a good experimental setup that was described by Isaac et al. (2014) regarded uneven geographical coverage of sampling. As the map in Figure 16 shows, the samples were quite scattered; some areas, such as the South-West of Norway, had densely clustered sampling. Other areas were left vastly uncovered; especially the northern parts of the country. It should be noted that there were relatively few participants for such a large scale study, and thus it cannot be expected that the sampling coverage would be as sufficient as preferred. The map is likely a reflection of the distribution of population density in the country, which is a phenomenon often seen in citizen science (Geldmann et al. 2016). This might also be something to note for future studies. If an adequate participation does not grant a sufficient geographical coverage, manual field work and sampling might be necessary (Mair and Ruete 2016). In the case of investigating azole resistance in Norway, trying to increase engagement or gathering samples manually in the northern parts of the country might be required in order to gain a better sampling coverage, and thus a more representable surveillance data. In terms of the geographical distribution of *A. fumigatus* and *A. niger* prevalence, it appeared to be lower further up North compared to South of Norway. This could be due to the warmer climate down South, which promotes proliferation of fungi (Kwon-Chung and Sugui 2013). This might imply that the South is more susceptible to become endemic than the North, but a better sampling coverage is needed in order to determine that.

Another violation Isaac et al. (2014) brought up is that the quality of the data is affected by the differences in effort among the participants. It is likely that the participants did not handle the equipment or followed instructions equally as thoroughly; e.g. if the spore traps were left out for the amount of time instructed (6 h), or if the setup was handled with equal care. There are no ways of

telling how significant these variables were, or how much they have affected the results. They are nevertheless something that must be taken into consideration upon analysing the data and drawing conclusions. This violation is also relevant in terms of the information provided by the participants; how the questionnaire was filled and interpreted seemed to be somewhat different between the participants. This has in turn made data sorting especially challenging. The participants in this sampling already had some experience from the first round, which could have contributed positively to the quality of the data. In future citizen science projects, it might be worth to consider providing forms that are clearer and even more on point with its questions to restrict any ambiguity. It is also important to communicate clearly with the participants; i.e. educate them and give them feedback on how they are operating in order to further limit noise and errors (Silvertown 2009).

Because of all the uncertainties that come with citizen science, there is a possibility that this sampling approach has affected some of the results. Some primary plates had minimal or no fungal growth; this was peculiar, given that farms are likely to harbour a lot of fungi. The cultivation methods selected for fungal species that could grow at 37 °C, so it is likely that there were many species on the film that were uncultivable under the conditions used. If sampling was done by the participants exactly as instructed, and the film had no fungal growth, it could mean that their farms were dominated by mesophilic fungi.

Despite all the potential errors that can come with citizen science, this approach seemed like a viable way of collecting data for microbiological studies such as this, which Shelton et al. (2020) also has concluded. With the two sampling rounds combined, a substantial amount of isolates were collected much quicker than if they were to be collected manually.

5.2 Species identification of the isolates revealed the importance of sequencing

In terms of morphological identification and isolation of *A. fumigatus* and *A. niger* colonies from the primary plates, both fungal species were relatively easy to recognise. Incubating the samples at 37 °C on DG18 potentially restricted the growth of several other species that could be mistaken for *A. fumigatus*, such as some *Penicillium* spp. Whenever there were any doubts, microscopy could reveal if a colony was an *Aspergillus* sp. or a *Penicillium* sp. Most often, microscopy was used to identify other fungal colonies as many of the *Penicillium* colonies looked quite different from *A. fumigatus* colonies; e.g. some *Penicillium* colonies had a yellow zone around it or was a different shade of green. While the target species were fairly recognisable, the identity of the colonies could not really be determined with any certainty beyond that they belonged to *Aspergillus* section *Fumigati* or section *Nigri*. Species within the same complex are most often only distinguishable from one another through molecular analyses (Alcazar-Fuoli et al. 2008, Gautier et al. 2016).

All the isolates that were morphologically identified as an *Aspergillus* belonging to section *Fumigati* turned out to be *A. fumigatus* in the sequence analysis. The sequence identification of the black *Aspergillus* isolates revealed that none of them were *A. niger*; 36 *Aspergillus tubingensis*, and 1 *Aspergillus welwitschiae*. This shows how important it is to use molecular tools in order to confirm the species identity of a strain; especially if there are several other variants with similar morphological and metabolic properties. This type of identification is especially crucial in certain industries, where strains within the same species can be either pathogenic or commercially beneficial (Frisvad et al. 2018). While *A. niger* is among the most common species to cause aspergillosis, *A. tubingensis* is also a very prevalent pathogenic species among the black *Aspergillus* spp. (Gautier et al. 2016, Toyotome et al. 2018). The results of the identification might also be an implication that *A. tubingensis* could be more prevalent in Norway than *A. niger*, or that *A. tubingensis* is more competitively fit. However, it is unknown how many people get infected by *A. tubingensis* and other black *Aspergillus* spp. in Norway annually.

Two genetic markers were used to identify the isolates; *caM* and *bT2*. The variety in BLAST top hits for *caM* was higher than *bT2*; this connotes to the calmodulin gene having a higher resolution in species identification for *Aspergillus* spp. than the β-tubulin gene (Alshehri and Palanisamy 2020). On the other hand, *caM* was more difficult to work with, and had a lower success rate in terms of PCR and sequencing than *bT2* had. From this experience, it is recommended to use *caM* as the primary genetic marker for a more distinct identification of *Aspergillus* spp., but to use *bT2* if *caM* fails. If the resolution is not crucial, and the only directive is to confirm the species, it is advised to use *bT2* due to its practical advantages.

Morphological observation is quick and reliable when identifying fungal species as all the isolates were later revealed to be of the *Aspergillus* complex that the target species belonged to. By restricting some growth conditions, the target species became easier to extract. However, molecular identification is still necessary to confirm the species, which was especially prominent when sequencing the black *Aspergillus* isolates because none of them were *A. niger*.

5.3 *A. fumigatus* and *A. niger* require different approaches in analyses due to biological differences

Even though *A. fumigatus* and *A. niger* belong in the same genera and share some biological attributes (Samson 2019, p. 130, 146), a few distinctions became especially noticeable during some of the analyses. These distinctions have shown that some of the protocols that were originally made for *A. fumigatus* were not optimal for all *Aspergillus* spp. Initially, *caM* was meant to be the only genetic marker for sample identification in this study. However, it was eventually decided to switch over to *bT2* for some of the isolates as all the black *Aspergillus* isolates produced several bands during *caM*

PCR. The first assumption was that there might have been some kind of contamination during the PCR, but because the negative samples were blank, this was not likely to be the case. Upon aligning the primer sequences (CMD5 and CMD6) with random *caM* sequences of *A. fumigatus, A. niger* and *A. tubingensis*, it became apparent that the primers were not optimal for all species; both primer sequences were mostly found in the *A. fumigatus caM* sequences. A suboptimal alignment between the primers and intended template flanks could have led to the primers annealing to other parts of the genome of the black aspergilli. A whole genome sequencing could reveal which parts of the genome these primers might have annealed to. In order to optimise *caM* PCR for black *Aspergillus* spp. in any future studies, a different set of primers that are more specific for the target species should be considered.

Another challenge that could possibly be related to dissimilarities between species appeared during PCR of *cyp51A*. The *cyp51A* PCR was mainly done on the clinical *A. fumigatus* strains, but one *cyp51A* PCR run was also attempted on one of the *A. tubingensis* isolates, which appeared to not yield any products at all. DNA from a random *A. fumigatus* isolate was used as a positive sample template during the PCR, which produced the correct amplicon. These results further implies that not all primers are universal for every *Aspergillus* spp.

During *cyp51A* PCR on the clinical isolates, an extra band at 1000 bp appeared on the gel, and it was necessary to extract the right PCR product through gel purification. No contamination appeared during PCR as the negative sample was blank (Figure 36). According to Henriksen (2021), where *cyp51A* PCR was done exclusively on environmental isolates, such extra bands did not appear. This might be an indication that there are some differences between clinical and environmental strains, even though they are of the same species. The host environment is very different from the outdoor and indoor environment, which tells how adaptable *A. fumigatus* is. It is expected that the transcriptional profile of clinical and environmental isolates are very different, but the genome should be the same. According to Debeaupuis et al. (1997), their analysis showed no genetic clustering between clinical and environmental samples. In other words, clinical and environmental strains are equally as genetically diverse from each other as strains within one of these groups are. Contamination during colony isolation as a possible explanation for the extra band forming in the *cyp51A* PCR could be considered, but this is uncertain. Extracting and sequencing the extra band might reveal where it originated from, though it was not prioritised in this study.

PCR was not the only step that displayed challenges with the protocol between different species. Black *Aspergillus* spp. are much more pigmented than *A. fumigatus*, and the phenotypic resistance screening methods relied on using absorbance (MF-values) as a measurement for spore concentration. It was therefore thought that there would be a difference in spore concentration between *A. fumigatus* and black *Aspergillus* spp. at the same MF-value, and the spore counting test showed that

there was a two-three folds difference at 0.5 MF. Still, it remains unclear how much of an impact this might have had on the results as the protocol was not adjusted for black *Aspergillus* spp. for the duration of the study. Nor is it clear if the difference is equally as significant at other MF-values.

If the differences in spore concentration affected the results of the VIPcheck™, then the black *Aspergillus* suspensions would have acted more susceptible to azoles because of the lower spore concentration (granted that the *Aspergillus* spp. respond similarly to the antifungals). Using a lower concentration than what was instructed in the protocol could potentially deem some resistant strains as non-resistant, and phenotypic resistance would go undetected. It was attempted to keep a consistent MF-value between isolates during the VIPcheck™. Some of the black *Aspergillus* isolates managed to grow in some of the azole wells, and there were also more black *Aspergillus* isolates than *A. fumigatus* isolates that passed the VIPcheck™. It is unknown why this was the outcome, considering the differences in spore concentration. It could be that black aspergilli are naturally more resilient to azoles, or that more spores managed to cluster together upon inoculation.

Lastly, the differences in spore concentration could affect the outcome of the E-test; black *Aspergillus* suspensions with a lower spore concentration could achieve a breaking point at a lower azole concentration. This difference might be a little more significant for the E-test as there is less leeway for MF-value used in this test. It is possible that this may have affected the results of the resistance screening, but the extent is unclear. The breakpoints for both species were evaluated equally throughout this study. The spore concentration in relation to MF-value is not the same between different species with different grades of pigmentation. This is something that might be worth investigating for future studies involving different *Aspergillus* spp. in order to design optimal protocols that are more species specific.

5.4 The differences between phenotypic resistance screening methods

Two phenotypic azole resistance screening methods were used in this study. In the first screening method, the VIPcheck™, two *A. fumigatus* isolates and eight black *Aspergillus* isolates had managed to grow in the azole wells. This resistance screening tool was quick and easy to set up, something that was especially convenient when handling a large number of isolates. The isolates that had a high score on the VIPcheck™ (++ or +++) were screened with the second screening method; the E-test, which showed what azole concentration the fungi would be growth inhibited (MIC). In reference to EUCAST (2022), none of the isolates exhibited any traits of azole resistance on the E-test. One uncertainty is that there are no standard MIC-values for *A. niger* nor *A. tubingensis* that distinguish between sensitive and resistant strains. This has made it challenging to determine if a black *Aspergillus* isolate can be considered resistant or not. However, the ECOFF-values to *A. niger* were higher than any of the MIC-values to the black *Aspergillus* isolates, which indicates that these isolates

are likely WT strains; that is, if *A. tubingensis* is similar to *A. niger* in terms of azole susceptibility. The *A. fumigatus* isolates also had MIC-values under the ECOFF-values, and none of them exceeded the threshold for resistance.

The VIPcheck™ seems to be quite sensitive because some isolates managed to grow on azole infused agar, but later showed no resistance on the E-test. This also seemed to be the case for Henriksen (2021). According to Buil et al. (2017), the VIPcheck™ is quite reliable and manages to discriminate between resistant and sensitive strains, and that minimal growth after 48 h should be used as a threshold for resistance. There were many false positives from the VIPcheck™ during this study. The VIPcheck™ is a qualitative test, and how the results are interpreted can be very subjective. This could explain why the experience with this screening method was so different from Buil et al. (2017). In the "BARNS" project, the threshold chosen was very low due to limited previous experience with the test; this was to make sure that no potential resistant strains were left undetected.

E-tests can sometimes be challenging to read when working with filamentous fungi, but in this study, the breakpoints were relatively easy to read after 24 h. Even so, the interpretation of the breakpoints can sometimes be different between persons. Letting more people read the results might alleviate any ambiguities. Due to the lack of resources and time at one point, RPMI plates and old ETEST® strips that were newly expired were utilised, which could have affected the results. Upon testing voriconazole ETEST® strips from a new and an old batch on the same isolate, there did not seem to be much noticeable differences. The RPMI plates were never used if they were one day or more past the expiration date, and it has likely not affected the results much.

The experimental setup of azole resistance screening used in this study was based on a "process of elimination". The VIPcheck™ served as the first layer in detecting resistance among the isolates. The sensitivity of the VIPcheck™ makes it less likely to leave resistant strains undetected, but some very minimal mycelial growth in the azole wells can most likely be negligible. The VIPcheck™ is also faster and easier to set up than the E-test. Time was saved as the VIPcheck™ made it no longer necessary to put every isolate through the E-test. Instead, the E-test served as the second layer in resistance screening, and only the isolates selected by the VIPcheck™ were screened. Resistant strains detected on the E-test further restricts the number of isolates needed to be screened for mutations on *cyp51A* and its promoter region. This streamline of assays proved to be an efficient technique when looking for azole resistance.

5.5 Mucorales contamination can be inconspicuous and persistent

There was one *A. fumigatus* isolate (G23H2S2) that managed to grow on agar with voriconazole on the VIPcheck™ (See Figure 43-B in Appendix 12). This mycelium was quite

filamentous, and it was first believed that this was a stress response from the fungus. However, upon attempting to inoculate an agar plate with the glycerol stock solution, it became apparent that the isolate was contaminated with Mucorales. It is unknown when the isolate was contaminated, because the secondary plate looked pure before the stock solution was made. The control well on the VIPcheck™ also looked like it only had an *A. fumigatus* colony and no network of filamentous mycelium. Furthermore, there were no apparent signs of contamination on the *caM* PCR product (See Figure 50-C in Appendix 16), or any noise in the sequence data (see Table 36 in Appendix 18).

There was Mucorales on the primary plate this isolate stemmed from, so it is most likely the contamination occurred during the isolation phase, and somehow went undetected. A similar phenomenon was also observed by Henriksen (2021). It seems that the Mucorales was unable to grow in the presence of *A. fumigatus*, but could lie dormant in its presence. Mucorales has been reported to be naturally resistant to azoles (Nordøy and Gaustad 2008). Once *A. fumigatus* was inhibited, Mucorales was able to germinate; this must have been the case in the voriconazole well on the VIPcheck™ plate. It is unknown how it managed to grow when the stock solution was cultivated.

It can be difficult to prevent such inconspicuous cross contaminations. The Mucorales did not seem to have affected the results significantly. The only preventative measures to take are to work as sterile as possible, and if contamination does appear, one should try and isolate the target fungus to the best of their ability (e.g. see section 3.1.3.2) and restart the analysis.

5.6 Very few resistant *A. fumigatus* isolates have been detected in Norway

The resistance screening showed that none of the *A. fumigatus* or black *Aspergillus* isolates collected during this study were resistant to itraconazole, voriconazole or posaconazole. It is unlikely that there are no resistant mutants in the environment due to evidence of resistant *A. fumigatus* with environmental mutations from patients in Norway (Skaar et al. 2019). The outcome of this study is most likely due to the small sample size compared to the scale of the project. With fewer isolates to work with, there is a lesser chance of detecting any resistant strains. A primary plate with multiple colonies of *A. fumigatus* and/or black *Aspergillus* spp. could potentially harbour resistant strains, but only a maximum of three colonies were isolated from each plate, and the pick was rather random.

Furthermore, the sampling size and the lack of azole resistant isolates make it impossible to find any correlations between prevalence and specific external factors. One such factor of particular interest was the farmers' use of pesticides, which was inquired in the questionnaire. Only two of the 37 participants used fungicides, and six were running an organic farm. None of the participants disclosed if these fungicides were of the DMI type. Even if resistant strains were found among the

isolates, the total data gathered would still not have been sufficient enough to observe any trends. A more specific questionnaire could potentially uncover more relevant information.

The three clinical isolates from NVI's strain collection were isolated from animal hosts (See Table 34 in Appendix 14) and had shown phenotypic azole resistance. The mutation screening on *cyp51A* and its promoter region revealed that all three strains harboured the TR_{34}/L98H mutation. This is a mutation is obtained through the environmental route of resistance and promotes overexpression of the *cyp51A* gene, but it is also one of the most common mutation among clinical azole resistant *A. fumigatus* samples (Buil et al. 2019). Oslo University Hospital stated that most of the resistant *A. fumigatus* isolates from their clinical strain library harboured the TR_{34}/L98H mutation. Additionally, one of their samples had the TR_{46}/Y121F/T289A mutation, and some had point mutations related to azole therapy in patients (C. T. Andersen, Personal Communication, 07.03.2022). Aspergillosis and other fungal infections in animals are likely underreported (Skaar et al. 2019), and likely is also the prevalence of azole resistant strains. The identification of the *cyp51A* genotype of the three clinical isolates along with what was reported by C. T. Andersen is consistent with the findings in other studies (Jensen et al. 2016, Verweij et al. 2016). It is also consistent with the leading hypothesis that environmental impacts have an effect on the development of azole resistant strains, which then lead to treatment failure in humans and animals that are infected.

In the first sampling round done by Henriksen (2021), three resistant strains were detected; both environmental mutations, TR_{34}/L98H and TR_{46}/Y121F/T289A, were identified, along with another resistance mutation not found on *cyp51A*. Henriksen (2021) also discloses that the resistant strains were found in different geographical locations, implying that environmental selection pressure is not restricted to one area of the country. There could be a small possibility that resistant strains have been imported from other parts of the world, but this is difficult to prove. It is likely that azole resistance emerged from the Norwegian environment, given the reproduction rate and adaptation capabilities of *A. fumigatus* (Latgé and Chamilos 2019).

Because of the small sampling size, it is limited what can be drawn as a final conclusion in terms of prevalence of azole resistant *Aspergillus* spp. in Norway. However, the fact that no resistant strains were found during the second sampling may serve as an indication of how low this prevalence may be compared to other countries. The number of resistant strains (3) uncovered in relation to the number of isolates found during both samplings (166) was fairly small. One of the initial hypotheses was that Norway would have a lower prevalence of azole resistance than other countries. The results of this study may seem consistent with it. Still, more extensive studies are needed in order to confirm this hypothesis.

5.7 Azole resistance is a One Health challenge

Several papers have reported azole resistant *A. fumigatus* in patients and linked the mutation to environmental selection pressure (Bromley et al. 2014, Buil et al. 2019, Prigitano et al. 2019). Azoles are important in health, industry and food production, and the emergence of resistant fungal strains has become a challenge in a One Health perspective (Skaar et al. 2019).

One Health is a model approach in which the health of humans, animals and the environment are closely linked together (Atlas 2013). For example, an environmental change can cause a rippling effect that will directly or indirectly affect humans and/or animals on a local or global scale (Shomaker et al. 2013). The term was first used somewhere between 2003 and 2004, but the concept can be traced back much further than that. One Health focuses on infectious diseases and how to contain, minimise or prevent them from advancing. The goal is to achieve the best public, veterinary and environmental health, which are affected by changes in the ecosystem along with other human interferences (Mackenzie and Jeggo 2019). It is a collaborative and multidisciplinary effort between several sectors and science fields, and they work together to maintain a balance that is beneficial for humans, plants and animals by reducing any impact that may interfere with it (Atlas 2013, Mackenzie et al. 2014, Mackenzie and Jeggo 2019).

In a One Health perspective, azole resistant fungal infections are linked to human impact on the environment. Fungicide use in agriculture and industry can mediate azole resistance in pathogenic fungal strains, which then can spread to humans and animals with infections that are difficult to treat. Azole resistance is developed in both patients and the environment, and while medicine and agriculture do not use the exact same azoles, cross resistance can occur (Snelders et al. 2012). The resistance obtained in the environment will then lead to treatment failure and increase mortality rates. Additionally, climate changes are expected to increase the prevalence of pathogenic fungi (Velásquez et al. 2018, Skaar et al. 2019), and with fungal infections expected to increase (Latgé and Chamilos 2019), the demand for effective drugs to manage these diseases will also increase.

It cannot be stated with certainty that agricultural fungicides are the main selection pressure event in the rise of azole resistant fungi in Norway, but there are implications, given that environment mediated resistance mutations, TR_{34}/L98H and TR_{46}/Y121F/T289A, have been uncovered in both clinical and environmental samples. Azole resistance in Norway and the rest of the world in a One Health perspective is something that should be investigated and addressed further.

5.8 Future approaches for azole resistance surveillance and management in Norway

Azole resistant pathogenic fungi are becoming an increasing problem all over the world (Verweij et al. 2016). While azole resistant *A. fumigatus* has been detected in both patients and the environment in Norway, the true extent of resistance in this country is still not fully understood yet; nor what the strongest driving forces are. In order to determine where Norway is currently at in the development, a higher sampling density is needed. Sampling over time is something that should also be considered in order to monitor changes, and see what directions they are taking. An increased sampling size is also needed in order to find any correlations with external factors and map out hotspots. While the results from the study imply that the prevalence of azole resistance in Norway is lower compared to other countries, management of fungal diseases should still be executed in order to prevent any serious endemics. Henceforth, a collaborative effort to close any knowledge gaps should be further enhanced so the authorities can implement helpful measures.

Trying to understand the resistance mechanisms and how it is proliferating is imperative in managing it, and so several aspects of the ecosystem should be investigated in order to map out the extent of the endemic. The prevalence of azole resistant *A. fumigatus* could imply that there might be other fungal pathogens that can have obtained azole resistance, or resistance against other fungicides, through the same routes. Black *Aspergillus* spp. were also screened for resistance during the study, and even though no resistance was detected, it is not unlikely that there are strains that have responded similarly to environmental impacts as *A. fumigatus* has.

Furthermore, expanding the approaches in laboratory analyses could be considered. While *cyp51A* has the most studied mutations that lead to azole resistance, screening for mutations on other relevant genes could be informative. Examples of such genes are *cyp51B*, which carries out similar functions as *cyp51A* in ergosterol biosynthesis (Dhingra and Cramer 2017). Another relevant gene is *cdr1B*, which is related to the efflux pump mechanism pathway when overexpressed (Hagiwara et al. 2016). Other screening methods for phenotypic resistance could also be considered if there are uncertainties with the E-test, such as Clinical and Laboratory Standards Institute (CLSI) broth microdilution. In this assay, a dilution series of an antifungal is tested across a dilution series of an isolate spore suspension in order to determine the MIC-value (Nascente et al. 2009). Similar setups can also be used to test several isolates and antifungals simultaneously, which could increase the efficiency of screening methods (Cox et al. 2009, Wedge et al. 2013).

The fact that most resistant clinical samples seem to harbour the TR_{34}/L98H environmental mutation (Buil et al. 2019) is another evidence for concern. It is important to make sure azole resistance do not spiral out of control while it is still manageable, which is why preventative measures should be implemented by the authorities. It is unlikely that azole use in industry and agriculture will

end completely, especially since food demands are increasing with the growing human population (Brauer et al. 2019, Verweij et al. 2020). It is therefore imperative to ration the resources we have in order to reserve its efficiency. Integration of new laws and regulations on use of azoles would enable more time to discover new families of drugs to combat fungal infections. Such regulations include using the lowest effective dose of azoles as well as rotating between the fungicide classes used in agriculture (Gossen et al. 2014, Lopez-Ruiz et al. 2020).

The "BARNS" project was a pilot run for a more elaborate AMR research project called Navazole, where a more extensive citizen science sampling with multiple rounds will be executed. The research project will investigate azole resistance in *Zymoseptoria tritici*, *Parastagonospora nodorum* and *A. fumigatus* from several sample types and different geographical areas in Norway. The research project also aims to identify hotspots for proliferation and resistance development and try to better understand azole resistance in a One Health perspective in order to implement the best preventative measures.

5.9 Conclusion

The prevalence of azole resistant *A. fumigatus* and black *Aspergillus* spp. in Norway is not yet fully understood. This is mostly due to the small sampling size and the one-sided focus on Norwegian farms in this study, but the results may imply that it is relatively low compared to other countries. It is still uncertain what driving forces are involved, but the results seem consistent with the leading hypothesis that environmental impacts affect the development of resistance. The results are also consistent with the initial hypothesis that farm buildings may be hotspots for *A. fumigatus* proliferation, but a clear conclusion cannot be drawn from this study because of the size and bias of the sampling. It is difficult to find any correlations between proliferation and the determining external factors that were investigated, such as seasonal temperature differences, but the presence and activity of animals could potentially have affected the outcome of this study. Furthermore, laboratory techniques used might need optimisation for different species in future studies. More extensive surveillance and sampling are needed in order to gain a better understanding of azole resistance development in Norway. Measures to preserve public health and food safety can be implemented once more is known about the pattern of fungal proliferation and development of resistance.

6. References

1. Alastruey-Izquierdo, A., Cadranel, J., Flick, H., Godet, C., Hennequin, C., Hoenigl, M., Kosmidis, C., Lange, C., Munteanu, O., Page, I. and Salzer, H. J. F. (2018). Treatment of Chronic Pulmonary Aspergillosis: Current Standards and Future Perspectives. *Respiration*, *96*(2), 159-170. DOI:10.1159/000489474
2. Alcazar-Fuoli, L. and Mellado, E. (2013). Ergosterol biosynthesis in Aspergillus fumigatus: its relevance as an antifungal target and role in antifungal drug resistance. *Frontiers in Microbiology*, *3*. DOI:10.3389/fmicb.2012.00439
3. Alcazar-Fuoli, L., Mellado, E., Alastruey-Izquierdo, A., Cuenca-Estrella, M. and Rodriguez-Tudela, J. L. (2008). Aspergillus section Fumigati: antifungal susceptibility patterns and sequence-based identification. *Antimicrobial agents and chemotherapy*, *52*(4), 1244-1251. DOI:10.1128/AAC.00942-07
4. Alcazar-Fuoli, L., Mellado, E., Garcia-Effron, G., Lopez, J. F., Grimalt, J. O., Cuenca-Estrella, J. M. and Rodriguez-Tudela, J. L. (2008). Ergosterol biosynthesis pathway in Aspergillus fumigatus. *Steroids*, *73*(3), 339-347. DOI:https://doi.org/10.1016/j.steroids.2007.11.005
5. Alshehri, B. and Palanisamy, M. (2020). Evaluation of molecular identification of Aspergillus species causing fungal keratitis. *Saudi Journal of Biological Sciences*, *27*(2), 751-756. DOI:https://doi.org/10.1016/j.sjbs.2019.12.030
6. ATCC. (2020). Use of The ATCC® Aspergillus Fumigatus Drug Testing Panel in Antifungal Drug Testing. In. University Boulevard Manassas, Virginia, USA: American Type Culture Collection.
7. Atlas, R. M. (2013). One Health: Its Origins and Future. In J. S. Mackenzie, M. Jeggo, P. Daszak and J. A. Richt (Eds.), *One Health: The Human-Animal-Environment Interfaces in Emerging Infectious Diseases: The Concept and Examples of a One Health Approach* (p. 1-13). Berlin, Heidelberg: Springer Berlin Heidelberg.
8. Balding, P. R., Porro, C. S., McLean, K. J., Sutcliffe, M. J., Maréchal, J. D., Munro, A. W. and de Visser, S. P. (2008). How do azoles inhibit cytochrome P450 enzymes? A density functional study. *J Phys Chem A*, *112*(50), 12911-12918. DOI:10.1021/jp802087w
9. Becher, R. and Wirsel, S. G. R. (2012). Fungal cytochrome P450 sterol 14α-demethylase (CYP51) and azole resistance in plant and human pathogens. *Applied Microbiology and Biotechnology*, *95*(4), 825-840. DOI:10.1007/s00253-012-4195-9
10. Benedict, K., Jackson, B. R., Chiller, T. and Beer, K. D. (2019). Estimation of Direct Healthcare Costs of Fungal Diseases in the United States. *Clin Infect Dis*, *68*(11), 1791-1797. DOI:10.1093/cid/ciy776
11. Berger, S., El Chazli, Y., Babu, A. F. and Coste, A. T. (2017). Azole Resistance in Aspergillus fumigatus: A Consequence of Antifungal Use in Agriculture? *Frontiers in Microbiology*, *8*. DOI:10.3389/fmicb.2017.01024
12. Bhagat, J., Singh, N., Nishimura, N. and Shimada, Y. (2021). A comprehensive review on environmental toxicity of azole compounds to fish. *Chemosphere*, *262*, 128335. DOI:https://doi.org/10.1016/j.chemosphere.2020.128335
13. bioMérieux. (2016). ETEST® Antifungal Susceptibility Testing. 15206C - en - 2016/08 ed. b. SA. 08/2016. Marcy-l'Étoile, France.
14. bioMérieux. (2018). Summary of ETEST® Performance, Interpretive Criteria and Quality Control Ranges. 15211L - 2018/12 ed. bioMérieux. 12/2018. Marcy-l'Etoile - France.
15. bioMérieux. (n.d.). ETEST®. Retrieved 31.05.2022, From https://www.biomerieux-usa.com/clinical/etest.
16. Bongomin, F., Gago, S., Oladele, R. O. and Denning, D. W. (2017). Global and Multi-National Prevalence of Fungal Diseases-Estimate Precision. *J Fungi (Basel)*, *3*(4). DOI:10.3390/jof3040057
17. Bonney, R., Phillips, T. B., Ballard, H. L. and Enck, J. W. (2016). Can citizen science enhance public understanding of science? *Public Underst Sci*, *25*(1), 2-16. DOI:10.1177/0963662515607406
18. Brauer, V. S., Rezende, C. P., Pessoni, A. M., De Paula, R. G., Rangappa, K. S., Nayaka, S. C., Gupta, V. K. and Almeida, F. (2019). Antifungal Agents in Agriculture: Friends and Foes of Public Health. *Biomolecules*, *9*(10), 521. DOI:10.3390/biom9100521
19. Bromley, M. J., van Muijlwijk, G., Fraczek, M. G., Robson, G., Verweij, P. E., Denning, D. W. and Bowyer, P. (2014). Occurrence of azole-resistant species of Aspergillus in the UK environment. *Journal of Global Antimicrobial Resistance*, *2*(4), 276-279. DOI:https://doi.org/10.1016/j.jgar.2014.05.004
20. Buil, J. B., Lee, H. A. L. v. d., Rijs, A. J. M. M., Zoll, J., Hovestadt, J. A. M. F., Melchers, W. J. G. and Verweij, P. E. (2017). Single-Center Evaluation of an Agar-Based Screening for Azole Resistance in Aspergillus fumigatus by Using VIPcheck. *Antimicrobial Agents and Chemotherapy*, *61*(12), e01250-01217. DOI:doi:10.1128/AAC.01250-17

21. Buil, J. B., Snelders, E., Denardi, L. B., Melchers, W. J. G. and Verweij, P. E. (2019). Trends in Azole Resistance in Aspergillus fumigatus, the Netherlands, 1994-2016. *Emerging infectious diseases*, *25*(1), 176-178. DOI:10.3201/eid2501.171925
22. Bultman, K. M., Kowalski, C. H. and Cramer, R. A. (2017). Aspergillus fumigatus virulence through the lens of transcription factors. *Medical Mycology*, *55*(1), 24-38. DOI:10.1093/mmy/myw120
23. Camps, S. M. T., Rijs, A. J. M. M., Klaassen, C. H. W., Meis, J. F., O'Gorman, C. M., Dyer, P. S., Melchers, W. J. G. and Verweij, P. E. (2012). Molecular epidemiology of Aspergillus fumigatus isolates harboring the TR34/L98H azole resistance mechanism. *Journal of clinical microbiology*, *50*(8), 2674-2680. DOI:10.1128/JCM.00335-12
24. Cao, D., Wang, F., Yu, S., Dong, S., Wu, R., Cui, N., Ren, J., Xu, T., Wang, S., Wang, M., Fang, H. and Yu, Y. (2021). Prevalence of Azole-Resistant Aspergillus fumigatus is Highly Associated with Azole Fungicide Residues in the Fields. *Environmental Science & Technology*, *55*(5), 3041-3049. DOI:10.1021/acs.est.0c03958
25. Chen, P., Liu, J., Zeng, M. and Sang, H. (2020). Exploring the molecular mechanism of azole resistance in Aspergillus fumigatus. *Journal de Mycologie Médicale*, *30*(1), 100915. DOI:https://doi.org/10.1016/j.mycmed.2019.100915
26. Chen, P., Liu, M., Zeng, Q., Zhang, Z., Liu, W., Sang, H. and Lu, L. (2020). Uncovering New Mutations Conferring Azole Resistance in the Aspergillus fumigatus cyp51A Gene. *Frontiers in Microbiology*, *10*. DOI:10.3389/fmicb.2019.03127
27. Chen, S. C. A. and Sorrell, T. C. (2007). Antifungal agents. *Medical Journal of Australia*, *187*(7), 404-409. DOI:https://doi.org/10.5694/j.1326-5377.2007.tb01313.x
28. Chowdhary, A., Kathuria, S., Xu, J., Sharma, C., Sundar, G., Singh, P. K., Gaur, S. N., Hagen, F., Klaassen, C. H. and Meis, J. F. (2012). Clonal Expansion and Emergence of Environmental Multiple-Triazole-Resistant Aspergillus fumigatus Strains Carrying the TR34/L98H Mutations in the cyp51A Gene in India. *PLOS ONE*, *7*(12), e52871. DOI:10.1371/journal.pone.0052871
29. Chowdhary, A., Sharma, C. and Meis, J. F. (2017). Azole-Resistant Aspergillosis: Epidemiology, Molecular Mechanisms, and Treatment. *The Journal of Infectious Diseases*, *216*(suppl_3), S436-S444. DOI:10.1093/infdis/jix210
30. Corry, J. E. L., Curtis, G. D. W. and Baird, R. M. (1995). Dichloran glycerol (DG18) agar. In J. E. L. Corry, G. D. W. Curtis and R. M. Baird (Eds.), *Progress in Industrial Microbiology* (Vol. 34, p. 300-302): Elsevier.
31. Cox, K. D., Quello, K., Deford, R. J. and Beckerman, J. L. (2009). A Rapid Method to Quantify Fungicide Sensitivity in the Brown Rot Pathogen Monilinia fructicola. *Plant Disease*, *93*(4), 328-331. DOI:10.1094/pdis-93-4-0328
32. Crossley, B. M., Bai, J., Glaser, A., Maes, R., Porter, E., Killian, M. L., Clement, T. and Toohey-Kurth, K. (2020). Guidelines for Sanger sequencing and molecular assay monitoring. *Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, *32*(6), 767-775. DOI:10.1177/1040638720905833
33. Cruz-Magalhães, V., Andrade, J., Figueiredo, Y., Marbach, P. and Souza, J. (2019). Sisal Bole Rot: An Important but Neglected Disease. In.
34. Da Silva Ferreira, M. E., Colombo, A. L., Paulsen, I., Ren, Q., Wortman, J., Huang, J., Goldman, M. H. S. and Goldman, G. H. (2005). The ergosterol biosynthesis pathway, transporter genes, and azole resistance in Aspergillus fumigatus. *Medical Mycology*, *43*(Supplement_1), S313-S319. DOI:10.1080/13693780400029114
35. Debeaupuis, J. P., Sarfati, J., Chazalet, V. and Latgé, J. P. (1997). Genetic diversity among clinical and environmental isolates of Aspergillus fumigatus. *Infection and Immunity*, *65*(8), 3080-3085. DOI:doi:10.1128/iai.65.8.3080-3085.1997
36. Delattin, N., Cammue, B. P. and Thevissen, K. (2014). Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. *Future Medicinal Chemistry*, *6*(1), 77-90. DOI:10.4155/fmc.13.189
37. Denning, D. W., Cadranel, J., Beigelman-Aubry, C., Ader, F., Chakrabarti, A., Blot, S., Ullmann, A. J., Dimopoulos, G. and Lange, C. (2016). Chronic pulmonary aspergillosis: rationale and clinical guidelines for diagnosis and management. *European Respiratory Journal*, *47*(1), 45. DOI:10.1183/13993003.00583-2015
38. Denning, D. W., Venkateswarlu, K., Oakley, K. L., Anderson, M. J., Manning, N. J., Stevens, D. A., Warnock, D. W. and Kelly, S. L. (1997). Itraconazole resistance in Aspergillus fumigatus. *Antimicrobial Agents and Chemotherapy*, *41*(6), 1364-1368. DOI:doi:10.1128/AAC.41.6.1364
39. Dhingra, S. and Cramer, R. A. (2017). Regulation of Sterol Biosynthesis in the Human Fungal Pathogen Aspergillus fumigatus: Opportunities for Therapeutic Development. *Frontiers in Microbiology*, *8*. DOI:10.3389/fmicb.2017.00092

40. Duong, T.-M. N., Le, T.-V., Tran, K.-L. H., Nguyen, P.-T., Nguyen, B.-P. T., Nguyen, T.-A., Nguyen, H.-L. P., Nguyen, B.-N. T., Fisher, M. C., Rhodes, J., Marks, G., Fox, G. J., Chen, S. C.-A., Walsh, M. G., Barrs, V. R., Talbot, J., Halliday, C. L., Sorrell, T. C., Day, J. N. and Beardsley, J. (2021). Azole-resistant Aspergillus fumigatus is highly prevalent in the environment of Vietnam, with marked variability by land use type. *Environmental Microbiology*, *23*(12), 7632-7642. DOI:https://doi.org/10.1111/1462-2920.15660
41. Dupont, S., Lemetais, G., Ferreira, T., Cayot, P., Gervais, P. and Beney, L. (2012). ERGOSTEROL BIOSYNTHESIS: A FUNGAL PATHWAY FOR LIFE ON LAND? *Evolution*, *66*(9), 2961-2968. DOI:https://doi.org/10.1111/j.1558-5646.2012.01667.x
42. Engel, T. G. P., Erren, E., Vanden Driessche, K. S. J., Melchers, W. J. G., Reijers, M. H., Merkus, P. and Verweij, P. E. (2019). Aerosol Transmission of Aspergillus fumigatus in Cystic Fibrosis Patients in the Netherlands. *Emerging infectious diseases*, *25*(4), 797-799. DOI:10.3201/eid2504.181110
43. Ermakova, E. and Zuev, Y. (2017). Effect of ergosterol on the fungal membrane properties. All-atom and coarse-grained molecular dynamics study. *Chemistry and Physics of Lipids*, *209*, 45-53. DOI:https://doi.org/10.1016/j.chemphyslip.2017.11.006
44. Espinel-Ingroff, A. and Turnidge, J. (2016). The role of epidemiological cutoff values (ECVs/ECOFFs) in antifungal susceptibility testing and interpretation for uncommon yeasts and moulds. *Revista Iberoamericana de Micología*. DOI:10.1016/j.riam.2016.04.001
45. EUCAST. (2022). MIC EUCAST distribution website. Retrieved 15.05.2022, From https://mic.eucast.org/.
46. EUCAST. (2022). Overview of antifungal ECOFFs and clinical breakpoints for yeasts, moulds and dermatophytes using the EUCAST E.Def 7.3, E.Def 9.4 and E.Def 11.0 procedures. 3.0, valid from 2022-01-18. Retrieved 29.05.2022, From https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/AFST_EUCAST_BP_ECOFF_v3.0_18_01_22.pdf.
47. Eveleigh, A., Jennett, C., Blandford, A., Brohan, P. and Cox, A. L. (2014). *Designing for dabblers and deterring drop-outs in citizen science*. Paper presented at the Proceedings of the SIGCHI Conference on Human Factors in Computing Systems, Toronto, Ontario, Canadaretrieved from https://doi.org/10.1145/2556288.2557262
48. Foley, K., Fazio, G., Jensen, A. B. and Hughes, W. O. H. (2014). The distribution of Aspergillus spp. opportunistic parasites in hives and their pathogenicity to honey bees. *Veterinary Microbiology*, *169*(3), 203-210. DOI:https://doi.org/10.1016/j.vetmic.2013.11.029
49. Fraczek, M. G., Bromley, M., Buied, A., Moore, C. B., Rajendran, R., Rautemaa, R., Ramage, G., Denning, D. W. and Bowyer, P. (2013). The cdr1B efflux transporter is associated with non-cyp51a-mediated itraconazole resistance in Aspergillus fumigatus *Journal of Antimicrobial Chemotherapy*, *68*(7), 1486-1496. DOI:10.1093/jac/dkt075
50. Frisvad, J. C. and Larsen, T. O. (2016). Extrolites of Aspergillus fumigatus and Other Pathogenic Species in Aspergillus Section Fumigati. *Frontiers in Microbiology*, *6*. DOI:10.3389/fmicb.2015.01485
51. Frisvad, J. C., Møller, L. L. H., Larsen, T. O., Kumar, R. and Arnau, J. (2018). Safety of the fungal workhorses of industrial biotechnology: update on the mycotoxin and secondary metabolite potential of Aspergillus niger, Aspergillus oryzae, and Trichoderma reesei. *Applied microbiology and biotechnology*, *102*(22), 9481-9515. DOI:10.1007/s00253-018-9354-1
52. Garcia-Rubio, R., Gonzalez-Jimenez, I., Lucio, J. and Mellado, E. (2020). Characterization of Aspergillus fumigatus cross-resistance between clinical and DMI azole drugs. *Appl Environ Microbiol*, *87*(5). DOI:10.1128/aem.02539-20
53. Gautier, M., Normand, A.-C., L'Ollivier, C., Cassagne, C., Reynaud-Gaubert, M., Dubus, J.-C., Brégeon, F., Hendrickx, M., Gomez, C., Ranque, S. and Piarroux, R. (2016). Aspergillus tubingensis : a major filamentous fungus found in the airways of patients with lung disease. *Medical Mycology*, *54*(5), 459-470. DOI:10.1093/mmy/myv118
54. Geldmann, J., Heilmann-Clausen, J., Holm, T. E., Levinsky, I., Markussen, B., Olsen, K., Rahbek, C. and Tøttrup, A. P. (2016). What determines spatial bias in citizen science? Exploring four recording schemes with different proficiency requirements. *Diversity and Distributions*, *22*(11), 1139-1149. DOI:https://doi.org/10.1111/ddi.12477
55. Glass, N. L. and Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol*, *61*(4), 1323-1330. DOI:10.1128/aem.61.4.1323-1330.1995
56. Gossen, B. D., Carisse, O., Kawchuk, L. M., Van Der Heyden, H. and McDonald, M. R. (2014). Recent changes in fungicide use and the fungicide insensitivity of plant pathogens in Canada. *Canadian Journal of Plant Pathology*, *36*(3), 327-340. DOI:10.1080/07060661.2014.925506

57. Goutam, U., Kukreja, S., Yadav, R., Salaria, N., Thakur, K. and Goyal, A. K. (2015). Recent trends and perspectives of molecular markers against fungal diseases in wheat. *Frontiers in Microbiology*, *6*. DOI:10.3389/fmicb.2015.00861
58. Greene, R. (2005). The radiological spectrum of pulmonary aspergillosis. *Medical Mycology*, *43*(Supplement_1), S147-S154. DOI:10.1080/13693780500064771
59. Guarro, J., GenéJ and Stchigel, A. M. (1999). Developments in fungal taxonomy. *Clinical microbiology reviews*, *12*(3), 454-500. DOI:10.1128/CMR.12.3.454
60. Guinea, J., Verweij, P. E., Meletiadis, J., Mouton, J. W., Barchiesi, F., Arendrup, M. C., Arendrup, M. C., Arikan-Akdagli, S., Barchiesi, F., Castanheira, M., Chryssanthou, E., Friberg, N., Guinea, J., Järv, H., Klimko, N., Kurzai, O., Lagrou, K., Lass-Flörl, C., Mares, M., Matos, T., Meletiadis, J., Moore, C. B., Mouton, J. W., Muehlethaler, K., Rogers, T. R., Andersen, C. T. and Velegraki, A. (2019). How to: EUCAST recommendations on the screening procedure E.Def 10.1 for the detection of azole resistance in Aspergillus fumigatus isolates using four-well azole-containing agar plates. *Clinical Microbiology and Infection*, *25*(6), 681-687. DOI:https://doi.org/10.1016/j.cmi.2018.09.008
61. Hagiwara, D., Takahashi, H., Fujimoto, M., Sugahara, M., Misawa, Y., Gonoi, T., Itoyama, S., Watanabe, A. and Kamei, K. (2016). Multi-azole resistant Aspergillus fumigatus harboring Cyp51A TR46/Y121F/T289A isolated in Japan. *Journal of Infection and Chemotherapy*, *22*(8), 577-579. DOI:https://doi.org/10.1016/j.jiac.2016.01.015
62. Hagiwara, D., Watanabe, A., Kamei, K. and Goldman, G. H. (2016). Epidemiological and Genomic Landscape of Azole Resistance Mechanisms in Aspergillus Fungi. *Front Microbiol*, *7*, 1382. DOI:10.3389/fmicb.2016.01382
63. Hashimoto, A., Hagiwara, D., Watanabe, A., Yahiro, M., Yikelamu, A., Yaguchi, T. and Kamei, K. (2017). Drug Sensitivity and Resistance Mechanism in Aspergillus Section *Nigri* Strains from Japan. *Antimicrobial Agents and Chemotherapy*, *61*(8), e02583-02516. DOI:doi:10.1128/AAC.02583-16
64. Heather, J. M. and Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA. *Genomics*, *107*(1), 1-8. DOI:10.1016/j.ygeno.2015.11.003
65. Hecker, S., Bonney, R., Haklay, M., Hölker, F., Hofer, H., Goebel, C., Gold, M., Makuch, Z., Ponti, M., Richter, A., Robinson, L., Iglesias, J. R., Owen, R., Peltola, T., Sforzi, A., Shirk, J., Vogel, J., Vohland, K., Witt, T. and Bonn, A. (2018). Innovation in Citizen Science – Perspectives on Science-Policy Advances. *Citizen Science: Theory and Practice*, *3(1), p.4*. DOI: http://doi.org/10.5334/cstp.114
66. Henriksen, E. M. N. (2021). *Pathogenic fungi in Norway - screening for azole-resistant Aspergillus fumigatus in Norwegian barns*. (Chemistry and biotechnology Master's Thesis). Norwegian University of Life Sciences, Ås, Norway. Retrieved from https://hdl.handle.net/11250/2984093 Brage NMBU database.
67. Hitchcock, C. A., Barrett-Bee, K. J. and Russell, N. J. (1987). The lipid composition and permeability to azole of an azole- and polyene-resistant mutant of Candida albicans. *Journal of Medical and Veterinary Mycology*, *25*(1), 29-37. DOI:10.1080/02681218780000041
68. Hof, H. (2001). Critical Annotations to the Use of Azole Antifungals for Plant Protection. *Antimicrobial Agents and Chemotherapy*, *45*(11), 2987-2990. DOI:doi:10.1128/AAC.45.11.2987-2990.2001
69. Hong, S. B., Go, S. J., Shin, H. D., Frisvad, J. C. and Samson, R. A. (2005). Polyphasic taxonomy of Aspergillus fumigatus and related species. *Mycologia*, *97*(6), 1316-1329. DOI:10.3852/mycologia.97.6.1316
70. Houpikian, P. and Raoult, D. (2002). Traditional and molecular techniques for the study of emerging bacterial diseases: one laboratory's perspective. *Emerging infectious diseases*, *8*(2), 122-131. DOI:10.3201/eid0802.010141
71. I. Diagnostics. (2018). *Roswell Park Memorial Institute Medium (RPMI medium) - Frequently asked questions*. [Pamphlet].
72. Ismaiel, W. F., Abdelazim, M. H., Eldsoky, I., Ibrahim, A. A., Alsobky, M. E., Zafan, E. and Hasan, A. (2021). The impact of COVID-19 outbreak on the incidence of acute invasive fungal rhinosinusitis. *American Journal of Otolaryngology*, *42*(6), 103080. DOI:https://doi.org/10.1016/j.amjoto.2021.103080
73. Isaac, N. J. B., van Strien, A. J., August, T. A., de Zeeuw, M. P. and Roy, D. B. (2014). Statistics for citizen science: extracting signals of change from noisy ecological data. *Methods in Ecology and Evolution*, *5*(10), 1052-1060. DOI:https://doi.org/10.1111/2041-210X.12254
74. Jensen, R. H., Hagen, F., Astvad, K. M. T., Tyron, A., Meis, J. F. and Arendrup, M. C. (2016). Azole-resistant Aspergillus fumigatus in Denmark: a laboratory-based study on resistance mechanisms and genotypes. *Clinical Microbiology and Infection*, *22*(6), 570.e571-570.e579. DOI:https://doi.org/10.1016/j.cmi.2016.04.001
75. Jordá, T. and Puig, S. (2020). Regulation of Ergosterol Biosynthesis in Saccharomyces cerevisiae. *Genes*, *11*(7), 795.

76. Joyce, L. F., Downes, J., Stockman, K. and Andrew, J. H. (1992). Comparison of five methods, including the PDM Epsilometer test (E test), for antimicrobial susceptibility testing of Pseudomonas aeruginosa. *Journal of Clinical Microbiology*, *30*(10), 2709-2713. DOI:doi:10.1128/jcm.30.10.2709-2713.1992
77. Jørgensen, L. N. and Heick, T. M. (2021). Azole Use in Agriculture, Horticulture, and Wood Preservation – Is It Indispensable? *Frontiers in Cellular and Infection Microbiology*, *11*. DOI:10.3389/fcimb.2021.730297
78. Kartverket (Cartographer). (n.d). Norgeskart. Retrieved 30.03.2022 from https://norgeskart.no/#!?project=norgeskart&layers=1005&zoom=3&lat=7197864.00&lon=396722.00
79. Kauffman, C. A. and Carver, P. L. (1997). Use of Azoles for Systemic Antifungal Therapy. In J. T. August, M. W. Anders, F. Murad and J. Coyle (Eds.), *Advances in Pharmacology* (Vol. 39, p. 143-189): Academic Press.
80. Kwon-Chung, K. J. and Sugui, J. A. (2013). Aspergillus fumigatus--what makes the species a ubiquitous human fungal pathogen? *PLoS pathogens*, *9*(12), e1003743-e1003743. DOI:10.1371/journal.ppat.1003743
81. Lamoth, F. (2016). Aspergillus fumigatus-Related Species in Clinical Practice. *Frontiers in Microbiology*, *7*. DOI:10.3389/fmicb.2016.00683
82. Latgé, J.-P. and Chamilos, G. (2019). Aspergillus fumigatus and Aspergillosis in 2019. *Clinical Microbiology Reviews*, *33*(1), e00140-00118. DOI:doi:10.1128/CMR.00140-18
83. Latgé, J. P. (1999). Aspergillus fumigatus and aspergillosis. *Clin Microbiol Rev*, *12*(2), 310-350. DOI:10.1128/CMR.12.2.310
84. Lepesheva, G. I. and Waterman, M. R. (2007). Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1770*(3), 467-477. DOI:https://doi.org/10.1016/j.bbagen.2006.07.018
85. Lestrade, P. P. A., Meis, J. F., Melchers, W. J. G. and Verweij, P. E. (2019). Triazole resistance in Aspergillus fumigatus: recent insights and challenges for patient management. *Clinical Microbiology and Infection*, *25*(7), 799-806. DOI:https://doi.org/10.1016/j.cmi.2018.11.027
86. Li, C., Zhou, J., Du, G., Chen, J., Takahashi, S. and Liu, S. (2020). Developing Aspergillus niger as a cell factory for food enzyme production. *Biotechnology Advances*, *44*, 107630. DOI:https://doi.org/10.1016/j.biotechadv.2020.107630
87. Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J. and Kauserud, H. (2013). Fungal community analysis by high-throughput sequencing of amplified markers – a user's guide. *New Phytologist*, *199*(1), 288-299. DOI:https://doi.org/10.1111/nph.12243
88. LO-Laboroptik. (n.d.). Information about COUNTING CHAMBER (HEMACYTOMETER). Retrieved, 02.06.2022, From http://www.lo-laboroptik.de/englisch/info/info.html.
89. Lopez-Ruiz, F., Mair, W., Thomas, G., Jayasena, K., Hills, A. and Martin, A. (2020). Azole resistance in spot-form net blotch in Western Australia. Retrieved 11.05.2022, From https://grdc.com.au/resources-and-publications/grdc-update-papers/tab-content/grdc-update-papers/2020/5/azole-resistance-in-spot-form-net-blotch-in-western-australia.
90. Lorenz, T. C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of visualized experiments : JoVE*(63), e3998-e3998. DOI:10.3791/3998
91. Luo, A., Ling, C., Ho, S. Y. W. and Zhu, C.-D. (2018). Comparison of Methods for Molecular Species Delimitation Across a Range of Speciation Scenarios. *Systematic Biology*, *67*(5), 830-846. DOI:10.1093/sysbio/syy011
92. Mackenzie, J. S. and Jeggo, M. (2019). The One Health Approach-Why Is It So Important? *Tropical medicine and infectious disease*, *4*(2), 88. DOI:10.3390/tropicalmed4020088
93. Mackenzie, J. S., McKinnon, M. and Jeggo, M. (2014). One Health: From Concept to Practice. *Confronting Emerging Zoonoses: The One Health Paradigm*, 163-189. DOI:10.1007/978-4-431-55120-1_8
94. Mair, L. and Ruete, A. (2016). Explaining Spatial Variation in the Recording Effort of Citizen Science Data across Multiple Taxa. *PLOS ONE*, *11*(1), e0147796. DOI:10.1371/journal.pone.0147796
95. Mellado, E., Diaz-Guerra, T. M., Cuenca-Estrella, M. and Rodriguez-Tudela, J. L. (2001). Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in Aspergillus fumigatus and other Aspergillus species. *J Clin Microbiol*, *39*(7), 2431-2438. DOI:10.1128/jcm.39.7.2431-2438.2001
96. Mellado, E., Garcia-Effron, G., Alcázar-Fuoli, L., Melchers, W. J. G., Verweij, P. E., Cuenca-Estrella, M. and Rodríguez-Tudela, J. L. (2007). A New *Aspergillus fumigatus* Resistance Mechanism Conferring In Vitro Cross-Resistance to Azole Antifungals Involves a Combination of *cyp51A*

Alterations. *Antimicrobial Agents and Chemotherapy*, *51*(6), 1897-1904. DOI:doi:10.1128/AAC.01092-06
97. Morrissey, I., Oggioni, M. R., Knight, D., Curiao, T., Coque, T., Kalkanci, A., Martinez, J. L. and the, B. C. (2014). Evaluation of Epidemiological Cut-Off Values Indicates that Biocide Resistant Subpopulations Are Uncommon in Natural Isolates of Clinically-Relevant Microorganisms. *PLOS ONE*, *9*(1), e86669. DOI:10.1371/journal.pone.0086669
98. Mortensen, K. L., Jensen, R. H., Johansen, H. K., Skov, M., Pressler, T., Howard, S. J., Leatherbarrow, H., Mellado, E. and Arendrup, M. C. (2011). Aspergillus species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on Aspergillus fumigatus azole resistance. *Journal of clinical microbiology*, *49*(6), 2243-2251. DOI:10.1128/JCM.00213-11
99. Munro, A. W., McLean, K. J., Grant, J. L. and Makris, T. M. (2018). Structure and function of the cytochrome P450 peroxygenase enzymes. *Biochemical Society Transactions*, *46*(1), 183-196. DOI:10.1042/bst20170218
100. Nascente, P. d. S., Meinerz, A. R. M., de Faria, R. O., Schuch, L. F. D., Meireles, M. C. A. and de Mello, J. R. B. (2009). CLSI broth microdilution method for testing susceptibility of Malassezia pachydermatis to thiabendazole. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, *40*(2), 222-226. DOI:10.1590/S1517-83822009000200002
101. Neofytos, D., Chatzis, O., Nasioudis, D., Boely Janke, E., Doco Lecompte, T., Garzoni, C., Berger, C., Cussini, A., Boggian, K., Khanna, N., Manuel, O., Mueller, N. J. and van Delden, C. (2018). Epidemiology, risk factors and outcomes of invasive aspergillosis in solid organ transplant recipients in the Swiss Transplant Cohort Study. *Transpl Infect Dis*, *20*(4), e12898. DOI:10.1111/tid.12898
102. Nordøy, I. and Gaustad, P. (2008). Resistensproblemer ved behandling av invasive soppinfeksjoner. *Tidsskriftet Den Norske Legeforening*(128: 2607-11).
103. Odds, F. C., Brown, A. J. P. and Gow, N. A. R. (2003). Antifungal agents: mechanisms of action. *Trends in Microbiology*, *11*(6), 272-279. DOI:https://doi.org/10.1016/S0966-842X(03)00117-3
104. Pappas, P. G., Alexander, B. D., Andes, D. R., Hadley, S., Kauffman, C. A., Freifeld, A., Anaissie, E. J., Brumble, L. M., Herwaldt, L., Ito, J., Kontoyiannis, D. P., Lyon, G. M., Marr, K. A., Morrison, V. A., Park, B. J., Patterson, T. F., Perl, T. M., Oster, R. A., Schuster, M. G., Walker, R., Walsh, T. J., Wannemuehler, K. A. and Chiller, T. M. (2010). Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clin Infect Dis*, *50*(8), 1101-1111. DOI:10.1086/651262
105. Parker, J. E., Warrilow, A. G. S., Cools, H. J., Martel, C. M., Nes, W. D., Fraaije, B. A., Lucas, J. A., Kelly, D. E. and Kelly, S. L. (2011). Mechanism of Binding of Prothioconazole to *Mycosphaerella graminicola* CYP51 Differs from That of Other Azole Antifungals. *Applied and Environmental Microbiology*, *77*(4), 1460-1465. DOI:doi:10.1128/AEM.01332-10
106. Parker, J. E., Warrilow, A. G. S., Price, C. L., Mullins, J. G. L., Kelly, D. E. and Kelly, S. L. (2014). Resistance to antifungals that target CYP51. *Journal of Chemical Biology*, *7*(4), 143-161. DOI:10.1007/s12154-014-0121-1
107. Paul, R. A., Rudramurthy, S. M., Dhaliwal, M., Singh, P., Ghosh, A. K., Kaur, H., Varma, S., Agarwal, R. and Chakrabarti, A. (2018). Magnitude of Voriconazole Resistance in Clinical and Environmental Isolates of Aspergillus flavus and Investigation into the Role of Multidrug Efflux Pumps. *Antimicrobial Agents and Chemotherapy*, *62*(11), e01022-01018. DOI:doi:10.1128/AAC.01022-18
108. Paul, S., Diekema, D. and Moye-Rowley, W. S. (2013). Contributions of Aspergillus fumigatus ATP-Binding Cassette Transporter Proteins to Drug Resistance and Virulence. *Eukaryotic Cell*, *12*(12), 1619-1628. DOI:doi:10.1128/EC.00171-13
109. Pérez-Cantero, A., López-Fernández, L., Guarro, J. and Capilla, J. (2020). Azole resistance mechanisms in Aspergillus: update and recent advances. *International Journal of Antimicrobial Agents*, *55*(1), 105807. DOI:https://doi.org/10.1016/j.ijantimicag.2019.09.011
110. Pontes, L., Beraquet, C. A. G., Arai, T., Pigolli, G. L., Lyra, L., Watanabe, A., Moretti, M. L. and Schreiber, A. Z. (2020). Aspergillus fumigatus Clinical Isolates Carrying CYP51A with TR34/L98H/S297T/F495I Substitutions Detected after Four-Year Retrospective Azole Resistance Screening in Brazil. *Antimicrobial Agents and Chemotherapy*, *64*(3), e02059-02019. DOI:doi:10.1128/AAC.02059-19
111. Prigitano, A., Esposto, M. C., Romanò, L., Auxilia, F. and Tortorano, A. M. (2019). Azole-resistant Aspergillus fumigatus in the Italian environment. *Journal of Global Antimicrobial Resistance*, *16*, 220-224. DOI:https://doi.org/10.1016/j.jgar.2018.10.017
112. Ramage, G., Rajendran, R., Sherry, L. and Williams, C. (2012). Fungal Biofilm Resistance. *International Journal of Microbiology*, *2012*, 528521. DOI:10.1155/2012/528521
113. Rhodes, J. C. (2006). Aspergillus fumigatus: Growth and virulence. *Medical Mycology*, *44*(Supplement_1), S77-S81. DOI:10.1080/13693780600779419

114. Riscili, B. P. and Wood, K. L. (2009). Noninvasive pulmonary Aspergillus infections. *Clin Chest Med*, *30*(2), 315-335, vii. DOI:10.1016/j.ccm.2009.02.008
115. Rudramurthy, S. M., Paul, R. A., Chakrabarti, A., Mouton, J. W. and Meis, J. F. (2019). Invasive Aspergillosis by Aspergillus flavus: Epidemiology, Diagnosis, Antifungal Resistance, and Management. *Journal of Fungi*, *5*(3), 55.
116. Samson, R. A., Hoekstra, E. S. and Frisvad, J. C. (2004). *Introduction to food- and airborne fungi* (Seventh ed.). Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures. p. 304
117. Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C., Andersen, B. (2019). *Food and Indoor Fungi* (Second ed.). Utrecht, The Netherlands: Westerdijk Fungal Biodiversity Institute. p. 130, 146
118. Samson, R. A., Visagie, C. M., Houbraken, J., Hong, S. B., Hubka, V., Klaassen, C. H. W., Perrone, G., Seifert, K. A., Susca, A., Tanney, J. B., Varga, J., Kocsubé, S., Szigeti, G., Yaguchi, T. and Frisvad, J. C. (2014). Phylogeny, identification and nomenclature of the genus Aspergillus. *Studies in Mycology*, *78*, 141-173. DOI:https://doi.org/10.1016/j.simyco.2014.07.004
119. Schauwvlieghe, A. F. A. D., Rijnders, B. J. A., Philips, N., Verwijs, R., Vanderbeke, L., Van Tienen, C., Lagrou, K., Verweij, P. E., Van de Veerdonk, F. L., Gommers, D., Spronk, P., Bergmans, D. C. J. J., Hoedemaekers, A., Andrinopoulou, E.-R., van den Berg, C. H. S. B., Juffermans, N. P., Hodiamont, C. J., Vonk, A. G., Depuydt, P., Boelens, J. and Wauters, J. (2018). Invasive aspergillosis in patients admitted to the intensive care unit with severe influenza: a retrospective cohort study. *The Lancet Respiratory Medicine*, *6*(10), 782-792. DOI:https://doi.org/10.1016/S2213-2600(18)30274-1
120. Segal, B. H. (2009). Aspergillosis. *New England Journal of Medicine*, *360*(18), 1870-1884. DOI:10.1056/NEJMra0808853
121. Sharma, C., Hagen, F., Moroti, R., Meis, J. F. and Chowdhary, A. (2015). Triazole-resistant Aspergillus fumigatus harbouring G54 mutation: Is it de novo or environmentally acquired? *Journal of Global Antimicrobial Resistance*, *3*(2), 69-74. DOI:https://doi.org/10.1016/j.jgar.2015.01.005
122. Sheehan, D. J., Hitchcock, C. A. and Sibley, C. M. (1999). Current and Emerging Azole Antifungal Agents. *Clinical Microbiology Reviews*, *12*(1), 40-79. DOI:doi:10.1128/CMR.12.1.40
123. Shelton, J. M. G., Collins, R., Uzzell, C. B., Alghamdi, A., Dyer, P. S., Singer, A. C. and Fisher, M. C. (2022). Citizen Science Surveillance of Triazole-Resistant Aspergillus fumigatus in United Kingdom Residential Garden Soils. *Applied and environmental microbiology*, *88*(4), e0206121-e0206121. DOI:10.1128/AEM.02061-21
124. Shelton, J. M. G., Fisher, M. C. and Singer, A. C. (2020). Campaign-Based Citizen Science for Environmental Mycology: the “Science Solstice” and “Summer Soil-stice” Projects to Assess Drug Resistance in Air and Soilborne *Aspergillus fumigatus*. *bioRxiv*, 2020.2006.2011.146241. DOI:10.1101/2020.06.11.146241
125. Shendure, J. and Ji, H. (2008). Next-generation DNA sequencing. *Nature Biotechnology*, *26*(10), 1135-1145. DOI:10.1038/nbt1486
126. Shomaker, T. S., Green, E. M. and Yandow, S. M. (2013). Perspective: One Health: A Compelling Convergence. *Academic Medicine*, *88*(1), 49-55. DOI:10.1097/ACM.0b013e31827651b1
127. Silva, D. M., Batista, L. R., Rezende, E. F., Fungaro, M. H. P., Sartori, D. and Alves, E. (2011). Identification of fungi of the genus Aspergillus section nigri using polyphasic taxonomy. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, *42*(2), 761-773. DOI:10.1590/S1517-838220110002000044
128. Silvertown, J. (2009). A new dawn for citizen science. *Trends in Ecology & Evolution*, *24*(9), 467-471. DOI:https://doi.org/10.1016/j.tree.2009.03.017
129. Skaar, I., Abdellaue, A., Andersen, C. T., Ardenrup, M. C., Bjørnholt, J. V., Christensen, E., Divon, H. H., Ficke, A., Gaustad, P., Strand, E., Sørum, H. and Verweij, P. E. (2019). *Knowledge and knowledge gaps on azole resistance in a One Health perspective* (3). N. V. Institute. Ås, Norway. Retrieved from
130. Snelders, E., Camps, S. M. T., Karawajczyk, A., Schaftenaar, G., Kema, G. H. J., van der Lee, H. A., Klaassen, C. H., Melchers, W. J. G. and Verweij, P. E. (2012). Triazole Fungicides Can Induce Cross-Resistance to Medical Triazoles in Aspergillus fumigatus. *PLOS ONE*, *7*(3), e31801. DOI:10.1371/journal.pone.0031801
131. Snelders, E., van der Lee, H. A. L., Kuijpers, J., Rijs, A. J. M. M., Varga, J., Samson, R. A., Mellado, E., Donders, A. R. T., Melchers, W. J. G. and Verweij, P. E. (2008). Emergence of Azole Resistance in Aspergillus fumigatus and Spread of a Single Resistance Mechanism. *PLOS Medicine*, *5*(11), e219. DOI:10.1371/journal.pmed.0050219
132. Stielow, J. B., Lévesque, C. A., Seifert, K. A., Meyer, W., Iriny, L., Smits, D., Renfurm, R., Verkley, G. J. M., Groenewald, M., Chaduli, D., Lomascolo, A., Welti, S., Lesage-Meessen, L., Favel, A., Al-Hatmi, A. M. S., Damm, U., Yilmaz, N., Houbraken, J., Lombard, L., Quaedvlieg, W., Binder, M., Vaas, L. A. I., Vu, D., Yurkov, A., Begerow, D., Roehl, O., Guerreiro, M., Fonseca, A., Samerpitak, K., van Diepeningen, A. D., Dolatabadi, S., Moreno, L. F., Casaregola, S., Mallet, S., Jacques, N., Roscini,

L., Egidi, E., Bizet, C., Garcia-Hermoso, D., Martín, M. P., Deng, S., Groenewald, J. Z., Boekhout, T., de Beer, Z. W., Barnes, I., Duong, T. A., Wingfield, M. J., de Hoog, G. S., Crous, P. W., Lewis, C. T., Hambleton, S., Moussa, T. A. A., Al-Zahrani, H. S., Almaghrabi, O. A., Louis-Seize, G., Assabgui, R., McCormick, W., Omer, G., Dukik, K., Cardinali, G., Eberhardt, U., de Vries, M. and Robert, V. (2015). One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia*, *35*, 242-263. DOI:10.3767/003158515X689135

133. Sugui, J. A., Peterson, S. W., Figat, A., Hansen, B., Samson, R. A., Mellado, E., Cuenca-Estrella, M., Kwon-Chung, K. J. and Warnock, D. W. (2014). Genetic Relatedness versus Biological Compatibility between Aspergillus fumigatus and Related Species. *Journal of Clinical Microbiology*, *52*(10), 3707-3721. DOI:doi:10.1128/JCM.01704-14
134. Szabo, B. G., Lakatos, B., Bobek, I., Szabo, E., Szlavik, J. and Vályi-Nagy, I. (2021). Invasive fungal infections among critically ill adult COVID-19 patients: First experiences from the national centre in Hungary. *Journal of Medical Mycology*, *31*(4), 101198. DOI:https://doi.org/10.1016/j.mycmed.2021.101198
135. Saad, A. H., DePestel, D. D. and Carver, P. L. (2006). Factors Influencing the Magnitude and Clinical Significance of Drug Interactions Between Azole Antifungals and Select Immunosuppressants. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *26*(12), 1730-1744. DOI:https://doi.org/10.1592/phco.26.12.1730
136. Taccone, F. S., Van den Abeele, A.-M., Bulpa, P., Misset, B., Meersseman, W., Cardoso, T., Paiva, J.-A., Blasco-Navalpotro, M., De Laere, E., Dimopoulos, G., Rello, J., Vogelaers, D., Blot, S. I. and on behalf of the Asp, I. C. U. S. I. (2015). Epidemiology of invasive aspergillosis in critically ill patients: clinical presentation, underlying conditions, and outcomes. *Critical Care*, *19*(1), 7. DOI:10.1186/s13054-014-0722-7
137. Tekaia, F. and Latgé, J.-P. (2005). Aspergillus fumigatus: saprophyte or pathogen? *Current Opinion in Microbiology*, *8*(4), 385-392. DOI:https://doi.org/10.1016/j.mib.2005.06.017
138. Thermo Fisher Scientific. (2019). GeneRuler 1 kb DNA Ladder. Pub. no. MAN0013004 ed. T. F. Scientific. 26.07.2019. Vilnius, Lithuania.
139. Thermo Fisher Scientific. (n.d.). Technical Resources, 11875 - RPMI 1640. Retrieved 04.06.2022, From https://www.thermofisher.com/no/en/home/technical-resources/media-formulation.114.html.
140. Toda, M., Beer, K. D., Kuivila, K. M., Chiller, T. M. and Jackson, B. R. (2021). Trends in Agricultural Triazole Fungicide Use in the United States, 1992–2016 and Possible Implications for Antifungal-Resistant Fungi in Human Disease. *Environmental Health Perspectives*, *129*(5), 055001. DOI:doi:10.1289/EHP7484
141. Totomoch-Serra, A., Marquez, M. F. and Cervantes-Barragán, D. E. (2017). Sanger sequencing as a first-line approach for molecular diagnosis of Andersen-Tawil syndrome. *F1000Research*, *6*, 1016-1016. DOI:10.12688/f1000research.11610.1
142. Toyotome, T., Hagiwara, D., Takahashi, H., Watanabe, A. and Kamei, K. (2018). Emerging Antifungal Drug Resistance in Aspergillus fumigatus and Among Other Species of Aspergillus. *Current Fungal Infection Reports*, *12*(3), 105-111. DOI:10.1007/s12281-018-0318-9
143. Valencia, C. A., Pervaiz, M. A., Husami, A., Qian, Y. and Zhang, K. (2013). Sanger Sequencing Principles, History, and Landmarks. In C. A. Valencia, M. A. Pervaiz, A. Husami, Y. Qian and K. Zhang (Eds.), *Next Generation Sequencing Technologies in Medical Genetics* (p. 3-11). New York, NY: Springer New York.
144. van der Linden, J. W. M., Camps, S. M. T., Kampinga, G. A., Arends, J. P. A., Debets-Ossenkopp, Y. J., Haas, P. J. A., Rijnders, B. J. A., Kuijper, E. J., van Tiel, F. H., Varga, J., Karawajczyk, A., Zoll, J., Melchers, W. J. G. and Verweij, P. E. (2013). Aspergillosis due to Voriconazole Highly Resistant Aspergillus fumigatus and Recovery of Genetically Related Resistant Isolates From Domiciles. *Clinical Infectious Diseases*, *57*(4), 513-520. DOI:10.1093/cid/cit320
145. Velásquez, A. C., Castroverde, C. D. M. and He, S. Y. (2018). Plant–Pathogen Warfare under Changing Climate Conditions. *Current Biology*, *28*(10), R619-R634. DOI:https://doi.org/10.1016/j.cub.2018.03.054
146. Verweij, P. E., Ananda-Rajah, M., Andes, D., Arendrup, M. C., Brüggemann, R. J., Chowdhary, A., Cornely, O. A., Denning, D. W., Groll, A. H., Izumikawa, K., Kullberg, B. J., Lagrou, K., Maertens, J., Meis, J. F., Newton, P., Page, I., Seyedmousavi, S., Sheppard, D. C., Viscoli, C., Warris, A. and Donnelly, J. P. (2015). International expert opinion on the management of infection caused by azole-resistant Aspergillus fumigatus. *Drug Resistance Updates*, *21-22*, 30-40. DOI:https://doi.org/10.1016/j.drup.2015.08.001
147. Verweij, P. E., Chowdhary, A., Melchers, W. J. G. and Meis, J. F. (2016). Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles? *Clinical*

infectious diseases : an official publication of the Infectious Diseases Society of America, *62*(3), 362-368. DOI:10.1093/cid/civ885
148. Verweij, P. E., et al. (2017). *Azole - resistance selection in Aspergillus fumigatus Final Report*.
149. Verweij, P. E., Lestrade, P. P. A., Melchers, W. J. G. and Meis, J. F. (2016). Azole resistance surveillance in Aspergillus fumigatus: beneficial or biased? *Journal of Antimicrobial Chemotherapy*, *71*(8), 2079-2082. DOI:10.1093/jac/dkw259
150. Verweij, P. E., Lucas, J. A., Arendrup, M. C., Bowyer, P., Brinkmann, A. J. F., Denning, D. W., Dyer, P. S., Fisher, M. C., Geenen, P. L., Gisi, U., Hermann, D., Hoogendijk, A., Kiers, E., Lagrou, K., Melchers, W. J. G., Rhodes, J., Rietveld, A. G., Schoustra, S. E., Stenzel, K., Zwaan, B. J. and Fraaije, B. A. (2020). The one health problem of azole resistance in Aspergillus fumigatus: current insights and future research agenda. *Fungal Biology Reviews*, *34*(4), 202-214. DOI:https://doi.org/10.1016/j.fbr.2020.10.003
151. Verweij, P. E., Snelders, E., Kema, G. H., Mellado, E. and Melchers, W. J. (2009). Azole resistance in Aspergillus fumigatus: a side-effect of environmental fungicide use? *Lancet Infect Dis*, *9*(12), 789-795. DOI:10.1016/s1473-3099(09)70265-8
152. Verweij, P. E., Zhang, J., Debets, A. J. M., Meis, J. F., van de Veerdonk, F. L., Schoustra, S. E., Zwaan, B. J. and Melchers, W. J. G. (2016). In-host adaptation and acquired triazole resistance in Aspergillus fumigatus: a dilemma for clinical management. *The Lancet Infectious Diseases*, *16*(11), e251-e260. DOI:https://doi.org/10.1016/S1473-3099(16)30138-4
153. VIPcheckTM. (2015). PROTOCOL VIPCHECKTM. Retrieved 18.10.2021, From https://www.vipcheck.nl/images/uploads/downloads/Protocol_VIPcheck_FUO_2015.pdf.
154. Vries, R. P. d. and Visser, J. (2001). Aspergillus Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. *Microbiology and Molecular Biology Reviews*, *65*(4), 497-522. DOI:doi:10.1128/MMBR.65.4.497-522.2001
155. Walker, D. M., Castlebury, L. A., Rossman, A. Y. and White, J. F. (2012). New molecular markers for fungal phylogenetics: Two genes for species-level systematics in the Sordariomycetes (Ascomycota). *Molecular Phylogenetics and Evolution*, *64*(3), 500-512. DOI:https://doi.org/10.1016/j.ympev.2012.05.005
156. Walsh, T. J. and Dixon, D. M. (1996). *Medical Microbiology* (S. Baron Ed. 4th ed.). Galveston (TX): University of Texas Medical Branch at Galveston. p. Chapter 75
157. Wangler, M. and Bellen, H. (2017). In Vivo Animal Modeling. In *Basic Science Methods for Clinical Researchers* (p. 211-234).
158. Wedge, D. E., Curry, K. J., Kreiser, B., Curry, A., Abril, M. and Smith, B. J. (2013). Fungicide Resistance Profiles for 13 Botrytis cinerea Isolates from Strawberry in Southeastern Louisiana. *International Journal of Fruit Science*, *13*(4), 413-429. DOI:10.1080/15538362.2013.789253
159. Yılmaz, M., Ozic, C. and Gok, İ. (2012). *Principles of Nucleic Acid Separation by Agarose Gel Electrophoresis, in Gel Electrophoresis - Principles and Basics*. London, United Kingdom: IntechOpen. p. 33-40
160. Zhu, T., Chen, X., Li, C., Tu, J., Liu, N., Xu, D. and Sheng, C. (2021). Lanosterol 14α-demethylase (CYP51)/histone deacetylase (HDAC) dual inhibitors for treatment of Candida tropicalis and Cryptococcus neoformans infections. *European Journal of Medicinal Chemistry*, *221*, 113524. DOI:https://doi.org/10.1016/j.ejmech.2021.113524
161. Zilberberg, M. D., Nathanson, B. H., Harrington, R., Spalding, J. R. and Shorr, A. F. (2018). Epidemiology and Outcomes of Hospitalizations With Invasive Aspergillosis in the United States, 2009–2013. *Clinical Infectious Diseases*, *67*(5), 727-735. DOI:10.1093/cid/ciy181

7. Appendix

Appendix 1 – Instruction manual

The English version can be seen in the next page.

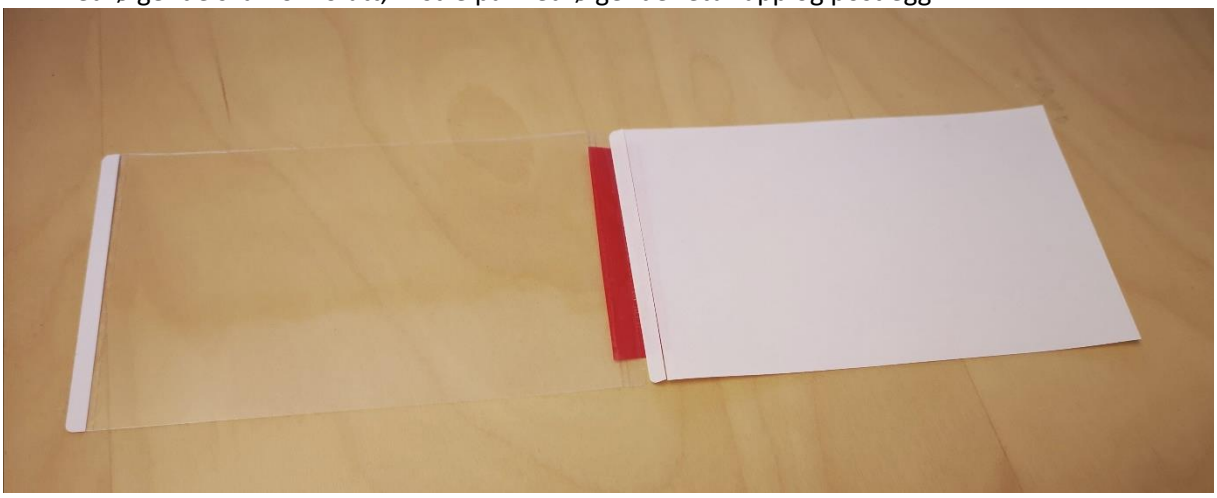
Resistente muggsopp i fjøset?

Veterinærinstituttet
Norwegian Veterinary Institute

Veiledning

Du har nå mottatt to plastfilmer. De er tapet sammen i den ene enden. Vennligst ikke fjern denne tapen da den skal gjøre det enklere å feste beskyttelsen tilbake på filmen (se bildet under).
Filmene skal eksponeres i to ulike rom. Hvis du har dyrehold skal én plasseres på fôrlageret (film merket L) og én i husdyrrommet (film merket H). Hvis du har korn/fôrproduksjon skal du bare bruke den ene filmen (L), og den skal plasseres på korn/fôrlageret.

1. Fyll ut medfølgende spørreskjema. Fyll ut med samme ID-nummer som angitt på filmen (G-x).
2. Prøv å utføre så sterilt som mulig: Trekk beskyttelsesfilmen forsiktig helt av uten å berøre innsidene og behold tapefestet på (se bilde). Slik blir det lettere å feste på beskyttelsesfilmen igjen etter endt eksponering.
3. Legg filmen med limsiden (eksponeringsflaten) opp på en flate midt i rommet ca. en meter over bakken hvor den kan ligge urørt i seks timer. Hvis du ikke finner egnet sted midt i rommet, legg den på et sted hvor det er litt luftsirkulasjon og nærhet til dyr/korn/fôr.
4. Etter endt eksponering, legger du beskyttelsesfilmen forsiktig og jevnt, men fast og så nøyaktig du kan, tilbake over eksponeringsflaten.
5. Legg plastfilmen(e) ned i medfølgende zip-lock pose. Legg posen og spørreskjemaet i medfølgende svarkonvolutt, klistre på medfølgende returlapp og postlegg.



Bilde1: Plastfilmen slik den skal eksponeres. Behold tapen på i enden slik at det blir lettere å feste beskyttelsen tilbake på filmen etter endt prøvetaking.

Tusen takk for hjelpen!

Resistant fungi in the barn?

Veterinærinstituttet
Norwegian Veterinary Institute

Instructions

You have now received two plastic films. They are taped together on one end. Please do not remove this tape as it is there to make it easier to put the protective sheet back over the film again (see picture below). The films are to be exposed in two separate rooms. If you have livestock, one film is to be placed in the animal feed storage room (film marked L) and one in the animal housing room (film marked H). If you produce grains/animal feed, then you are to only use one film (L), and it is to be placed in the storage room.

2. Fill out the questionnaire that came with the sampling set. Fill in with the same ID-number you see on the films (G-x).
3. Try to work as sterile as possible: Carefully peel off the protective sheet fully without touching the inside and keep the tape on (see picture). That way, it will be easier to put the protective sheet back on again after air exposure.
4. Place the film with the sticky side (adhesive side) up on a surface in the middle of the room about a meter above the ground where it is to be left untouched for six hours. If there are no ideal surfaces in the middle of the room, place it somewhere where there is air circulation and close to animals/grains/feed.
5. After exposure, place the protective sheet carefully and evenly, but also firmly and as precise as you can, back over the adhesive side.
6. Place the plastic film(s) in the zip-lock bag that was included. Place the bag and the questionnaire in the return envelope, stick on the return label that was included and mail it.

Figure1: The plastic film the way it is to be exposed. Keep the tape at the end on so it becomes easier to place the protective sheet back over the film after sampling.

Thank you so much!

Appendix 2 – Questionnaire

The English version can be seen in the next page.

Resistente muggsopp i fjøset?

Veterinærinstituttet
Norwegian Veterinary Institute

Dersom du vil motta resultatene fra gården din trenger vi ditt samtykke (se baksiden). *Det er helt frivillig*. Dersom du vil delta anonymt trenger du ikke å fylle ut kontaktinformasjon. **ID-nummeret på filmen og postnummeret MÅ oppgis.**

ID-NUMMER PÅ FILMEN*	
Postnummer*	

Navn	
Adresse	
Telefon	
E-post	

DRIFTSTYPE

Økologisk drift? **JA** ☐ **NEI** ☐

DYREHOLD	**Antall (totalt)**	**Besetningstype (Kryss av i skravért rute)**				**Type strø (sett kryss)**			
						Flis	**Halm**	**Talle**	**Annet**
Melkekyr		Bås		Løsdrift					
Kjøttfe		Ammeku		Framfôring					
Småfe		Sau		Geit					
Gris		Avlsbesetn		Slaktegris					
Hest									
Annet									

KORN	**Areal (# mål)**	**Bruker du sprøytemidler? Hvis ja; spesifisér hvilket produkt**			
		Bruker ikke	**Mot sopp**	**Mot ugress**	**Mot insekter**
Havre					
Hvete					
Bygg					
Rug					
Annet					

HUSDYRROM (H-FILM)			**LAGERROM (L-FILM)**	
Temperatur i rommet			**Temperatur i rommet**	
Hva slags dyr?			**Hva lagres her?**	
			Tørrhøy	
			Ensilasje	
			Annet (spesifisér)	

Skriv på baksiden om du trenger mere plass.

Resistant fungi in the barn?

Veterinærinstituttet
Norwegian Veterinary Institute

If you would like to receive the results from your farm, we will need your consent (see next page). *This is completely voluntary*. You do not need to fill out contact information if you would like to stay anonymous. **ID-number on the films and post code MUST be included.**

ID-NUMBER ON THE FILM*	
Post code*	

Name	
Address	
Phone	
E-mail	

TYPE OF FARM

Organic farm? **YES** ☐ **NO** ☐

ANIMALS	**Number (total)**	**Type of management (Set an X on the shaded area)**				**Type of bedding (Set an X)**			
						Woodchips	**Straw**	**Talle ***	**Other**
Dairy cow		Booth		Open housing					
Meat cattle		Nursing cow		Artificial feeding					
Sheep/goat		Sheep		Goat					
Pig		Breeding		Butcher hogs					
Horse									
Other									

GRAIN	**Area (# 1000 m²)**	**Do you use pesticides? If yes; please specify which product**			
		Not using	**Fungicide**	**Herbicide**	**Insecticide**
Oat					
Wheat					
Barley					
Rye					
Other					

ANIMAL HOUSING ROOM (H-FILM)		**STORAGE ROOM (L-FILM)**	
Temperature in the room		**Temperature in the room**	
What type of animal?		**What is stored here?**	
		Dry hay	
		Silage	
		Other (specify)	

Use the back of this page if you need more space to write.

*Talle = a mix of bedding, animal urine and faeces

Appendix 3 – Instruments

Table 22: Devices and equipment used during the study.

Instruments/equipment	Manufacturer	Application
MicroAmp® Clear Adhesive Films	Thermo Fisher Scientific, Waltham, Massachusetts	Adhesive film used as spore traps.
Labmodul Greenlife PRO	Labmodul	Airflow cabinet to restrict cross contamination while working.
Nikon Stereo Microscope SMZ1270i	Nikon	Macroscope used for morphologic identification of colonies.
Olympus BX-50	Olympus	Microscope used to identify samples by morphology and spore counting.
Sellotape Crystal Clear ultra transparent premium quality tape	Sellotape	Clear tape used to prepare microscopy slides.
WypAll® X60	Kimberly-Clark® Professional	Tissue used for microscopy slide preparation.
DEN-1B McFarland Densitometer	Grant Instruments	Instrument for measuring density of spore suspensions in McFarland.
Mixer Mill MM400	RETSCH®	Machine that homogenises spore suspensions and breaks cells mechanically for DNA extraction.
Thermomixer Comfort, 2 ml	Eppendorf®	Instrument that stirs and heats DNA samples.
Heraeus Pico 21 centrifuge	Thermo Scientific, Waltham, Massachusetts	Spins samples for separation or droplet collection.
Vortex Mixer	VELP Scientifica	For mixing samples.

Mini Star Whiteline	VWR	Spins samples to collect droplets.
QIAcube Connect	QIAGEN®, Vienna, Austria	Machine that automatically extracts DNA with buffers (See Table 27 in Appendix 4).
NanoDrop™ One	Thermo Fisher Scientific, Waltham, Massachusetts	Measures DNA concentration.
96-well T100™Thermal Cycler	BIO-RAD	PCR machine.
Veriti 96 Well Thermal Cycler	Thermo Fisher Scientific, Waltham, Massachusetts	PCR machine.
Azure c150	Azure™ biosystems	Geldoc to capture gel images with UV-light.
Telkamer Bürker-Türk bright line, with clamps, double net ruling	Marienfeld, Berlin, Germany	Used to count spores and find spore concentration in spore/cell suspensions.

Appendix 4 – Compounds

Table 23: Types of water used throughout the study. All were prepared by NVI's media production department.

Water	Description
Distilled water	Distilled water is treated by adding 5-10 ml into test tubes before autoclaving them under temperatures of 134 °C for 20 minutes.
Milli-Q water	Milli-Q water is free of any nucleic acids and DNases, and is made by running distilled water through a filter with reverse osmosis.
Physiological saltwater	Physiological saltwater is distilled water that contains 0.85% NaCl (9.0 g NaCl in 1.0 L distilled water). It grants better growing conditions for the fungi and prevents spores from potentially rupturing.

Table 24: Ethanol solutions used throughout the study.

Ethanol	Manufacturer	Description/application
70% EtOH	VWR	For sterilising the work bench and preparing microscopy slides. Diluted 96 % EtOH in lab.
96% EtOH	VWR	For sterilising equipment
100% EtOH	VWR	For molecular steps of the analysis

Table 25: Media and their composition used in this study. Recipe for DG18, MEA and SAB were prepared by NVI's media production department, and the recipes stated are for ca. 1 L medium. The RPMI medium was prepared by Oslo University Hospital, and the recipe stated is for ca. 4 L medium.

Medium	Application	Components	Product/ manufacturer	Measurements
DG18[1]	Promotes growth of fungi with lower water activity and inhibits growth of bacteria and fast growing fungi such as Mucorales.	Dichloran-Glycerol Agar Base	Oxoid CM729	31.5 g
		Glycerol 85%	Merck 1.04094	220 g
		Distilled/RO-water		1.0 L
		Trace metal solution for CZID and others	KA_K 0009	1.0 ml
		Chloramphenicol (50 mg/ml)	KA_K 0007	1.0 ml
		Chlortetracycline (5 mg/ml)	KA_K 0010	10.0 ml
MEA[1]	For cultivating fungi.	Malt extract	Oxoid LP0039	20.0 g
		Bacto Peptone	Difco 211677	1.0 g
		Glucose	Merck 1.08337	20.0 g
		Distilled/RO-water		1.0 L
		Agar Bacteriological	Oxoid LP0011	20.0 g
SAB[1]	For cultivating fungal isolates. It also inhibits bacterial growth.	Neopeptone	Difco 211681	10.0 g
		D(+) Glucose	Merck 1.08337	20.0 g
		Bacto Yeast extract	Difco 212750	5.0 g
		Distilled/RO-water		1.0 L
		Chloramphenicol (50 mg/ml)	KA_K 0007	0.1 ml
		Agar Bacteriological	Oxoid LP0011	20.0 g

RPMI-1640[2]	For cultivating cells and promote healthy cell growth.	Bacto agar	Difco	60 g
		RO-water		4000 ml
		RPMI-1640	Sigma R1383	33.6 g
		MOPS	Sigma	138 g
		D(+) Glucose	Prolabo	80 g
		NaOH 10%		112 ml

1. Retrieved from the Norwegian Veterinary Institute media production catalogues.
2. Retrieved from Oslo University Hospital.

Table 26: Phenotypic resistance screening tools and their manufacturers – VIPcheck™ plates and ETEST® strips.

Azole asset	Manufacturer	Description
VIPcheck™	Mediaproducts BV, Groningen, The Netherlands	A four-well agar plate where one well contains 4 mg/L itraconazole (1), one with 2 mg/L voriconazole (2), one with 0.5 mg/L posaconazole (3) and one without azoles for control (4).
ETEST® strips	bioMérieux, Marcy-l'Étoile, France	Plastic strips with a gradient concentration of azoles (itraconazole, voriconazole, posaconazole) 0.002 mg/L – 32 mg/L.

Table 27: List of compounds and their manufacturers used for the analyses throughout the study.

Compound	Manufacturer	Description	
Microbiology			
PBS, 0.1% Tween® 20	NVI Mediaproduction	Phosphate buffered saline. Maintains a stable pH of 7,4 and prevents cells from rupturing. Tween® 20 prevents cells from clumping together. Composition for 30 L solution is listed below.	
		NaCl	240.0 g
		KH_2PO_4	6.0 g
		Na_2HPO_4 x $2H_2O$	44.7 g
		KCl	6.0 g
		Tween® 20	30.0 ml
		Distilled water	30.0 L
Lactofuchsin	NVI Mediaproduction	A compound with a deep magenta colour that was used for staining samples on microscopy slides. The solution both preserves the fungal structures as well as accentuates certain characteristics for light microscopy.	
10% Glycerol	NVI Mediaproduction	This solution lowers the water activity and protects the spores in lower temperatures.	
DNA extraction			
Proteinase K, 20 mg/ml	Sigma-Aldrich, St. Louis, Missouri	Enzyme that breaks down proteins	
AL	QIAGEN®, Vienna, Austria	Lysis buffer that contains enzymes like chitinase to break down cell walls.	
AW1	QIAGEN®, Vienna, Austria	Wash buffer that washes away proteins from the spin column.	
AW2	QIAGEN®, Vienna, Austria	Wash buffer that washes away salts from the spin column.	

AE	QIAGEN®, Vienna, Austria	Elusion buffer that detaches nucleic acids from the spin column.
PCR		
27955702 illustra™ PuReTaq™ Ready-To-Go™	GE Healthcare, Buckinghamshire, UK	PCR kit. It contains beads with necessary components for the PCR reaction except the primers, template and MQ-water.
10x Dream Taq buffer	Thermo Fisher Scientific, Waltham, Massachusetts	Buffer to maintain the right conditions for PCR.
dNTP	Thermo Fisher Scientific, Waltham, Massachusetts	Building blocks for DNA synthesis.
Dream Taq	Thermo Fisher Scientific, Waltham, Massachusetts	DNA polymerase.
Gel electrophoresis/ gel purification		
TBE	NVI Mediaproduction	Tris-borat-EDTA (ethylenediaminetetraacetic acid). It is a running buffer for the electrophoresis; it conduct electricity and creates conditions in which DNA obtains the highest total negative charge.
Agarose, universal, peqGOLD	VWR, Spain	Compound used to create the gel for gel electrophoresis.
GelRed™ Nucleic Acid Stain 10,000X Water	Sigma-Aldrich, St. Louis, Missouri	Fluorescent dye that stains nucleic acids in gel electrophoresis.
6X DNA Loading Dye	Thermo Fisher Scientific, Vilnius, Lithuania	A coloured buffer that binds to nucleic acids and increases their density as well as dying them for better visibility.

GeneRuler 1 kb DNA Ladder SM0311	Thermo Fisher Scientific, Lithuania	A mix of nucleic acid chains of different, specific lengths and dye.
Isopropanol	VWR	Isopropanol helps DNA to bind to the spin column.
QG	QIAGEN®, Vienna, Austria	Buffer that solubilises the agarose gel and acts as a pH indicator.
PE	QIAGEN®, Vienna, Austria	Wash buffer that removes salts out of the spin column.
EB	QIAGEN®, Vienna, Austria	Buffer that elutes DNA from the spin column.

Appendix 5 – Software

Table 28: Software used to process data and compose this thesis.

Program	Manufacturer	Application
Geneious Prime	Biomatters, New Zealand	For aligning and assembling sequences and perform BLAST search (NCBI).
Microsoft Word	Microsoft, Washington	For writing
Microsoft Excel	Microsoft, Washington	For data sorting, volume/concentration calculations, making diagrams.
Microsoft PowerPoint	Microsoft, Washington	For making figures.

Appendix 6 – Household sampling

According to Figure 19, the percentage of primary plates that had *A. fumigatus* was at around 40% in animal housing and storage rooms respectively. The percentage plates that had *A. niger* appearing was at about 20-30%. In order to get an idea of how common it is for these two fungal species to appear in an agricultural setting compared to a household setting, a small test was conducted. A set of 19 samples were taken from random households in Norway and cultivated in the same manner as the samples from the farms (see section 2.1.4). The samples came from the southern and western parts of the country as well as around the Oslo area. There was generally less fungal growth on the plates from the households than the plates from the farms. Of the 19 plates, 2 had *A. fumigatus*, and none had *A. niger* growth. The results are listed in Table 29.

Table 29: Participant information and fungal growth of air samples from households.

Sample no.	Residents	Pets	Room	Temp. (C°)	Findings
HUS1	16	None	Kitchen	23	No growth
HUS2	1	Two dogs	Kitchen/living room	20	4 *Penicillium* (three types), yeast, 1 unknown, fuzzy, white colony
HUS3	1	Two dogs	Sleeping room	19	4 *Penicillium*
HUS4	4	Three dogs, one cat	Sleeping room	22	No growth
HUS5	4	Three dogs, one cat	Wash room/dog room	22	1 *Penicillium*, two types of yeast
HUS6	2	None	Living room	21	No growth
HUS7	1	One dog	Kitchen/living room	22	No growth
HUS8	1	One dog	Sleeping room	20	No growth
HUS9	2	None	Studio apartment	19	Two types of yeast
HUS10	3	None	Kitchen/living room	22	1 *A. fumigatus*
HUS11	1	None	Living room	19	No growth
HUS12	3	None	Living room	19	No growth
HUS13	2	One cat	Kitchen	21	Mucorales, yeast
HUS14	2	None	Kitchen/living room	21	No growth
HUS15	2	Two cats	Living room	22	No growth
HUS16	5	One cat	Living room	24	2 white unknown colonies
HUS17	2	One cat	Kitchen	17	1 *A. fumigatus*
HUS18	5	None	Sleeping room	20	No growth
HUS19	1	None	Kitchen/living room	17	No growth

Appendix 7 – Participant information

Table 30: Information about the participants gathered from the questionnaire in Appendix 2. Information from G11 is missing.

Farm no.	Ecologic	Number of animals	Animal	Production form	Bedding	Crop	Plot area (1000 m^2)	Pesticide	Temperature H2 (C°)	Animals kept in H2	Temperature L2 (C°)	Storage items in L2
G4	No	14	Dairy cow	Loose housing	Woodchips				16	Cow calves	14	Dry hay, feed
G5	No	50	Dairy cow	Loose housing	Woodchips				10-15	Cattle	10-15	Ensilage, straw, vitamins, tools
G7	Yes	103	Meat cattle	Fed	Woodchips, talle	Oat	100	None	15	Cattle	10	Dry hay, ensilage, grains, straw
						Others	50	None				
G8	No	23	Dairy cow	Loose housing	Woodchips, straw				15-20	Cattle	2-10	Sheep, silo, hay
		20	Sheep									
G9	No		Dairy cow	Loose housing	Woodchips					Dairy cow		Dry hay, ensilage, silo
			Meat cattle	Fed	Woodchips							
			Sheep									
		1	Horse									
G10	No	25	Dairy cow	Pen	Woodchips				15	Cattle	8	Bedding
G11												
G13	No	16	Dairy cow	Pen	Woodchips				10	Cow, calves (no animals inside during sampling)	10	Feed in silo, straw, tools
G14	No	33	Dairy cow	Loose housing						Cattle		Ensilage
		15	Ox	Loose housing								
G15	No		Dairy cow	Loose housing					15	Cow, calves	12	Feed, baleage
			Meat cattle (Nursing)									
G18	Yes	46	Dairy cow	Loose housing	Woodchips, straw	Barley	39		12	Cow, calves	10	Ensilage, woodchips, potatoes
G19	No	270	Sheep						7	Sheep, 30 lambs inside	7	Silos with woodchips, used for sheep
G21	No		Meat cattle (Nursing)	Fed, loose housing	Woodchips				15	Cattle	10	Equipment, feed
G22	No	21	Meat cattle (Nursing)		Woodchips, straw, talle	Oat	10	Herbicide, insecticide	10	Nursing cow (no animals inside during sampling)	10	Grains, wheat
						Wheat	50	Fungicide, herbicide, insecticide				
G23	No	23	Sheep		Talle				15	Horse, sheep	10	Dry hay, ensilage, hay, hay bales
		2	Horse		Woodchips							
G25	Yes	20	Dairy cow	Loose housing	Woodchips, others			None	15	Cattle	10	Ensilage, straw
G26	No	22	Dairy cow	Pen	Woodchips				20	Cattle	13	Feed
		8	Meat cattle									
G27	Yes	65	Goat		Talle				10	Goat	10	Dry hay, ensilage

G28	No	25	Dairy cow	Loose housing	Woodchips				13	Cow, calves	10	Baleage, woodchips
		25	Young meat cattle		Woodchips, straw							
G29	No	40	Meat cattle (Nursing)	Fed	Woodchips				15	Cattle	12	Dry hay, ensilage, fertilisers, tools
		2	Horse		Woodchips							
G32	No	24	Meat cattle (Nursing)		Straw				8	Young animals	7	Dry hay, chicken
G33	No					Others	26	Fungicide, herbicide			12	Dry hay, hay
G34	No	40	Dairy cow	Loose housing	Woodchips				15	Cattle	12	Ensilage in silo, stray, baleage
		120	Sheep									
G35	Yes	150	Sheep		Woodchips				10	Sheep, cat	10	Dry hay, various items
		1	Horse									
			Chicken, quail									
G36	No	10	Sheep		Woodchips, talle				17	Sheep (no animals inside during sampling)	17	Dry hay, woodchips
G37	No					Hay	100	None			10	Dry hay
G38	No	30	Sheep		Talle				14	Sheep	14	Dry hay, hay, woodchips
		2	Horse									
G39	Yes	16	Dairy cow	Pen	Woodchips				16	16 dairy cows, 5 meat cattle, 10 calves (mechanical ventilation)	8	Dry hay, feed in silo and basin
G40	No	27	Dairy cow	Pen	Woodchips, straw				20	Cattle	15	Woodchips, straw, feed
G42	No	50	Dairy cow	Loose housing	Woodchips, straw				13	Cow, calves	12	Straw, feed
G43	No	60	Dairy cow	Loose housing	None				12	Oxes (sporetrap fell with sticky side down at one point)	15	Dairy cow
G44	No	7500	Chicken	Free range	Other				21	Chicken	21	Chicken feed
G45	No		Dairy cow	Loose housing	Woodchips					Dairy cow, meat cattle, calves, oxes		Silo, straw
G47	No	20	Dairy cow	Pen	Woodchips				15	Pigs	10	Ensilage, feed
		120	Pig		Woodchips							
G48	No	15000	Chicken		Woodchips				20	Chicken	12	Chicken feed
G52	No	60	Dairy cow	Loose housing	Woodchips, straw				15	Cattle	15	Feed, baleage
			Meat cattle	Fed								
G53	No	34	Dairy cow	Pen	Woodchips				18		14	

Talle = a mix of bedding, animal urine and faeces.

Appendix 8 – Contamination test photos

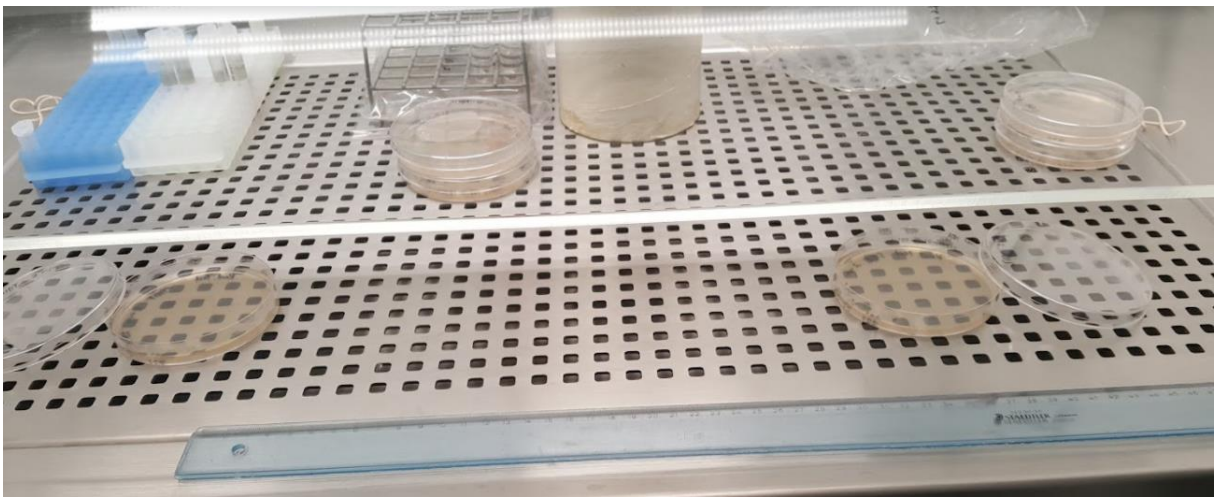


Figure 38: Setup of the contamination test. One plate was placed where secondary plates were processed. The other plate was placed 30-40 cm to the side.

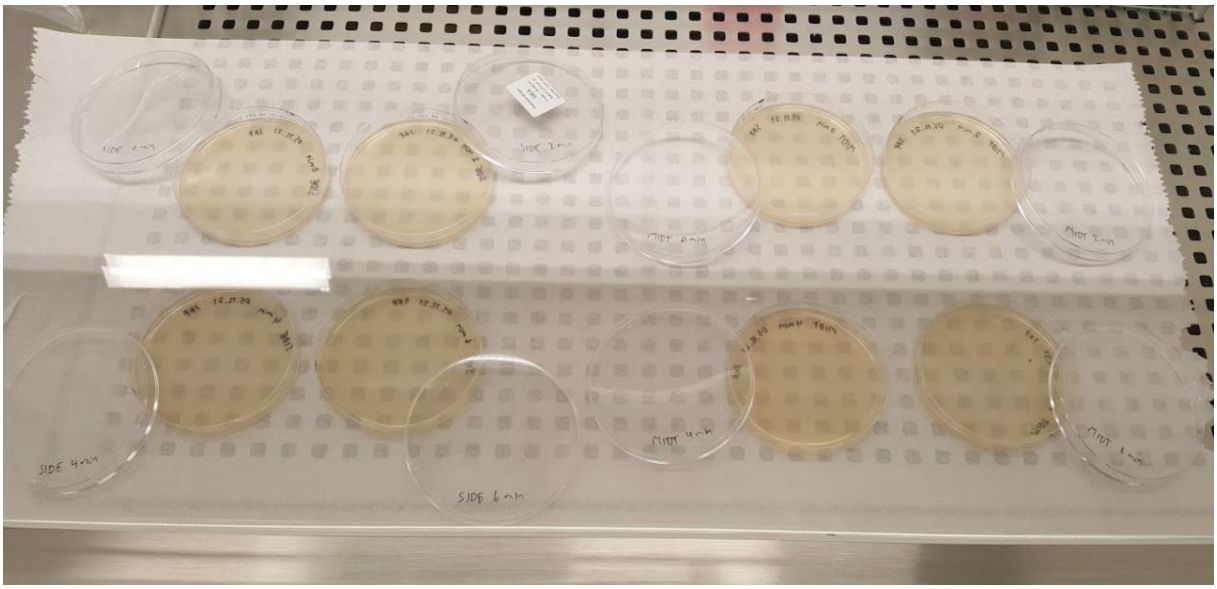


Figure 39: Results of contamination test. *A. fumigatus* – MEA, round 1.

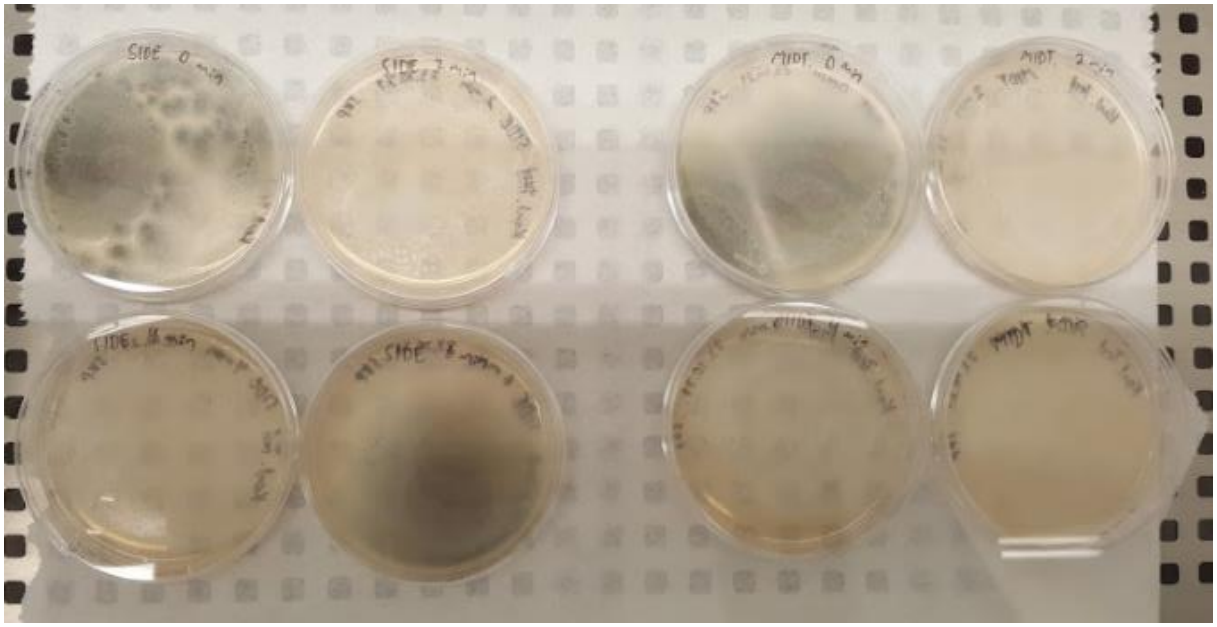


Figure 40: Results of contamination test. *A. fumigatus* – MEA round 2.

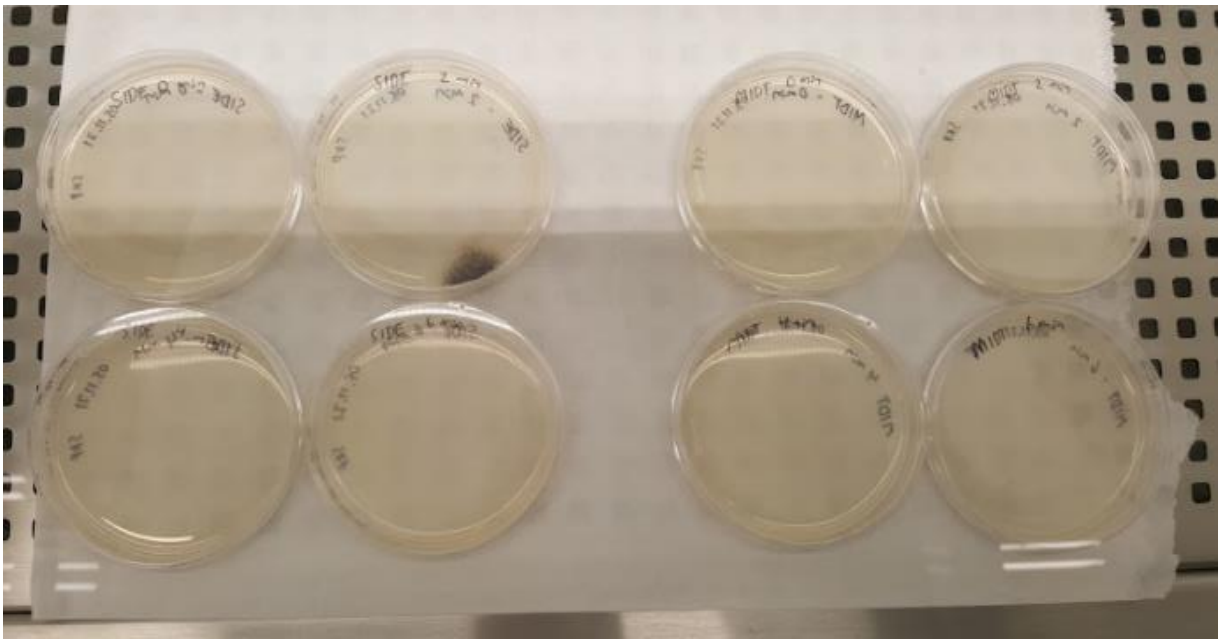


Figure 41: Results of contamination test. *A. niger* - DG18.

Appendix 9 – Findings on primary plates

Table 31: A list of fungi identified on each primary plate and the number of isolates extracted from them.

Farm no.	H2/ L2	Findings	Isolates	
			A. fumigatus	*A. niger*
G4	H2	**Yeast**		
	L2	**Yeast**, **Mucorales**, 1 ***Penicillium***		
G5	H2	**Yeast**		
	L2	2 white/pink unidentified colonies		
G7	H2	1 ***A. niger***, **Mucorales** coverage		1
	L2	1 ***A. fumigatus***, 1 ***A. niger***, **yeast**, **Mucorales** cover	1	1
G8	H2	**Yeast**		
	L2	**Yeast**, 2 white **Mucorales**		
G9	H2	**Yeast**, 1 white **Mucorales**, 10 white/light green ***Penicillium***		
	L2	**Yeast**, 3 white **Mucorales**, 2 white + 1 light green ***Penicillium***		
G10	H2	***A. fumigatus*** coverage	3	
	L2	50+ ***A. fumigatus***	3	
G11	H2	**Yeast**, 8 ***Penicillium***, 1 ***Scopulariopsis brevicaulis***		
	L2	**Yeast**, 1 **Mucorales** (inhibited), 2 light green unidentified colonies		
G13	H2	**Yeast**		
	L2	1 white unidentified colony		
G14	H2	2 ***A. fumigatus***, 1 green + 2 hvite/ light green ***Penicillium***,	2	
	L2	Two types of **yeasts**, 1 white **Mucorales**		
G15	H2	Two types of **yeasts**, unidentified white colonies		
	L2	No growth		
G18	H2	12 (growth inhibited) ***A. fumigatus***, 1 ***A. niger***, **yeast**, two types of **Mucorales**, ***Penicillium***	3	1
	L2	10 inhibited ***A. fumigatus***, **yeast**, **Mucorales**, several ***Penicillium***	3	
G19	H2	No growth		
	L2	**Yeast**, ***Penicillium*** at the edge		

G21	H2	**Yeast**, 3 ***Scopulariopsis***		
	L2	**Yeast**, 6 ***Scopulariopsis***, 1 unidentified yellow colony.		
G22	H2	***A. fumigatus*** coverage, 20 ***A. niger***, 3 **Mucorales**, ***Penicillium***	3	3
	L2	50+ ***A. fumigatus***, 1 ***A. niger***, several ***Penicillium***	3	1
G23	H2	40+ ***A. fumigatus***, 2 ***A. niger***, three types of **Mucorales**, 8 ***A. flavus***, 1 ***A. tritici***, 5 ***A. nidulans***	3	2
	L2	13 ***A. fumigatus***, 1 ***A. niger***, two types of **Mucorales**, 4 ***A. nidulans***, 4 ***A. flavus***, 1 ***A. tritici***	3	1
G25	H2	1 ***A. fumigatus***, 2 ***A. niger***, **yeast**, two types of **Mucorales**, ***A. tritici***, ***Peacilomyces***	1	2
	L2	1 ***A. fumigatus***, 1 ***A. niger***, **yeast**, 1 **Mucorales**, 27 ***Paecilomyces***, ***A. tritici***	1	1
G26	H2	**Yeast**		
	L2	1 ***A. fumigatus***, two types of **yeast**, 1 ***Scopulariopsis***	1	
G27	H2	1 ***A. niger***, **yeast**, **Mucorales**, ***Scopulariopsis***		1
	L2	1 ***A. niger***, **yeast**, **Mucorales**, several ***Scopulariopsis***		1
G28	H2	50+ ***A. fumigatus***	3	
	L2	44 ***A. fumigatus***, **yeast**.	3	
G29	H2	1 ***A. fumigatus***	1	
	L2	No growth		
G32	H2	**Mucorales**, 50+ ***A. flavus***		
	L2	**Mucorales**, 3 ***A. flavus***, 1 white unidentified colony		
G33	L2	1 ***A. fumigatus***, several ***A. niger***, **Mucorales**, ***A. flavus***	1	3
G34	H2	Two types of **yeast**, 3 **Mucorales**		
	L2	1 ***A. fumigatus***, two types of **yeast**, 1 ***Scopulariopsis***, 1 ***Penicillium***	1	
G35	H2	Two types of **Mucorales**, 20+ ***Scopulariopsis***		
	L2	**Mucorales**, 8 ***Scopulariopsis***		
G36	H2	1 unidentified white colony		
	L2	1 unidentified colony		
G37	L2	Some **yeast** on the edge		
G38	H2	***A. fumigatus***, 50+ ***A. niger***, **Mucorales**, 3 ***A. nidulans***	3	3

	L2	24 ***A. fumigatus***, 50+ ***A. niger***, 2 **Mucorales**, 1 ***A. flavus***, 1 ***A. nidulans***	3	3
G39	H2	**Yeast**, 2 white/light green ***Penicillium***		
	L2	**Yeast**		
G40	H2	**Yeast**, **Mucorales**, 2 ***Scopulariopsis***, 1 unidentified ***Aspergillus*** sp., 2 unidentified colonies with a yellow zone		
	L2	**Yeast**, 1 ***Scopulariopsis***, 1 unknown ***Aspergillus*** sp., 2 unknown colonies with yellow zone		
G42	H2	3 ***A. niger***, **yeast**, **Mucorales**		3
	L2	1 ***A. niger***, **yeast**, three types of **Mucorales**, 2 ***A. flavus***		1
G43	H2	1 ***A. niger***, **yeast**, 2 ***A. glaucus***		1
	L2	Two types of **yeast**, 9 ***A. glaucus***		
G44	H2	1 ***A. fumigatus***, **yeast**, **Mucorales**, 15 ***A. glaucus***, two types of unidentified ***Aspergillus*** spp.	1	
	L2	**Yeast**, 50+ ***A. glaucus*** (mostly at the edges), 6 unknown ***Aspergillus*** sp.		
G45	H2	Two types of **yeast**		
	L2	**Yeast**, 1 ***A. flavus***		
G47	H2	1 ***A. fumigatus***, **yeast**, 50+ ***A. flavus***	1	
	L2	**Yeast**, 2 ***A. glaucus***, 8 ***Scopulariopsis***		
G48	H2	5 ***A. fumigatus***, 2 ***A. niger***, **yeast**, **Mucorales**, 14 ***A. flavus*** on the film's edge, 1 unidentified colony with yellow conidia	3	2
	L2	**Yeast**, 3 ***A. flavus*** at the edges, 5 unknown white colonies (one with yellow zone)		
G52	H2	**Yeast**, on the edge of the film: 1 ***A. fumigatus***, 2 **Mucorales** (one in the middle of the plate), 1 ***A. nidulans***, 1 ***A. flavus***	1	
	L2	**Yeast**, at the edges: 1 ***A. fumigatus***, 1 **Mucorales**, 2 ***A. flavus***	1	
G53	H2	28 ***A. fumigatus***, 23 ***A. niger***, **yeast**, **Mucorales**, 2 ***A. tritici***, 1 ***A. flavus***	3	3
	L2	50+ ***A. fumigatus***, 11 ***A. niger***, 2 ***A. nidulans***	3	3

Appendix 10 – Pale *A. fumigatus*

Figure 42: *A. fumigatus* isolate (G22H2S2) on MEA after incubation at 37 °C for three days. The colonies were very pale and were fusing at the edges. It had produced very little conidia, but the microscopy and *caM* sequencing confirmed that it was *A. fumigatus*. See Figure 40 in Appendix 8 and Figure 20-A for comparison.

Appendix 11 – VIPcheck™ scores

Table 32: VIPcheck™ score results of all *A. fumigatus* isolates. Isolates marked in grey were screened with E-test.

		VIPCHECK 24 H				VIPCHECK 48 H			
FARM NO.	MF-value	Itraconazole	Voriconazole	Posaconazole	Control well	Itraconazole	Voriconazole	Posaconazole	Control well
G7L2S1	0.96	-	-	-	+++	-	-	-	+++
G10H2S1	0.95	-	-	-	+++	-	-	-	+++
G10H2S2	1.15	-	-	-	+++	-	-	-	+++
G10H2S3	1.07	-	-	-	+++	-	-	-	+++
G10L2S1	1.15	-	-	-	+++	-	-	-	+++
G10L2S2	1.15	-	-	-	+++	-	-	-	+++
G10L2S3	1.06	-	-	-	+++	-	-	-	+++
G14H2S1	1.19	-	-	-	+++	-	-	-	+++
G14H2S2	1.11	-	-	-	+++	-	-	-	+++
G18H2S1	1.40	-	-	-	+++	-	-	-	+++
G18H2S2	1.03	-	-	-	+++	-	-	-	+++
G18H2S3	1.01	-	-	-	+++	-	-	-	+++
G18L2S1	1.05	-	-	-	+++	-	-	-	+++
G18L2S2	1.02	-	-	-	+++	-	-	-	+++
G18L2S3	1.09	-	-	-	+++	-	-	-	+++
G22H2S1	1.39	-	-	-	+++	-	-	-	+++
G22H2S2	0.62	-	-	-	+++	-	-	-	+++
G22H2S3	1.07	-	-	-	+++	+	-	-	+++
G22L2S1	1.19	-	-	-	+++	-	-	-	+++
G22L2S2	1.03	-	-	-	+++	-	-	-	+++
G22L2S3	1.11	-	-	-	+++	-	-	-	+++
G23H2S1	1.26	-	-	-	+++	-	-	-	+++
G23H2S2	1.19	-	+++	-	+++	+	+++	-	+++
G23H2S3	1.02	-	-	-	+++	-	-	-	+++
G23L2S1	1.14	-	-	-	+++	-	-	-	+++
G23L2S2	1.19	-	-	-	+++	-	-	-	+++
G23L2S3	1.43	-	-	-	+++	-	-	-	+++
G25H2S1	1.00	-	-	-	+++	-	-	-	+++
G25L2S1	1.03	-	-	-	+++	-	-	-	+++
G26L2S1	1.20	-	-	-	+++	+	-	-	+++
G28H2S1	1.50	-	-	-	+++	-	-	-	+++
G28H2S2	1.31	-	-	-	+++	-	-	-	+++
G28H2S3	1.18	-	-	-	+++	-	-	-	+++
G28L2S1	0.96	-	-	-	+++	-	-	-	+++
G28L2S2	1.02	-	-	-	+++	-	-	-	+++
G28L2S3	1.15	-	-	-	+++	-	-	-	+++
G29H2S1	1.12	-	-	-	+++	-	-	-	+++
G33L2S1	1.07	-	-	-	+++	-	-	-	+++
G34L2S1	1.10	+	+	-	+++	+	+	-	+++
G38H2S1	1.15	-	-	-	+++	-	+	-	+++
G38H2S2	1.07	-	-	-	+++	-	-	+	+++
G38H2S3	0.96	+	-	-	+++	+	-	-	+++
G38L2S1	1.24	+	-	-	+++	++	-	+	+++
G38L2S2	0.98	-	-	-	+++	-	-	-	+++
G38L2S3	1.30	-	-	-	+++	+	-	+	+++
G44H2S1	1.73	-	-	-	+++	-	-	-	+++
G47H2S1	1.21	-	-	-	+++	-	-	-	+++
G48H2S1	1.17	-	-	-	+++	-	-	-	+++
G48H2S2	1.09	-	-	-	+++	-	-	-	+++
G48H2S3	1.26	-	-	-	+++	-	-	-	+++
G52H2S1	1.22	-	-	-	+++	-	-	-	+++
G52L2S1	1.27	-	-	-	+++	-	-	-	+++
G53H2S1	1.19	-	-	-	+++	-	-	-	+++
G53H2S2	1.26	-	-	-	+++	-	-	-	+++
G53H2S3	1.11	-	-	-	+++	-	-	-	+++
G53L2S1	1.15	-	-	-	+++	-	-	-	+++
G53L2S2	1.30	-	-	-	+++	-	-	-	+++
G53L2S3	1.12	-	-	-	+++	+	-	-	+++

Table 33: VIPcheck™ score results of all *A. niger* isolates. Isolates marked in grey were screened with E-test.

		VIPCHECK 24 H				VIPCHECK 48 H			
FARM NO.	**MF-value**	**Itraconazole**	**Voriconazole**	**Posaconazole**	**Control well**	**Itraconazole**	**Voriconazole**	**Posaconazole**	**Control well**
G7H2A1	1.16	-	-	-	+++	-	-	+	+++
G7L2A1	1.08	-	-	-	+++	-	-	+	+++
G18H2A1	1.42	+	-	-	+++	++	-	++	+++
G22H2A1	1.24	-	-	-	+++	+	-	-	+++
G22H2A2	1.14	-	-	-	+++	+	-	-	+++
G22H2A3	1.24	-	-	-	+++	-	-	-	+++
G22L2A1	1.07	-	-	-	+++	-	-	-	+++
G23H2A1	1.11	+	-	-	+++	++	-	+	+++
G23H2A2	1.27	-	-	-	+++	-	-	+	+++
G23L2A1	1.59	+	-	-	+++	++	-	-	+++
G25H2A1	0.98	-	-	-	+++	-	-	+	+++
G25H2A2	1.15	-	-	-	+++	+	-	+	+++
G25L2A1	1.11	-	-	-	+++	+	-	+	+++
G27H2A1	1.07	-	-	-	+++	++	-	+	+++
G27L2A1	1.55	-	-	-	+++	++	-	+	+++
G33L2A1	1.19	+	-	+	+++	++	-	+++	+++
G33L2A2	1.16	-	-	-	+++	-	-	+	+++
G33L2A3	1.03	-	-	-	+++	-	-	+	+++
G38H2A1	1.03	-	-	-	+++	+++	-	-	+++
G38H2A2	1.07	-	-	-	+++	+	-	-	+++
G38H2A3	1.15	-	-	-	+++	-	-	-	+++
G38L2A1	1.15	-	-	-	+++	-	-	-	+++
G38L2A2	0.99	-	-	-	+++	+	-	-	+++
G38L2A3	1.18	-	-	-	+++	-	-	-	+++
G42H2A1	1.28	-	-	-	+++	+	-	-	+++
G42H2A2	1.85	-	-	-	+++	+	-	-	+++
G42H2A3	1.17	-	-	-	+++	-	-	-	+++
G42L2A1	1.10	-	-	-	+++	-	-	-	+++
G43H2A1	1.16	-	-	-	+++	+	-	-	+++
G48H2A1	1.68	-	-	-	+++	-	-	-	+++
G48H2A2	1.08	-	-	-	+++	+	-	+	+++
G53H2A1	1.30	-	-	-	+++	-	-	-	+++
G53H2A2	1.07	-	-	-	+++	+	-	-	+++
G53H2A3	1.02	-	-	-	+++	-	-	-	+++
G53L2A1	1.07	-	-	-	+++	-	-	-	+++
G53L2A2	1.19	-	-	-	+++	-	-	-	+++
G53L2A3	1.15	-	-	-	+++	-	-	-	+++

Appendix 12 – VIPcheck™ photos

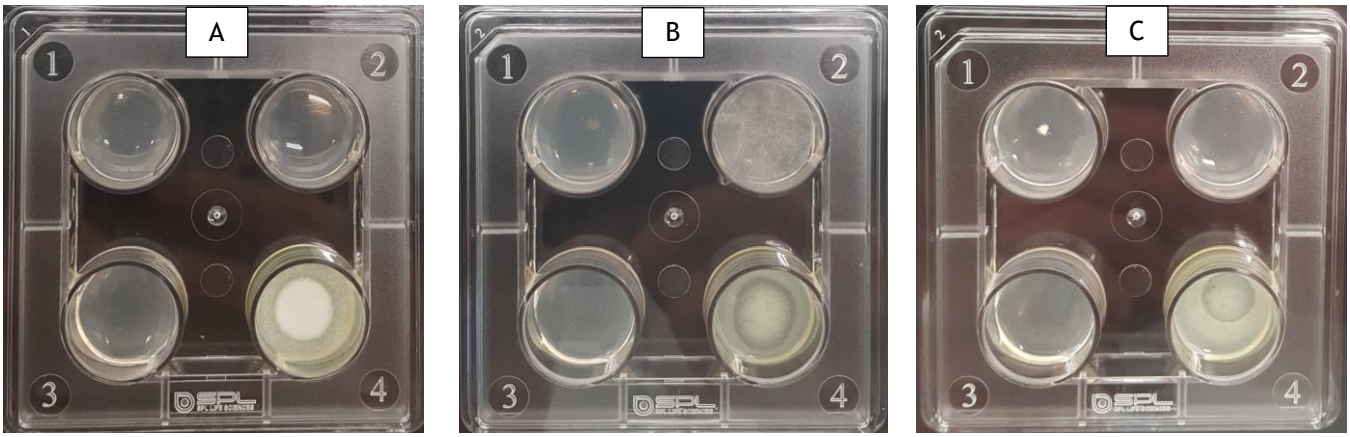


Figure 43: VIPcheck™ plates of *A. fumigatus* after 48 h in 37 °C. These isolates were further screened for resistance with the E-test. Well 1; 4 mg/L itraconazole, well 2; 2 mg/L voriconazole, well 3; 0.5 mg/L posaconazole, well 4; control. **A**; G22H2S2, **B**; G23H2S2, **C**; G38L2S1.

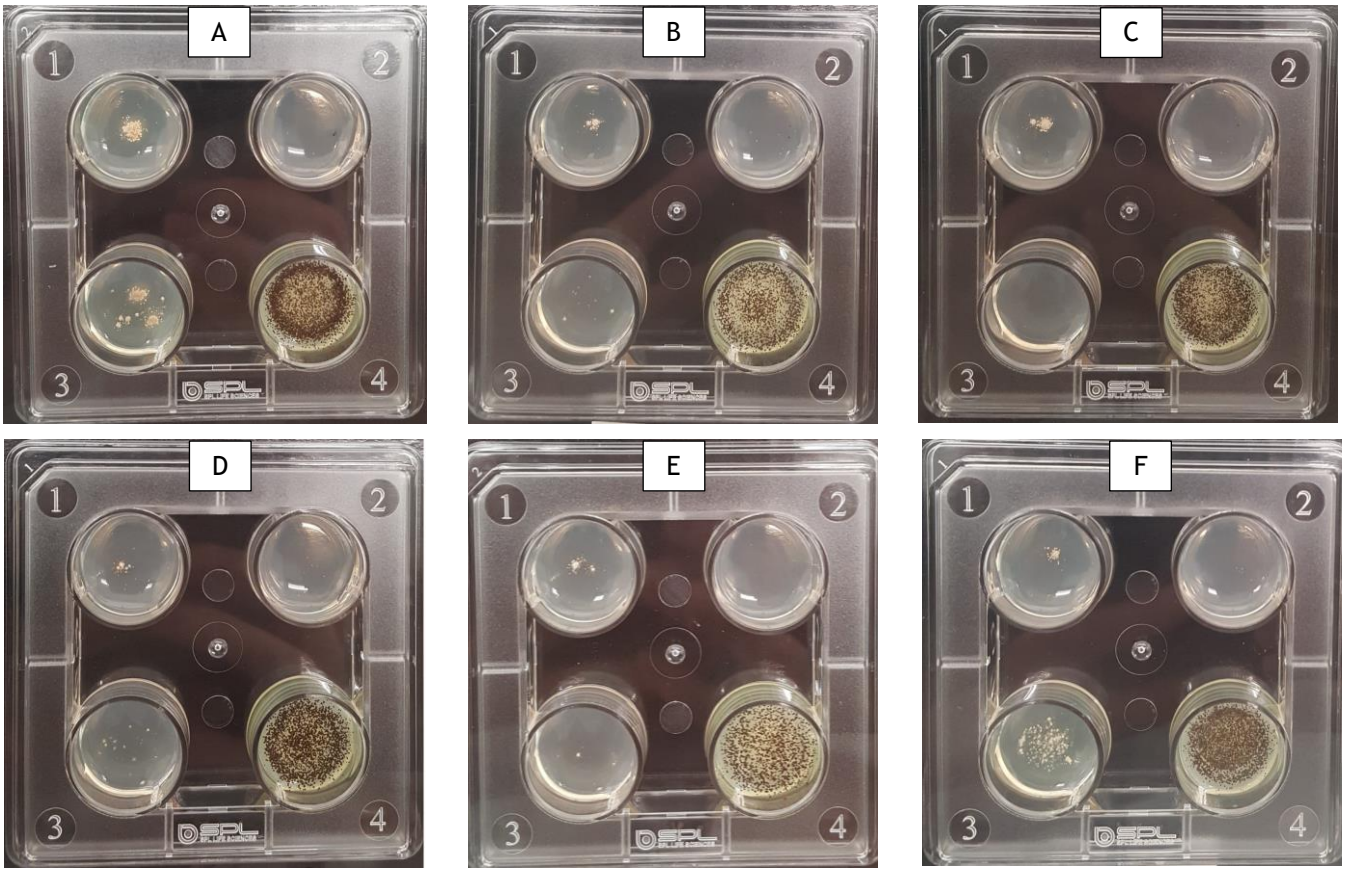


Figure 44: VIPcheck™ plates of *A. niger* after 48 h in 37 °C. These isolates were further screened for resistance with the E-test. Well 1; 4 mg/L itraconazole, well 2; 2 mg/L voriconazole, well 3; 0.5 mg/L posaconazole, well 4; control. **A**; G18H2A1, **B**; G23H2A1, **C**; G23L2A1, **D**; G27H2A1, **E**; G27L2A1, **F**; G33L2A1, **G**; G38H2A1, **H**; G42H2A2.

Appendix 13 – E-test results photos

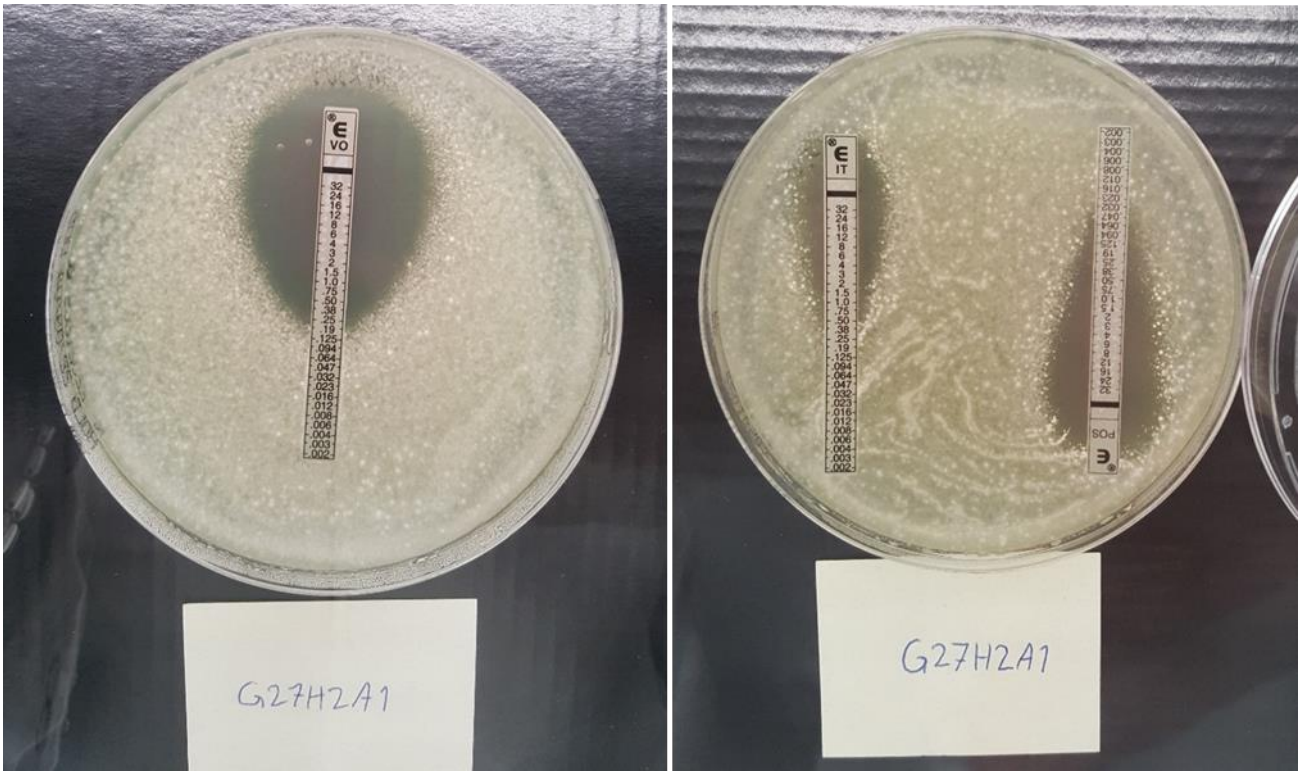


Figure 45: E-test results of G27H2A1. This *A. niger* isolate shows no resistance against any of the azoles. ETEST® strips from left to right: voriconazole, itraconazole and posaconazole. See Table 19 for the readings.

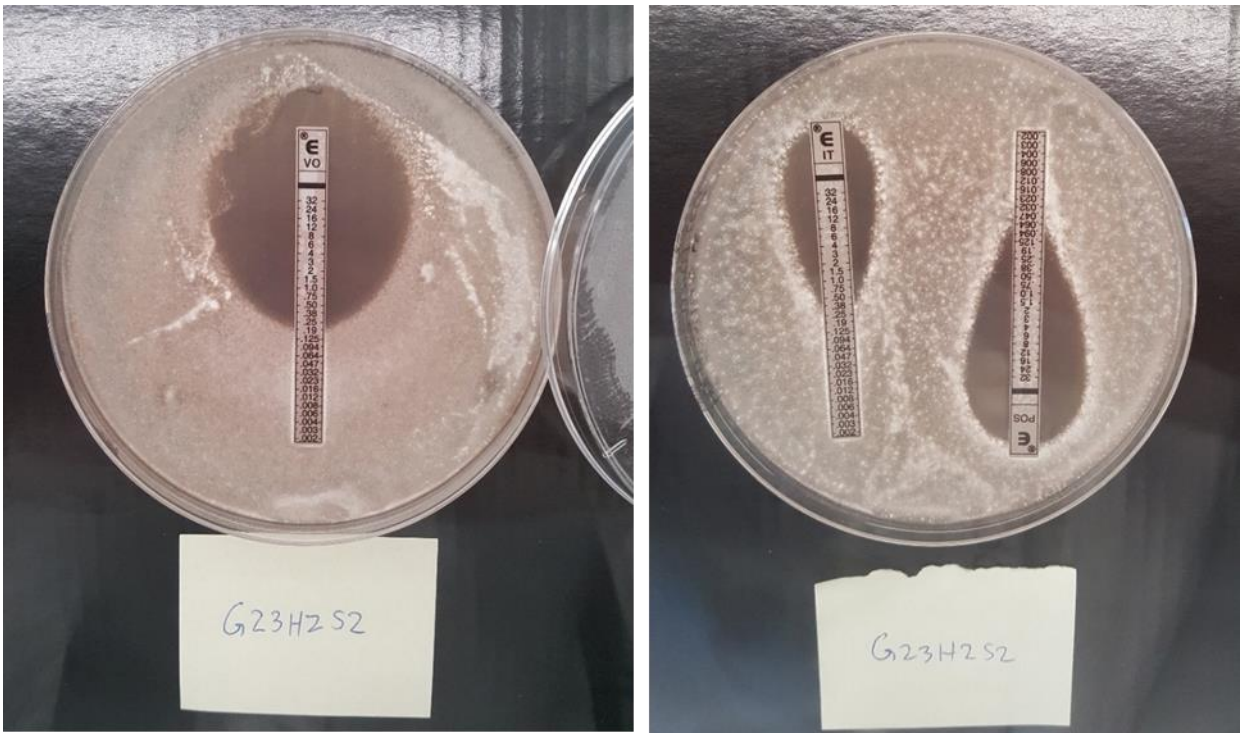


Figure 46: E-test results of G23H2S2. This *A. fumigatus* isolate shows no resistance against any of the azoles. ETEST® strips from left to right: voriconazole, itraconazole and posaconazole. See Table 19 for the readings.

Figure 47: E-test results of the clinical *A. fumigatus* isolate, VI06245. ETEST® strips from left to right: posaconazole, voriconazole and itraconazole. See Table 19 for the readings.

Figure 48: E-test results of the clinical *A. fumigatus* isolate, VI06584. ETEST® strips from left to right: posaconazole, voriconazole and itraconazole. See Table 19 for the readings.

Figure 49: E-test results of the clinical *A. fumigatus* isolate, VI06658. ETEST® strips from left to right: voriconazole, posaconazole and itraconazole. See Table 19 for the readings.

Appendix 14 – Clinical sample information

Table 34: Information of the clinical *A. fumigatus* isolate from NVI's strain collection.

Sample no.	Species	Host	E-test (2018) Itracon azole	E-test (2018) Voricon azole	E-test (2018) Posacon azole	VIPcheckTM (2018) - 24 h				VIPcheckTM (2018) - 48 h			
						Itracon azole	Voricon azole	Posacon azole	Control	Itracon azole	Voricon azole	Posacon azole	Control
VI 06245	*Aspergillus fumigatus*	Cattle, placenta	32 **R**	1 **S**	1/0,5 **R**	-	-	-	++	++	+	+	++
VI 06584	*Aspergillus fumigatus*	Dog, sinuses	4 **R**	0,5 **S**	0,5 **R**	++	-	-	++	++	++	++	++
VI 06658	*Aspergillus fumigatus*	Dog, sinuses	>32 **R**	1 **S**	0,5 **R**	+	-	-	++	++	+	+	++

S = sensitive

R= resistant

Appendix 15 – Consensus of *cyp51A* references used mutation screening

>"Wild-type A. fumigatus (AF338659)"

ATAATCGCAGCACCACTTCAGAGTTGTCTAGAATCACGCGGTCCGGATGTGTGCTGAGCCGAATGAAA
GTTGCCTAATTACTAAGGTGTAGTTCCAGCATACCATACACCCTAACTCATACTACGGTAGGTAGATC
TACTTACCTATGAACCTATATTGGTAGGTAGGTGAATATAAAATACAGCATGGAACATGTTTTTCATT
AGCTGGTCTCTCATTCGTCCTTGTCCTAGGCCTTAAGGAATCCAGTATATGAAATAATCCCTCTTATC
CATTTTCCTCCTATTCTTTTTCATTTCCCTCATCACTGCAACTCTAATCCTCGGGCTCACCCTCCCTG
TGTCTCCTCGAAATGGTGCCGATGCTATGGCTTACGGCCTACATGGCCGTTGCGGTGCTGACGGCAAT
CTTGCTCAATGTTGTTTATCAATTATTCTTTCGGCTTTGGAACCGAACAGAACCGCCAATGGTCTTTC
ATTGGGTCCCATTTCTGGGTAGTACCATCAGTTACGGGATTGATCCCTACAAGTTCTTCTTTGCGTGC
AGAGAAAAGGCAAGTCTCAAGATTGTAGTTTGACATTCATTCCTGGGCGCATTGCTGAGTATTGCTTT
CTTAACCGGCAGTATGGCGATATCTTCACTTTTATACTGTTGGGTCAAAAAACCACAGTCTACCTGGG
CGTTCAGGGGAACGAGTTTATTCTCAACGGCAAGCTCAAGGATGTCAATGCGGAAGAGGTCTATAGTC
CATTGACGACCCCCGTTTTCGGATCGGACGTGGTGTATGATTGTCCCAATTCCAAGCTGATGGAGCAG
AAAAAGTTCATCAAGTACGGCTTGACTCAGTCTGCGTTAGAGTCTCATGTGCCACTTATTGAGAAGGA
GGTTTTGGACTATCTGCGCGATTCACCGAACTTTCAAGGCTCGTCCGGCCGGATGGACATCTCTGCGG
CAATGGCTGAGATTACCATTTTTACCGCTGCTCGAGCCCTCCAAGGCCAGGAAGTTCGTTCCAAACTC
ACGGCTGAGTTCGCTGACCTCTATCATGACCTGGACAAGGGCTTTACTCCCATCAATTTTATGCTACC
GTGGGCCCCATTGCCGCATAACAAGAAGCGAGATGCTGCTCATGCGCGCATGAGGTCAATCTACGTTG
ACATCATCAATCAGCGCCGTCTTGACGGTGACAAGGACTCTCAGAAATCAGACATGATATGGAACCTG
ATGAACTGCACATACAAAAACGGCCAGCAAGTGCCTGATAAAGAGATTGCGCACATGATGATAACCCT
GTTGATGGCTGGTCAGCATTCGTCTTCGTCCATCAGCGCCTGGATTATGCTGAGACTGGCCTCACAGC
CAAAAGTCCTCGAAGAGCTGTATCAGGAACAGCTGGCCAATCTTGGCCCCGCCGGGCCAGACGGCAGT
CTTCCTCCGCTCCAGTACAAGGATCTTGACAAACTTCCCTTCCATCAACATGTTATTCGTGAAACCTT
ACGGATTCACTCCTCTATTCACTCTATCATGCGCAAGGTGAAAAGCCCCTTGCCCGTTCCCGGGACCC
CTTACATGATTCCTCCCGGTCGCGTGCTCCTTGCTTCACCTGGAGTGACAGCCCTCAGCGACGAACAC
TTCCCCAATGCTGGGTGCTGGGATCCCCATCGCTGGGAGAACCAGGCTACTAAGGAGCAGGAGAACGA
CGAGGTTGTCGACTACGGTTACGGCGCCGTCTCCAAGGGCACGTCAAGTCCCTATCTTCCGTTTGGTG
CTGGCCGACACCGCTGTATCGGCGAGAAATTCGCTTATGTCAACCTTGGTGTGATTCTGGCGACCATT
GTGCGCCACCTGCGACTTTTCAACGTGGATGGAAAGAAAGGAGTCCCTGAAACTGACTATTCATCCCT
CTTTTCGGGCCCCATGAAGCCAAGCATCATCGGCTGGGAGAAGCGGTCGAAAAACACATCCAAGTGAG
ACTGTTGTAACCATCGAGGACTTCAAAGGATTTGGTGTGATCGGAATAGGTGTATTATACTTAATTCA
CCCCTCGA

>"TR34/L98H"

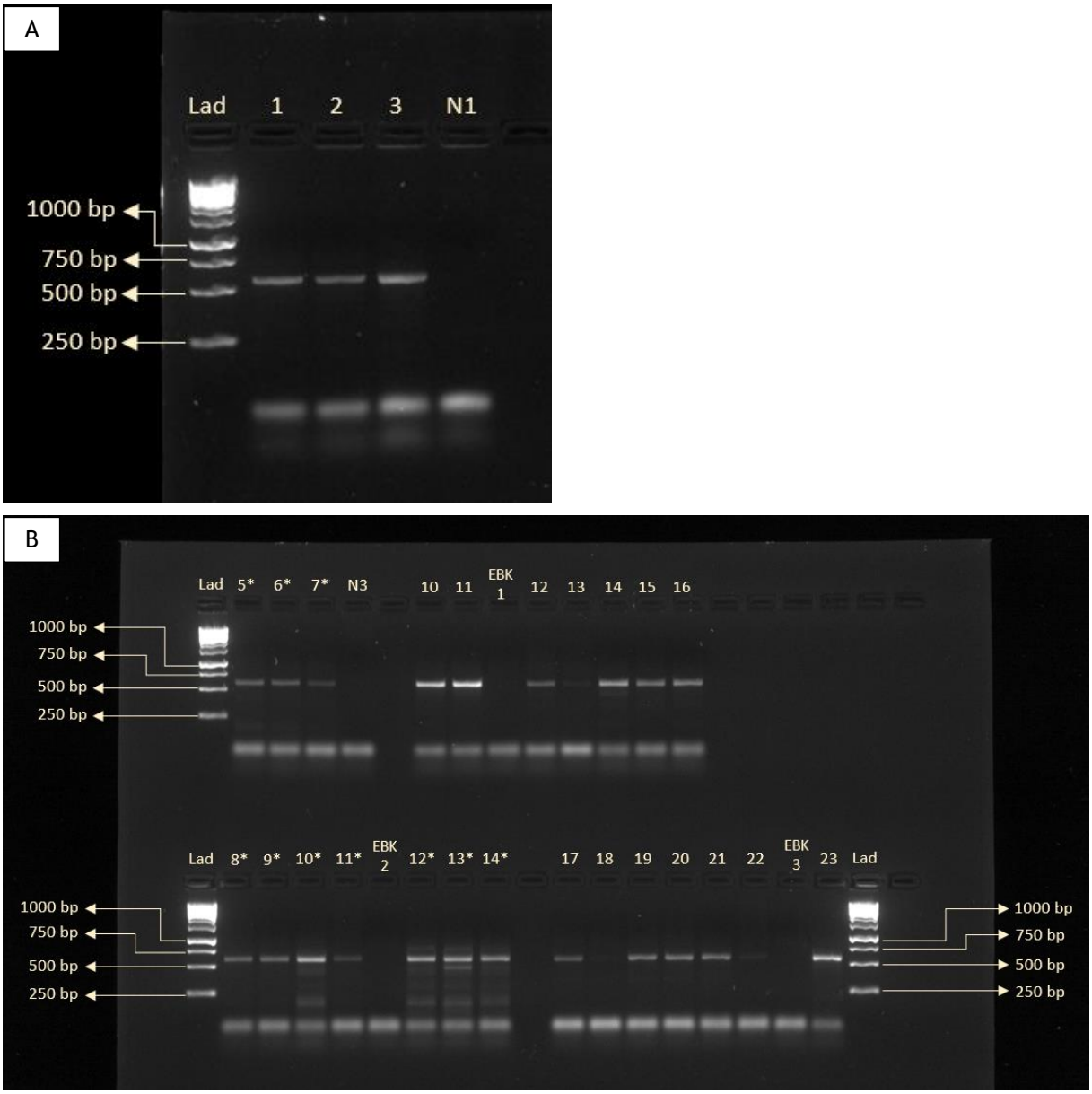
ATAATCGCAGCACCACTTCAGAGTTGTCTAGAATCACGCGGTCCGGATGTGTGCTGAGCCGAATGAAT
CACGCGGTCCGGATGTGTGCTGAGCCGAATGAAAGTTGCCTAATTACTAAGGTGTAGTTCCAGCATAC
CATACACCCTAACTCATACTACGGTAGGTAGATCTACTTACCTATGAACCTATATTGGTAGGTAGGTG

AATATAAAATACAGCATGGAACATGTTTTTCATTAGCTGGTCTCTCATTCGTCCTTGTCCTAGGCCTT
AAGGAATCCAGTATATGAAATAATCCCTCTTATCCATTTTCCTCCTATTCTTTTTCATTTCCCTCATC
ACTGCAACTCTAATCCTCGGGCTCACCCTCCCTGTGTCTCCTCGAAATGGTGCCGATGCTATGGCTTA
CGGCCTACATGGCCGTTGCGGTGCTGACGGCAATCTTGCTCAATGTTGTTTATCAATTATTCTTTCGG
CTTTGGAACCGAACAGAACCGCCAATGGTCTTTCATTGGGTCCCATTTCTGGGTAGTACCATCAGTTA
CGGGATTGATCCCTACAAGTTCTTCTTTGCGTGCAGAGAAAAGGCAAGTCTCAAGATTGTAGTTTGAC
ATTCATTCCTGGGCGCATTGCTGAGTATTGCTTTCTTAACCGGCAGTATGGCGATATCTTCACTTTTA
TACTGTTGGGTCAAAAAACCACAGTCTACCTGGGCGTTCAGGGGAACGAGTTTATTCTCAACGGCAAG
CACAAGGATGTCAATGCGGAAGAGGTCTATAGTCCATTGACGACCCCCGTTTTCGGATCGGACGTGGT
GTATGATTGTCCCAATTCCAAGCTGATGGAGCAGAAAAAGTTCATCAAGTACGGCTTGACTCAGTCTG
CGTTAGAGTCTCATGTGCCACTTATTGAGAAGGAGGTTTTGGACTATCTGCGCGATTCACCGAACTTT
CAAGGCTCGTCCGGCCGGATGGACATCTCTGCGGCAATGGCTGAGATTACCATTTTTACCGCTGCTCG
AGCCCTCCAAGGCCAGGAAGTTCGTTCCAAACTCACGGCTGAGTTCGCTGACCTCTATCATGACCTGG
ACAAGGGCTTTACTCCCATCAATTTTATGCTACCGTGGGCCCCATTGCCGCATAACAAGAAGCGAGAT
GCTGCTCATGCGCGCATGAGGTCAATCTACGTTGACATCATCAATCAGCGCCGTCTTGACGGTGACAA
GGACTCTCAGAAATCAGACATGATATGGAACCTGATGAACTGCACATACAAAAACGGCCAGCAAGTGC
CTGATAAAGAGATTGCGCACATGATGATAACCCTGTTGATGGCTGGTCAGCATTCGTCTTCGTCCATC
AGCGCCTGGATTATGCTGAGACTGGCCTCACAGCCAAAAGTCCTCGAAGAGCTGTATCAGGAACAGCT
GGCCAATCTTGGCCCCGCCGGGCCAGACGGCAGTCTTCCTCCGCTCCAGTACAAGGATCTTGACAAAC
TTCCCTTCCATCAACATGTTATTCGTGAAACCTTACGGATTCACTCCTCTATTCACTCTATCATGCGC
AAGGTGAAAAGCCCCTTGCCCGTTCCCGGGACCCCTTACATGATTCCTCCCGGTCGCGTGCTCCTTGC
TTCACCTGGAGTGACAGCCCTCAGCGACGAACACTTCCCCAATGCTGGGTGCTGGGATCCCCATCGCT
GGGAGAACCAGGCTACTAAGGAGCAGGAGAACGACGAGGTTGTCGACTACGGTTACGGCGCCGTCTCC
AAGGGCACGTCAAGTCCCTATCTTCCGTTTGGTGCTGGCCGACACCGCTGTATCGGCGAGAAATTCGC
TTATGTCAACCTTGGTGTGATTCTGGCGACCATTGTGCGCCACCTGCGACTTTTCAACGTGGATGGAA
AGAAAGGAGTCCCTGAAACTGACTATTCATCCCTCTTTTCGGGCCCCATGAAGCCAAGCATCATCGGC
TGGGAGAAGCGGTCGAAAAACACATCCAAGTGAGACTGTTGTAACCATCGAGGACTTCAAAGGATTTG
GTGTGATCGGAATAGGTGTATTATACTTAATTCACCCCTCGA

>"TR46/Y121F/T289A"
ATAATCGCAGCACCACTTCAGAGTTGTCTAGAATCACGCGGTCCGGATGTGTGCTGAGCCGAATGAAA
GTTGTCTAGAATCACGCGGTCCGGATGTGTGCTGAGCCGAATGAAAGTTGCCTAATTACTAAGGTGTA
GTTCCAGCATACCATACACCCTAACTCATACTACGGTAGGTAGATCTACTTACCTATGAACCTATATT
GGTAGGTAGGTGAATATAAAATACAGCATGGAACATGTTTTTCATTAGCTGGTCTCTCATTCGTCCTT
GTCCTAGGCCTTAAGGAATCCAGTATATGAAATAATCCCTCTTATCCATTTTCCTCCTATTCTTTTTC
ATTTCCCTCATCACTGCAACTCTAATCCTCGGGCTCACCCTCCCTGTGTCTCCTCGAAATGGTGCCGA
TGCTATGGCTTACGGCCTACATGGCCGTTGCGGTGCTGACGGCAATCTTGCTCAATGTTGTTTATCAA
TTATTCTTTCGGCTTTGGAACCGAACAGAACCGCCAATGGTCTTTCATTGGGTCCCATTTCTGGGTAG

TACCATCAGTTACGGGATTGATCCCTACAAGTTCTTCTTTGCGTGCAGAGAAAAGGCAAGTCTCAAGA
TTGTAGTTTGACATTCATTCCTGGGCGCATTGCTGAGTATTGCTTTCTTAACCGGCAGTATGGCGATA
TCTTCACTTTTATACTGTTGGGTCAAAAAACCACAGTCTACCTGGGCGTTCAGGGGAACGAGTTTATT
CTCAACGGCAAGCTCAAGGATGTCAATGCGGAAGAGGTCTATAGTCCATTGACGACCCCCGTTTTCGG
ATCGGACGTGGTGTTTGATTGTCCCAATTCCAAGCTGATGGAGCAGAAAAAGTTCATCAAGTACGGCT
TGACTCAGTCTGCGTTAGAGTCTCATGTGCCACTTATTGAGAAGGAGGTTTTGGACTATCTGCGCGAT
TCACCGAACTTTCAAGGCTCGTCCGGCCGGATGGACATCTCTGCGGCAATGGCTGAGATTACCATTTT
TACCGCTGCTCGAGCCCTCCAAGGCCAGGAAGTTCGTTCCAAACTCACGGCTGAGTTCGCTGACCTCT
ATCATGACCTGGACAAGGGCTTTACTCCCATCAATTTTATGCTACCGTGGGCCCCATTGCCGCATAAC
AAGAAGCGAGATGCTGCTCATGCGCGCATGAGGTCAATCTACGTTGACATCATCAATCAGCGCCGTCT
TGACGGTGACAAGGACTCTCAGAAATCAGACATGATATGGAACCTGATGAACTGCACATACAAAAACG
GCCAGCAAGTGCCTGATAAAGAGATTGCGCACATGATGATAGCCCTGTTGATGGCTGGTCAGCATTCG
TCTTCGTCCATCAGCGCCTGGATTATGCTGAGACTGGCCTCACAGCCAAAAGTCCTCGAAGAGCTGTA
TCAGGAACAGCTGGCCAATCTTGGCCCCGCCGGGCCAGACGGCAGTCTTCCTCCGCTCCAGTACAAGG
ATCTTGACAAACTTCCCTTCCATCAACATGTTATTCGTGAAACCTTACGGATTCACTCCTCTATTCAC
TCTATCATGCGCAAGGTGAAAAGCCCCTTGCCCGTTCCCGGGACCCCTTACATGATTCCTCCCGGTCG
CGTGCTCCTTGCTTCACCTGGAGTGACAGCCCTCAGCGACGAACACTTCCCCAATGCTGGGTGCTGGG
ATCCCCATCGCTGGGAGAACCAGGCTACTAAGGAGCAGGAGAACGACGAGGTTGTCGACTACGGTTAC
GGCGCCGTCTCCAAGGGCACGTCAAGTCCCTATCTTCCGTTTGGTGCTGGCCGACACCGCTGTATCGG
CGAGAAATTCGCTTATGTCAACCTTGGTGTGATTCTGGCGACCATTGTGCGCCACCTGCGACTTTTCA
ACGTGGATGGAAAGAAAGGAGTCCCTGAAACTGACTATTCATCCCTCTTTTCGGGCCCCATGAAGCCA
AGCATCATCGGCTGGGAGAAGCGGTCGAAAAACACATCCAAGTGAGACTGTTGTAACCATCGAGGACT
TCAAAGGATTTGGTGTGATCGGAATAGGTGTATTATACTTAATTCACCCCTCGA

Appendix 16 – Gel electrophoresis images of *caM, bT2* and *cyp51A* amplicons



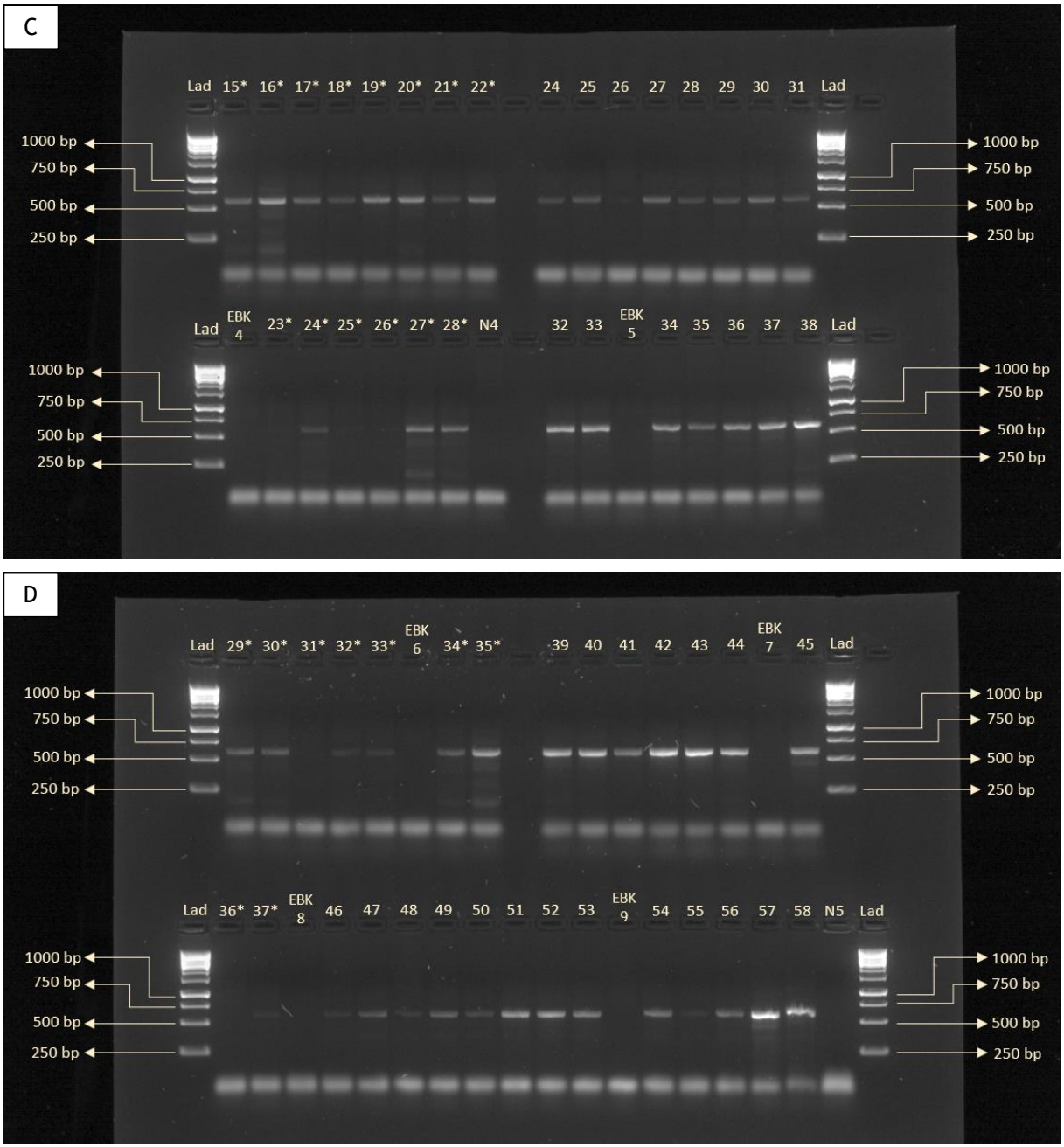


Figure 50: Gel image of *caM* PCR product (between 500 bp and 750 bp). Samples are numbered; numbers with * are from *A. niger*, and samples without are from *A. fumigatus*. EBKs are extraction blank controls, and N are negative controls, which were all blank. Band sizes are marked on the ladders (Lad). Sample details are in Table 35 in Appendix 17.

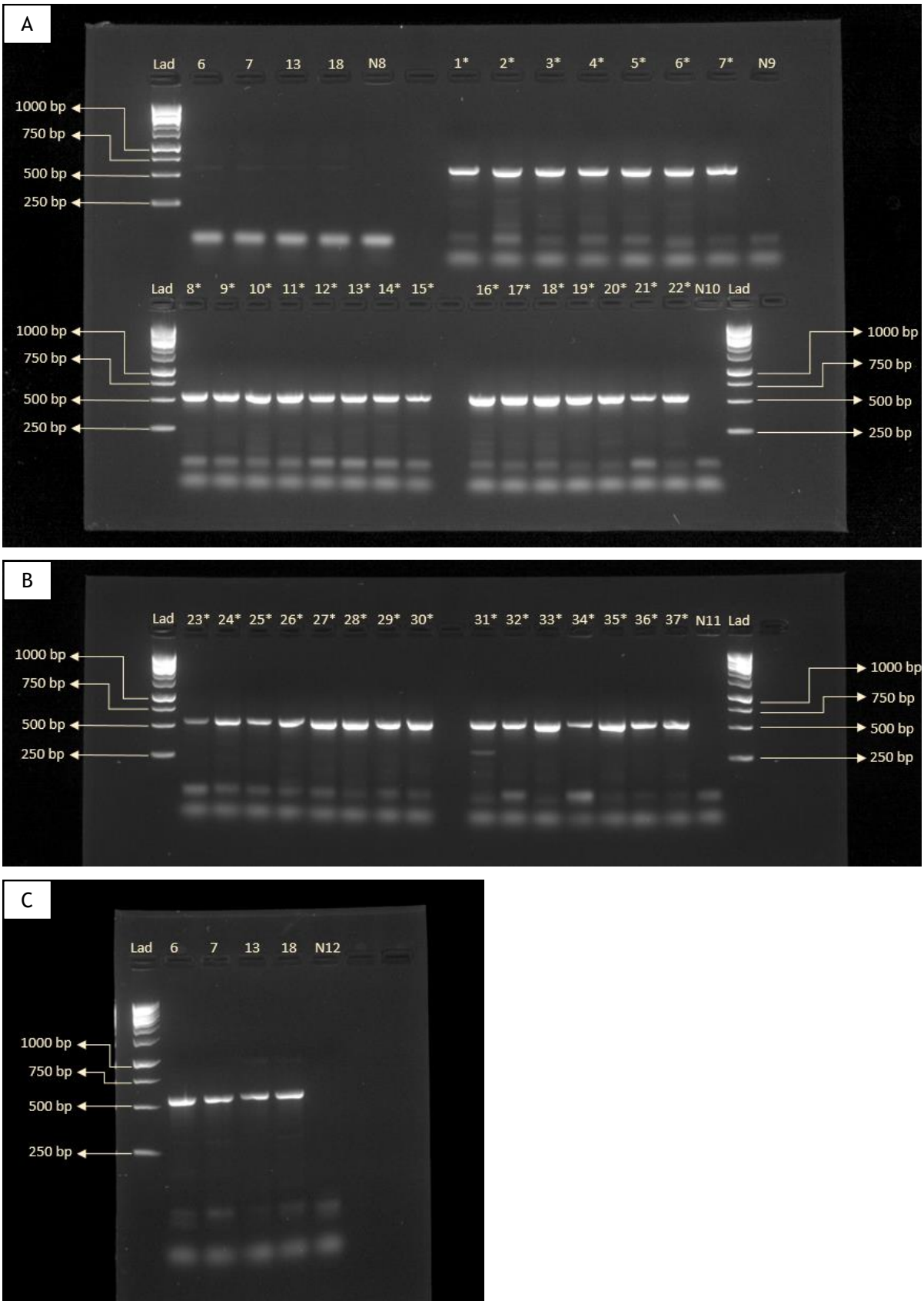


Figure 51: Gel image of *A. fumigatus caM* PCR products (samples in **A** without *), *A. niger* bT2 PCR products (samples in **A**, **B** and **C** marked with *) and *A. fumigatus bT2* PCR products (samples in **C** without *). All negative controls (N) were blank, and *caM* products were faint (between 500 bp and 750 bp). All *bT2* products were very clear (at around 500 bp) . Band sizes are marked on the ladders (Lad). Sample details are in Table 35 in Appendix 17.

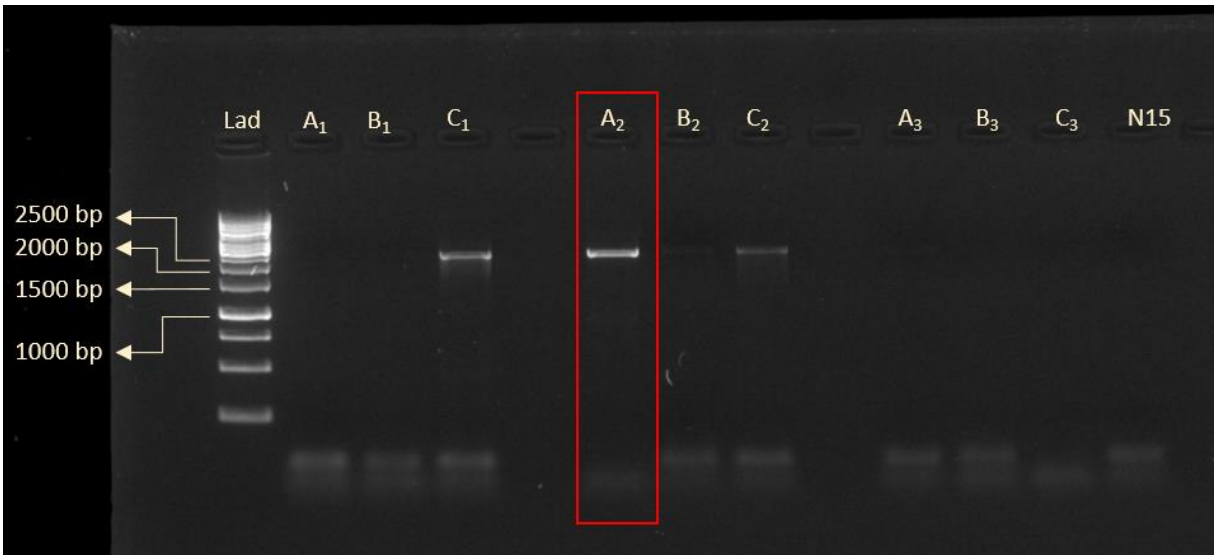


Figure 52: Gel image of the second attempt at *cyp51A* PCR on the three resistant clinical *A. fumigatus* isolates from NVI's strain collection (between 2000 bp and 2500 bp). Three parallels of the reaction were running at the same time. Lad is the ladder, and N15 is the negative control. Unfortunately, the reaction mix evaporated during the PCR, which resulted in a poor yield. However, sample A in the second parallel (marked with the red box) seemed promising and had no traces of the persistent extra band. This PCR product was therefore sent for sequencing without going through gel purification beforehand. Samples are listed in Table 21.

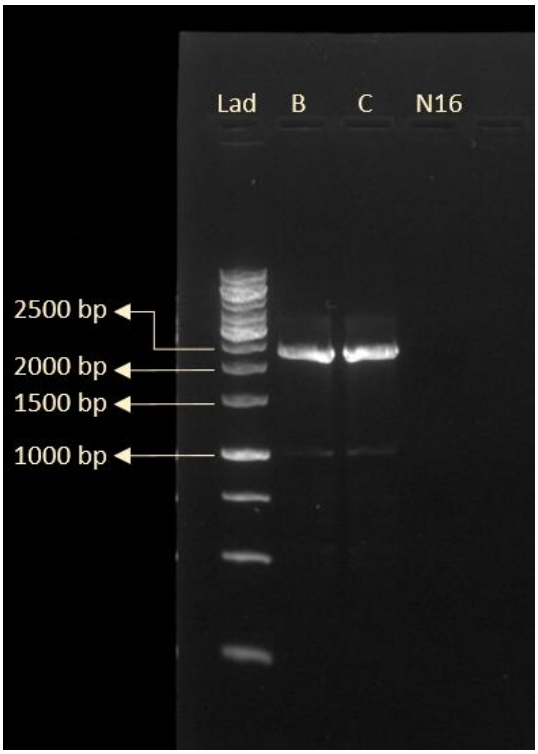


Figure 53: Gel image of the third attempt at *cyp51A* PCR on the resistant clinical *A. fumigatus* isolates from NVI's strain collection (between 2000 bp and 2500 bp). Lad is the ladder, and N16 is the negative control. The extra band at 1000 bp still persisted, and it was therefore decided to run gel purification on these two isolates before sequencing. Samples are listed in Table 21.

Appendix 17 – Molecular sample numbers

Table 35: Sample numbers and their corresponding DNA numbers seen on the gel images. *A. fumigatus* are marked in blue, and *A. niger* are marked in orange.

Farm no.	DNA no.	Farm no.	DNA no.	Farm no.	DNA no.
G7L2S1	1	G23H2S2	33	G22H2A2	7*
G10H2S1	2	G23H2S3	34	G22H2A3	8*
G10H2S2	3	G23L2S1	35	G22L2A1	9*
G10H2S3	4	G23L2S2	36	G18H2A1	10*
G10L2S1	5	G23L2S3	37	G48H2A1	11*
G10L2S2	6	G26L2S1	38	G48H2A2	12*
G10L2S3	7	G29H2S1	39	G27H2A1	13*
G22L2S1	8	G34L2S1	40	G27L2A1	14*
G22L2S2	9	G38H2S1	41	G33L2A1	15*
G22L2S3	10	G38H2S2	42	G33L2A2	16*
G28H2S1	11	G38H2S3	43	G33L2A3	17*
G28H2S2	12	G38L2S1	44	G23H2A1	18*
G28H2S3	13	G38L2S2	45	G23H2A2	19*
G28L2S1	14	G38L2S3	46	G23L2A1	20*
G28L2S2	15	G47H2S1	47	G38H2A1	21*
G28L2S3	16	G52H2S1	48	G38H2A2	22*
G25H2S1	17	G52L2S1	49	G38H2A3	23*
G25L2S1	18	G44H2S1	50	G38L2A1	24*
G18H2S1	19	G14H2S1	51	G38L2A2	25*
G18H2S2	20	G14H2S2	52	G38L2A3	26*
G18H2S3	21	G53H2S1	53	G42H2A1	27*
G18L2S1	22	G53H2S2	54	G42H2A2	28*
G18L2S2	23	G53H2S3	55	G42H2A3	29*
G18L2S3	24	G53L2S1	56	G42L2A1	30*
G22H2S1	25	G53L2S2	57	G53H2A1	31*
G22H2S2	26	G53L2S3	58	G53H2A2	32*
G22H2S3	27	G7H2A1	1*	G53H2A3	33*
G48H2S1	28	G7L2A1	2*	G53L2A1	34*
G48H2S2	29	G25H2A1	3*	G53L2A2	35*
G48H2S3	30	G25H2A2	4*	G53L2A3	36*
G33L2S1	31	G25L2A1	5*	G43H2A1	37*
G23H2S1	32	G22H2A1	6*		

Appendix 18 – Sequence information

Table 36: Sequence and BLAST result information for each *A. fumigatus* isolate.

Sample no.	Genetic marker	Strands	Contig length (bp)	Length prior to trim (bp)	% HQ	% GC	E-value	Bit score	% Hit grade	Specie
G7L2S1	*caM*	1 (CMD6)	511	561	99,0 %	48,5 %	0	944,758	100,0 %	*A. fumigatus*
G10H2S1	*caM*	2	580		99,0 %	49,4 %	0	1072,18	100,0 %	*A. fumigatus*
G10H2S2	*caM*	2	515		100,0 %	48,5 %	0	946,605	99,9 %	*A. fumigatus*
G10H2S3	*caM*	2	578		99,8 %	49,1 %	0	1061,1	99,9 %	*A. fumigatus*
G10L2S1	*caM*	1 (CMD5)	467	568	97,6 %	49,5 %	0	863,506	100,0 %	*A. fumigatus*
G10L2S2	*bT2*	2	547		100,0 %	52,1 %	0	1011,24	100,0 %	*A. fumigatus*
G10L2S3	*bT2*	2	535		100,0 %	51,8 %	0	989,078	100,0 %	*A. fumigatus*
G14H2S1	*caM*	1 (CMD6)	523	560	98,1 %	48,9 %	0	966,918	100,0 %	*A. fumigatus*
G14H2S2	*caM*	2	565		100,0 %	48,6 %	0	1033,4	99,8 %	*A. fumigatus*
G18H2S1	*caM*	2	578		100,0 %	48,8 %	0	1057,4	99,8 %	*A. fumigatus*
G18H2S2	*caM*	2	577		99,8 %	49,7 %	0	1055,56	99,8 %	*A. fumigatus*
G18H2S3	*caM*	2	577		99,5 %	48,9 %	0	1055,56	99,8 %	*A. fumigatus*
G18L2S1	*caM*	2	575		100,0 %	48,7 %	0	1051,86	99,8 %	*A. fumigatus*
G18L2S2	*caM*	2	566		100,0 %	49,2 %	0	1046,32	100,0 %	*A. fumigatus*
G18L2S3	*caM*	2	545		98,5 %	48,7 %	0	1007,54	100,0 %	*A. fumigatus*
G22H2S1	*caM*	2	549		99,6 %	49,0 %	0	1014,93	100,0 %	*A. fumigatus*
G22H2S2	*caM*	2	291		100,0 %	51,1 %	1,68E-147	534,802	99,9 %	*A. fumigatus*
G22H2S3	*caM*	2	578		99,0 %	49,0 %	0	1061,1	99,9 %	*A. fumigatus*
G22L2S1	*caM*	2	551		99,6 %	48,8 %	0	1018,62	100,0 %	*A. fumigatus*
G22L2S2	*caM*	2	577		99,7 %	49,0 %	0	1066,64	100,0 %	*A. fumigatus*
G22L2S3	*caM*	2	566		99,8 %	49,2 %	0	1046,32	100,0 %	*A. fumigatus*
G23H2S1	*caM*	2	565		99,5 %	49,2 %	0	1044,48	100,0 %	*A. fumigatus*
G23H2S2	*caM*	2	565		100,0 %	49,4 %	0	1044,48	100,0 %	*A. fumigatus*
G23H2S3	*caM*	2	577		98,8 %	48,8 %	0	1055,56	99,8 %	*A. fumigatus*
G23L2S1	*caM*	2	555		100,0 %	49,3 %	0	1026,01	100,0 %	*A. fumigatus*
G23L2S2	*caM*	2	577		99,8 %	49,1 %	0	1066,64	100,0 %	*A. fumigatus*
G23L2S3	*caM*	2	578		100,0 %	49,4 %	0	1066,64	100,0 %	*A. fumigatus*
G25H2S1	*caM*	2	523		100,0 %	48,7 %	0	966,918	100,0 %	*A. fumigatus*
G25L2S1	*bT2*	2	536		100,0 %	52,0 %	0	990,925	100,0 %	*A. fumigatus*
G26L2S1	*caM*	2	556		100,0 %	49,0 %	0	1027,86	100,0 %	*A. fumigatus*
G28H2S1	*caM*	2	576		99,0 %	49,1 %	0	1064,79	100,0 %	*A. fumigatus*
G28H2S2	*caM*	2	577		100,0 %	49,2 %	0	1066,64	100,0 %	*A. fumigatus*
G28H2S3	*bT2*	2	537		100,0 %	52,2 %	0	992,771	100,0 %	*A. fumigatus*
G28L2S1	*caM*	2	577		100,0 %	48,8 %	0	1055,56	99,8 %	*A. fumigatus*
G28L2S2	*caM*	2	577		100,0 %	49,1 %	0	1066,64	100,0 %	*A. fumigatus*
G28L2S3	*caM*	2	577		99,7 %	49,1 %	0	1066,64	100,0 %	*A. fumigatus*
G29H2S1	*caM*	2	555		100,0 %	49,6 %	0	1016,78	99,5 %	*A. fumigatus*
G33L2S1	*caM*	2	577		100,0 %	49,3 %	0	1066,64	100,0 %	*A. fumigatus*
G34L2S1	*caM*	2	566		99,3 %	49,2 %	0	1046,32	100,0 %	*A. fumigatus*
G38H2S1	*caM*	2	565		100,0 %	49,2 %	0	1044,48	100,0 %	*A. fumigatus*
G38H2S2	*caM*	2	553		100,0 %	49,1 %	0	1022,32	100,0 %	*A. fumigatus*
G38H2S3	*caM*	2	568		100,0 %	49,1 %	0	1050,02	100,0 %	*A. fumigatus*
G38L2S1	*caM*	2	553		100,0 %	49,0 %	0	1016,78	99,7 %	*A. fumigatus*
G38L2S2	*caM*	2	544		100,0 %	48,9 %	0	1005,7	100,0 %	*A. fumigatus*
G38L2S3	*caM*	2	576		97,4 %	49,0 %	0	1064,79	100,0 %	*A. fumigatus*
G44H2S1	*caM*	2	462		97,8 %	48,3 %	0	854,272	100,0 %	*A. fumigatus*
G47H2S1	*caM*	2	577		99,7 %	49,0 %	0	1066,64	100,0 %	*A. fumigatus*
G48H2S1	*caM*	2	507		99,4 %	48,4 %	0	937,372	100,0 %	*A. fumigatus*
G48H2S2	*caM*	2	576		99,1 %	49,0 %	0	1064,79	100,0 %	*A. fumigatus*
G48H2S3	*caM*	2	577		99,8 %	49,0 %	0	1066,64	100,0 %	*A. fumigatus*

G52H2S1	*caM*	2	578		100,0 %	49,0 %	0	1062,94	99,9 %	*A. fumigatus*
G52L2S1	*caM*	2	566		99,8 %	49,3 %	0	1035,24	99,8 %	*A. fumigatus*
G53H2S1	*caM*	2	578		100,0 %	49,1 %	0	1068,48	100,0 %	*A. fumigatus*
G53H2S2	*caM*	2	577		99,3 %	49,0 %	0	1066,64	100,0 %	*A. fumigatus*
G53H2S3	*caM*	2	577		97,2 %	49,4 %	0	1066,64	100,0 %	*A. fumigatus*
G53L2S1	*caM*	2	567		99,3 %	49,1 %	0	1048,17	100,0 %	*A. fumigatus*
G53L2S2	*caM*	2	551		100,0 %	49,3 %	0	1018,62	100,0 %	*A. fumigatus*
G53L2S3	*caM*	2	551		100,0 %	49,3 %	0	1018,62	100,0 %	*A. fumigatus*

Table 37: Sequence and BLAST result information for each *A. niger* isolate. All turned out to be *A. tubingensis* except sample G53H2A1 (marked in the table), which was *A. welwitschiae*.

Sample no.	Genetic marker	Strands	Contig length (bp)	Length prior to trim (bp)	% HQ	% GC	E-value	Bit score	Hit grade	Specie
G7H2A1	*bT2*	2	529		100,0 %	52,9 %	0	977,998	100,0 %	*A. tubingensis*
G7L2A1	*bT2*	2	530		100,0 %	53,4 %	0	979,845	100,0 %	*A. tubingensis*
G18H2A1	*bT2*	2	530		100,0 %	52,8 %	0	979,845	100,0 %	*A. tubingensis*
G22H2A1	*bT2*	2	533		100,0 %	52,6 %	0	985,385	100,0 %	*A. tubingensis*
G22H2A2	*bT2*	2	527		100,0 %	52,9 %	0	974,305	100,0 %	*A. tubingensis*
G22H2A3	*bT2*	2	532		100,0 %	52,9 %	0	983,538	100,0 %	*A. tubingensis*
G22L2A1	*bT2*	2	531		100,0 %	52,9 %	0	981,691	100,0 %	*A. tubingensis*
G23H2A1	*bT2*	2	526		100,0 %	53,3 %	0	972,458	100,0 %	*A. tubingensis*
G23H2A2	*bT2*	2	469		98,7 %	54,2 %	0	867,199	100,0 %	*A. tubingensis*
G23L2A1	*bT2*	2	527		100,0 %	53,0 %	0	974,305	100,0 %	*A. tubingensis*
G25H2A1	*bT2*	2	528		100,0 %	52,8 %	0	976,151	100,0 %	*A. tubingensis*
G25H2A2	*bT2*	2	532		100,0 %	53,1 %	0	983,538	100,0 %	*A. tubingensis*
G25L2A1	*bT2*	2	534		100,0 %	52,7 %	0	987,231	100,0 %	*A. tubingensis*
G27H2A1	*bT2*	2	531		100,0 %	52,8 %	0	981,691	100,0 %	*A. tubingensis*
G27L2A1	*bT2*	2	533		100,0 %	52,8 %	0	985,385	100,0 %	*A. tubingensis*
G33L2A1	*bT2*	2	530		100,0 %	53,1 %	0	979,845	100,0 %	*A. tubingensis*
G33L2A2	*bT2*	2	529		100,0 %	53,1 %	0	977,998	100,0 %	*A. tubingensis*
G33L2A3	*bT2*	2	530		100,0 %	53,0 %	0	979,845	100,0 %	*A. tubingensis*
G38H2A1	*bT2*	2	531		100,0 %	52,8 %	0	981,691	100,0 %	*A. tubingensis*
G38H2A2	*bT2*	2	530		100,0 %	53,0 %	0	979,845	100,0 %	*A. tubingensis*
G38H2A3	*bT2*	2	546		100,0 %	52,4 %	0	1009,39	100,0 %	*A. tubingensis*
G38L2A1	*bT2*	2	533		100,0 %	52,6 %	0	985,385	100,0 %	*A. tubingensis*
G38L2A2	*bT2*	2	531		100,0 %	52,2 %	0	981,691	100,0 %	*A. tubingensis*
G38L2A3	*bT2*	2	533		100,0 %	52,6 %	0	985,385	100,0 %	*A. tubingensis*
G42H2A1	*bT2*	2	531		100,0 %	53,0 %	0	981,691	100,0 %	*A. tubingensis*
G42H2A2	*bT2*	2	527		100,0 %	53,0 %	0	974,305	100,0 %	*A. tubingensis*
G42H2A3	*bT2*	2	529		100,0 %	52,9 %	0	977,998	100,0 %	*A. tubingensis*
G42L2A1	*bT2*	2	448		100,0 %	51,3 %	0	828,419	100,0 %	*A. tubingensis*
G43H2A1	*bT2*	2	528		100,0 %	53,3 %	0	976,151	100,0 %	*A. tubingensis*
G48H2A1	*bT2*	2	528		100,0 %	52,8 %	0	976,151	100,0 %	*A. tubingensis*
G48H2A2	*bT2*	2	532		100,0 %	52,8 %	0	983,538	100,0 %	*A. tubingensis*
G53H2A1	*bT2*	2	533		100,0 %	52,5 %	0	985,385	100,0 %	*A. welwitschiae*
G53H2A2	*bT2*	2	533		100,0 %	52,6 %	0	985,385	100,0 %	*A. tubingensis*
G53H2A3	*bT2*	2	529		100,0 %	53,1 %	0	977,998	100,0 %	*A. tubingensis*
G53L2A1	*bT2*	2	528		100,0 %	52,6 %	0	976,151	100,0 %	*A. tubingensis*
G53L2A2	*bT2*	2	525		100,0 %	53,3 %	0	970,611	100,0 %	*A. tubingensis*
G53L2A3	*bT2*	2	531		100,0 %	52,8 %	0	981,691	100,0 %	*A. tubingensis*

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway