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Pathogenic fungi in Norway - screening for azole-resistant *Aspergillus fumigatus* in Norwegian barns

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Preface

This thesis of 30 ECTS concludes my master's degree in Chemistry and Biotechnology at the Norwegian University of Life Sciences (NMBU). The work was done at the Norwegian Veterinary Institute (NVI). In addition to being a very exciting period, working at NVI gave me a lot of experience and insight into the work that happens there.

I want to thank my main supervisor Ida Skaar who gave me help and constructive criticism, even on short notice. I want to thank my co-supervisor Hege Hvattum Divon for a lot of help and a lot of proofreading and for being very patient with me. I also want to thank Elin Rolén and Ellen Christensen for tutoring me and sharing their knowledge on fungi. Special thanks to Lonny Margrethe Kløvfjell who not only helped me a great deal but was always available 24/7 and saved me three hours of travel for ten minutes of work a couple of times.

Lastly, I want to thank my mother, father, brother, and sister for kind words, shelter, and food.

Abstract

Azoles are used today against fungal infections both clinically in humans and in agriculture to great effect. However, recent years have seen the rise of azole-resistant fungi. The two most common mutations causing the increased resistance are the TR_{34}/L98H and TR_{46}/Y121F/T289A mutations located in the *cyp51A* gene. One of the species with increasing azole resistance is the common pathogen *Aspergillus fumigatus.* While other countries have conducted surveys to gauge the level of azole resistant *A. fumigatus,* Norway has not. The BARNES project sought to provide preliminary data on how widespread azole-resistant *A. fumigatus* are in farms across Norway, in addition to serving as a pilot project for a larger study. Citizen science was used to sample *A. fumigatus* from farms in Norway. These samples were identified as *A. fumigatus* by calmodulin sequencing and screened for resistance to itraconazole, voriconazole, and posaconazole with VIPCheck™ and then E-tests. The *cyp51A* gene was sequenced in resistant isolates. Of the 108 isolates of *A. fumigatus* gathered, four (3,7%) were resistant to all three azoles. Two of the resistant isolates had mutations in the *cyp51A* gene, one with the TR_{34}/L98H mutation and the other with the TR_{46}/Y121F/T289A mutation. This is the first time the TR_{46}/Y121F/T289A mutation has been proven to exist environmentally in Norway. The other two isolates did not have any mutations in the *cyp51A* gene. While one is most likely actually a sensitive isolate, the other must owe its resistance to a mechanism other than a *cyp51A* mutation. While the percentage of resistant isolates are in line with data from other countries, a study with a larger data sample is needed to accurately reflect the occurrence of azole-resistant *A. fumigatus* in Norway.

Abstrakt

Azoler er i dag brukt mot soppinfeksjoner bade klinisk i mennesker og i jordbruk til stor nytte. I de siste årene har mengden azole resistente sopper økt. De to vanligste mutasjonene som forårsaker den økte resistensen er TR_{34}/L98H og TR_{46}/Y121F/T289A mutasjonene som befinner seg i *cyp51A* genet. Én av artene med økene resistens er den vanlige patogene *Aspergillus fumigatus.* Mens andre land har gjort undersøkelser for å anslå nivået av azole resistent *A. fumigatus*, har ikke Norge gjort det. BARNS prosjektet søker å skaffe preliminær data om hvor utbredt *A. fumigatus* er på gårder rundt i Norge, i tillegg til å være et pilotprosjekt for en større undersøkelse. Folkeforskning ble brukt til å ta prøver av *A. fumigatus* fra gårder i Norge. Disse prøvene ble identifisert som *A. fumigatus* ved å sekvensere kalmodulin genet, og ble testet for resistens mot itraconazole, voriconazole, og posaconazole med VIPCheck™ og så E-tester. I resistente isolater ble *cyp51A* genet sekvensert. Av de 108 isolatene av *A. fumigatus* som ble samlet, var fire (3.7%) resistent mot all tre azolene. To av de resistente isolatene hadde mutasjoner i *cyp51A* genet, den ene med TR_{34}/L98H mutasjonen og den andre med TR_{46}/Y121F/T289A mutasjonen. Dette er første gang TR_{46}/Y121F/T289A har blitt vist til å eksistere i naturen i Norge. De to andre isolatene hadde ingen mutasjoner i *cyp51A* genet. Det ene isolatet er mest sannsynlig egentlig sensitivt, mens det andre må ha en annen resistens mekanisme annet enn mutasjon i *cyp51A* genet. Selv om prosentandelen av resistente isolater stemmer overens med data fra andre land, vil det trenges en undersøkelse med en større mengde prøver for å få et nøyaktig bilde av situasjonen med azole resistente *A. fumigatus* i Norge

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1 Introduction

Pathogenic fungi levy a high cost on society. In agriculture, fungi are responsible for most plant diseases (Labandeira and Prevec, 2014), while clinically, fungi are responsible for over 1,6 million human deaths every year (Bongomin et al., 2017). One of the most used antifungals to combat pathogenic fungi are azoles. Azoles have historically been very effective especially since the introduction of triazoles (Andes and Dismukes, 2011). However, an increase in azole-resistant fungi threaten the usefulness of azoles. The leading theory of how azole resistance is increasing, is that the use of azoles in agriculture unintentionally selects for resistance mutations in the fungi surrounding the crop fields. Overuse of azoles has therefore developed azole-resistant fungi in the same way overuse of antibiotics has in antibiotic-resistant bacteria (CDC, 2021). Although the use of azoles in Norway is low (NORM/NORM-VET, 2018), there exists very little data on the state of azole-resistant fungi in Norway. For example, the level of azole resistance in the very common human pathogen *Aspergillus fumigatus* is unknown in Norway. To address this, the Norwegian Agriculture Agency has through the "Research funding for agriculture and food industry" (FFL/JA) funded the BARNS project (Full title: Pathogenic fungi in Norwegian barns - are they resistant to fungicides?) as a pilot project to look for azole-resistant *A. fumigatus* in farms. This thesis is the execution of the BARNS project and therefore its purpose is to assess the level of azole-resistant *A. fumigatus* in farms across Norway. In addition, the thesis will look at improvements that can be made when a larger study is performed.

2 Background

2.1 Fungi

Fungi are a kingdom of eukaryotic organisms divided into multicellular filamentous fungi (molds) and unicellular yeasts. They are important as symbionts and decomposers in nature, and are used by humans in for example industry, research, and food (Deacon, 2005). However, not all fungi are benign to suman society. While genera like *Fusarium* is responsible for major crop losses due to plant disease (Perincherry et al., 2019), there are major human pathogens that have in recent years become a cause for concern (Verweij et al., 2020).

The most common fungal infections in humans are superficial, affecting skin, nails, and hair. However, many fungal infections cause major impact on human lives and are potentially fatal. While nearly a billion people suffer from superficial infections, 150 million suffer from more severe infections and over 1.6 million die each year. This is comparable to the mortality rate of tuberculosis or three times the mortality rate of malaria (Bongomin et al., 2017). Among fungal human pathogens are the *Candida* and *Aspergillus* genera especially of note. *Aspergillus* are responsible for the most fungal infections from molds in the world and among them the species *Aspergillus fumigatus* is the most common human pathogen *(Webster and Weber, 2007).*

2.3 *Aspergillus fumigatus*

A. fumigatus is a saprophytic filamentous fungus belonging to the phylum Ascomycota. Being saprophytic means the fungus feeds on dead organic matter, so *A. fumigatus* exists in the soil among dead plants and grows especially well on compost (RIVM, 2017) (Verweij et al., 2020). As an ascomycete it reproduces asexually with conidia, small spores with a diameter of 2.5 µm (Latgé and Chamilos, 2019). Until recently, only the asexual reproduction cycle of *A. fumigatus* was known. However, in 2009 a sexual reproduction cycle involving ascospores created from complementary mating types was discovered (O'Gorman et al., 2009).

A. fumigatus produces enough airborne conidia in a day to have concentrations of 1 to 100 conidia per m^3 in most indoor and outdoor environments (Latgé and Chamilos, 2019). *A. fumigatus* is also a ubiquitous fungus, growing in many different habitats (Ashu et al., 2017). This means that most humans will inhale hundreds of *A. fumigatus* conidia every day (RIVM, 2017). Healthy individuals will usually not show any reaction to this as their immune system will handle any attempted infection immediately. However, *A. fumigatus* conidia can in

allergic or immunocompromised patients cause respiratory diseases of varying degrees collectively called aspergillosis (Latgé and Chamilos, 2019).

2.4 Aspergillosis

Non-invasive aspergillosis usually develops in immunocompetent or atopic patients. Immunocompetent patients can experience various forms of chronic pulmonary aspergillosis (CPA). One form of CPA is aspergilloma, where a mass of fungal cells colonizes a cavity in the lungs. Aspergilloma is often preceded by pulmonary tuberculosis, and it is estimated that over 350,000 new CPA cases complicate treated pulmonary tuberculosis. Atopic patients can develop allergic bronchopulmonary aspergillosis (ABPA), especially patients with chronic asthma or cystic fibrosis (Latgé and Chamilos, 2019).

Invasive aspergillosis (IA) usually develops in immunocompromised patients. This includes patients with chronic diseases, patients undergoing medical therapy (for example organ transplant recipients or patients undergoing chemotherapy), and patients who are critically ill. About 50% or more of patients with IA die (Kousha et al., 2011, Shah et al., 2018, Latgé and Chamilos, 2019).

2.5 Antifungals

Antifungals are drugs used to treat fungal infections, both in agriculture and clinically. Based on the targets of the antifungal drugs, they can be classified into seven groups: inhibitors of ergosterol biosynthesis, fungal membrane disruptors, fungal cell wall disruptors, inhibitors of sphingolipid synthesis, inhibitors of nucleic acid synthesis, inhibitors of protein synthesis, and inhibitors of microtubule synthesis (Campoy García and Adrio, 2017). One of the most developed and used classes of antifungals are the azoles, belonging to the group of inhibitors of ergosterol biosynthesis.

2.5.1 Azoles

Azoles are the most common antifungal drugs in clinical use (Vandeputte et al., 2012). Azoles have at least one five-atom heterocycle which contains at least one nitrogen atom. Their method of action is to bind to and disrupt the enzyme Cyp51A, a sterol 14α-demethylase (figure 1). Cyp51A is important in the synthesis of C14-demethyl-lanosterol from lanosterol (Campoy García and Adrio, 2017). Without C14-demethyl-lanosterol the fungi cannot make ergosterol, which is vital for its cell membrane.

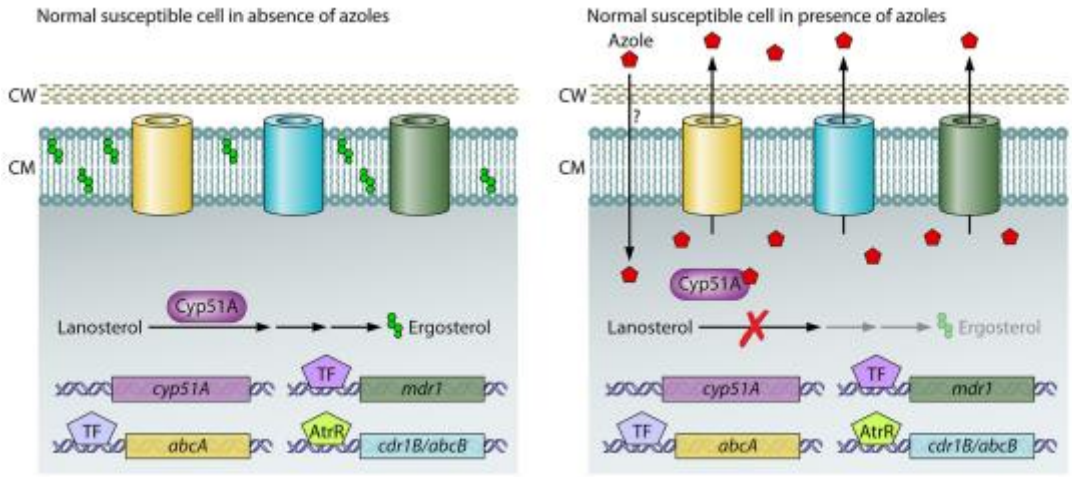

Figure 1: Synthesis of ergosterol in a sensitive fungal cell in the absence and in the presence of azoles*. In the absence of azoles, the synthesis of ergosterol continues as normal. In the presence of azoles the Cyp51A enzyme is inhibited and ergosterol synthesis stops (Dudakova et al., 2017).*

The first developed azoles, the imidazoles, had high toxicity, severe side effects and interacted with several other drugs. They were later replaced with the safer triazoles, azoles where the five-atom heterocycles have three nitrogen atoms. There are several azoles available to date, but the first generation triazole itraconazole, and the second generation triazole voriconazole and posaconazole are mainly used for treatment of aspergilloses (figure 2) (Campoy García and Adrio, 2017, Dudakova et al., 2017).

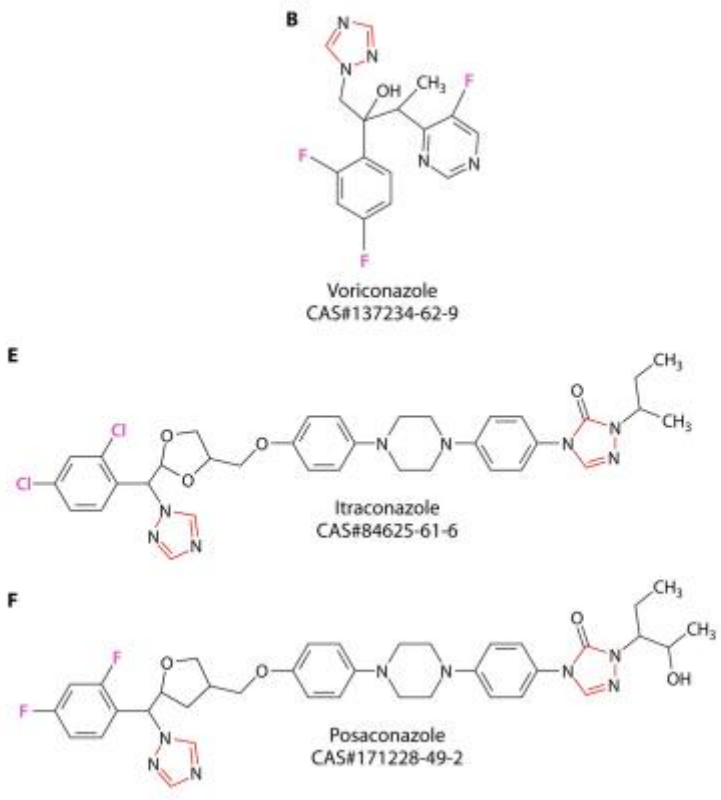

Figure 2: Chemical structure of voriconazole, itraconazole, and posaconazole (Dudakova et al., 2017).

2.7 Azole resistance

Several resistance mechanisms are thought to contribute to the resistance of azoles in *A. fumigatus* and are summarized in figure 3. Reduced interaction affinity is the most common resistance mechanism and comes from mutations in the *cyp51A* coding region altering the structure of the Cyp51A enzyme so that it has less affinity for azoles (Hagiwara et al., 2016). Examples are the G54W and M220I mutations that are associated with clinical *A. fumigatus* isolates (Verweij et al., 2020).

Overexpression of Cyp51A is another mechanism and is the result of introduction of tandem repeats (TR) in the promotor region of the *cyp51A* gene. In resistant isolates, TR of two different lengths are found in combination with one or more single point mutations that lower the azole affinity for Cyp51A (Hagiwara et al., 2016). Examples are the very common TR_{34}/L98H mutation and the TR_{46}/Y121F/T289A mutation which confers high voriconazole resistance (van der Linden et al., 2013). Both TR mutations are associated with environmental *A. fumigatus* isolates (Verweij et al., 2020).

Apart from alterations in the *cyp51A* gene there are other putative resistance mechanisms. An increase in the expression of efflux pumps is one such resistance mechanism, most likely resulting from overexpression of the *cdr1B* gene. The increased number of efflux pumps will transport azoles out of the cell before they can bind to Cyp51A. Additional resistance mechanisms may exist, such as degradation of azoles by the cell or circumventing the need for Cyp51A with an alternative pathway, but there is little knowledge about these mechanisms (Hagiwara et al., 2016).

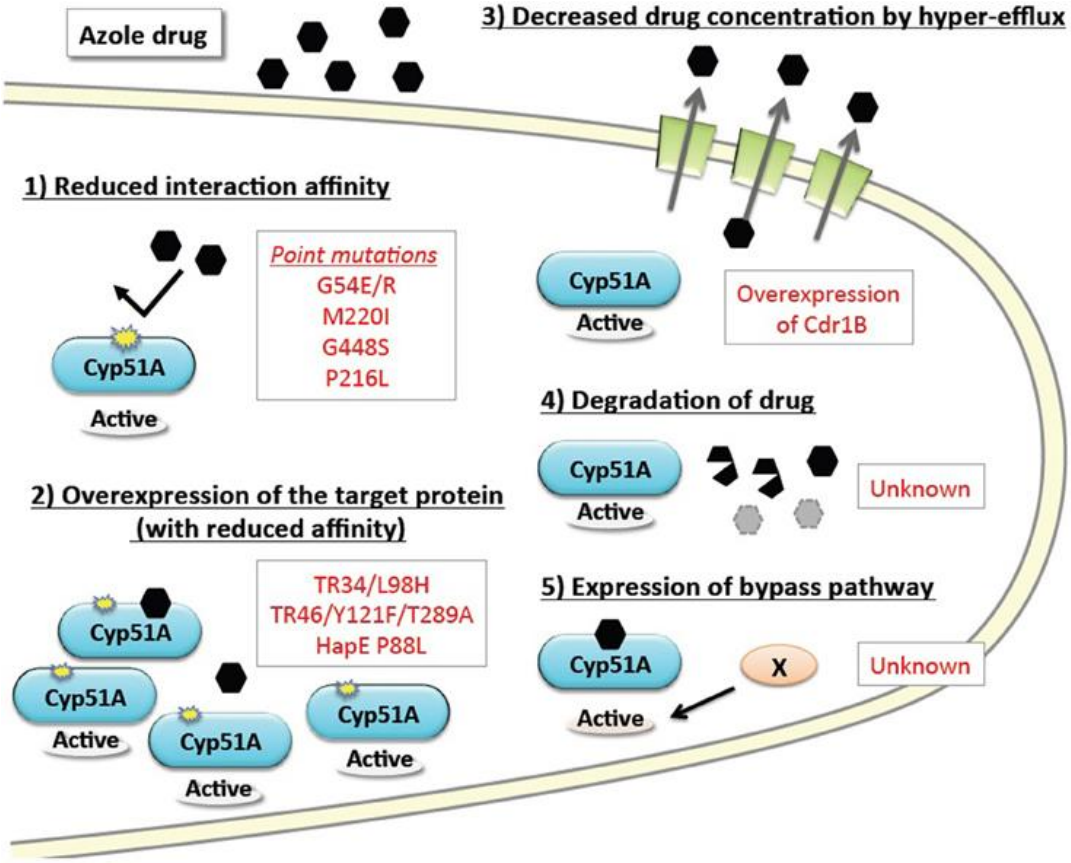

Figure 3: Resistance mechanisms against azoles in A. fumigatus. *Reduced interaction affinity (1) is a mechanism where mutations in the cyp51A gene changes the structure of the Cyp51A enzyme, reducing the affinity azoles have for the enzyme. Overexpression of target protein (2) is a mechanism where tandem repeats in the cyp51A gene promotes expression of the Cyp51A enzyme, creating more Cyp51A enzymes. Hyper-efflux (3) is a mechanism where genes coding for azole efflux pumps are overexpressed, resulting in an increased number of pumps transporting azoles out of the cell before they can affect the Cyp51A enzymes. There may also be mechanisms involving degradation of azoles by the cell (4) or other pathways that bypass Cyp51A (5), but there is little knowledge about these mechanisms as of yet (Hagiwara et al., 2016).*

The identification of azole resistant *A. fumigatus* strains increased in the Netherlands during the 1990s, and multi-azole-resistant A. fumigatus was observed in 1999. The most common mutations found in clinical cases were the TR_{34}/L98H and TR_{46}/Y121F/T289A mutations.

These are mutations that are found in the environment; hence, the increase is thought to be a side effect of azole fungicides used in agriculture. The *A. fumigatus* living in the soil surrounding the farms will be unintentionally selected for resistance (Verweij et al., 2009, Verweij et al., 2020).

Multi-azole-resistant *A. fumigatus* isolates are documented globally, with percentage of azole resistant isolates varying from 1% to over 10% (Figure 4) (Lestrade et al., 2019). Some areas of Vietnam have even shown azole resistance in over half of the *A. fumigatus* samples collected (Duong et al., 2021). In Norway, however, there is currently no data on the presence of azole resistant *A. fumigatus* (Nordøy et al., 2018).

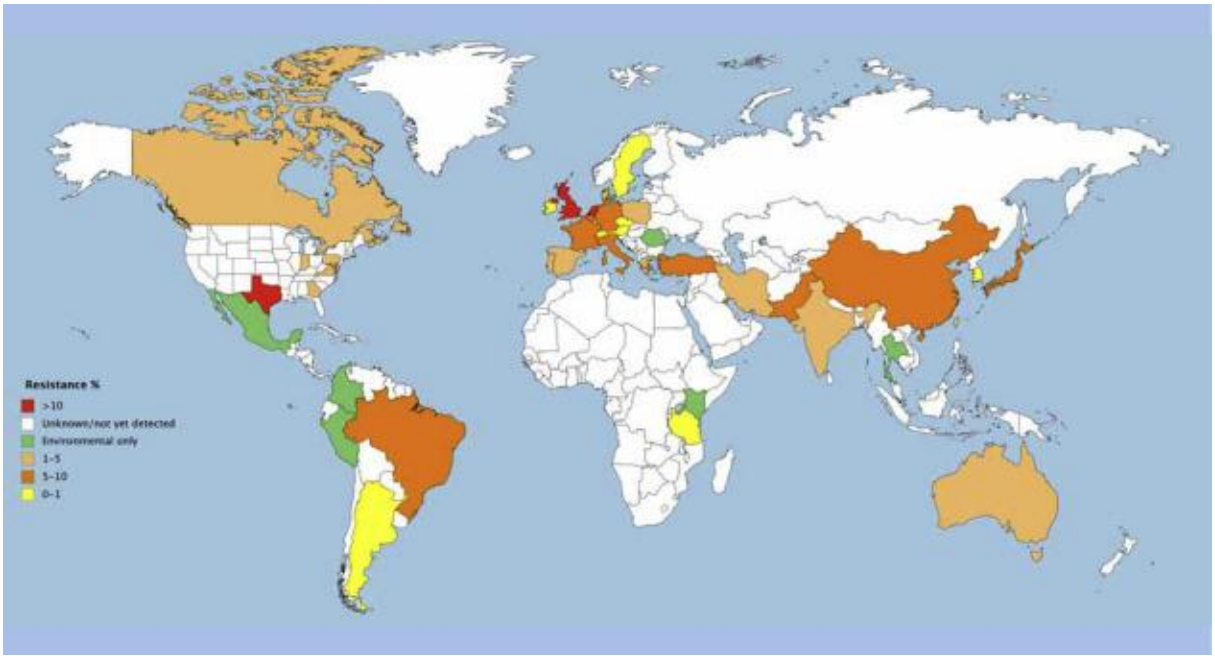

Figure 4: Epidemiology of azole resistant A. fumigatus. *Countries that did not document azole resistance but had cases of A. fumigatus infections were classified as 0-1% (Lestrade et al., 2019).*

2.8 Method theory

2.8.1 Methods for resistance testing in culture

VIPCheck™ is an assay used to screen for *A. fumigatus* strains resistant to voriconazole, itraconazole and/or posaconazole. It consists of a plate with four wells, containing RPMI 1640–2% glucose agar, numbered from one to four (figure 5). Well number one to three

contains 4 mg/L itraconazole, 2 mg/L voriconazole, and 0.5 mg/L posaconazole, respectively. The fourth well contains no azoles and functions as a control. Screening is done by inoculating all wells with fungal spores. Sensitive isolates will only grow in the fourth well, while resistant isolates will, in addition, show growth on one or more of the other wells. (Arendrup et al., 2017, VIPCheck™, Mediaproducts BV, Groningen, Netherlands)

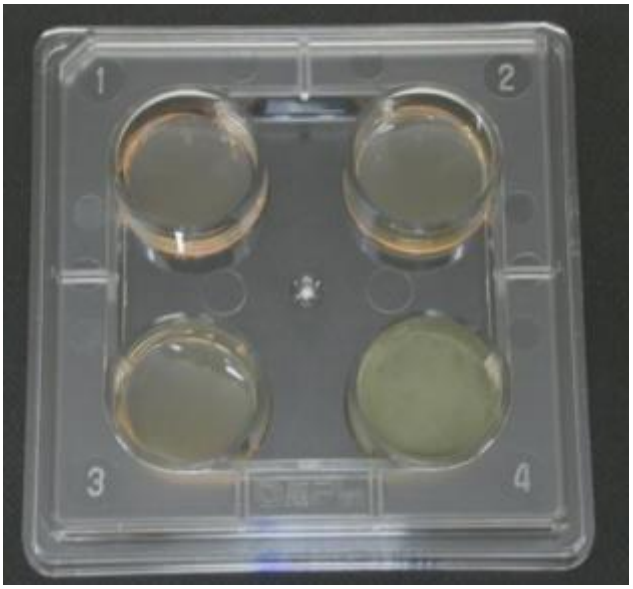

Figure 5: A VIPCheck™ plate. *Wells are numbered one to four. Well number one contains 4 mg/L itraconazole, well number two contains 2 mg/L voriconazole, well number three contains 0.5 mg/L Posaconazole, and well number four is control.*

Studies have shown that VIPCheck™ has high sensitivity and specificity (Arendrup et al., 2017, Buil et al., 2017). It has been used during the late 2010s to screen for resistant *A. fumigatus* in hospitals (Griffin et al., 2019, Montesinos et al., 2017) and more recently to screen for resistant *A. fumigatus* in patients suffering from COVID-19 (Meijer et al., 2021, van Someren Gréve et al., 2021)

The E-test was developed by AB Biodisk as a method of testing antimicrobial susceptibility. The E-test is a thin, narrow strip that has a gradient of an antimicrobial agent along the underside of the strip. By laying an E-test on a Petri dish containing agar, the antimicrobial agent will diffuse into the agar around the E-test. Microbial cells growing on the agar will only grow where the antimicrobial gradient is under the cells' minimum inhibitory concentration (MIC), forming an inhibition zone around the higher gradient. The concentration of the gradient is printed on the upper surface of the E-test in µg/ml, allowing

the MIC value to be read by looking at where the inhibition zone intersects the E-test (see figure 6) (Baker et al., 1991). The MIC-value can be compared to the breakpoint values given by EUCAST to see if a microbe is sensitive or resistant (EUCAST, 2020).

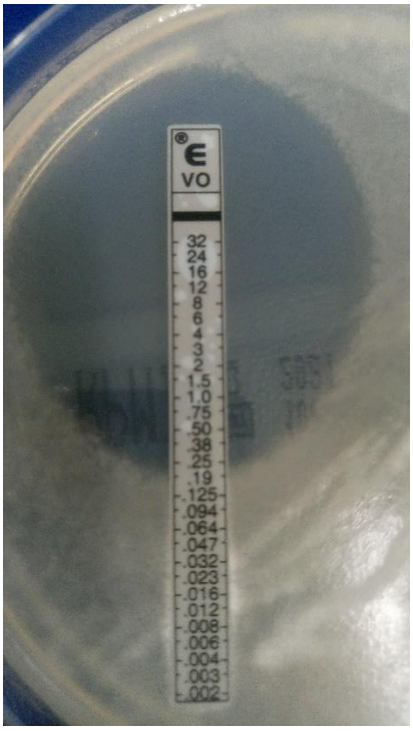

Figure 6: An E-test of voriconazole used on agar containing A. fumigatus. *The intersect is between 0.19 µg/ml and 0.25 µg/ml. The MIC-value is always set as the highest if it is in between two values, which means the MIC-value here is 0.25 µg/ml.*

2.8.2 Molecular characterization of resistance

PCR is a powerful technique for amplification of DNA sequences invented by Kary Mullis in 1983 (Mullis, 1990), and is widely used by both clinicians and researchers. Qualitative PCR is used to amplify DNA and can by extension be used to detect, for example, the presence of pathogens. Quantitative PCR can also determine the amount of DNA in a sample. (Garibyan and Avashia, 2013).

A PCR assay requires template DNA, primers specific for the sequence of interest, nucleotides, and DNA polymerase. The template DNA contains the target DNA that is to be amplified. The primers are short strands of DNA complementary to the target DNA. The nucleotides are the four bases that make up DNA: adenine (A), thymine (T), guanine (G), and cytosine (C). The DNA polymerases are thermophilic enzymes that adds nucleotides to a growing DNA strand using a complementary strand. These components are mixed into a master mix (Garibyan and Avashia, 2013).

The master mix is put into a thermal cycler, where the amplification takes place. The thermal cycler heats up to above the melting point of the two template DNA strands, which separates the strands. This is called denaturation. The next step is annealing, where the temperature is lowered to let the primers bind to the target DNA. The last step is elongation, where temperature is raised to the optimal temperature for the DNA polymerases. The polymerases then create a new DNA strand starting from the primers and using the old strand as the template. This creates two pairs of the double-stranded target DNA. The cycle of denaturation-annealing-elongation is repeated, each time doubling the amount of target DNA (Garibyan and Avashia, 2013).

2.8.3 Gel electrophoresis

Gel electrophoresis is a method of separating proteins, DNA or RNA by size or charge. The gel used in the electrophoresis is usually made from either polyacrylamide or agarose. Agarose gels have the advantage of being easy to create, handle and use (Yılmaz et al., 2012), and are most effective at separating DNA fragments between 100 bp and 25 kb (Lee et al., 2012). Polyacrylamide gels are best for separating DNA fragments smaller than 100 bp. (Lee et al., 2012). In this thesis, agarose was used to create gels to separate DNA.

Separation starts when the DNA is loaded into wells in the gel and an electric current is applied to the gel. Since DNA is negatively charged because of its negatively charged phosphate groups, the DNA will begin to move towards the positive anode. The movement speed of the DNA is dependent on its mass/charge ratio. Since the amount of phosphate groups, and therefore charge, increase with the mass of the DNA, the mass/charge ratio is equal between all DNA molecules. However, smaller DNA molecules move faster through the pores in the gel. Thus, over time the smaller DNA molecules will travel further into the gel than larger ones will (Lee et al., 2012). After separation, the bands of DNA molecules in the gels can be visualized by UV-light by having the gel stained during casting in for example gelred (VWR, Pennsylvania, USA).

2.8.4 Sequencing (Sanger)

Sanger sequencing is a method for identifying the nucleotide sequence of DNA, developed by Frederick Sanger in 1977. It consists of three steps (Sigma-Aldrich).

Firstly, a DNA template such as PCR amplified DNA is used in a type of PCR (see above) with one primer and chain-termination. A small number of modified dNTPs called dideoxyribonucleotides (ddNTPs) are added into the PCR mix. ddNTPs lack the 3'-OH group required for continued elongation of the newly created DNA strands, and they are fluorescently labeled. Because a ddNTP will be introduced into a growing DNA strand by random chance, this PCR will create copies of the template strand terminated at random lengths. Because the ddNTP terminates the sequence, it will always be the last nucleotide in the copies (Sigma-Aldrich).

Secondly, all the copies of various lengths are separated by gel electrophoresis (see above).

Thirdly, since the four kinds of ddNTPs are fluorescently labeled and a ddNTP is always the last nucleotide in a copy, the gel bands can be read from smallest to largest to determine the original template sequence. This is done by a computer that excites the fluorescent tags in each band with a laser and then registers the color output (Sigma-Aldrich).

3 Materials and methods

Figure 7 shows the entire workflow from the sampling of fungal spores from farms, to processing and analysis of *A. fumigatus* strains.

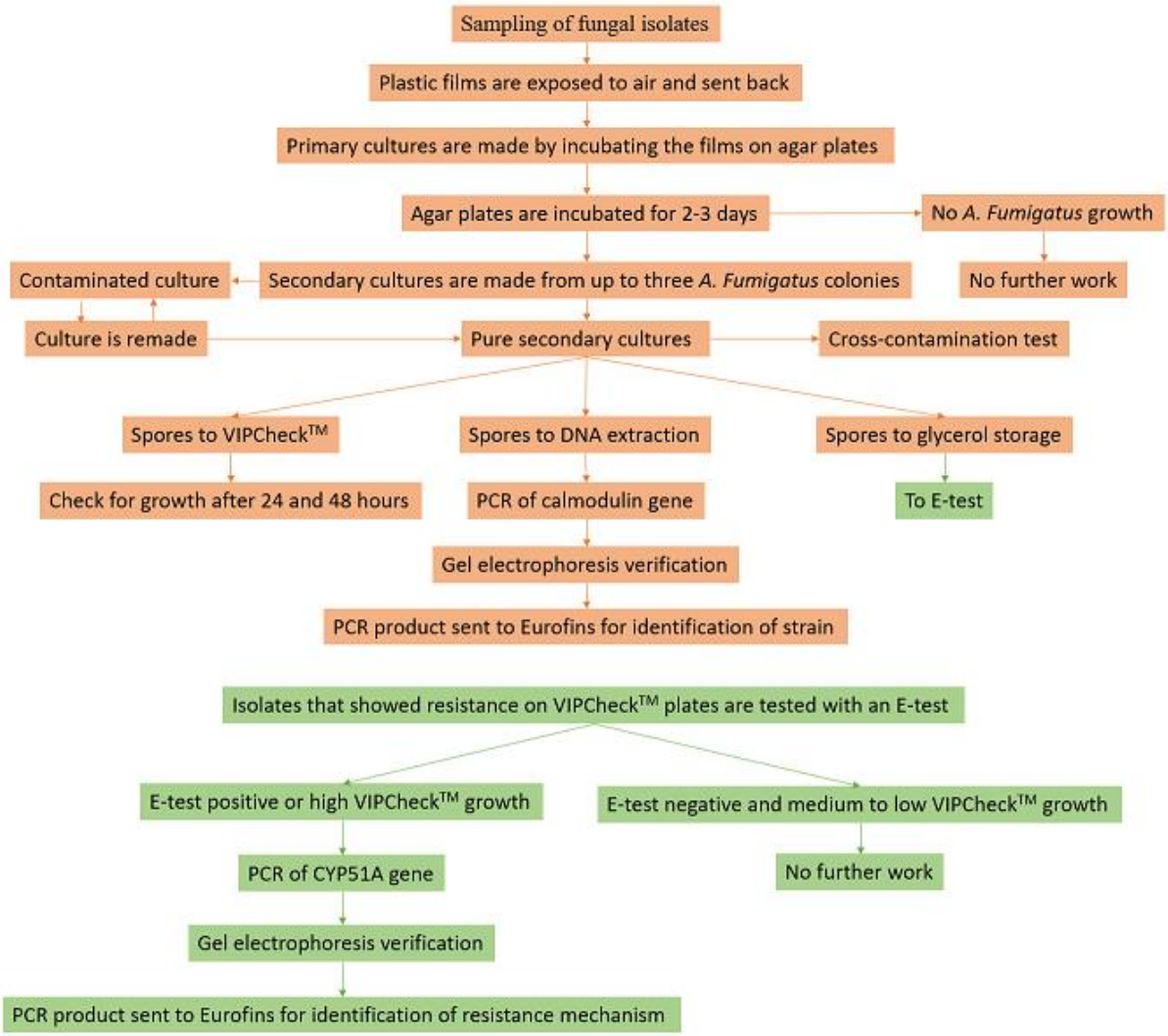

Figure 7: Illustration of the workflow from start to finish.

3.1 Sampling of fungal isolates

Farmers across Norway were asked to participate in the BARNS project as a citizen science engagement. The farmers that participated received by mail a user manual (Appendix 1), and two MicroAmp Clear Adhesive Film (Thermo Fisher Scientific, Massachusetts, USA) plate

foils marked “L” (Lagerrom, English: granary) and “H” (Husdyrrom, English: room where farm animals are housed). After removing the protection cover of each foil, they were to leave the “L” film exposed to the air in the granary, and “H” film exposed in the building where they kept the animals. After six hours, the plastic films were glued back to the protective cover to prevent further exposure. The films were then sent back to the Norwegian Veterinary Institute (NVI).

Along with the films, each of the participants were sent a questionnaire (Appendix 2), either in paper form or digitally. The purpose of the questionnaire was to document what kind of farming operations the participants were preforming, and the conditions of the rooms in which the plastic films were exposed.

Each farm participating in the BARNS project will in this thesis only be linked to a municipality in Norway, and not to its exact location, as to comply with the General Data Protection Regulation (GDPR) (European Parliament and Council of the European Union, 2016).

3.2 Primary cultures

When the adhesive PCR plate foils reached the NVI, the cover was removed, and the foils were laid out on square DG18 agar plates (Appendix 3) with the sticky side towards the agar. The plates were incubated at 37°C for 24 hours. The films were then removed, and the plates were further incubated for 2-3 days, or until sufficient fungal growth was observed. The number of fungal colonies on the plate was documented and, if possible, the genus and/or species was identified based on morphology (visual inspection only). If present, up to three *A. fumigatus* colonies were plated over to new DG18 agar plates using sterile inoculation loops, to make three pure secondary cultures. If a plate had more than three *A. fumigatus* colonies, the three most visually different colonies were chosen to enhance the chance of selecting clonally different fungi. An example of a primary culture is shown in figure 8.

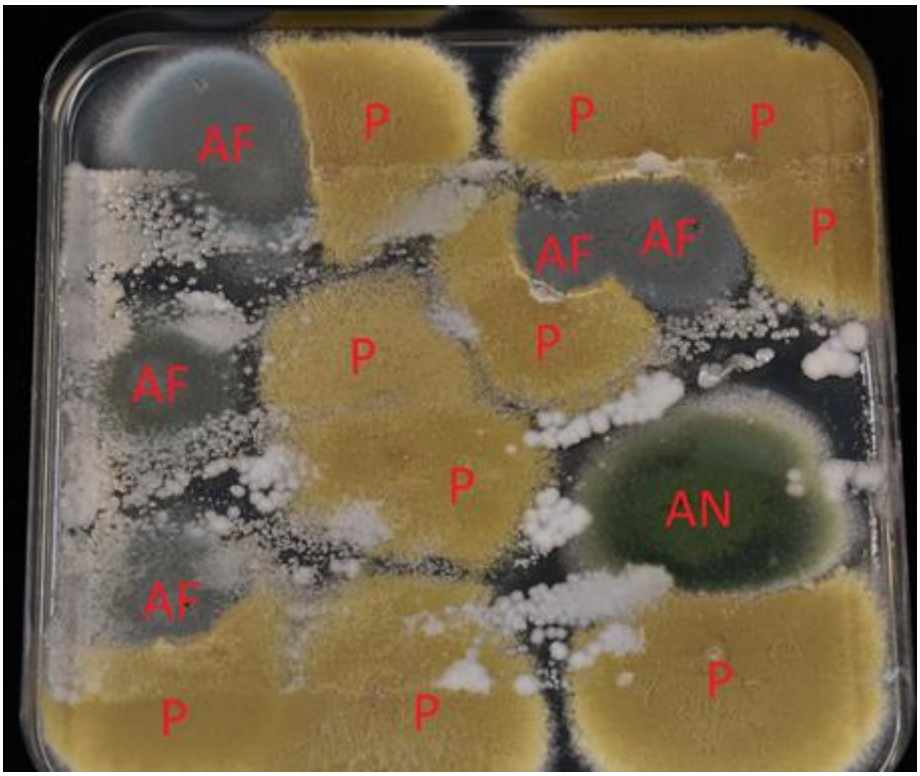

Figure 8: A primary culture of fungi collected from one adhesive foil. *It contains 5 colonies of A. fumigatus (AF), 1 Aspergillus nidulans (AN), and 10 Paecilomyces (P). It also contains an abundance of small white colonies of yeast. The area covered with yeast indicates where the plastic film laid.*

3.3 Secondary pure cultures

Pure secondary cultures were made by transferring *A. fumigatus* spores with a sterile plastic inoculation loop from one colony from the primary culture plate to a new petri dish containing DG18 agar (Appendix 3). The secondary cultures were incubated at 37°C for 2-3 days or until sufficient growth was observed. The cultures were checked for contaminants. If a secondary culture contained any other fungus than *A. fumigatus*, a new secondary culture was made. If a secondary culture was a pure isolate, it was kept at 4°C ± 1°C for short-term storage until further work.

3.4 Glycerol storage

To store *A. fumigatus* isolates from the secondary cultures, spores from each culture were transferred with a sterile cotton swab to two tubes, each filled with 10% glycerol water. One of the glycerol tubes were stored at -4°C ± 1°C and used for E-testing. The other tube was frozen at -80°C for long-term backup and archive.

3.5 Cross-contamination test

A small experiment was performed to see how long *A. fumigatus* spores could be a source of contamination in the sterile laminar flow hood workspace. Inside the laminar hood, *A. fumigatus* spores were moved approximately 30 cm, from one plate to another plate three times. Right after, two open agar plates were put where the movement of the spores had taken place. One was placed in the center of where the spore transfer had occurred, while the other plate was positioned about 30 cm to the left of the first. The intention was to see how far spores would travel in the air after transfer. The plates remained for two minutes before being removed, whereupon two new open agar plates were put in their place. This was done four times, resulting in four pairs of agar plates being exposed for two minutes to the air from zero, two, four, and six minutes after the spore movement. These plates were incubated at 37°C for 2-3 days to see if there would be any *A. fumigatus* colonies (in this case regarded as cross-contaminants) growing on them. The results of this test were taken into consideration when working with the *A. fumigatus* spores during resistance testing to avoid cross-contamination.

3.6 Resistance testing

3.6.1 VIPCheck™

All secondary cultures were tested with VIPCheck™ (Mediaproducts BV, Groningen, Netherlands) according to the manufacturer's description. This was accomplished by first moving spores with a sterile cotton swab from a pure *A. fumigatus* culture into a test tube of 6 mL sterile reverse osmosis (RO) purified water (made in-house) to make a suspension. The suspension was then measured in a densitometer (Grant Instruments Ltd, Cambridgeshire, United Kingdom) and adjusted to a McFarland value between 0.5-2. After this, a single drop (about 25µL) of the suspension was placed in each well on the VIPCheck™ plate. The plates were incubated at 37°C, and they were checked for growth after 24 and 48 hours.

Growth in the wells on the VIPCheck™ plates were separated into four categories: No growth (-), very low growth (+), low to medium growth (++), and high growth (+++) (See figure 9). Only isolates with low to high growth (++ and +++) were registered as positive and selected for E-test.

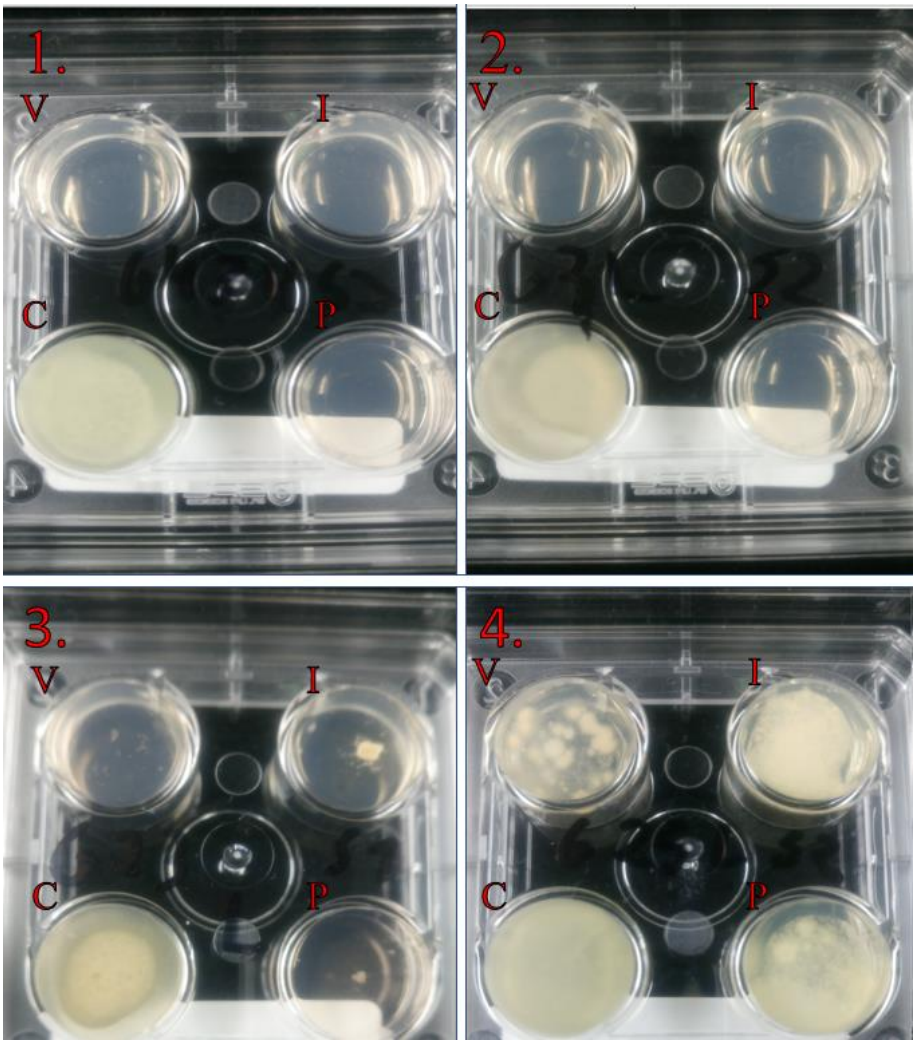

Figure 9: Examples of the four VIPCheck™ growth categories (1-4). *The pictures are taken from underneath the VIPCheck™ plates. The wells on each plate are voriconazole (V), itraconazole (I), control (C), and posaconazole (P). The 1. picture has no growth (-) on all three wells, the 2. picture has very low growth (+) on the two top wells (almost imperceptible), the 3. picture has low to medium growth (++) on all three wells, and the 4. picture has high growth (+++) on all three wells.*

3.6.2 E-test

All VIPCheck™ positive isolates were tested with E-tests for itraconazole (Biomérieux, Marcy-l'Étoile, France) voriconazole (Biomérieux), and posaconazole (Biomérieux). Spores from glycerol stocks of VIPCheck™ positive isolates were inoculated on DG18 agar (Appendix 3) by dipping an inoculation loop in the glycerol and streaking it on the agar. After 2-3 days, or when sufficient spores had been produced, a sterile cotton swab was used to transfer spores into a test tube with 6 mL sterile physiological saline solution (9g of NaCl per liter of distilled water) to make a suspension. The suspension was allowed to settle for 10 minutes before 3 mL, taken from the middle of the suspension, were pipetted to a new test tube, and the rest was discarded. The suspension was then measured in a densitometer (Grant

Instruments Ltd) and adjusted to a McFarland value between 0.5-0.6. The adjusted suspension was poured into two RPMI agar plates (Biomérieux), covering the whole plate. Excess spore suspension was then quickly removed from the RPMI plates with a pipette and discarded. The three E-tests were placed on the two RPMI plates, itraconazole and voriconazole on one, and posaconazole on the other. The RPMI plates were incubated at 37°C, and the MIC-value was registered after 24 hours.

An E-test would be considered positive for itraconazole or voriconazole if the MIC-value was >1 mg/L. An E-test would be considered positive for posaconazole if the MIC-value was either > 0.25 mg/L, or 0.25 mg/L for posaconazole and > 1 mg/L for itraconazole. This is in accordance with breakpoints set by EUCAST (EUCAST, 2020).

3.7 DNA extraction

Genomic DNA was extracted from all secondary cultures. First, spores were taken from a pure culture with a sterile cotton swab and suspended in a 2 mL Eppendorf tube containing 450 µL AL buffer (QIAGEN, Hilden, Germany) and one 3 mm steel bead. The tubes were shaken in a MM400 Retsch mixer mill (Retsch, Haan, Germany) at frequency setting 25 hz for 300 seconds to lyse the fungal spores. After the shaking, the tube was centrifuged in an Eppendorf Centrifuge 5415 R at 12000 g for 10 seconds. 10 µL of proteinase K (20 ng/mL) (VWR, Pennsylvania, USA) were added, and the tube was incubated on an Eppendorf Comfort (Eppendorf, Hamburg, Germany) at 56°C with 550 rpm shaking for 30 minutes. The tube was then centrifuged at 12000 g for 5 minutes. 200 µL of the supernatant were pipetted to a new 2 mL Eppendorf tube and put into a QIACube machine (QIAGEN) for further DNA purification using the program: "DNA mini nr 4 → tissue". DNA was stored at -18°C until further work.

3.8 Polymerase chain reaction (PCR)

For molecular identification of *Aspergillus*, part of the calmodulin gene was amplified by PCR and for identification of resistance mechanism the *cyp51A* gene was amplified. Primers used in the PCR are shown in table 1. A negative control sample containing no DNA template were added to each PCR to detect contamination.

Table 1: The primers used for PCR amplification and sequencing.

PCR	Primer	Use	Sequence
Calmodulin	CMD5 (Forward)[1]	PCR and sequencing	CCGAGTACAAGGAGGCCTTC
	CMD6 (Reverse)[1]	PCR and sequencing	CCGATAGAGGTCATAACGTGG
cyp51A	P-A07(Forward)[2][3]	PCR and sequencing	TCATATGTTGCTCAGCGG
	P-A04 (Forward)[2]	Sequencing	CAGACATGATATGGAACC
	cyp51A_2F (Forward)[3]	Sequencing	CGGCAATCTTGCTCAATG
	P450.2 (Reverse)[2]	Sequencing	CTGTCTCACTTGGATGTG
	Cyp51A_1R (Reverse)[3]	Sequencing	CATTGAGCAAGATTGCCG
	Cyp51A_2R (Reverse)[3]	Sequencing	GGTGAATCGCGCAGATAGT
	Cyp51A_3R (Reverse)[3]	Sequencing	GTCAAGATCCTTGTACTGGAGC
	cyp51A_4R (Reverse)[3]	PCR	CCTATTCCGATCACACCAAA

1. (Hong et al., 2005)
2. (Mellado et al., 2001)
3. (Mortensen et al., 2011)

3.8.1 Calmodulin PCR

Calmodulin PCR was performed on all *A. fumigatus* isolates. Samples were made by adding 2µL of template DNA from each isolate to a master mix as shown in table 2. The samples were spun down, before they were put into a T100 Thermal Cycler (Bio-Rad Laboratories, California, USA). PCR program used in the thermal cycler is shown in table 3.

Table 2: Components in the mix used for PCR amplification of the calmodulin gene.

Components	Volume (µL) / Reaction
Forward primer CMD5[1] stock 5µM	3
Reverse primer CMD6[1] stock 5µM	3
MilliQ-water (made in-house)	17
PuReTaq Ready-To_Go PCR beads[2]	-
DNA template	2
Total	25

1. Table 1
2. (VWR)

Table 2: PCR program for amplification of the calmodulin gene.

Operation	Temperature	Time (min)	Cycles
Heating and activation	95°C	4:00	1x
Denaturation	95°C	1:00	35x
Annealing	55°C	1:00	
Elongation	72°C	2:00	
Final elongation	72°C	8:00	1x
Cooling	8°C	∞	1x

3.8.1 *cyp51A* PCR

The *cyp51A* PCR was based on Mellado et al. (2001) and Mortensen et al. (2011). The *cyp51A* PCR was performed on isolates that were E-test positive and/or had high growth (+++) on VIPCheck™. Samples were made by adding 4 µL of template DNA from each isolate to a master mix as shown in table 4. The samples were spun down, before they were put into a T100 Thermal Cycler (Bio-Rad Laboratories). PCR program used in the thermal cycler is shown in table 5.

Table 4: Components in the mix used for PCR amplification of the cyp51A gene.

Components	Volume (µL) / Reaction
10x Dream Taq buffer[2]	5
dNTP mix (10 mM)[2]	1
Forward primer P-A07[1] stock 10 uM	1
Reverse primer cyp51A_4R[1] stock 10 uM	1
Dream Taq (5 U/ul)[2]	0,25
MilliQ-water (made in-house)	37,75
DNA template	4
Total	50

1. Table 1
2. (Thermo Fisher Scientific)

Table 5: PCR program for amplification of the cyp51A gene.

Operation	Temperature	Time (min)	Cycles
Heating and activation	95°C	2:00	1x
Denaturation	95°C	0:30	35x
Annealing	58°C	0:30	
Elongation	72°C	1:30	
Final elongation	72°C	7:00	1x
Cooling	8°C	∞	1x

3.9 Verification of PCR products

To verify that the PCR was successful, gel electrophoresis was performed on all PCR products. To make the 1% agarose gels, 1 g of agarose universal powder (VWR) was mixed with 100 mL of 1x TBE buffer (Thermo Fisher Scientific) in an Erlenmeyer flask. This flask was heated in a microwave for 30 seconds and then gently stirred by hand. This was repeated until all agarose powder was dissolved. Then, 7 µL of gelred (VWR) were added to the mixture, and it was poured into a gel tray. Two gel combs were placed into the tray and the mixture was left to solidify. When the gel was solid, it was moved over to a gel electrophoresis chamber, the combs were pulled out and the chamber was filled with 1x TBE buffer. The first well of the gel was loaded with 3,5 µL 1 kB PeqGold ladder (VWR) for calmodulin PCR product, and 100 bp DNA Ladder (VWR) for *cyp51A* PCR product. From each PCR product, 5 µL were mixed with 1 µL of 6x loading dye (VWR) and loaded unto the gel. Separation was achieved by applying 90 V for 45 min. After this, the gel was visualized under UV light in a Biorad Universal Hood II using the Image Lab software (Bio-Rad Laboratories) to take a picture of the gel. For amplification of the calmodulin gene, expected length of the PCR product was 580 bp. For *cyp51A*, expected amplicon length was 2048 bp.

3.10 Quantification and sequencing of PCR product

Unpurified PCR products were sent to Eurofins Genomics (Eurofin Genomics, Ebersberg, Germany) for Sanger sequencing using the primers shown in table 1. According to Eurofins Genomics' protocol, for calmodulin, a concentration of 10 ng/µL DNA and 15 µL PCR product were needed, and for *cyp51A*, a concentration of 20 ng/µL DNA and 20 µL were needed. The concentration of 15 random samples of calmodulin PCR product and all *cyp51A*

PCR products were verified with a Qubit 4 Fluorometer (Thermo Fisher Scientific) to determine concentration. No altering of concentration was needed before shipping to Eurofins Genomics. The Sanger sequencing produced .ab1-files containing the sequences.

3.11 Sequence analysis

The .ab-files were read using the software Geneious prime (Biomatter, Auckland, New Zealand). Each .ab1-file contained one read of one of the isolates. For the calmodulin reads, the CMD5 and CMD6 (table 1) reads for each isolate were combined into a consensus sequence and for the *cyp51A* reads, the seven primer reads (table 1) for each isolate were combined into a consensus sequence. Assembly into a consensus sequence was performed with the De Novo Assemble function (Align/Assemble → De Novo Assemble) with Geneious as assembler on the highest sensitivity. To identify the isolates as *A. fumigatus*, a BLAST (Basic Local Alignment Search Tool) search was performed on the calmodulin consensus sequences. This was done in Geneious prime using the BLAST function with the Megablast program on the nucleotide collection database. To look for mutations in the *cyp51A* consensus sequences, the sequence for the wildtype *cyp51A* gene, the TR_{34}/L98H mutation, and the TR_{46}/Y121F/T289A mutation were added to Geneious (Add → new sequence). These sequences were compared to the isolates consensus sequences with multiple alignment (Align/Assemble → Multiple alignment) using the MUSCLE Alignment with re-alignment and 20 iterations.

4 Results

4.1 Citizen science samples and metadata

Initially, 54 farms received the films to sample fungal spores and the questionnaires regarding farming operations and conditions. Of these, 46 farms completed sampling and sent the films and questionnaires to the NVI. Out of the 46 farms, three had no livestock, resulting in 46 films from rooms used as granary (marked with “L”) and 43 films from rooms used for animal housing (marked with “H”). Of these, nine (4 “H” and 5 “L”) films showed no growth at all. Approximately 50% of the spore trap films gave primary cultures with one or several *A. fumigatus* colonies (i.e., 22 “L” films and 20 “H” films). From these, a maximum of three *A. fumigatus* colonies were isolated from each plate, resulting in 53 “L” colonies and 55 “H” colonies, hence a total of 108 *A. fumigatus* pure isolates secondary cultures. Figure 10 shows a map of the farms that provided *A. fumigatus* samples along with where the resistant *A. fumigatus* isolates were found. Most of the samples are from southern Norway, with many

samples from Viken and from areas around Stavanger and Trondheim. The isolates will in this thesis be designated as “Farm number (1-54) + L/H + colony number (1-3)”.

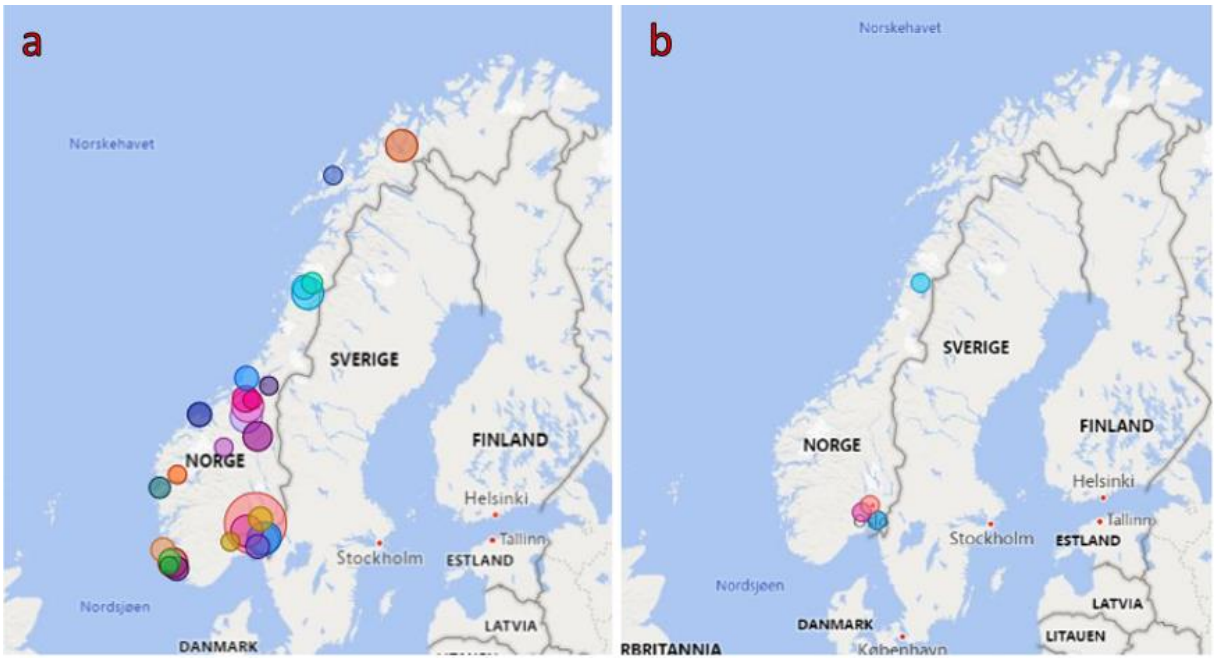

Figure 10: Geographic distribution of the A. fumigatus samples taken from farms across Norway. ***a)*** *All A. fumigatus samples.* ***b)*** *The azole-resistant isolates. Each colored circle is a farm that produced A. fumigatus samples. The size of a circle is proportional to the number of A. fumigatus isolates from each farm.*

4.2 Cross-contamination test

A cross-contamination test was performed to better understand how spore transfers could be done efficiently but with no cross-contamination. Results of the cross-contamination test are shown in figure 11. One of the two plates had *A. fumigatus* growth at every time interval. The growth was always on the plate positioned in the center of where spore transfer occurred.

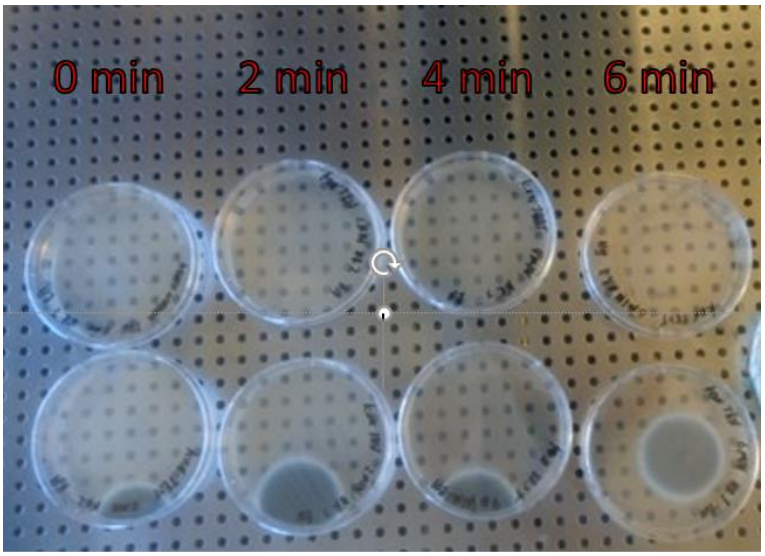

Figure 11: The four pairs of agar plates from the cross-contamination test. *From left to right: zero, two, four and six minutes after spore transfer. The plates on the bottom row were positioned in the center of the spore transfer, while the top plates were put 30 cm to the left of the center.*

4.3 Resistance testing of isolates against azoles

4.3.1 VIPCheck™

As a first screen for azole resistance all 108 isolates were screened using VIPCheck™ to determine possible resistance to voriconazole, itraconazole and/or posaconazole. Of the 108 isolates, 35 showed some degree of growth on at least one azole and were categorized as VIPCheck™ positive. Of the 35 VIPCheck™ positive, eight isolates showed especially high growth on one or several azoles as shown in figure 12.

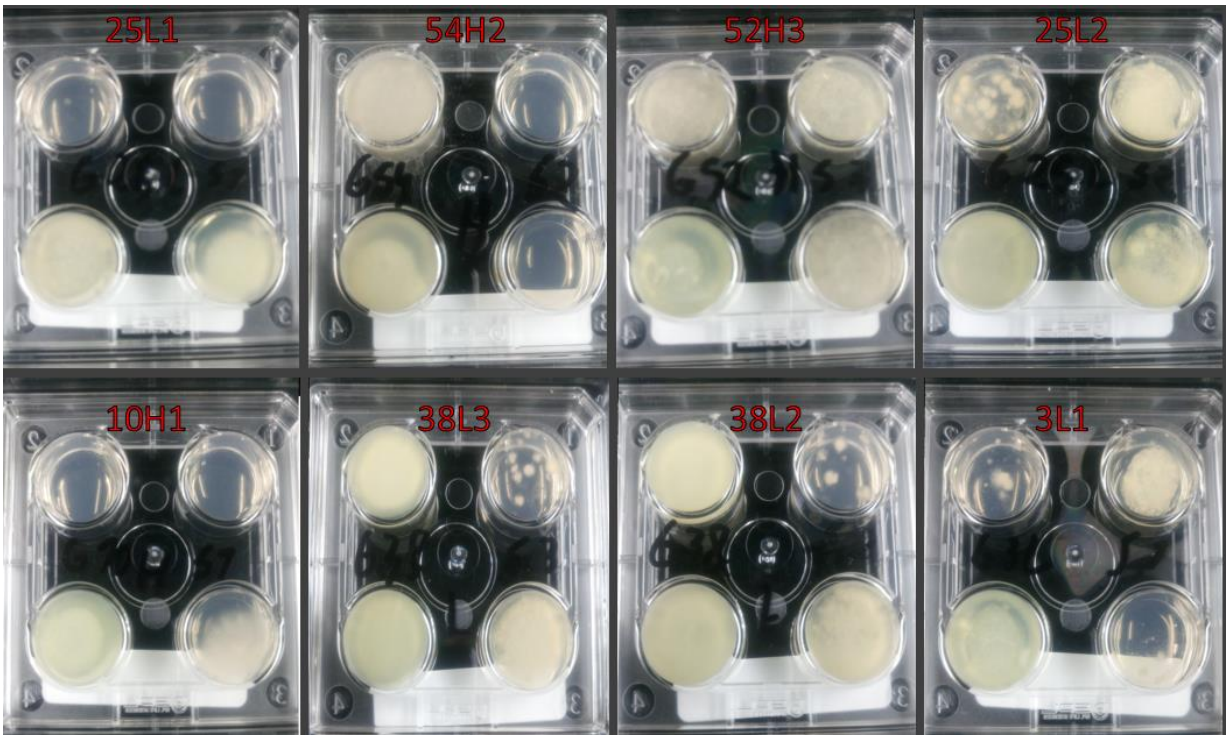

Figure 12: The eight isolates with high growth on at least one VIPCheck^{TM} azole well.

Isolate 54H2 had a unique morphology as it produced vast amounts of aerial hyphae that grew up through and slightly out of its well and is shown in more detail in figure 13.

Figure 13: Front view of the 54H2 isolate VIPCHECK™ plate. *This isolate has no growth on itraconazole or posaconazole, but on voriconazole it grows up and out of the well, bending off at the lid.*

4.3.2 E-test

All 35 VIPCheck™ positive isolates were tested with E-tests for resistance against the same three azoles to verify the results from VIPCheck™. The results are summarized in table 6.

Table 6: Results from the E-tests of the 35 VIPCheck™ positive isolates. *Values exceeding the breakpoint values are colored red.*

Isolate	Itraconazole MIC (mg/L) Breakpoint: >1 mg/L	Voriconazole MIC (mg/L) Breakpoint: >1 mg/L	Posaconazole MIC (mg/L) Breakpoint: >0.25 mg/L
3L1	4	2	1
4H1	0.50	0.25	0.125
5H1	0.50	0.25	0.094
5H3	0.50	0.25	0.094
7H1	0.75	0.38	0.125
7L3	0.50	0.25	0.19
10H1	0.38	0.19	0.094
10H3	0.38	0.19	0.125
18H3	0.38	0.19	0.094
23L1	0.38	0.19	0.064
24L1	0.38	0.19	0.094
25L1	0.50	0.25	0.094
25L2	4	1,5	0.50
25L3	0.50	0.19	0.19
26H1	0.38	0.19	0.094
26H2	0.38	0.125	0.064
28H2	0.75	0.38	0.19
28L1	0.50	0.38	0.094
28L2	0.50	0.38	0.19
31H1	0.50	0.25	0.19
31H2	0.38	0.19	0.094
31H3	0.50	0.25	0.19
33L1	0.50	0.19	0.19
38H1	0.50	0.25	0.125
38L2	0.50	0.25	0.19
38L3	4	>32	2
43H1	0.50	0.38	0.094
45H2	0.50	0.19	0.094
48L2	0.38	0.19	0.032
52H1	0.38	0.19	0.125
52H2	0.75	0.19	0.19
52H3	0.50	0.19	0.125
53L1	0.50	0.25	0.125
53L3	0.50	0.19	0.125
54H2	0.50	0.25*	0.38

*The isolate 54H2 showed an inhibition zone on voriconazole but had hyphae growing on top of the inhibition zone.

Four of the 35 isolates were resistant on an E-test according to EUCAST breakpoint values (EUCAST, 2020). Three of these were resistant to all three azoles. The fourth was only resistant to posaconazole even though it did not show any growth on the posaconazole well on VIPCheck™. Of special note were isolate 38L3 that was extremely resistant to voriconazole (>32mg/L), and the 54H2 isolate that seemed to be sensitive to voriconazole but would grow on top of the voriconazole inhibition zone. These isolates are shown in figure 14.

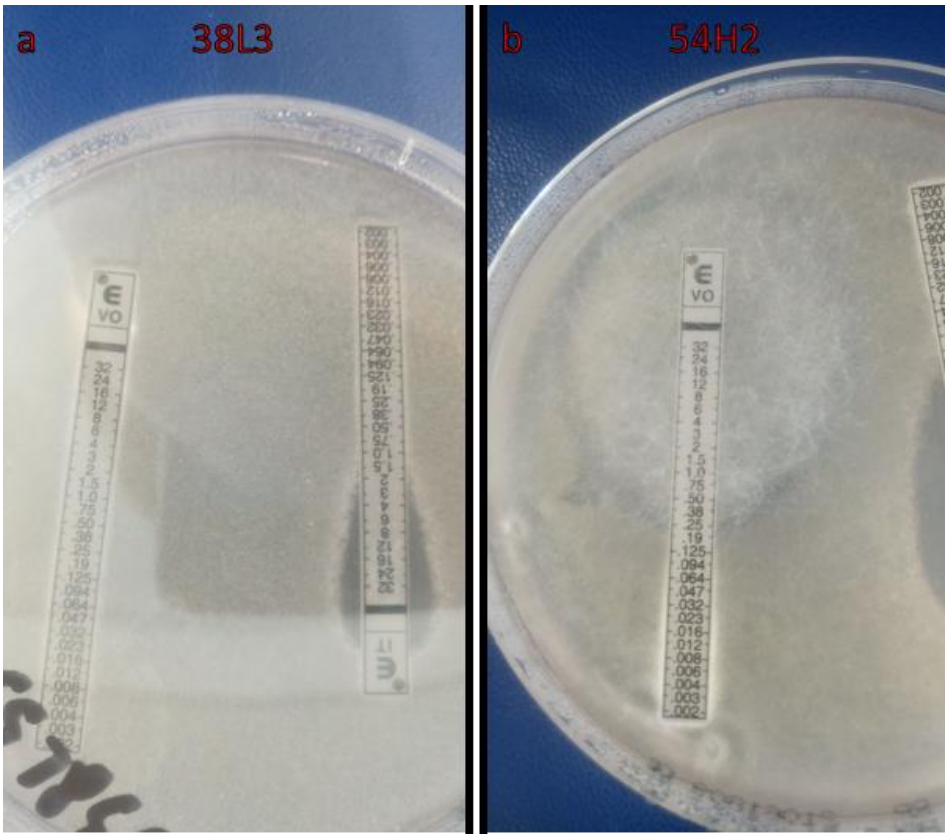

Figure 14: Unique results from the E-tests. a) *The 38L3 isolate that had no inhibition on the voriconazole E-test.* ***b)*** *The 54H2 isolate that grew on top of the inhibition zone.*

Because of its unique reaction to voriconazole, a new secondary culture was made from the hyphae growing on top of the voriconazole from the 54H2 isolate. The secondary culture is shown in figure 15. The morphology of the secondary culture shows that it is not *A.* fumigatus but possibly a *Lichtheimia corymbifera*. The primary culture the 54H2 isolate originated was documented to have extensive Mucorales growth.

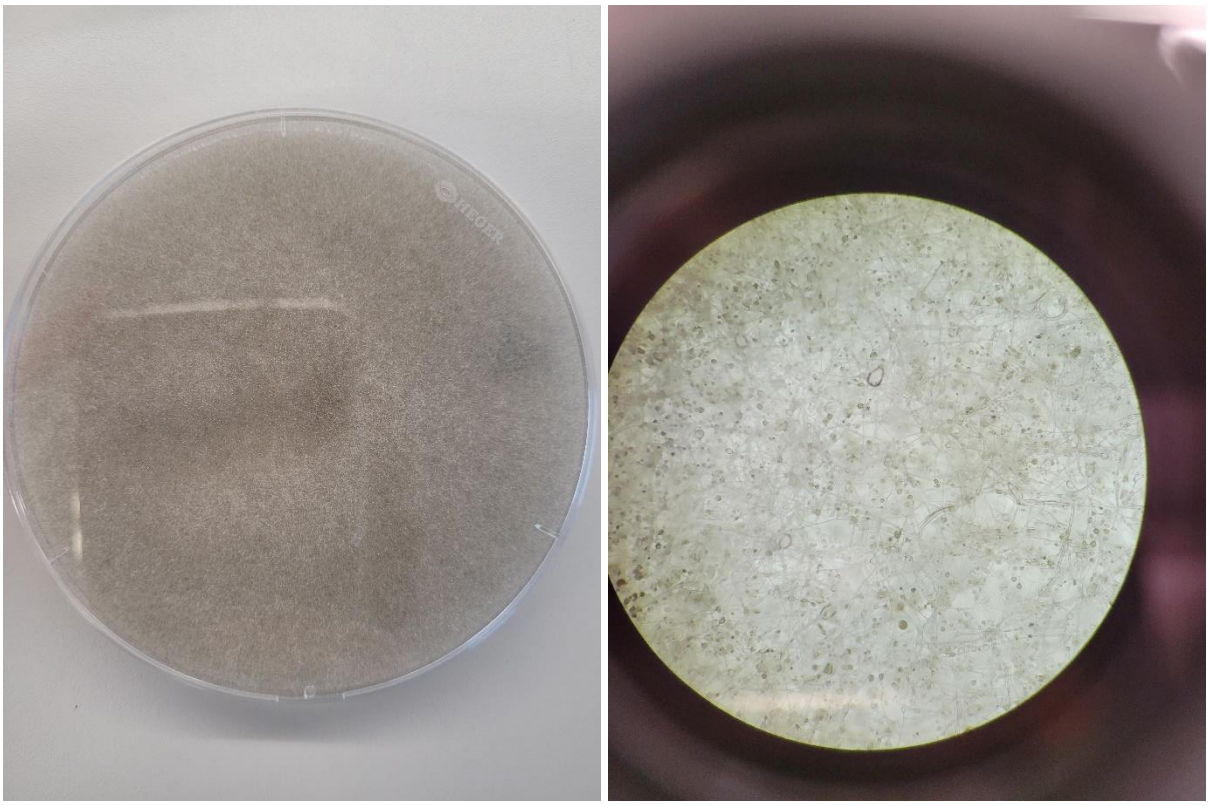

Figure 15: Culture made from the hyphae growing on the voriconazole in isolate 54H2. a) *Macroscopic picture of the culture.* ***b)*** *Picture of the culture taken trough a stereo microscope.*

4.5 Molecular analysis

4.5.1 Molecular identification with calmodulin

To verify that all 108 isolates were *A. fumigatus* a part of the calmodulin gene was sequenced. The target sequence was PCR amplified using calmodulin primers and PCR products were verified with gel electrophoresis. An example is shown in figure 16. Samples that did not show a calmodulin band were redone in a second round of PCR.

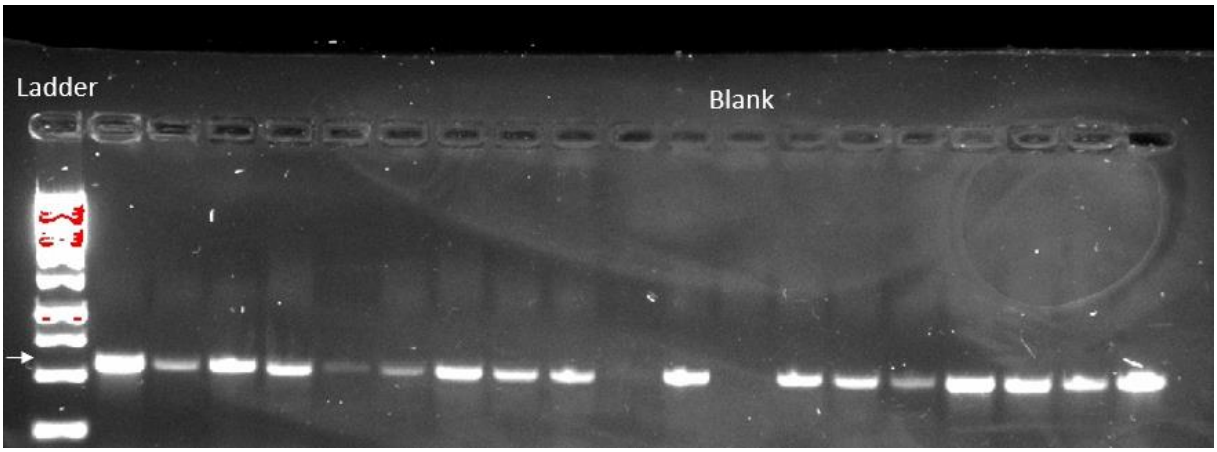

Figure 16: Gel electrophoresis of the first 18 calmodulin PCR samples. *The 1 kB ladder is in the first well, while the blank (negative control) is in the 13th well. The white arrow on the left shows the approximate size of the calmodulin gene.*

After two rounds of PCR, 88 isolates were successfully sequenced, while the remaining 17 isolates failed sequencing. However, all these 17 isolates were sensitive to all three azoles on VIPCheck™, so a third round of PCR was not deemed necessary. Of the 88 successfully sequenced isolates, 83 were identified as *A. fumigatus* with a 99% or higher similarity in a BLAST search (Appendix 4). Five isolates were identified as *A. fumigatus* with a slightly lower similarity of around 97%. These five isolates were sensitive to all three azoles on VIPCheck™, so resequencing was not deemed necessary.

4.5.2 *cyp51A* gene

To establish the molecular mechanism behind the azole resistant isolates the *cyp51A* gene was sequenced in the eight isolates that showed high growth on VIPCheck™. Gel electrophoresis confirmed PCR products for all isolates. An example is shown in figure 17.

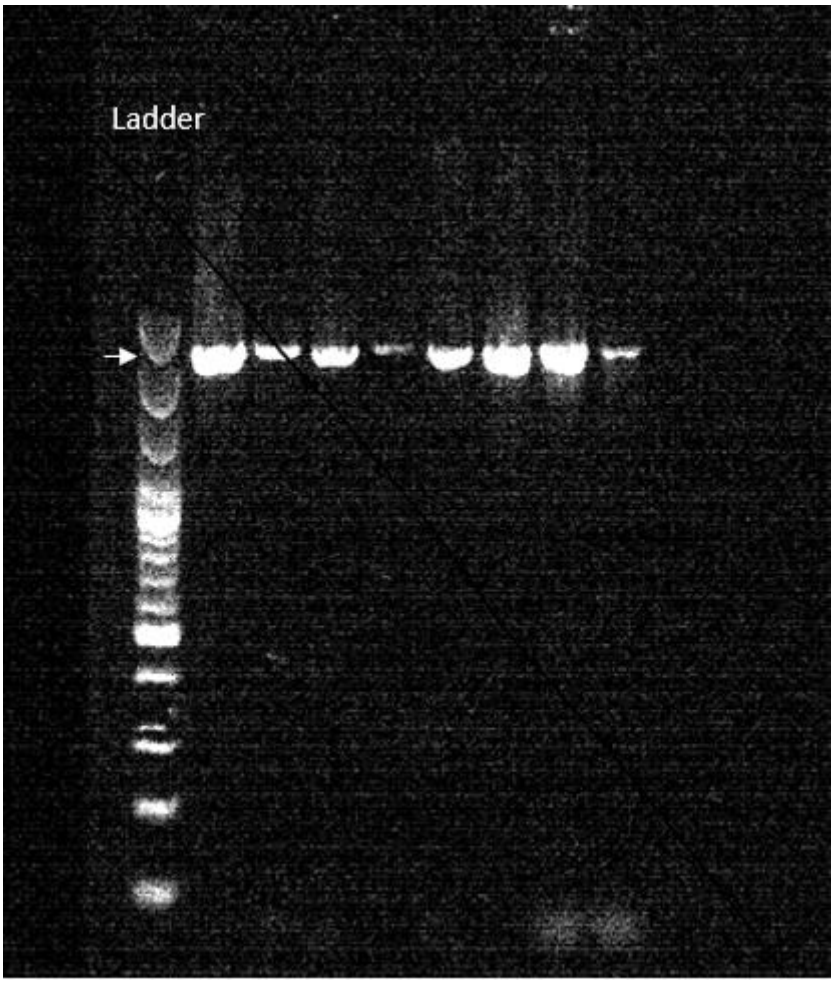

Figure 17: Gel electrophoresis of the cyp51A PCR samples. *The 100 bp ladder is in the first well. The white arrow in the left shows where the part of the cyp51A gene should be.*

The 3L1 isolate had the TR34/L98H mutation while the 38L3 isolate had the TR46/Y121F/T289A mutation. The remaining six isolates had a wildtype *cyp51A* gene. The two wildtype isolates 25L2, and 54H2 were positive on E-test. Therefore, 25L2, and 54H2 must have another resistance mechanism different from a *cyp51A* mutation. The four wildtype isolates: 10H1, 38L2, 25L1, and 52H3 were only positive on VIPCheck™ but not on the E-tests. These isolates are therefore considered sensitive. Sequencing results for *cyp51A* are shown in figure 18.

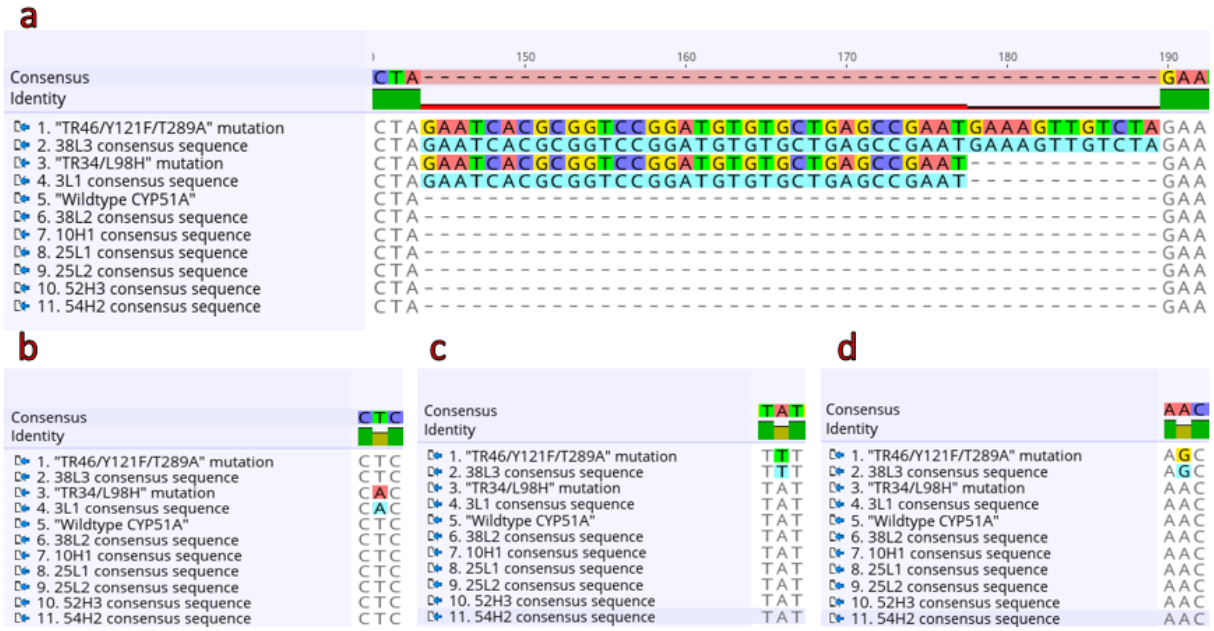

***Figure 18: Sequencing results for* cyp51A. a)** Promotor region showing tandem repeats. There is a 46 tandem repeat in the TR46/Y121F/T289A mutation reference sequence and the 38L3 consensus sequence. There is also a 34 tandem repeat in the TR34/L98H mutation reference sequence and the 3L1 consensus sequence. **b)** The L98H mutation indicated in red for the reference and in blue for isolate 3L1. **c)** The Y121F mutation indicated in green for the reference and in blue for isolate 38L3. **d)** The T289A mutation indicated in yellow for the reference and in blue for isolate 38L3.

5 Discussion

5.1 Citizen science sampling

This study is the first to investigate the occurrence of azole resistance in *A. fumigatus* in Norway, and the sampling was organized as a citizen science project. While citizen science puts sampling into the hands of untrained people whose sampling cannot be accurately documented, it has several advantages. Firstly, it is very cost effective, as the travel cost is substituted for the cheaper shipping costs. Secondly, it is very time efficient as many people will sample faster than a few. Thirdly, because of the lower cost and time, citizen science will often yield more samples than manual sampling will. However, since participation is optional, anyone could sign up to participate and then fail to deliver samples. In this project about a seventh of participants (14.8%) signed up but did not send in any samples. So future projects utilizing citizen science should expect a decently lowered sample size than the potential size. Citizen science also results in most participation from the most populated areas as demonstrated in figure 10. If a less populated area is to be sampled, some manual sampling may be required.

After cultivating the spore trap films on agar plates approximately 50% of the primary cultures had one or several *A. fumigatus* colonies. This is in line with the results of a similar citizen science study air sampling from Great Britain. In this study the amount of air samples that contained *A. fumigatus* ranged between 36-57% (Shelton, 2020).

Nine of the 89 films received had no fungal growth at all. Any primary culture where there was no fungal growth was most likely due to an error in the sampling, as it is unlikely that no fungal spores would land on a film for six hours. A higher error rate is to be expected when sampling is done with citizen science, as every sample is done by different people with no experience in spore sampling. In this thesis, the error rate was about ten percent. If common mistakes could be investigated, instructions on how to sample spores could be improved to reduce the error rate. For instance, the questionnaire could also ask the participant if any of the instructions were unclear.

The metadata collected from the questionnaires could not show any trends among the farms with resistant *A. fumigatus* isolates. This was mainly because of two reasons. Firstly, since only four isolates were confirmed as resistant on E-tests, there were only a very small sample size of farms with resistant *A. fumigatus*. Any trends in the metadata could therefore be from random chance. Secondly, because some of the questionnaires sent with the films were either not filled in or not sent back, two of the four farms with resistant isolates did not have any metadata. One of the main trends to look for would have been if farms with resistant *A. fumigatus* also used fungicides on their crops. This would agree with the leading theory of *A. fumigatus* resistance developing from fungicide use from farms. However, since only three of the 46 farms answered that they used fungicides there wouldn't have been enough data to suggest a link. A larger *A. fumigatus* azole resistance project would need to put more emphasis on participants answering the questionnaire to be able to investigate metadata trends.

5.2 Molecular Identification of *A. fumigatus* isolates

A. fumigatus colonies were selected from primary plates by visual inspection, and secondary cultures were then subject to sequencing of a fragment of the calmodulin gene to confirm the species. The calmodulin gene was successfully sequenced in 88 of the 108 isolates, including all resistant isolates, and identified all isolates as *A. fumigatus.* Sequencing failed, however, for 17 isolates. Isolates who failed sequencing showed an empty or very faded band on the gel electrophoresis, indicating that either the DNA extraction or the PCR had failed. As these isolates were VIPCheck™ negative molecular identification was not pursued.

5.3 Contamination

The results from the cross-contamination test indicated that the *A. fumigatus* spores would stay in the air for some time (at least six minutes), but also that the spores would not travel 30 cm from their transfer path. Meaning that the risk of cross-contamination during spore transfer would be low if successive spore transfers either were performed after at least six minutes had passed, or at least 30 cm away from where the previous spore transfer was done. Therefore, when working on the secondary cultures the workspace inside the hood was divided into three parts: left, middle and right. Spore transfer of one culture would be done on the left part, transfer of the next culture would take place on the middle part, then the next culture on the right part. After this the workspace was cleaned with a 70% ethanol solution and left for 10 minutes. This was done to minimize the chance for cross-contamination while still working through the cultures efficiently.

To assess the degree of cross-contamination, fungal growth pattern on VIPCheck™ plates was compared for the VIPCheck™ positive isolates. The isolates seemed to produce a visually unique VIPCheck™ growth pattern in the wells. That is, when one isolate was re-tested on another VIPCheck™ plate, it had the same growth pattern as before. Considering this, visually different VIPCheck™ plates can indicate different isolates while VIPCheck™ plates that are visually similar may indicate cross-contamination. All but two of the 35 VIPCheck™ positive isolates had different morphology, which signals a low, if not nonexistent, amount of cross-contamination during VIPCheck™ screening.

Isolates 38L2 and 38L3 had a similar growth pattern on their VIPCheck™ plates indicating the possibility of cross-contamination. However, isolate 38L3 was inoculated after 38L2, and 38L2 was E-test negative while 38L3 was E-test positive for all azoles. Since the VIPCheck™ plates were removed from the workspace after inoculation, it is impossible for the growth on the 38L2 plate to be from contamination by the spores from isolate 38L3. The similar morphology could instead be the result of the two isolates being from the same film from the same farm, meaning they could have very similar genotypes. In addition, they were inoculated and incubated on the same day at the same conditions. This highlights a drawback of using VIPCheck™ morphology to look for cross-contamination.

The Isolate 54H2 was first thought to show some unique way of resisting voriconazole, but on further inspection turned out to be contaminated with most likely *L. corymbifera* from the

Mucorales order. The primary culture from where 54H2 was sampled had Mucorales on it and the contamination most likely originated there. However, the fact that the *L. corymbifera* did not grow at all a secondary culture before the E-test is unexpected. Especially since it grew very fast when given its own agar plate after the E-test (figure 15). One possibility is that the 54H2 isolate was contaminated with a very low amount of *L. corymbifera* spores in relation to the *A. fumigatus* spores. The *A. fumigatus* would then outcompete the *L. corymbifera*, but the *L. corymbifera* is very resistant to voriconazole so when voriconazole was introduced the *L. corymbifera* spores had access to nutrition without *A. fumigatus* interfering. The fact that the calmodulin and *cyp51A* sequencing was successful on the 54H2 isolate is mostly likely caused by the differences in calmodulin and *cyp51A* between *A. fumigatus* and *L. corymbifera* (Uniprot, 2021). This could lead to the primers not binding to the *L. corymbifera* DNA, only amplifying and sequencing *A. fumigatus*.

5.4 Resistance screening

As a screening method, VIPCheck™ plates are less accurate than E-tests, but are much less time consuming. Screening an isolate with an E-test took about 20 times as long as screening with VIPCheck™. For even the small sample size of 108 isolates, screening with VIPCheck™ first made the process much more efficient. For larger projects with larger sample sizes VIPCheck™ should be considered as a way of narrowing down samples before a more precise method is used, i.e., E-tests. There are however more precise ways of determining MIC-values than E-tests. E-test loses precision because the MIC-value is rounded up when growth is between two values. Isolate 54H2 showed no growth on the posaconazole well on VIPCheck™ but was higher than the breakpoint resistance value on an E-test because its value was rounded up so it may have shown to be sensitive on a more accurate test. A more accurate, albeit more time consuming, method is for example a twofold dilution series to determine MIC-value, which is what EUCAST uses to set breakpoint values (EUCAST, 2020).

Most of the VIPCheck™ positive isolates did not show azole resistance on an E-test. One reason for the inconsistency between methods is that in this thesis even a low to medium growth (++) on a VIPCheck™ plate was considered VIPCheck™ positive. After testing all 35 isolates with E-test, only isolates with a high growth (+++) on a VIPCheck™ plate showed a MIC-value above resistance breakpoints on E-tests. Therefore, in future studies I would recommend to only examine isolates with a high growth on VIPCheck™ plates further.

Nevertheless, of the eight isolates that had high growth on VIPCheck™, four were negative on E-tests. This is surprising, as VIPCheck™ has shown high specificity in the past (Buil et al., 2017, Griffin et al., 2019). One possible reason for this is that the VIPCheck™ plates are supposed to be refrigerated before use, but the plates used in this thesis were stopped at the Norwegian customs for two weeks and the condition they were kept in is unknown. Another possibility is the wide range of concentration of spores you can use according to the VIPCheck™ protocol. This range is between 0.5-2 McFarland, while for E-tests the protocol has a range of 0.5-0.6 McFarland. The increased concentration in the VIPCheck™ tests might have given a level of resistance that the lower concentration in the E-test could not replicate. During future screenings with VIPCheck™ the McFarland values should be documented for each plate to see if high values can result in false positives.

5.5 Azole resistance in Norway

Four resistant *A. fumigatus* isolates were found from the farms. Three were in the county of Viken while the last one was in the county of Nordland. The sample size is too small to say anything definite about the geographical distribution of resistant *A. fumigatus* in Norway. However, the results show that resistant *A. fumigatus* exist at multiple farms in Norway and not just from one area. While the sample size is fairly small, taken at face value the amount of azole-resistant *A. fumigatus* found is relatively low (3.7%) and on the level of countries like Spain and Australia. This correlates with Norway's low use of azoles. Still, a larger study is required to confirm if these findings are representative of the real situation.

Both of the most common azole resistance mutations in the *cyp51A* gene, TR_{34}/L98H and TR_{46}/Y121F/T289A, were found. The TR_{34}/L98H mutation has previously been identified from one environmental isolate in Norway. However, the TR_{46}/Y121F/T289A mutation has not been found in an environmental isolate in Norway before (Chowdhary and Meis, 2018) The TR_{46}/Y121F/T289A mutation is especially troubling from a resistance perspective, as it in practice eliminates voriconazole as a treatment option completely (van der Linden et al., 2013). The isolate 38L3, the isolate containing the TR_{46}/Y121F/T289A mutation, had a MIC-value of over 32 mg/L, demonstrating its heavy voriconazole resistance.

The isolate 25L2 was resistant to all three azoles on both VIPCheck™ and the E-tests but did not have a mutation in the *cyp51A* gene. This is an unsurprising find, as many documented cases of resistant *A. fumigatus* do not show any mutations in the *cyp51A* gene (Chowdhary

and Meis, 2018). This could be caused by any of the currently unknown mechanisms. However, it may be of interest in future studies to sequence the *cdr1B* gene as it is a known azole resistance mechanism.

6 Conclusion

The data gathered in the BARNS project reveals that azole-resistant *A. fumigatus* exists environmentally in Norway at geographically different locations. Both most common *cyp51A* mutations are confirmed to also exist environmentally and at least one, so far, unknown mutation. Norway's level of azole resistant *A. fumigatus* seems to be low (3.7%), however a larger study needs to be performed to accurately know the situation. Such a study should consider sequencing *cdr1B* in addition to *cyp51A* and if VIPCheck™ plates are used, document the McFarland values. In addition, if many samples show a low to medium growth on VIPCheck™ tests, cost and time could be reduced by only doing further tests on VIPCheck™ plates with high growth as they seem to be the only resistant isolates.

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8 Appendix

Appendix 1: User manual for plastic films. Shown first in Norwegian then in English. The user manual was only translated to English in this thesis and was only sent out in Norwegian.

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 tilsendte elektroniske spørreskjema eller medfølgende papirversjon. Fyll ut med samme prøvenummer som angitt på filmen (G-x). Lenken til det elektroniske skjemaet er tilsendt på e-post. Du kan også skrive inn denne nettadressen manuelt: https://form.jotform.com/203422195208146.
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 (hvis du har fylt inn analogt) i medfølgende svarkonvolutt, klistre på returlapp fra posten 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 mold in the barn?

Veterinærinstituttet
Norwegian Veterinary Institute

Instructions

You have now received two plastic films. They are taped together at one end. Please do not remove this tape as it will make it easier to put the protective cover back on the film (See picture below).

Expose the films in two separate rooms. If you have animals, place one film in the granary (film marked as L) and one in the room where the animals are (film marked H). If you produce grain/feed you will only use one film (L) in the granary.

1. Complete the electronic questionnaire or the paper version included. Use the same sample number as on the films (G-x). The link to the electronic questionnaire is sent to you at your e-mail. You can also write the web page manually https://form.jotform.com/203422195208146.
2. Try to do this as sterile as possible: Pull the protective film carefully off without touching the inside and keep the end with the tape on (see picture). That way it will be easier to put the protective film on again after exposure.
3. Put the film down with the adhesive side up on a surface about one meter over the floor in the middle of the room where it can lie undisturbed for six hours. If you can't find an appropriate surface in the middle of the room, put the film in a place close to the animals/grain/feed and where there is a bit of air circulation.
4. After the exposure, put the protective film carefully and uniformly, but as fast and accurately as you can back over the exposed film.
5. Put the plastic film(s) in the included zip-lock bag. Put the bag and the questionnaire (if you used the paper version) in the included envelope, glue on the return label from the postal service and mail it.

Picture 1: The plastic film as it should look when exposing. Keep the tape end connected to the protective film so that it will be easier to put the protective film back on the exposed film.

Thank you for the assistance!

Appendix 2: Questionnaire form relating to farming operations. Shown first in Norwegian then in English. The questionnaire was only translated to English in this thesis and was only sent out in Norwegian.

Resistente muggsopp i fjøset?

Veterinærinstituttet
Norwegian Veterinary Institute

Dersom du vil motta resultatene fra gården din trenger vi ditt samtykke. *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		Framføring					
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 mold in the barn?

Veterinærinstituttet
Norwegian Veterinary Institute

We need your consent if you want to receive the results from your farm. *This is completely optional*. If you want to be anonymous, don't fill in your contact information. **You MUST include the ID-number on your film and your postal code**.

THE FILMS ID-NUMBER*	
Postal code*	

Name	
Address	
Telephone	
E-post	

TYPE OF FARM

Organic farm? **YES** ☐ **NO** ☐

ANIMALS	**Amount (total)**	**Type of management (Set an X in the shaded box)**				**Type of moisture absorber and bedding (Set an X)**			
						Saw dust	**Straw**	**Talle**	**Other**
Dairy cows		Booths		Outdoors					
Cattle for meat		Milk from cows		Artificial feed					
Sheep or goats		Sheep		Goat					
Pigs		Breeding		Butcher hogs					
Horses									
Other									

GRAIN	**Area (# 1000 square meters)**	**Du you use pesticides? If yes; specify which product**			
		Do not use	**Against fungi**	**Against weeds**	**Against insects**
Oat					
Wheat					
Barley					
Rye					
Other					

ROOM WITH ANIMALS (H-FILM)		**GRANARY (L-FILM)**	
Temperature in the room		**Temperature in the room**	
What kind of animals?		**What is stored here?**	
		Dry hay	
		Silage	
		Other (specify)	

Write on the back if you need more space.

Appendix 3: DG18 agar made at the Norwegian Veterinary Institute.

Components	Amount
Casein enzymatic digest[1]	5 g/L
D-Glucose[1]	10 g/L
Potassium dihydrogen phosphate[1]	1 g/L
Magnesium sulphate[1]	0.5 g/L
Dichloran[1]	0.002 g/L
Chloramphenicol[1]	0.1 g/L
Agar[1]	15 g/L
Glycerol solution (85%)	220 g
Distilled water / RO-water	1,0 L
Trace metal solution	1.0 mL
Chloramphenicol solution (50 mg/mL)	1,0 mL
Chlortetracycline solution (5 mg/mL)	10,0 mL

1. (Thermo Fisher Scientific)

Appendix 4: Example of a BLAST search for a confirmed *A. fumigatus* isolate.

Bit-Score	E Value	Grade ▼	Hit start	Hit end	Name	Description
1,026.01	0	100.0%	14	568	KJ175558	Aspergillus fumigatus isolate M 56 calmodulin (cmdA) gene, partial cds
1,020.47	0	99.7%	13	564	MT420414	Aspergillus fumigatus strain DTO 402-H1 calmodulin (cmdA) gene, partial cds
1,020.47	0	99.7%	8	559	MG991481	Aspergillus fumigatus strain CMXY25241 calmodulin (cmdA) gene, partial cds
1,020.47	0	99.7%	5	556	MG991440	Aspergillus fumigatus strain CMXY16512 calmodulin (cmdA) gene, partial cds
1,016.78	0	99.7%	1	553	MK451402	Aspergillus fumigatus strain CMV003C9 calmodulin gene, partial cds
1,016.78	0	99.7%	27	579	LR993220	Aspergillus fumigatus partial CaM gene for calmodulin, isolate FWiP_29
1,016.78	0	99.7%	28	580	LR993219	Aspergillus fumigatus partial CaM gene for calmodulin, isolate FWiP_11
1,016.78	0	99.7%	27	579	LR993218	Aspergillus fumigatus partial CaM gene for calmodulin, isolate FWiP_4
1,016.78	0	99.7%	27	579	LR993217	Aspergillus fumigatus partial CaM gene for calmodulin, isolate FWiP_3
1,016.78	0	99.7%	27	579	LR993216	Aspergillus fumigatus partial CaM gene for calmodulin, isolate FWiP_75
1,016.78	0	99.7%	26	578	LC589319	Aspergillus fumigatus NRRL 163 CaM gene for calmodulin, partial cds

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