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No man's land - An investigation of development of azole resistance in *Aspergillus fumigatus* within border zones surrounding golf fields.

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Acknowledgement

The work as a monument of personal achievement, as the larger part of the work related was done during a time in my life dominated by mental challenges that influenced daily life.
It is also a personal reminder that help will be given to those who seek it, and that accepting help from those willing to give it is not a sign of weakness.

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Chapter 1: Introduction

1.1 Background

Fungi exhibit remarkable diversity of form and function which allows them to thrive in almost every environment on Earth. This group of eukaryotic organisms play important roles in our ecosystems in the form of decomposers or regulators of nutrient cycles in soil (1, 2), as well as providing a rich habitat and food for other organisms (3).

However, fungi can also be harmful to animals and humans as its diversity also includes poisonous mushrooms, parasites, or pathogenic molds and yeasts (4, 5). Poisonous mushrooms

are one of the most well-known examples of harmful fungi (6). Mushroom poisoning can range in severity from mild gastrointestinal irritations to life-threatening damage to vital organs (7, 8). The fact that some of the most lethal species of poisonous mushrooms found in Norway (*Cortinaruis rubellus* and *Amanita virosa*) (https://www.helsenorge.no/giftinformasjon/sopp/norges-giftigste-sopper/) can be mistaken for edible species (*Cantarellus tubaeformis* and species in genera genera *Lycoperdon* and *Agaricus*) adds to the potential hazardous nature of these mushrooms. According to the Norwegian Health Informatics (NHI) there are an approximated 200 reported cases of mushroom poisoning annually (*https://nhi.no/forstehjelp/forgiftninger/giftig-sopp-inntak-av/*).

Pathogenic fungi can cause infections (mycoses). Superficial fungal infections are confined to the skin and hair, while invasive infections can be life threatening, affecting internal organs and leading to an annual mortality rate comparable to that of malaria and tuberculosis (9, 10, 11). Members of the genera *Candida* and *Aspergillus* represent the most common fungal pathogens worldwide. Such infections are commonly treated with antifungal drugs from the azole group (12, 13, 14, 15). There has been observed an emergence of azole resistance among potential lethal species within these genera since the end of the 1990s (13, 16, 17). This trend is of great concern as it can limit the options of treatments for such infections, and cause complications for patients with compromised immune systems such as patients undergoing chemotherapy and victims of HIV/AIDS, as well as patients receiving transplants partly due to immunosuppressive drugs being part of the post-transplant treatment (18, 19). Noteworthy, use of azoles is not limited to use in veterinary and medical practices, they are widely used in the environment to combat fungal pathogens attacking crops in the field or grass on the greens of golf courses (20, 21, 22, 23, 24). Reports of resistant strains of fungi isolated from patients with no previous treatments with azole drugs, suggest a resistance development in the environment being the dominant path of resistance development compared with the clinical path of resistance (25).

A particularly concerning member of the *Aspergillus* genus (that is showing an increase in azole resistance) is *Aspergillus fumigatus*, a ubiquitous saprophytic mold that causes allergic, chronic, and acute invasive diseases in humans and animals (26, 27, 28). The prevalence of azoleresistant fungal strains in the natural environment in Norway has not been studied so far, but azole-resistant *A. fumigatus* has been isolated from drinking water, air, animals and humans.

1.2 Significance of the study

Even with its significant annual mortality, fungal infections seem to be a threat to human health that is overlooked by the general eye (29). There are several emerging studies pointing toward azole resistance being an issue that needs attention, but there is limited knowledge about the mechanics that drive the development of resistance (26). There is also limited research on possible hotspots of resistance development. These knowledge gaps are equally evident within the research landscape of Norway. The current project, as a part of the research project *Navigating the threat of azole resistance development in human, plant and animal pathogens in Norway (Navazole)*, funded by the Norwegian Research Council, aims towards contributing

insights into the development and dispersal of azole resistant *A. fumigatus* in soil from border zones of azole treated areas, and thereby illuminate neglected parts of antimicrobial research within the One Health framework.

1.3 Research objectives and hypothesis

This study is performed as a part of the Navazole project, where the overall aims are to explore how fungi develop resistance to fungicides and to identify and understand Norwegian conditions where the risk of resistance development is particularly high. Furthermore, the Navazole project aims towards developing methods for surveillance and diagnostics related to antifungal resistance, providing protocols that can aid in the preservation of environmental, animal, and human health.

The main objective of the current study will be to investigate the presence of azole resistant *A. fumigatus* in the soil of border zones surrounding golf courses in Viken county, with the purpose to contribute to insights into the development and dispersal of azole resistant *A. fumigatus* in the environment and illuminate neglected parts of antimicrobial research within the One Health framework. This will be done by isolating and identifying fungi from soil samples gathered in these areas pre and post antifungal application, and determine *in vitro* susceptibility to different types of azoles using VIPcheck and E-test. Full sequence characterization of the CYP51a gene and promoter region will be performed on any resistant strains in order to examine resistance mechanisms.

The hypothesis is that the conditions in the soil in border zones surrounding golf greens are potential hot-spots for development of azole resistance. It is further hypothesized that levels of resistance vary relative to the time the azole antifungal are applicated to the golf greens.

Chapter 2: Literature review

2.1 Fungi

The term 'fungi' was coined in the 18th century by Carl Linnaeus (30) to cover a group of diverse organisms known nowadays as molds, yeasts, and mushrooms. Yet, Linnaeus, as many other scientists of that time, considered fungi as a members of the plant kingdom. With advances in cell biology, microscopy and availability of specimens new insights were possible. In the late 19th century, de Bary published a ground-breaking work for understanding fungi, where he shared his observations of the distinction between fungi and plants (31). In the early 21st century, based on molecular analyses, an estimate of the origin of the fungal kingdom was given as the range from 760 million years ago to 1.06 billion years ago (32). Though fungi share several characteristics with plants, both being non-motile eukaryotes anchored to their substrate, there are several structural and physiological differences that separate the two kingdoms. Among the

most significant differences are the fact that fungi have a unique chitinous cell wall, heterotrophic mode of nutrition through osmotrophy, and are lacking chlorophyll (33).

Fungi can be divided into two main groups – macrofungi, commonly understood as mushrooms, and microfungi. The term macrofungi refers to fungi with a solid spore-bearing fruiting body that is visible above the substrate on which the fungus is growing, and microfungi refers to smaller fungi that does not produce large multicellular fruiting bodies. The latter group includes fungi such as moulds and yeasts. Molds are multicellular filamentous fungi that reproduce through the production of sexual and asexual spores, and yeasts are unicellular fungi that do not produce hyphae and reproduces through asexual mitosis.

2.2 Taxonomy

The description and naming of fungi have been subject of much speculation and underwent numerous changes from its foundation set by pioneers such as Carl Linnaeus, Christiaan Hendrik Persoon, and Anton de Bary, and with advancement in methods of molecular methods of identification the controversy seems to be far from over. Thanks to the increase of accessibility of polymerase chain reaction (PCR) and sequencing services, we are witnessing a rapid molecular revolution in the understanding of taxonomy (Hibbet et al. 2007). The latest reported number of named and accepted species of fungi are at around 150 000, and a common estimate of the fungal species in existence is around 1.5 million (34)However, some studies claim that this number can be as high as 2.2-3.8 million estimated species based on host association and 11.7-13.2 million species using high-throughput sequencing, which means that we have roughly named described between <1% and 4% of all species (34, 35).

Though there are some controversies related the current state of the fungal tree of life (AFTOL), an up-to-date phylogenetic classification of fungi comprises nine major lineages – Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Basidiomycota and Ascomycota (33).

2.2.1 Ascomycota

The Ascomycota is the largest and most diverse phylum with an impressive amount of variation in habitats and life modes. It contains approximately 2/3 of the known fungal kingdom, with ca. 110 000 described species (33, 36). Fungi belonging to this phylum can be recognized by dikaryote hyphae which allows for the production of asci – containing asexual spores sac-like structures. These are not to be confused with the spore bearing, club-like basidia of the other member of Dikarya and the second richest phylum, Basidiomycota. Ascomycota can be further divided into three subphyla: Taphrinomycotina, Saccharomycotina, and Pezizomycotina.

Taphrinomycotina is known to be the poorest of the Ascomycetes with regards to species, but have a relative broad diversity among its representatives that mostly consists of plant pathogens and saprotrophic yeasts. Fungi classified as Taphrinomycotina have a various morphology types

– ranging from intracellular parasites to fungi with a complex morphological structures (33). This class makes up for its lack of numbers in species by presenting us with the objectively beautiful genus Neolecta (fig. 1).

FIGURE 1 NEOLECTA SP.

A well-known representative from Saccharomycotina is *Saccharomyces cerevisiae*, commonly known as bakers or brewer's yeast. This species has an important role in the process of brewing alcoholic beverages and making dough rise when baking, as it possess the ability to produce CO_2 and alcohol.

The subphylum Pezizomycotina harbors the largest diversity of species, which have been used in everything from ancient medicine to modern genetic. Those fungi are mostly filamentous saprobes or lichens, but also parasites, mycorrhizal fungi, or endophytes. Approximately 40% of this class consists of lichenized fungi, and 98% of all lichens have mycobionts from the Pezizomycota class (33). Lichens are a stable symbiosis between a fungus (mycobiont) and a green algae or cyanobacteria (photobiont). The mycobiont provides shelter, while receiving nutrients from the photobiont. This strikingly diverse group of organisms grows on everything from concrete to trees and do not degrade the substrate they grow on. They absorb water from the moisture in the air rather than through roots and these characteristics make them valuable ecological indicators that can aid in the assessment of factors such as air quality or rate of disturbance in forested areas. Another member of this class is the fungus known under the name *Cordyceps*. This fungus was made known and popular in the video game, and later TV-series, “The Last of Us” and in the novel “The girl with all the gifts". Although the depiction of this parasitic fungi is rather exaggerated, it does zombify its host in a similar way by growing its mycelium throughout the hots tissue, and later produce fruiting bodies that emerge from the host in a macabre display of the circle of life. Fascinating examples from this group include also mycotoxin producing fungi, including a potentially lethal pathogens accommodated in the genus *Aspergillus*.

2.3 Fungal pathogens

Fungal pathogens refers to members of the kingdom Fungi with the ability to cause diseases in a diverse selection of hosts, including plants, animals, and humans. Throughout history, these pathogens have had profound effects on human and animal health, as well as food safety, and continue to pose current-day threats. Some examples of fungal pathogens are listed in table 1.

Fungal pathogens can be divided as obligate – when they are unable to survive without a host, or opportunistic pathogens – if it requires a host with impaired immunity (37, 38, 39). Infections caused by fungal pathogens are commonly referred to as mycoses, and can be categorized as superficial, cutaneous, subcutaneous, or systemic dependent on the depth and extent of the infection. These pathogens rely on their capacity to colonize and invade their hosts, leading to a range of infections and often posing significant health threats.

Among these fungal pathogens, genus *Aspergillus* stands out as a clinically relevant and ubiquitous species known to cause invasive aspergillosis, particularly in immunocompromised individuals. Aspergillosis refers to a group of infections that most commonly manifests as respiratory system disorders.

TABLE 1. EXAMPLES OF FUNGAL PATHOGENS WITH HISTORICAL SIGNIFICANCE. LATIN NAME AND COMMON NAME, AS WELL AS DESCRIPTION OF COMMON HOSTS AND HOW THE PATHOGENS MANIFEST (39)

Fungi responsible	Common name	Symptoms	Common hosts
Aspergillus spp.	Aspergillosis	Infection in respiratory system	Human
Candida spp.	Candidiasis	Infection in oral cavity, digestive system, genitals, skin, and nails	Human
Pseudogymnoascus destructans	White-nose syndrome	Damage epidermal tissues, erode fat reserves, breaking of hibernation, death.	Hibernating bats
Batrachochytrium dendrobatidis **and** ***Batrachochytrium salamandrivorans***	"The Amphibian Apocalypse"	Degradation of permeable skin, death	Wide range of Amphibian species
Histoplasma **sp.**	Histoplasmosis	Infection in respiratory system	Human
Geaumannomyces graminis **var.** ***tritici***	Take-all disease	Attacks roots and stem base, prevent movement and uptake of water.	Wheat and barley
Phytophthora infestans	Late blight	Kills leaves and rots tissue of tubers	Potato

2.4 Genus *Aspergillus*

The genus *Aspergillus* (Pezizomycotina) is very diverse and rich in species of a high impact on our lives. It comprises a diverse assembly of filamentous fungi that was originally described by the Italian mycologist Pier Antonio Micheli in 1729. The name of the genus was inspired by the characteristic morphology of the conidiophores, resembling a tool used for spreading holy water called an aspergill.

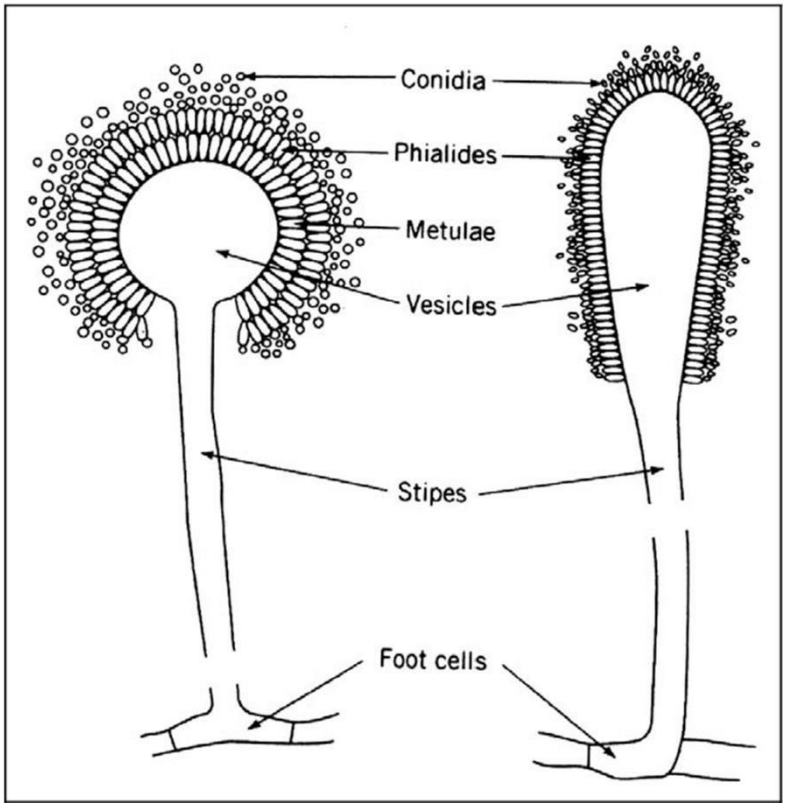


FIGURE 2 ASPERGILLUS CONIDIOPHORES WITH STRUCTURES LABELED (KLICH 2009).

This ubiquitous genera is responsible for processes such as food spoilage, bioproduction of valuable metabolites, such as antibiotics, medicines or enzymes (40), but is also famous producent of mycotoxins and often reported as animal and human pathogens (41, 42). Using phylogenetic tools, quite recently, a new insight into the genus *Aspergillus* was provided. Currently, the genus is divided into 6 subgenera, 27 sections and 75 series, with a total of 446 accepted species (43).

The *Aspergillus* genus (fig. 2) can be recognized by macromorphology – morphology of its colonies as a whole, taking into account parameters such as colony growth rates, texture, degree of sporulation, production of sclerotia or cleistothecia, colors of mycelium, production of pigments and exudates, and by micromorphology – morphology visible in the microscopic slides with parameters including shape of conidial heads, branching, color of stipes, as well as presence of and dimensions, shapes and textures of stipes, vesicles, metulae, phialides and conidia (40).

Prominent species within this group include *A. niger*, utilized in industrial production of organic acid, and *A. oryzae*, employed in traditional fermentation processes of food and beverage. Notably, *A. niger*, alongside *A. flavus*, can act as pathogens, responsible for aspergillosis, and can contaminate stored food with carcinogenic aflatoxins.

Another interesting species of concern regarding human and animal health, and the focus of this study, is *Aspergillus fumigatus*.

2.5 *Aspergillus fumigatus*

Aspergillus fumigatus is a saprotrophic mold of ecologic importance and well defined morphological characteristics. This species plays an important role in the degradation of organic matter and has an impact on the carbon and nitrogen cycling in a wide variety of ecosystems (44, 45, 46) This species produces a massive amount of small, lightweight spores that are easily airborne.

When grown on agar, colonies of *A. fumigatus* are usually slightly raised in elevation, circular in shape with an entire margin, and greyish green in colour with a white margin (fig. 3). Some key features of this fungi are the club-shaped conidiophore with uniseriate phialides on the upper 2/3 of conical vesicles. The globose conidia are small, lightweight, and easily airborne, and are produced in long chains. The morphology of the conidial heads makes these chains stick together as columns growing from the terminal vesicles.

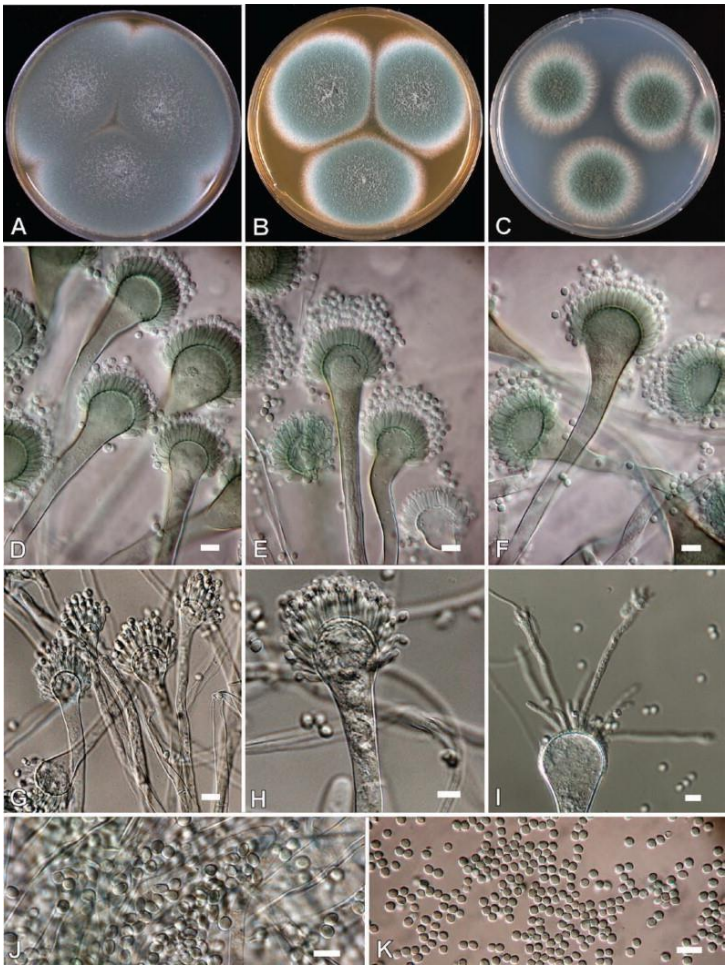


FIGURE 3 ASPERGILLUS FUMIGATUS. A-C. COLONIES ON VARIOUS MEDIA – A. CYA. B. MEA. C. CYA 37 °C; D-I. CONIDIOPHORES; J-K. CONIDIA. SCALE BARS = 10 MM. (ORIGINAL FIGURE FROM SAMSON ET AL. 2007)

This species of fungi is known to be thermotolerant with optimal growth observed at 37° C - 42° C. The conidia can survive at very high temperatures, though reduced ability to germinate has been observed with prolonged exposure to such temperatures above 55° C. The ability to tolerate high temperatures can be considered a contributing factor for the capability to colonize a diverse selection of environments.

2.5.1 *Aspergillus fumigatus* as pathogen

A. fumigatus is known for being one of the most prevalent opportunistic fungal pathogens in immunocompromised hosts. The massive number of small spores produced by this species contributes to the invasion of potential hosts through inhalation where the small spores penetrate deeply into airways where it can reproduce.

A. fumigatus has the ability to grow and germinate at temperatures as high as 45-55° C, and the conidia is able to remain intact at temperatures as high as 70° C (47). This fungus is known to thrive in compost piles where temperatures can reach above 40°C, and show high rates of growth and germination at 37-42°C *in vitro* (1, 48). This adaptation makes the human body a favorable habitat for *A. fumigatus* (47, 49).

Having a favored carbon source can be a factor that reduces virulence in fungal pathogens by reducing competitiveness. Wild types of *A. fumigatus* are prototrophs able to make more complex nutrients while growing on minimal medium, and show a higher virulence compared with auxotroph mutants growing on similar medium. *A. fumigatus* has a responsive metabolic programming system adapting to quality and quantity of carbon and nitrogen sources, a quality that is enabling the fungus to be a successful opportunistic pathogen (1).

Studies have highlighted 18 secondary metabolites produced by *A. fumigatus* that serve as a chemical defence system (50). Among these are the fungal toxin with potential immunosuppresive properties, classified as an epithiodioxopiperazin, called gliotoxin. This mycotoxin is characterized by the presence a di-sulfide bridge linking between carbon 3 and 6 of a diketopiperazine core structure (51, 52, 53, 54). Studies show that a large amount of clinical isolates produces this toxin, and that clinical isolates produce a larger amount of toxin compared with environmental isolates (42, 55). Although *A. fumigatus* primarily infects host with already compromised immune system, evidence points towards production of gliotoxin being one of significant factors for its virulence by further inhibition of immune responses (53, 56)

2.5.2 Types of Aspergillosis

As already mentioned the term aspergillosis refers to a range of infections produced by *Aspergillus* species. They can be divided based on the scale and seriousness of the infection into noninvasive and invasive forms (57, 58).

The non-invasive forms of aspergillosis are:

- **A**llergic **B**ronch**o****p**ulmonaric **A**spergillosis (ABPA)
  ABPA refers to an exaggregated immune response to the presence of conidia from Aspergillus in the airways. Individuals suffering from asthma or cystic fibrosis are particularly susceptable to this type of infection. The infection manifests primarly as symptoms such as recurring lung infections, shortness of, breath and coughing;
- **A**llergic **F**ungal **R**hino**s**inusitis (AFRS)
  AFRS is analogous to ABPA by being a chronic inflammatory disorder of the respiratory tract. The symtoms include chronic rhinosinusitis with nasal polyps, inhalant atopy, and sinus-obstructing inspissates that contain sparse fungal hyphae.

The invasive forms of aspergillosis include:

- **I**nvasive **A**spergillosis (IA)
  A potentially mortal infection that most commonly affects individuals with severly compromized immune systems such as advanced HIV/AIDS, or patients undergoing transplants or chemotherapy. IA occurs when the fungus infects the bloodstream or hosts tissue, and can spread as a systemic infection or localize as a cerebral or pulmonary infection;
- aspergilloma

This infection can occur in lung tissue has been previously damaged from diseases such as tubercolosis or chonic obstructive pulmonary disease, and manifests as a ball-like structure in the damaged parts of the lung that can cause recurrent respiratory infections, chest pains and coughing up blood.

Aspergillosis can also, in rare cases, manifest as cutaneous aspergillosis of the skin or soft tissues. These infections typically bring symptoms such as persistent nasal congestion and discharge and facial pain, or skin lesions and ulcers respectively. Similar to other variants of aspergillosis, these infections occur typically in individuals with already compromised immune system or undergoing invasive medical procedures.

2.6 Azole overview

Azoles are a class of organic antifungal compounds characterized by five-membered rings containing carbon and nitrogen atoms. These compounds exhibit robust efficacy against fungal pathogens and are widely used clinically and agriculturally (13). Their incorporation into clinical practice began in the mid to late 1900s, introducing first and second-generation azole drugs. Challenges including limited spectrum, pharmacokinetic interactions, and bioavailability arose, accompanied by side effects like nausea and cardiotoxicity due to formulation difficulties (59).

Triazoles emerged as a transformative breakthrough by replacing imidazole with a triazole ring, enhancing efficacy, expanding the spectrum of action, and reducing side-effect risks. Yet, some triazoles faced ineffectiveness against certain moulds such as *Aspergillus* and induced resistance in *Candida* species. Advancements since the early 2000s have refined the pharmacokinetics of triazole as well as safety profiles, and broad activity spectrum against resistant fungal infections. Itraconazole, voriconazole, and posaconazole are notable azole drugs demonstrating significant progress in antifungal development, displaying broad action spectra, and potency against moulds and resistant infections. The study will focus on investigating the interactions between these three antifungals and strains of *A. fumigatus*

2.6.1 Azole function and resistance

Azoles inhibit the growth of fungi by impeding the synthesis of ergosterol, a crucial component of the cell membrane, responsible for its fluidity. The cytochrome P450 sterol 14alphademethylase enzyme, encoded by homologous genes CYP51A and CYP51B, possesses an essential heme cofactor facilitating the conversion of lanosterol into ergosterol. Azoles attach to the enzyme's active site, obstructing the demethylation of the ergosterol precursor. This interruption halts ergosterol production, resulting in an accumulation of 14-methyl sterols like lanosterol, which acts as toxic intermediates. This accumulation negatively affects the cell membrane through the development of carbohydrate patches reaching the membrane and disrupts membrane structure and fluidity due to the lack of ergosterol. Consequently, the fungal cell cycle is impeded, leading to cell destruction and hindering fungal growth, ultimately causing the demise of existing fungi at the application site (60).

Research demonstrates a substantial increase in azole-resistant *A. fumigatus* strains since the late 1990s, with the frequency of resistant isolates rising from negligible levels to as high as 40% (61, 62)The prevalent mechanisms of environmental azole-resistance in *A. fumigatus* involve tandem repeat upregulation (TR) in the promoter region of cyp51a coupled with a point mutation in the CYP51A gene (63). Modifications in the target site of the 14alpha-demethylase enzyme, achieved through amino acid substitutions, reduce binding efficacy to the enzyme, consequently diminishing the antifungal effectiveness. Another mechanism linked to the CYP51A gene involves heightened gene expression, enabling successful ergosterol conversion despite the presence of azole drugs. This maintains the integrity of the cell wall, membrane, and fungal growth. Beyond the frequent CYP51A mutations, gene-phenotype associations have been observed on chromosomes 1, 8, and 71, as well as azole-resistant isolates lacking CYP51A polymorphisms. This suggests the existence of additional resistance mechanisms that might interact with established azole resistance pathways associated with alleles within CYP51A on chromosome 4. A mutation indirectly linked to the CYP51A gene but contributing to resistance involves the overexpression of efflux pumps. This mutation heightens azole resistance by reducing intracellular azole concentrations.

While the direct spread of resistant *A. fumigatus* strains from patients to the environment lacks conclusive evidence, genetic resemblances between environmental samples and clinical isolates indicate that aspergillosis involving resistant *A. fumigatus* strains is likely acquired from the environment. Thus, resistance development is more likely to occur within the environmental pathway rather than the clinical one (63, 64, 65).

2.7 Golf courses

The activity of golf has its roots back to 14th and 15th century Europe, where a combination of a moist and climate and terrain with native turf grasses made an environment for long distance games using balls and sticks. A close equivalent to modern golf was played in16th century Scotland, where the emergence of the first golfing societies with established rules. The terrain required virtually no maintenance to serve as courses for the activity as a result of grazing animals and climatic influences(22). As golf gained popularity during the 18th century, it spread into geographical regions that did not possess the climatic and cultural conditions to form similar terrains. This led to a demand for export of turf grasses to these areas. However, this required maintenance in the shape of fertilizer, mowing, and pesticides due to the lack of grazing animals and local conditions regarding nutrients and pests that the non-native turfgrasses did not have an adaptation to utilize properly or defend against.

The pesticides used on golf courses have been categorized as fungicides, herbicides, insecticides, and plant growth regulators, and there are ongoing attempts to calculate the posed risk for each of these categories (22). There are potential risks posed by pesticides in the shape of contamination of soil, aquatic ecosystems and groundwater, mortality in non-target species of pollinators, human health risks for workers and players, as well as a potential development of resistance by the use of broad-spectrum pesticides.

Fungicides are used on golf courses in Scandinavia, primarily to combat pink snow mold (*Microdochium nivale)*, and some fungicides containing azoles are approved for usage in Norway (66). According to a plant manager (personal communication) at The Norwegian Golf Association, the usage of azole fungicides are restricted to the golf greens, which constitutes around 2% of the total area of the golf course. The frequency of fungicide application is also restricted to twice a year, typically in early September and mid-October.

A study investigating potential pesticide risks linked with golf courses has identified fungicides as the category of pesticide with the highest amount of risk, and greens as one of the top parts of the golf fields linked with risk in Norway, as well as in parts of North America (20, 21, 22). The potential risks linked with pesticides leaching from golf courses and the highlighted hazards of fungicide usage on golf greens, makes the border zones surrounding golf courses a potential hot-spot linked with resistance development in fungi.

2.8 Antimicrobial testing

The World Health Organisation provides a definition of antimicrobial resistance (AMR) as a situation in which due to changes microbes '*no longer respond to medicines making infections harder to treat and increasing the risk of disease spread, severe illness and death*'. As a result, antimicrobial substances become ineffective and illnesses become increasingly difficult to treat (www.who.int).

Resistance screening in fungi is of great importance considering the increasing threat of resistant fungal pathogens to human and animal health and food safety. Resistance screening in fungi is attracting more focus in recent years as a response to constantly occurring reports pointing out cases of loss of susceptibility to popular medicines. Systematic screening of fungal isolates allows for timely identification of resistant strains, and facilitates the adaptation of treatment strategies as well as research into underlying mechanisms of resistance.

2.8.1 VIPcheck

VIPcheck is a simple and effective *in vitro* screening tool for determining resistance to a selection of azoles by observing the growth of colonies on agar (www.vipcheck.nl). The toolkit consists of a square plate with 4 numbered wells of agar containing the azole drugs itraconazole (1), voriconazole (2), posaconazole (3), and one growth control without azoles (4). This has been described as an effective way of screening resistance in samples of *A. fumigatus*, and several isolates can potentially be examined on the same plate. This tool provides only visual representation of resistance, and it is recommended to determine minimum inhibitory concetration (MIC) for isolates that develop colonies in VIPcheck wells 1-3.

2.8.2. E-test

E-test (https://www.biomerieux-usa.com/clinical/etest) is a valuable quantitative method used for determining the Minimum Inhibitory Concentration (MIC) of various antimicrobial compounds, including azoles. The E-test consists of a plastic strip containing an exponential

and continuous gradient of antimicrobial concentrations, which is placed on a dedicated agar medium. The agar plate, prior to placing the test stripe, has to be evenly covered with the tested microorganism.

After a certain growth time a drop-shaped inhibition zone is created around the strip by the antimicrobial diffusing into the agar, and levels of MIC can be determined where the inhibition zone intersects the test strip. Breakpoints of resistance for specific species has been established by EUCAST (https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals), and if the MIC established by the E-test exceeds the EUCAST breaking points, it can be determined that the microorganism is resistant to tested antimicrobial substance.

Spores from pure isolates of *A. fumigatus* can be tested using strips containing various compounds of azoles with this method, which makes it appropriate for determining MIC for VIPcheck pre-selected isolates.

2.8.3 Molecular methods of resistance screening

Molecular methods of resistance screening involve the detection of specific genetic markers or mutations associated with resistance to specific antimicrobial compounds. Polymerase chain reaction (PCR) is a technique used to amplify specific DNA sequences and often combined with sequencing techniques, can be used to determine the presence of elements related to resistance by targeting genes or specific mutations. This process has become a fundamental tool in molecular biology.

Sanger sequencing, also known as chain termination sequencing, is used to determine the precise order of nucleotide bases in a DNA molecule through the process of DNA sequencing. The process involves the use of DNA polymerase to amplify the target DNA region through PCR. During DNA synthesis, chain-terminating dideoxynucleotides (ddNTPs) that are fluorescently labelled are incorporated, representing each of the four nucleotide bases (A, T, C, G). This incorporation results in the synthesis of a series of DNA fragments of varying lengths, each terminating with a specific ddNTP. Capillary gel electrophoresis is then employed to organise the labelled fragments by size, making it possible for the instrument to read the fluorescence. By analysing fluorescent signals, the DNA sequence is determined. While newer sequencing technologies have emerged, Sanger sequencing remains a fundamental technique for studying precise sequences of genes, identifying genetic mutations, and analyzing entire genomes, making significant contributions to genetics research, diagnostics, and various fields of molecular biology.

2.9 Identification of fungi

Identification of fungi refers to the process of determining the taxonomic affinity of a specimen in question. This can be done through observation of morphology on various levels of magnification, or by investigating specific genetic markers, also known as barcoding sequences, or composition of genome sequence as a whole.

The visual, morphological methods of identification can be a favorable starting point in the identification process. This approach is based on observation of the morphology of the specimen, and comparison with established descriptions of a suspected species. Macroscopic observations deal with traits that is visible to the naked eye, which can include shape, size, color and texture of a specimen. Microscopic observations involve the observation of smaller parts of the fungi such as the shape of conidia, and structure of hyphae and reproductive structures, usually in form of microscopic slides observed under the microscope.

Molecular identification based on sequencing of molecular markers combines previously described PCR and sequencing techniques. There are a range of recommended molecular markers used for identification of fungi (67, 68). These molecular methods represent precise identification of fungi, and amplification specific genes using PCR has been successfully used to identify fungal species such as aspergilli down to at least species level.

Several molecular barcodes are recommended for identification of fungi from genus *Aspergillus*. The complete internal transcribed spacer region (ITS) is a universal fungal DNA barcode, and often a first choice for fungal identification (Stielow et al. 2015). Yet, in case of some taxons, including *Aspergillus* genus, sole ITS sequence does not provide sufficient information for identification. It is recommended to use a combination of molecular markers or techniques (morphological and molecular analysis). Recent analysis of the Aspergillus genus showed that use of molecular markers such as beta-tubulin (BenA, tub2 or beta-tub), calmodulin (CaM or cal) gene can provide sufficient information for *Aspergillus* isolates(43).

BenA gene encodes polypeptide proteins that form part of the microtubules in fungal cells. Due to its conservation across fungal species and the presence of informative sequence variations, it serves as a valuable genetic marker for fungal identification and phylogenetic studies.

CaM is a small, acidic protein that acts as a calcium receptor in eukaryotic organisms, including fungi. It plays a crucial role in regulating various cellular processes, including metabolic pathways and gene expression. This gene is used as a molecular marker to study the diversity and phylogenetic relationships among different fungal species (69).

Chapter 3: Materials and methods

3.1 Sample collection

The selection of golf courses was based on factors such as the use of azole fungicides in managing turfgrass, preference for 18-hole facilities, and geographical location. Most of the chosen courses were situated near untreated larger areas, providing well-defined border zones for investigation.

Due to constraints of antifungal usage like proximity to sources of drinking water or artificial grass for putting greens, confirmation of azole antifungal treatment was vital. Information from the Norwegian Golf Association's plant manager confirmed that the golf courses selected for this study were indeed treated with azole antifungals two times annually, and that azole

antifungal use was restricted to putting greens. This accounts for approximately 2% of a golf course's total area. Consequently, the soil sample collection was focused on the border zones surrounding the putting greens.

The annual azole antifungal treatment is administered in two rounds, common practice starts with initial application taking place in early September and the second application in the middle of October. In order to examine any contrast in azole susceptibility prior and post antifungal treatment, the soil samples were collected in two rounds; first round post azole treatment (round1), and second round prior to azole treatment (round2).

The samples were collected using a standard tablespoon that was sterilized between samples, using 80% alcohol and a lighter. The spoon was enveloped in paper towel soaked in 80% alcohol and placed within a plastic bag to maintain a relative sterile environment during transport. Given that transportation occurred on foot or using public transit like buses, trains, or trams, preserving sterility was critical. Certain aspects of the fieldwork took place during the operational hours of the golf courses. Precautionary measure in the shape of a PRO TEC Classic Certified helmet was put in place to mitigate the risk of potential head injuries resulting from the impact of a golf ball, which could considerably impede the capacity to conduct further examination of the gathered samples.

Soil samples were collected from the border zone, around 10-20 meters away from the selected putting greens, and stored in small cardboard boxes. In optimal scenarios, the soil was gathered at a depth of 2-4 cm from an exposed dry soil area measuring 1m x 1m. However, due to the wet weather encountered during early autumn, certain samples contained a notable amount of moisture. These samples needed thorough drying before progressing to the subsequent phases of isolating and cultivating fungal colonies. In some instances, sections of the border zone was partially or completely covered in grass, and required some removal of grass to access the underlying soil. Regardless of the extent of grass coverage and moisture, a 1m x 1m area was selected for all samples in order to keep true to the “ideal conditions”-protocol. Once samples were collected, they were stored at temperatures between 0-5°C until the complete round of field work was finished and the samples had dried sufficiently for the subsequent stages of analysis.

3.2 Isolation and subculturing

A small portions of the soil samples were transferred into a mortar and lightly grounded into a fine sand texture with a pestel. Two gram of the homogenized sample was put into a 10 ml Falcone tube together with 8 ml of MilliQ water in order to make a spore solution. The solution was vortexed and left for sedimentation to occur for 15-20 minutes, before gathering 100 µl of supernatant onto a DG18 agar plate (ingedients per 1L: Dichloran-Glycerol Agar Base – 31,5g; glycerol 85% – 220 g; trace metal solution for CZID and others – 1 ml; chloramphenicol (50 mg/ml) – 1 ml; chlortetracycline (5 mg/ml) – 10 ml). The solution was spread uniformly onto the plate using an L-stick and let dry completely in order for the solution to be absorbed into the agar before incubating at 42º C. Samples were monitored for emerging colonies every 24

hours. The type of agar and temperature for incubation allowed selective cultivation of thermotolerant fungi from soil.

Emerging colonies resembling *A. fumigatus* macroscopic morphology were examined by pressing a clear tape with the adhesive side towards the youngest parts of a fungal colony, washing away excess spores with 70% EtOH, and coloring the collected fungal parts with lactofucsine in order to observe microscopic morphology (fig. 4). Colonies with both macro- and microscopic morphology matching the known characteristics of *A. fumigatus* were subcultured into malt extract agar (MEA) plates (ingredients per 1L: malt extract – 20.0 g; bactopeptone – 1 g; glucose – 20 g; agar – 20 g) using a sterile swab and monitored for further emerging colonies.

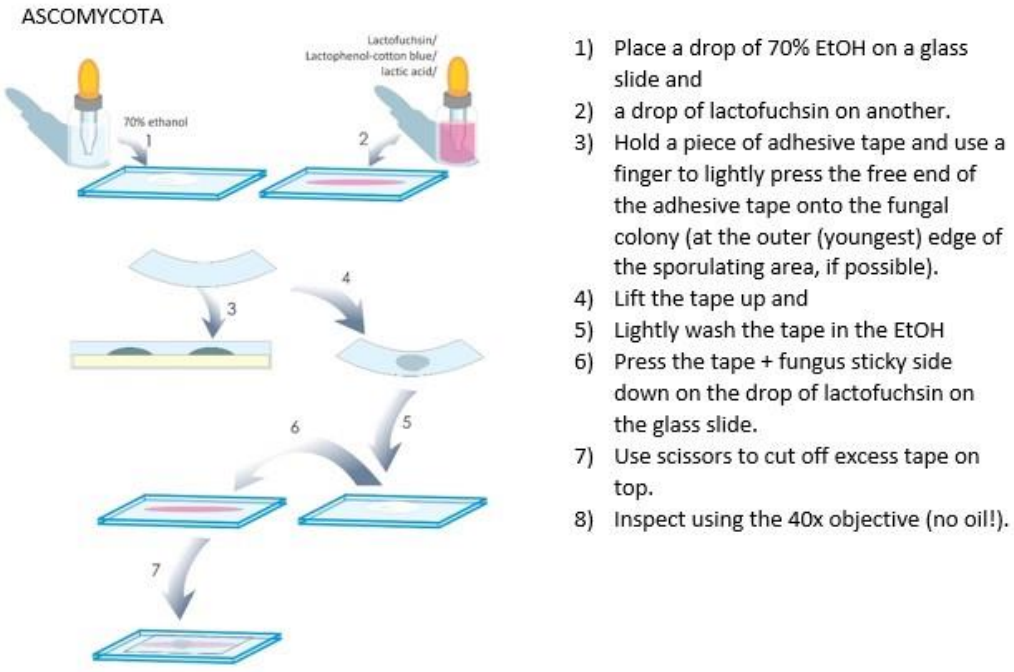


FIGURE 4 TAPE-TOUCH METHOD DESCRIPTION (MADE BY I. SKAAR)

Pure subcultures of each isolate were stored in two 1 ml tubes and 10% glycerol; one at 5±1º C for further examination in the lab, and another at -75±1º C for long term storage. Pure colonies of the stored fungi can be grown from the spore solution for approximately 24 months when stored in such conditions.

3.3 VIPcheck

VIPcheck is an uncomplicated tool for detecting azole resistance in *A. fumigatus*. Spores from the pure secondary cultures were used to prepare a spore solution. Preparation steps included gathering spores with a sterile swab and suspending them in 5 ml tubes with MilliQ water until the solution reached a value between 0.5 and 2 MacFarlane measured using a densitometer. A single drop of the solution was transferred to each of the four wells using a pipette, and incubated at 30±1°C. The wells were monitored after 24 hours and 48 hours to check for growth.

Levels of growth in the test wells were compared with growth in the control well for all samples and graded on a scale from no growth (0), some growth (1), medium growth (2), and high growth (3).

3.3.1 Pooling methods

Round 2 from the field work was screened for resistance using 1 VIPcheck per sample, and round 1 was screened using a method of pooling several samples into one VIPcheck. Due to the lack of a proper protocol and the drive to minimize the medium necessary for future screenings, two different methods of pooling were developed and tested. Both methods used followed the standard protocol of resistance screening with VIPcheck, but with individual modifications.

The method of pooling several samples works on the standard principle that the azoles will prohibit growth of susceptible samples, and only the resistant strains will emerge. Growth of fungi in test well has to be considered accordingly – as an indicator of resistance of one or more samples mixed together in the tested solution. Such samples need to be further screened separately using E-test to identify the resistant isolate.

In both tested methods, five isolates were pooled for the VIPcheck test. In method1 5 individual spore solutions were prepared, one for each isolate, and mixing equal volumes into one microtube at a given concentration measured using a densitometer. Afterwards, one drop of the pooled solution was transferred onto the different wells in the VIPcheck plate.

Similarly, in method2 five different spore solutions at the same concentration were prepared. Instead of pooling the solutions, 20 µl from each solution was transferred onto each of the VIPcheck wells. The volume was chosen based on an estimation that the volume of a standard drop is 100 µl, thus transfer of 5 × 20 µl provides a standardized test volume in each of the wells. The samples selected for the testing of methods included 4 strains from round 2 of screening, previously identified as a susceptible for azoles present in the VIPcheck, combined with (1) a resistant strain from a previously conducted project (the BARNS project), (2) a positive control – a reference resistant strain, and (3) a negative control – a reference susceptible strain. Results from both methods were compared in order to identify the one that provides the most accurate results.

3.4 E-test

Performance of the batch of agar and E-test stripes had to be confirmed prior to analysis of the samples obtained through the project.

Controls were prepared using *Candida parapsilopsis* ATCC22019, *Candida albicans* CCUG 3272 and *Candida crusei* CCUG 35869 of known MIC values. If the MIC values were within the provided value range batches of both – agar and E-test were considered effective.

Using spores from the glycerol spore stock, new pure colonies on agar plates were prepared. After ca. 48 hours of growth a thick spore solution (>3-5 MacFarland) was prepared by using a sterile cotton swab to collect spores from each colony and suspending them in sterile salt water. New solutions at 0.6 MacFarland were made by pipetting small volumes of the thick solution into new tubes of sterile salt water until reaching the target optical density. The 0.6 MacFarland solution was poured onto two RPMI agar plates and the excess liquid was removed using a pipette. Plates were left to dry in a laboratory cabinet until the spore solution had been absorbed into the agar.

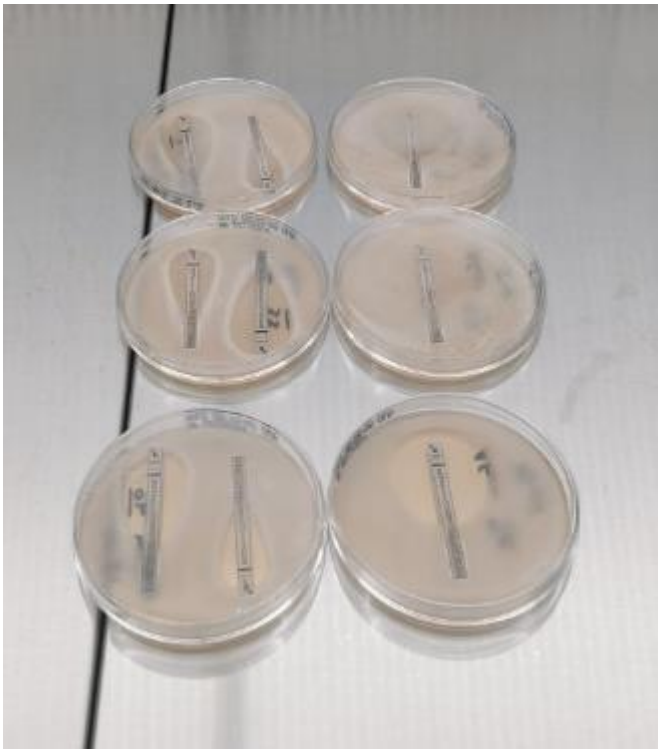


FIGURE 5 E-TEST ON RPMI PETRI DISHES WITH A. FUMIGATUS

E-test strips containing itraconazole and posaconazole were placed with an antifungal gradient oriented in opposite directions (fig. 5) on the same agar plate and one strip containing voriconazole was placed in the middle of another plate. This was repeated for each of the isolates. Plates were incubated in plastic bags with a wet piece of tissue at 37±1° C and examined for growth after 24 and 48 hours.

3.5 Molecular identification of strains

3.5.1 DNA extraction

Spore solution from the glycerol spore stock were incubated at 25±1° C on MEA agar until favorable amount of growth and sporulation. Into a 2 ml test tubes 350 µl of AL-buffer (Qiagen) and a single sterile steel ball were added. Sterile cotton swabs were soaked in a buffer in the test tubes to allow easier collection of the fungal material (mostly spores) from the fungal colony surface. Swab with collected material was inserted into the prepared test tubes and spinned in order to release fungal spores into the buffer. A control extraction blank control (EBK) containing MilliQ water instead of fungal matter was prepared for every 11th sample.

The solution was homogenized using a RETSCH® TissueLyser at 25 Hz for 3 min. After a short spin, a 10 µl of 20 mg/ml proteinase K was added, and the tubes are placed into the thermomix at 56° C, at 550 rpm for 25 min. The tubes were centrifuged at 12000 x g for 5 min, and 200 µl of the supernatant was transferred into a fresh 1.5 ml tube. If necessary the samples were stored at –20±1° C until further steps in the extraction protocol.

The QIAcube Connect by QIAGEN® was used to perform the extraction of DNA from the fungal prepared fungal matter. The program was set to QIAcube DNA mini kit – Tissue – Standard. Tubes with homogenized fungal matter and the control EBK, as well as the necessary reagents were place as instructed by the manufacturer protocol. Extracted DNA was stored at –20±1° C prior to next steps.

3.5.2 PCR and visualization

Betatubuline was selected as a target gene for molecular confirmation of fungal identity. Primer set Bt2A and Bt2B was used. A master mix containing MilliQ water and both primers (concentration of 5 µM) was prepared using a chart in excel to calculate the amounts of each reagent according to the table 2.

TABLE 2 COMPOSITION OF MASTER MIX USED FOR BENA AMPLIFICATION

Reagent	Volume [µl]
MilliQ water	7
Bt2A	3
Bt2B	3
PuReTaqTM Ready-To-GoTM PCR -	-
DNA template	2
	25

TABLE 3 PCR CYCLING CONDITIONS FOR BENA AMPLIFICATION

Step		Temperature	Time
Initial denaturation		95° C	5 min
Denaturation	Repeat 35 times	95° C	30 sec
Annealing		58° C	20 sec
Elongation		72° C	30 sec
Final elongation		72° C	5 min

Into microtubes provided in the PCR kit GE Healthcare Illustra ™ PuReTaq Ready-To-Go™ PCR 23 µl of master mix and 2 µl of DNA template were added. The microtubes were placed in Bio-Rad 96-well T100TM Thermal Cycler PCR and the PCR reaction was conducted as presented in table 3. PCR products were stored at –20±1° C until further analysis.

3.5.3. Gel electrophoresis

Gel electrophoresis is, among other applications, a common method of verifying success of PCR reactions as well as measuring the size of DNA fragments. This is done by using an electric

current to disperse DNA fragments inside a solidified agarose gel matrix, and then comparing the location of the dispersed fragments and intensity of the bank to a DNA ladder containing a defined DNA fragments. The viscous drag of the gel causes smaller molecules to travel faster across the gel compared with larger molecules.

The gel was prepared by mixing the agarose and TBE buffer into an Erlenmeyer flask at a ratio of 1.5 g agarose per 100 ml of TBE buffer and microwaving the mix until obtaining a clear solution. 7 µl of GelRedTM (Sigma-Aldrich, St. Louis, Missouri) per 100ml gel was added to the flask, the well-forming gel comb was inserted into the cassette, and the contents of the flask are poured into the cassette.

After solidification (ca. 20-25 min) the gel was set into electrophoresis tank filled with TBE buffer. Three microliters of DNA-ladder were loaded into the first and last well of the gel. Five microliters of a PCR product mixed with 1 µl of 6x loading buffer were added to the remaining wells. The electrophoresis was run at 90V for 30-60 minutes. Afterwards, a picture of the gel was taken with AzureTM c150 Geldoc instrument.

3.5.3 Sequencing and sequence analysis

Sequencing of each successfully amplified product was done by Eurofins Genomics (Ebersberg, Germany). PCR solutions were diluted with MilliQ water until reaching a total volume of 30 µl, and subsequently were divided into two equal volumes and pipetted into a 96tubes test plate labelled with a barcode (provided by Eurofins Genomics). Sequencing was performed with the same primer pair as for the PCR reaction in both directions.

3.6 Phylogenetic analysis

After quality control and trimming of the ends of one-direction sequences, the forward (FWD) and reverse (REV) sequences were used to create consensus sequences with Geneious Prime 2023.2 software. Consensus sequences were used as a query for Basic Local Alignment Search Tool – BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) that compared the obtained sequences with a sequences deposited in GenBank. Multiple sequence alignments (MSA) were performed using MAFFT v7.490 plugin in Geneious Prime software for sequences obtained as a result of this project and selected sequences from Houbraken et al., 2020 (mainly section Fumigati).

For phylogenetic analysis the CIPES Science Gateway was used (https://www.phylo.org/). First the best substitution model was calculated with ModelTest-NG (0.1.7). MSA file was used for phylogenetic interference with RAxML-NG (1.20), using calculated substitution model. Resulting phylogenetic tree was visualized and edited using FigTree v1.4.4 and InkSkype 1.3.

Chapter 4: Results

4.1 Isolation of Samples and Identification

A total of 86 soil samples were collected in two rounds from 11 different golf courses. Among these samples, 37 were obtained from 9 courses in the district of Follo, Viken county, while 6 samples were collected from 2 different courses located in Bodø municipality, Nordland county. Each golf course provided between 6 to 10 soil samples for analysis.

During the primary isolation process, numerous mould and yeasts colonies grew on the agar plates from the soil samples. However, microorganisms not relevant to the study and were not further identified. Through the observation of colony morphology as a whole and microscopic infection, colonies of *A. fumigatus* were identified in at least one sample from each golf course, resulting in a total of 77 strains (fig. 6).

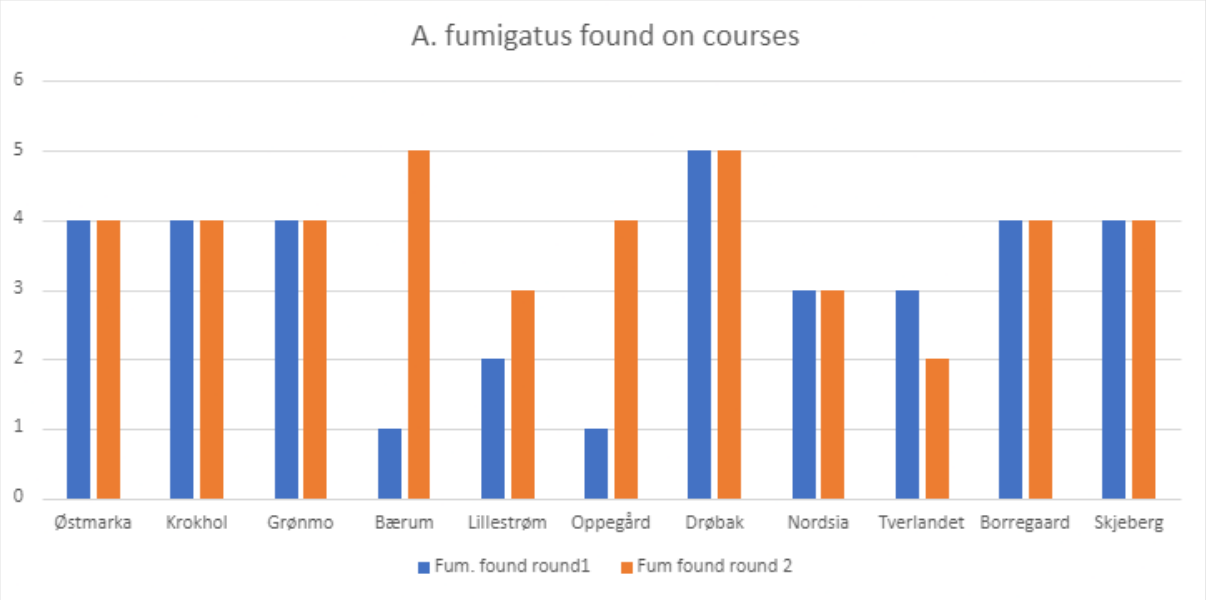


FIGURE 6 CHART COMPARING AMOUNT OF SAMPLES OF ASPERGILLUS FUMIGATUS ISOLATED FROM SOIL SAMPLES FOUND IN ROUND1 AND ROUND2 OF FIELD WORK.

The genetic marker BenA was amplified as a step in molecular verification of the collected samples. Validation of the PCR amplification across all samples was visualized and confirmed with gel electrophoresis (fig. 7). After the first PCR series, single bands were obtained from all specimens, except for samples 9A and 1B. Consequently, a subsequent round of PCR was carried out on these particular samples. These results indicated the absence of a band for EBK, while the PCR products displayed bands exhibiting slight variations of brightness, but consistent with the established size of BenA placing between 400-600 bp DNA marker bands.

Obtained sequences of BenA were between 479 and 577 bp length. The consensus sequences generated in Geneious Prime were subjected to analysis using BLAST, resulting in a 100% match with sequences that showed significant alignment with *A. fumigatus*.

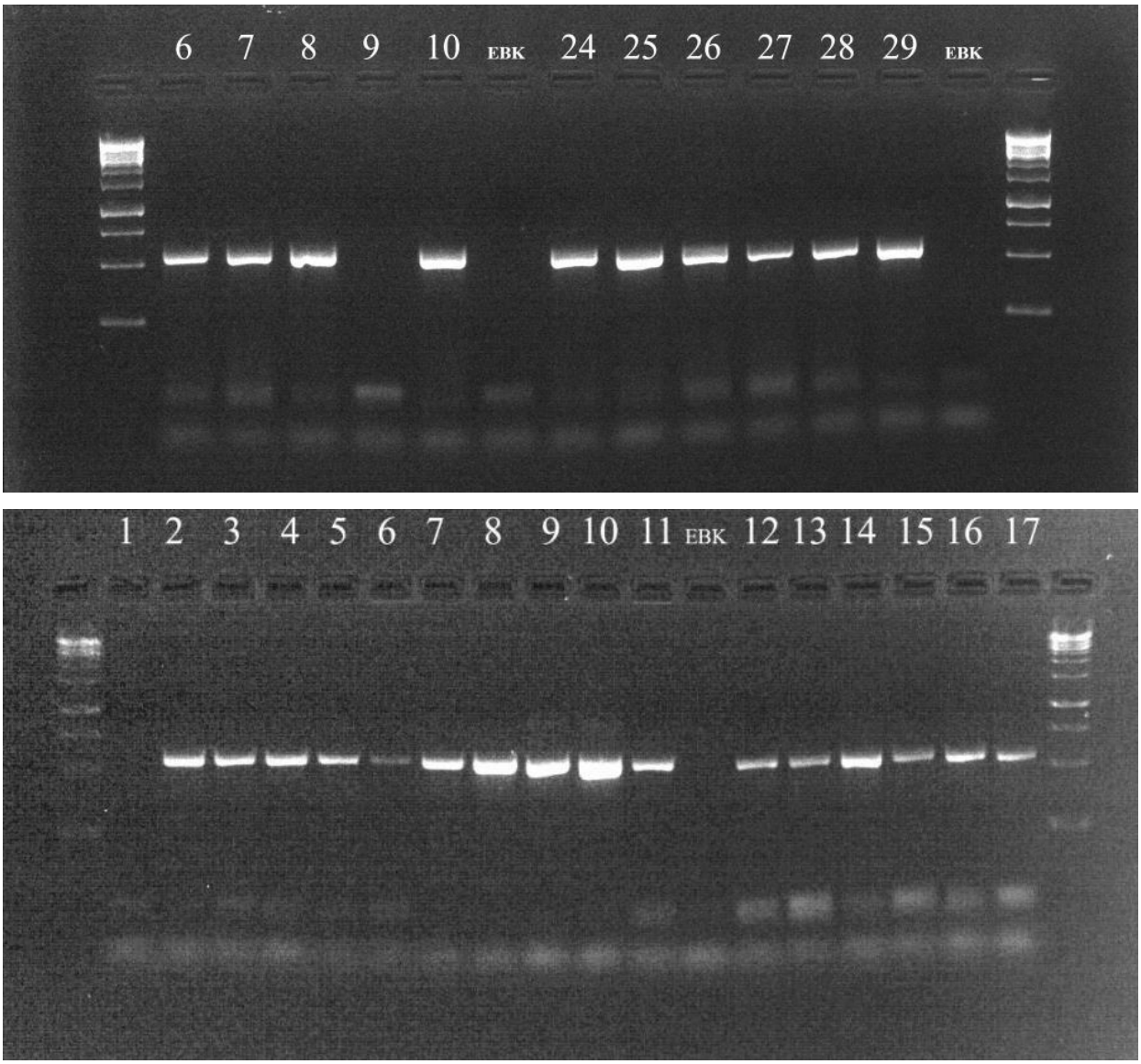


FIGURE 7 IMAGES OBTAINED FROM GELDOC (FIND PROPER NAME HERE) SHOWING RESULTS OF INITIAL ROUND OF PCR FOR ELECTED SAMPLES FROM ROUND 1 (A), AND ROUND 2 (B), AS WELL AS THE EXTRACTUIN BLANK CONTROL(EBK) CONTAINING MILLIQ WATER WITHOUT ANY EXTRACTED DNA.

A maximum likelihood tree was created using selected sequences from Houbraken 2020 (fig. 8). A total of 150 sequences, including sequences generated in the course of this project, and sequences representing section Fumigati were used. Additionally, *A. cejpii* CBS 157.66 (section Vargarum) sequence was used as an outgroup.

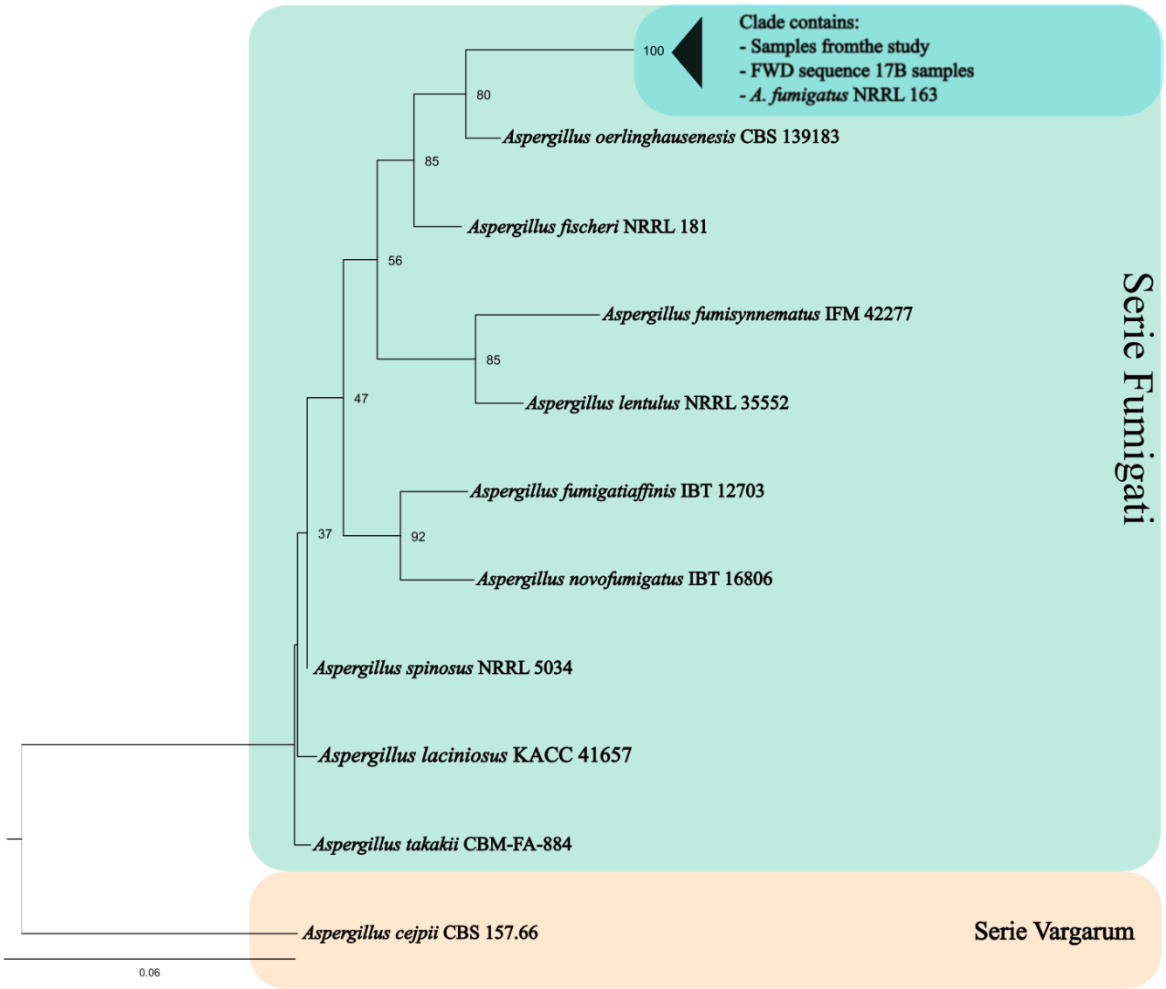


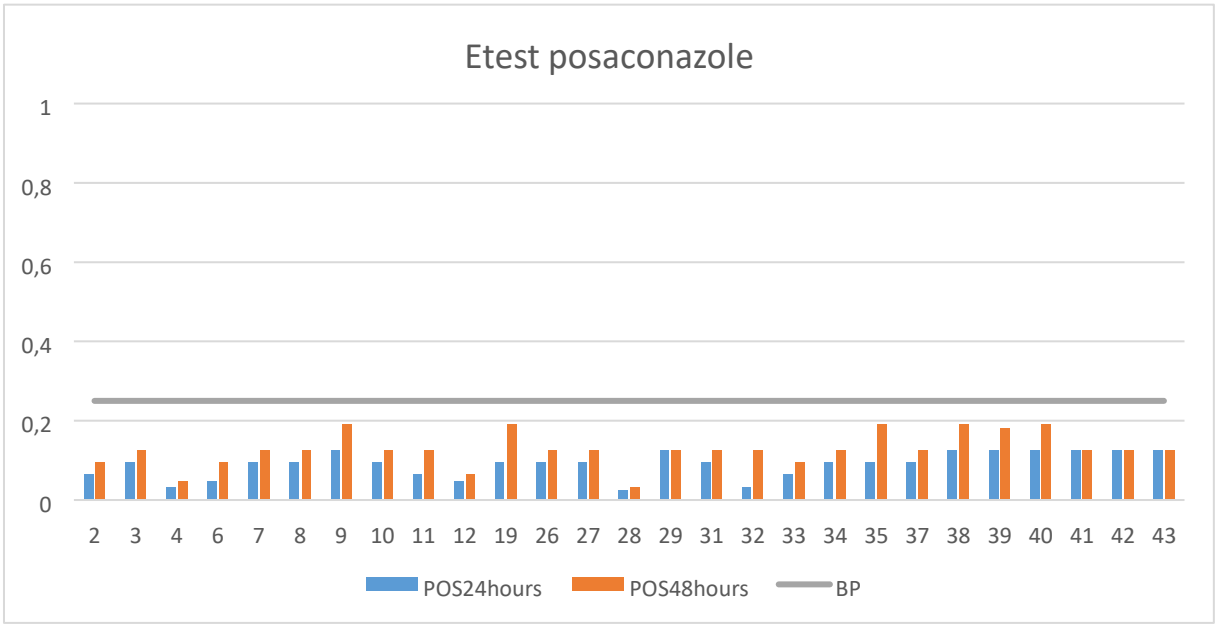
FIGURE 8 MAXIMUM LIKELIHOOD TREE. NUMBERS NEXT TO THE NODES REPRESENT THE BOOTSTRAP VALUE.

4.2 Resistance screening

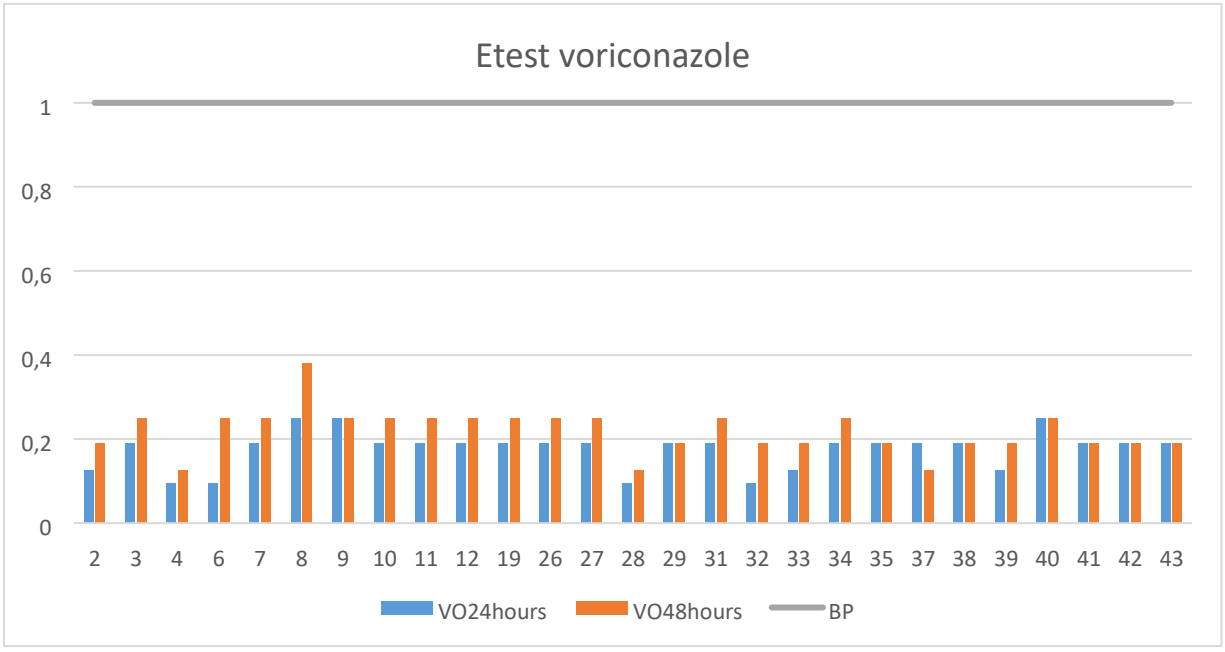
In the screening of samples isolated during round 1, resistance was detected in 27 samples using the VIPcheck method. However, when subjected to E-test, none of these samples showed growth above the resistance breakpoint for any of the tested azoles (fig. 9). Nonetheless, 13 samples exhibited growth close to the breaking point of resistance for itraconazole, with growth at or above the value of 0.75 µg/ml. Conversely, the resistance screening of isolates from round 2 did not reveal any growth, indicating that they were all susceptible to the azoles present in the VIPcheck. While a few samples displayed development of minute colonies in some wells, subsequent E-test analysis showed no growth near the resistance breakpoint.

In summary, no observations of resistance were made in any of the collected samples during the investigation.

A)


Etest posaconazole
1
0,8
0,6
0,4
0,2
0
2 3 4 6 7 8 9 10 11 12 19 26 27 28 29 31 32 33 34 35 37 38 39 40 41 42 43
POS24hours
POS48hours
BP

B)


Etest voriconazole
1
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VO48hours
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C)

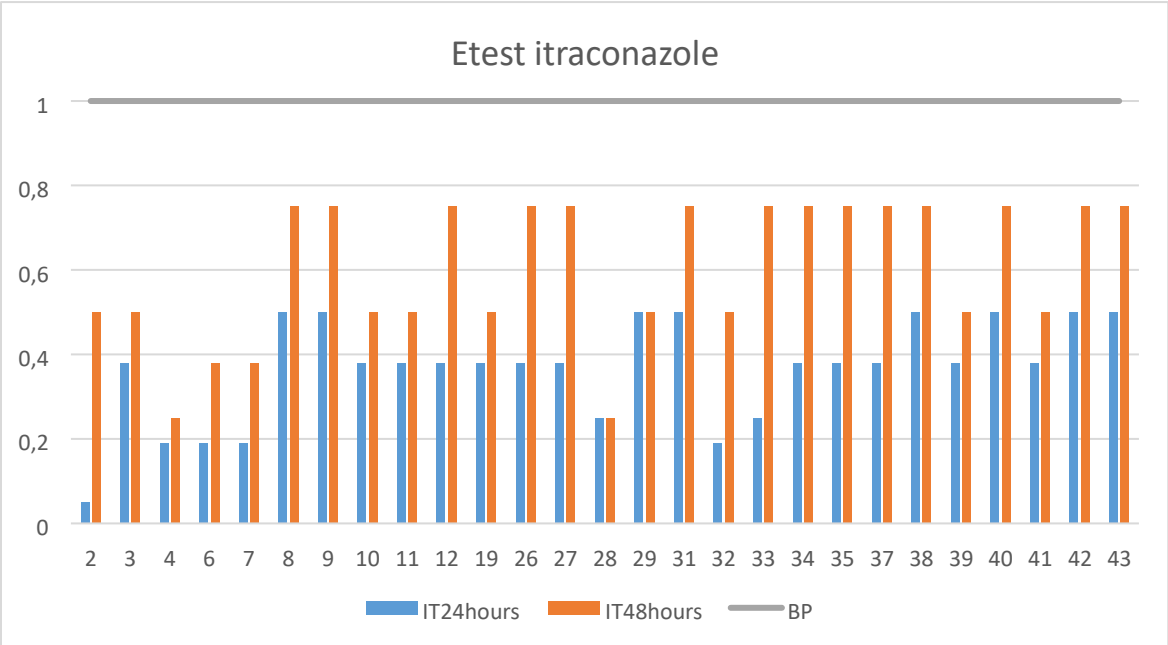

FIGURE 9 CHARTS SHOWING LEVELS OF SUSCEPTIBILITY IN SAMPLES SELECTED FROM ROUND1 TO A) POSACONAZOLE (POS), B) VORICONAZOLE (VO), AND C) ITRACONAZOLE (IT), MEASURED AFTER 24 HOURS AND 48 HOURS. BREAKPOINT FOR RESISTANCE IS MARKED WITH THICK LINE (BP)

4.2.1 Results pooling methods

The different methods of pooling samples had an impact on the ability to detect resistant samples when susceptible and resistant samples are combined. Method 2 proved superior by detecting both of the resistant strains combined with the susceptible ones, in contrast with the results produced by Method 1 that did not detect any of the resistant strains.

However, method 2 only detected the resistant strain in well 2 containing voriconazole and the control well, and no growth indicating resistance was detected in well number 1 and 3. There were no differences in detection of resistant strains between the different concentrations used. The VIPchecks containing solutions of 0.5 MacFarland detected the resistant strain with equal rate of success, compared with the VIPchecks were 1 and 1.5 MacFarland was applied.

Chapter 5: Discussion

The Navazole project description indicates that there are gaps in understanding the epidemiology of azole resistance in Norway. Evidence suggests that the primary route of resistance development is through environmental pathways rather than clinical ones, as

indicated by van der Linden in 2013(70). There have been instances of identifying resistant strains of *A. fumigatus* both in the environment and among patients(71). This emphasizes the significance of monitoring possible hotspot areas to enhance our comprehension of the mechanisms driving the development of resistance.

The findings from this study did not indicate the presence of resistant strains in the border zones surrounding the greens. However, the susceptibility pattern displayed from does imply that fungi in these areas show decreased vulnerability to azoles after azole treatment. The potential hazards associated with pesticide usage on golf courses have been evaluated in prior research conducted by Bekken in 2023, 2021, and 2022 (20, 21, 22). These studies have classified both putting greens and antifungals as potential high-risk categories in terms of area and type of pesticide, respectively. Taking into account the previously mentioned risk assessments along with the outcomes of this study, there is a valid reason to initiate further investigations into the fungal communities both within and around the green areas of the courses.

It was hypothesised that there would be fluctuations in the susceptibility profile of *A. fumigatus* that are associated with the timing of antifungal application in a specific area. Such patterns emerged in this investigation, where nearly all samples in round1 exhibited growth indicative of resistance. This stands in stark contrast to the outcomes of round 2 where no substantial growth was observed in wells containing azoles. The administration of the E-test to samples in round 2 was carried out almost as a procedural formality, intended to assess susceptibility levels in any samples with traces of growth. Upon initial inspection, drawing on previously collected data, this pattern suggests that susceptible strains of *A. fumigatus* might have been eradicated by the treatment, leaving a majority of resistant samples to prevail. This underscores the critical importance of sustained, long-term surveillance within these regions, aimed at examining the annual susceptibility profile of the fungal community within areas treated with azoles.

The method of pooling samples within one VIPcheck provided a method of resistance screening that saved both time and resources during the resistance screening performed in this study. The contrasting results produced by method1 and method2 did provide evidence that the method of pooling does impact the ability of the VIPcheck to detect resistant strains when combined with susceptible strains. A weakness in this method could be that the amount of spore solution applied exceeds the recommended volume, thus producing false positives. In this study, there was a noticeable amount of samples that were close to the breaking point of resistance. Perhaps the combination of 5 strains with low susceptibility could produce results mimicking that of a

resistant strain. The fact that method 2 performed equally with concentrations as low as 0.5 MacFarlane points towards a low amount of spores being necessary to detect resistant strains. Building on these results, one could hypothetically overcome issues regarding volume-based positives by using a lower volume per sample than in this study. Regardless, there are undeniable potential in reducing both time and resources spent by using a pooling-style method for resistance screening with VIPcheck.

Studies points out that *A.fumiga*tus thrives in compost heaps, and exposure to residual levels of azoles can create conditions that develop resistance (48, 72). During my fieldwork I observed several compost heaps in the border zones of the golf courses containing grass from the golf field. If we assume that these heaps contain grass from the greens as well as the rest of the course, we have favourable conditions for growth of *A. fumigatus* as well as exposure to residual levels of azoles which can drive development of resistant strains. A study into development of resistance within compost heaps containing trimmings from the golf courses could provide a beneficial contribution to the identification of hotspots of azole-resistance in these areas, and perhaps urge a request to find alternative handling of trimmings from golf courses. It is suggested that *A. fumigatus* does not survive forms of aerobic composting, as temperatures in such an environment can reach above 70° C (72). This is above the known thermotolerance for conidia of *A. fumigatus* at around 50°C(73, 74). Such methods of handling compost containing azole residues could provide solutions to potential hazards related to present day methods of handling golf course compost.

There were contrasting results both regarding resistance profile and amount of *A. fumigatus* found on the different courses in the different round of fieldwork. However, in opposition to the susceptibility profiles, there were a larger amount of *A. fumigatus* found in the second round of sample collection. One theory suggests that the utilization of antifungal agents eliminates a significant portion of the fungal population following their application, much like the presumed reasons behind the distinct susceptibility patterns. The diminished presence of *A. fumigatus* in the soil would likely manifest as a lower amount of colonies appearing after a primary isolation from soil samples. Temperature could be another factor behind the contrasting results. Some of the soil samples were gathered at temperatures approaching 0°C, and there has been reported a reduced sporulation in species of *Penicillium* and *Aspergillus* at these temperatures (75).

Reduced presence of *A. fumigatus* after antifungal treatment combined with reduced sporulation in the remaining fungi could very well explain the reduced numbers observed in round 1

compared with round2. However, it is interesting to observe that the golf course with the lowest amount of positive samples in round 1 (Bærum) had subjectively warmer conditions compared with other golf courses where the soil was practically frozen at the time soil extraction. This could point towards other factors such as soil moisture or composition being dominating factors for the composition of the fungal communities.

Chapter 6: Conclusions

In this study, we have investigated the effects of the use of azole antifungals on resistance in *A.fumigatus* in the border zones of golf courses. The results of the study did not reveal any resistant strains of *A.fumigatus.* However, the results did point towards there being a fluctuation both in amount of *A. fumigatus*, as well as susceptibility profile of the fungi found in these areas relative to the time of antifungal application. These findings are consistent with previous studies in the field suggesting such fluctuation, but does not support the main hypothesis of azole treatment driving development of resistance in the border zones of golf courses. Worth noting are studies emphasizing the favourable conditions within compost piles, alongside the notable presence of compost piles along the golf course periphery during sample collection, does point towards a point of interest for future studies.

The observations provided in this study underlines the demand for surveillance of such areas in order to identify hotspots for development of resistance, and aid in developing protocols for responsible use of antifungals.

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