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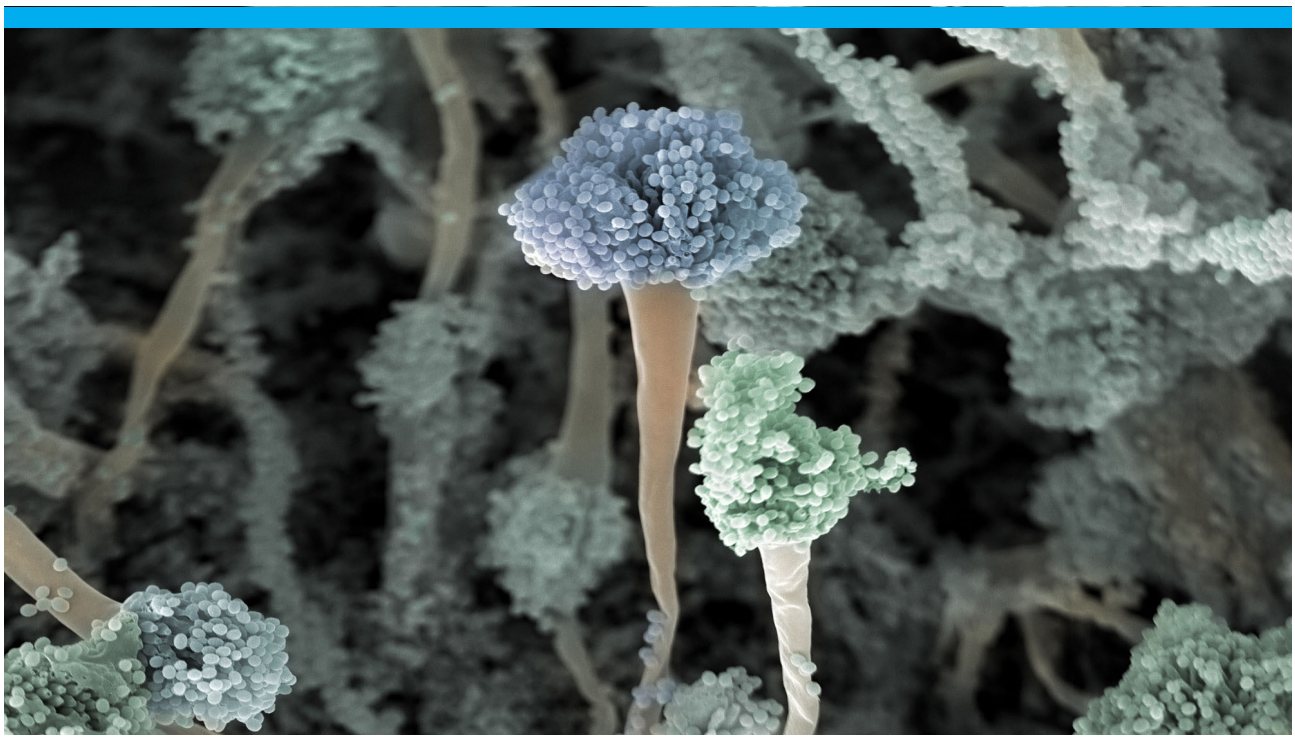
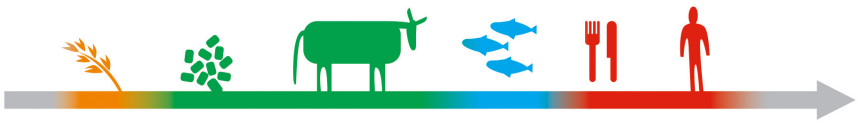
Knowledge and knowledge gaps on azole resistance in a One Health perspective





Veterinærinstituttet
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Azole resistance in a One Health perspective

Content

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Summary

The term antibiotic (which means "opposing life") refers to any substance produced by or derived from microorganisms that in dilute solutions can destroy or inhibit the growth of other microorganisms, including bacteria and fungi, in the treatment of infectious diseases [1-3].

This report is written as a part of the network project «Interdisciplinary think tank to minimize the emergence and spread of antifungal resistance» (ResAzoleNet) financed by the Norwegian Research Council. The report aims to give short overview of the current knowledge on azole resistance in a One Health perspective and to point out some major knowledge gaps from a Norwegian perspective.

When bacteria, fungi, viruses, and parasites are exposed to antimicrobial drugs, they are forced to attempt to develop resistance against the drugs developed to kill them. Although there is intense spotlight put on antimicrobial resistance (AMR), the focus is almost solely on antibacterial resistance. However, fungal infections and development of antifungal resistance are also emerging public health concerns as well [4]. It is thus crucial to immediately take action to prevent resistance emergence as we have witnessed in bacteria.

Azoles are efficient fungicides commonly used both to treat and prevent fungal diseases in humans and animals, as well as in food production, horticulture and wood industry. Residues of azoles in nature are regarded as environmental toxins and are suggested to have general endocrine disrupting properties. However, equally important, azoles in the environment drive the development of cross resistance in human pathogenic fungi, such as *Aspergillus fumigatus*; an observation that is confirmed by several international studies over the last decade [5-7].

Fungal infections caused by opportunistic fungi such as *A. fumigatus* kill more than one million people annually worldwide; at least as many as killed by tuberculosis or malaria [8]. Without effective antimicrobials for the prevention and treatment of infections, we will loose the advances achieved in modern medicine. Azole resistance is increasingly recognized as a problem in fungal infections and is consequently of growing concern globally.

Case fatality of patients with culture-positive azole-resistant invasive aspergillosis varies between 50% and 100% [8]. It is therefore crucial to immediately take measures against fungal resistance to prevent a situation such as the one we see in the development of resistance in bacteria. Unfortunately, awareness of fungal infections and fungal resistance as a global health problem is generally low. In Norway, this awareness is completely lacking, and the authorities focus primarily on antibacterial resistance in their action plans.

Climate changes are expected to lead to wilder, wetter and warmer weather. This means better conditions for fungi, and we can expect both new species and higher rates of fungal infections in plant production. Increased infection pressure will place higher demands on effective disease control in agriculture to reduce crop losses and to close the yield gap and feed the growing population by 2050. Since health, agriculture and industry are all heavily dependent on azole use, azole resistance is a true one health challenge. Surveillance studies have shown that, in areas to which azole resistance in *Aspergillus* is endemic, the environmental route of resistance selection contributes to >90% of resistance mechanisms in azole-resistant *Aspergillus* diseases [6, 9]. By understanding how azole resistance develops and establishes in the environment, effective measures that prevent resistance development can be designed and implemented. We have in this report identified some knowledge gaps and important measures that need to be addressed. Our work is intended to contribute to a necessary global initiative for revised regulations for the use of azoles in therapeutic treatments, agriculture and other purposes.

Summary in Norwegian – Norsk sammendrag

Begrepet antibiotika (som betyr "mot liv") refererer til ethvert stoff produsert av eller avledet fra mikroorganismer og som i fortynnede løsninger kan ødelegge eller hemme veksten av andre mikroorganismer, inkludert bakterier og sopp, i behandlingen av infeksjonssykdommer [1-3].

Denne rapporten er skrevet som en del av nettverksprosjektet «Tverrfaglig tenketank for å minimere fremvekst og spredning av antifungal resistens» (ResAzoleNet), finansiert av Forskningsrådet. Rapporten tar sikte på å gi en kort oversikt over den nåværende kunnskapen om azolresistens i et Én helse-perspektiv og påpeke noen store kunnskapshull fra et norsk ståsted.

Når bakterier, sopp, virus og parasitter eksponeres for antimikrobielle legemidler, drives de til å utvikle motstand mot legemidlene som er utviklet for å drepe dem. Selv om det er intenst søkelys på antimikrobiell resistens (AMR), er fokuset nesten utelukkende på *antibakteriell* resistens. Soppinfeksjoner og utvikling av resistens mot soppmidler er imidlertid også et folkehelseproblem av økende betydning [4]. Det er derfor avgjørende å iverksette tiltak umiddelbart for å forhindre antifungal resistens i å utvikle seg i et omfang slik vi har vært vitne til hos bakterier.

Azoler er effektive soppdrepende midler som benyttes til å behandle og forhindre soppsykdommer hos mennesker og dyr, samt i matproduksjon, hagebruk og treindustri. Rester av azoler i naturen betraktes som miljøgifter med mulige hormonforstyrrende egenskaper. Minst like viktig er det imidlertid at azoler i miljøet forårsaker et seleksjonspress som er en driver i utviklingen av resistens hos sopp som forårsaker infeksjoner hos mennesker og dyr. Dette er bekreftet i flere internasjonale studier i løpet av det siste tiåret [5-7].

Soppinfeksjoner forårsaket av opportunistiske sopp som *Aspergillus fumigatus* dreper mer enn 1 million mennesker årlig over hele verden; det er minst så mange som dør av tuberkulose eller malaria [8]. Uten effektive anti-mikrobielle midler for forebygging og behandling av infeksjoner mister vi de fremskrittene som er oppnådd i moderne medisin. Azolresistens er i stadig oftere et problem ved soppinfeksjoner, og er følgelig en kilde til økende bekymring globalt. Dødeligheten hos pasienter med kultur-positiv azolresistent invasiv aspergillose varierer mellom 50% og 100% [8]. Dessverre er bevisstheten om soppinfeksjoner og soppmiddelresistens som et globalt helseproblem generelt lav. I Norge mangler denne bevisstheten helt, og myndighetene fokuserer primært på antibakteriell resistens i sine handlingsplaner.

Klimaendringer forventes å føre til villere, våtere og varmere vær. Det betyr bedre forhold for soppen, og vi kan forvente både nye arter og flere soppinfeksjoner både hos planer, dyr og mennesker. Høyere smittepress vil stille større krav til effektiv sykdomsbekjempelse for å redusere avlingstap og redusere avstanden til avkastningsmålet som trengs for å fø den voksende befolkningen fram mot 2050. Siden helsevesenet, landbruket og industrien alle er sterkt avhengige av azolbruk, er azolresistens en sann Én helse-utfordring. Overvåkingsstudier har vist at i områder der azolresistens i *Aspergillus* er endemisk, bidrar miljødrevet resistensutvkling til > 90% av resistensmekanismene ved azolresistente aspergillusinfeksjoner. Ved å forstå hvordan azolresistens utvikles og etableres i miljøet kan effektive tiltak som forhindrer resistensutvikling utformes og implementeres. Vi har pekt på noen kunnskapshull og anbefalt viktige tiltak som kan bidra til et nødvendig globalt initiativ for reviderte forskrifter for bruk av azoler som medikamenter og som fungicider i landbruket og til andre formål.

Fungal infections

Fungi are a group of eukaryotic microorganisms characterized by growth as either yeasts or filamentous fungi. They are a natural part of our environment, either as commensals (*Candida* species) or in our environment where the moulds are present as spores and hyphae in air, soil and water.

Fungal infections in humans

In human medicine the majority of fungal infections are superficial, like vaginal or oral thrush and dermatophyte skin infections, while only a small number of severely ill hospitalized patients with underlying conditions and immune-deficiencies, are affected by invasive infections. Nevertheless, yeasts are among the top ten most common causes of nosocomial bloodstream infection [10] and fungi cause severe and deadly infections in millions of people each year worldwide; cryptococcal meningitis in HIV infected patients or chronic pulmonary aspergillosis (CPA) affecting patients with underlying lung disease or sequela after tuberculosis. Establishing estimates of the burden of fungal infections is an on-going global attempt and all countries have been encouraged to participate, facilitated by the Leading International Fungal Education (LIFE) portal [11]. In Norway we have a national surveillance of bloodstream infections of *Candida* species since 1991, with susceptibility data collected in the same time period. About 200 infections are registered annually [12].

No data on other fungal infections and no surveillance of other fungal species like *A. fumigatus* exist in Norway. Nevertheless, yeasts and moulds isolated from patients with severe infections are sent to the Norwegian Mycological Reference Laboratory at Oslo University Hospital for identification and susceptibility testing, but the number of specimens which are sent to the reference laboratory does not reflect the true burden of disease or the prevalence of antifungal resistance. To ameliorate the absence of systematic surveillance, estimates of burden of fungal disease in Norway were calculated [13].

Aspergillus

Aspergillosis is one of the most common fungal diseases in human medicine. Normally the fungus do not cause disease in humans, but individuals may develop allergy to the spores of *Aspergillus* or rarely an infection. One disease entity, allergic bronchopulmonary aspergillosis (ABPA), predominantly affects asthma patients, but also patients with cystic fibrosis and bronchiectasis may be affected. CPA affects patients with an underlying lung disease such as tuberculosis, previously treated lung cancer, sarcoidosis, emphysema and COPD [14, 15], whereas invasive aspergillosis (IA) affects severe immune-compromised patients secondary to cancer therapy or transplantation. Previously healthy influenza patients may also develop IA, during their intensive care stay, a feared and emerging complication [16, 17].

Fungal infections in animals

As in human medicine, the majority of fungal infections in animals are superficial. A wide variety of mycoses are seen also in Norwegian animals, including infections caused by dermatophytes, yeasts, Mucorales and *Aspergillus* species. At the Norwegian Veterinary Institute, fungal infections are diagnosed regularly in dogs, cats, cattle, horses, pigs and birds. Even though a broad range of infections are seen, ear infections (otitis externa) are probably the most common fungal animal disease that is treated with antifungal drugs. Traditionally, mainly superficial infections in animals were diagnosed and thus treated with antifungals, but the development in companion animal medicine have changed this practice. Especially sinonasal aspergillosis in dogs are regularly diagnosed and treated. Fungal infections are likely heavily underreported in animals, and we expect that the rapidly increasing quality in diagnostic tools in veterinary clinics in Norway will change the picture.

Plant diseases

Plant diseases have been threatening food production since biblical times. Blights and mildews were already mentioned by the Greek philosopher Theophrastus in 300 BC and mentioned in the Old Testament in ca 750 BC. They were feared as much as human diseases and war. It was not until the 1800s, that researchers found the cause of such plant diseases and applied the first inorganic compounds to reduce smut in wheat and mildews in grapes. The human famine caused by late blight of potatoes in 1845 to 1846 lead to the death of hundreds of thousands of people and the emigration of more than one and a half million people from Ireland to the United States of America. Until this very day, control of plant diseases is a high priority for food security and safety globally, as an estimated 16% of crop loss globally is associated with plant diseases.

Antifungal drugs

Only four major classes of antifungal drugs are available to treat invasive fungal infections in humans and animals. They include 1) pyrimidine analogs, 2) polyenes, 3) triazoles, and 4) echinocandins, of which only the latter three are available without restrictions in Norway. Allylamines, a fifth antifungal drug class, is licensed for superficial dermatophytic infections only.

Azoles

The azole antifungal drugs were first discovered in the early 1940s, with ketoconazole being the first orally active azole. Azoles are effective because they interfere with ergosterol biosynthesis by binding to one of the key ergosterol biosynthetic enzymes, Cyp51A. Ergosterol is a vital component of the fungal cell membrane, and without it the membrane fluidity is altered leading to fungal death.

Most medical antifungal drugs need to be prescribed by a physician, but fluconazole tablets (one-pack) and topical agents like clotrimazole or nystatin used to treat superficial infections are available over-the-counter (OTC) in Norway. The triazole fluconazole, by far the most widely used antifungal agent, has effect on yeasts and dermatophytes and is available as intravenous and oral formulation. The other drugs in the triazole class have broad-spectrum activity and voriconazole and isavuconazole have emerged as first-line therapies for IA. Posaconazole is widely used as mould prophylaxis in high-risk patient groups and itraconazole is used for chronic *Aspergillus* infections. A variety of antifungals is available for use in veterinary medicine. Ketoconazole, itraconazole, fluconazole, voriconazole, and posaconazole are all used to treat fungal infections in animals.

Antifungal use

The total use of antifungals in humans in Norway is low compared to the use in other industrialized countries [18]. Eighty percent of the antifungals are used *outside* hospitals and 90 % of the total use is due to fluconazole, 77 defined daily doses (DDD)/1000 inhabitants/year in 2017. The *total* use of systemic antifungals nearly doubled between 2004 and 2015, but decreased in 2017. Fluconazole use in the primary care increased between 2008 and 2017 (Figure 1). Not surprisingly the use of broad-spectrum antifungals tripled from 2008, but is still low and seems now to be fairly stable [18]. There are unfortunately no reliable estimates for veterinary use of azoles.

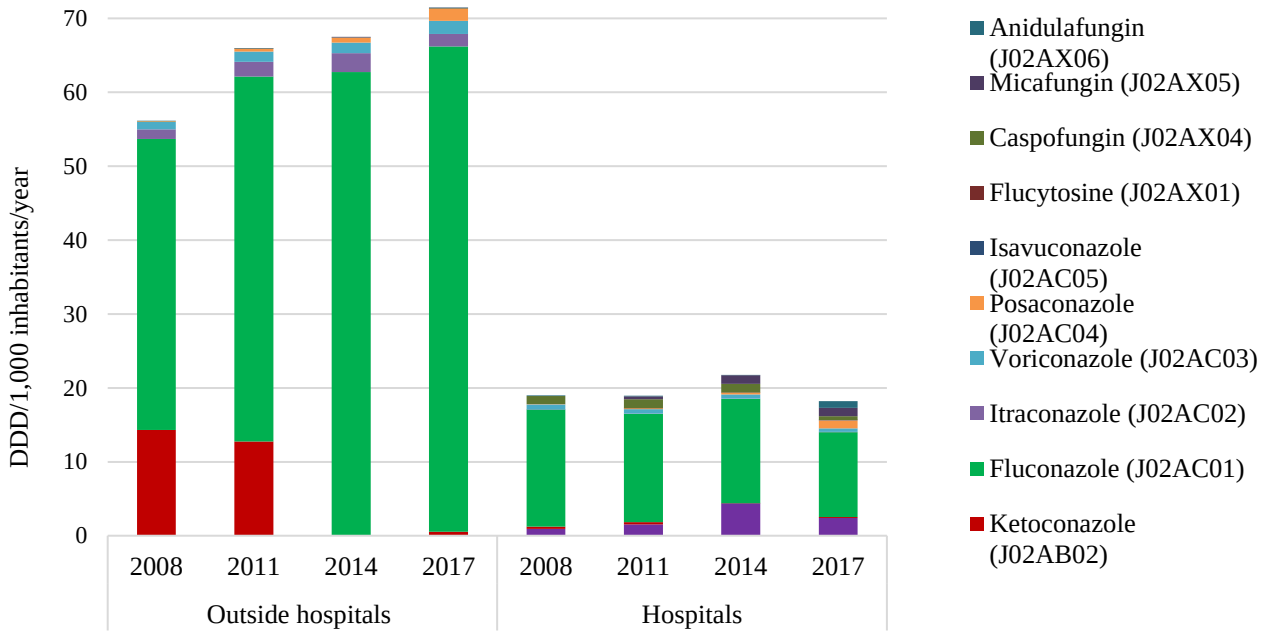

Figure 1. Consumption of antifungals for systemic use (ATC group J02) in Norway for primary care and hospitals 2008, 2011, 2014 and 2017, in DDD/1,000 inhabitants/year. From NORM/NORM-VET 2017 [18].

Azoles in agriculture

While application of broad-spectrum fungicides such as Copper and Sulphur containing products could be very effective, they had considerable non-target effects, were not rain-proof and were only effective on contact with the disease-causing agent. In 1968, the first azole was marketed to be used against fungal pathogens in a variety of crops, Benomyl™ (also known as Benlate™) a fungicide belonging to the benzimidazole group. The azoles were the first widely used organic compounds known to be taken up by the plant itself and highly specific to kill fungal pathogens. Benomyl was introduced to Norway in 1977 and mostly used on fruit trees, berries and vegetables. Seed treatment with azoles started shortly thereafter with Fuberidazol™ (a fungicide also belonging to the benzimidazole group) for cereals, but it was not before 1980, that an azole containing product was widely applied against leaf pathogens in cereals, Bayleton™ (triadimefon)Only a few years after the introduction of site-specific fungicides globally, loss of activity was observed in some, including the azoles.

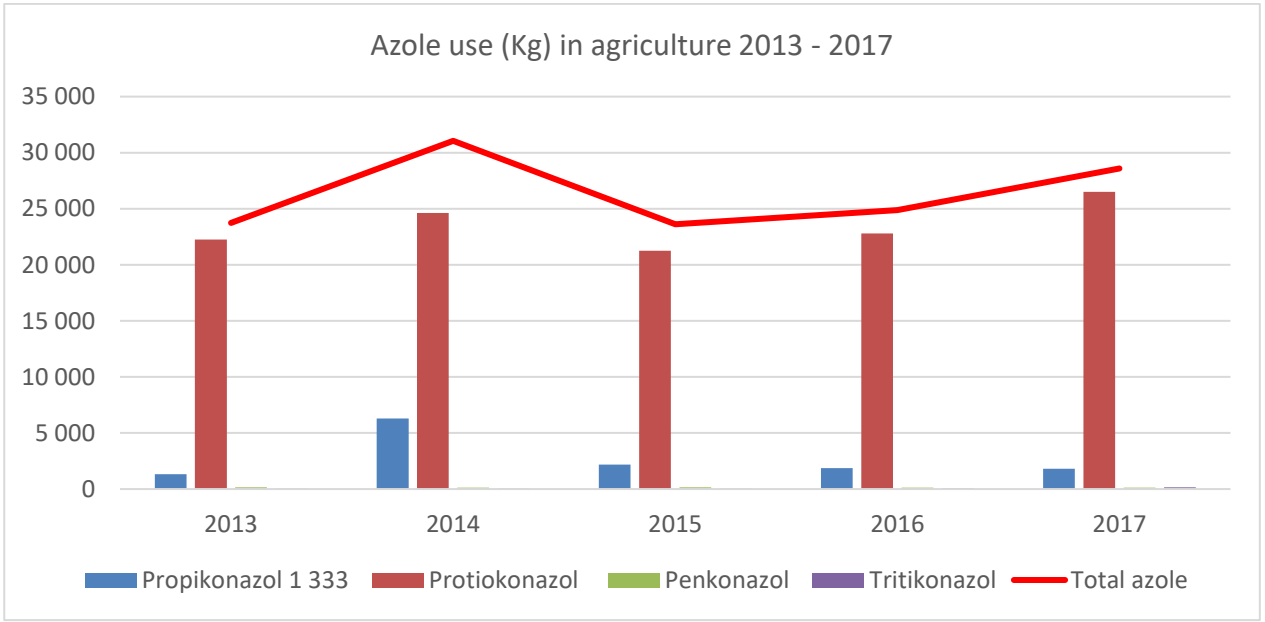

Figure 2. Azole use in agriculture 2013 -2017. Annual sales data import/producer to distributor in Norway [19]

Prothioconazol belongs to the azole group and is an active ingredient in Proline™, Siltra Xpro™ and Aviator Xpro™, fungicides registered for use in Norway. Other azoles, such as propioconazole, the active ingredient in Bumper™ (called Tilt™ earlier), difenoconazole active ingredient in the seed treatment Celeste™, and tridiconazole, active ingredient in the seed treatment Kinto™, were traded in much lower volumes in 2017 (2.693 kg, 1.501 kg, 58 kg, respectively).

A comparison of the usage of systemic azoles in medicine and agriculture (in Kg) is illustrated in Figure 3.

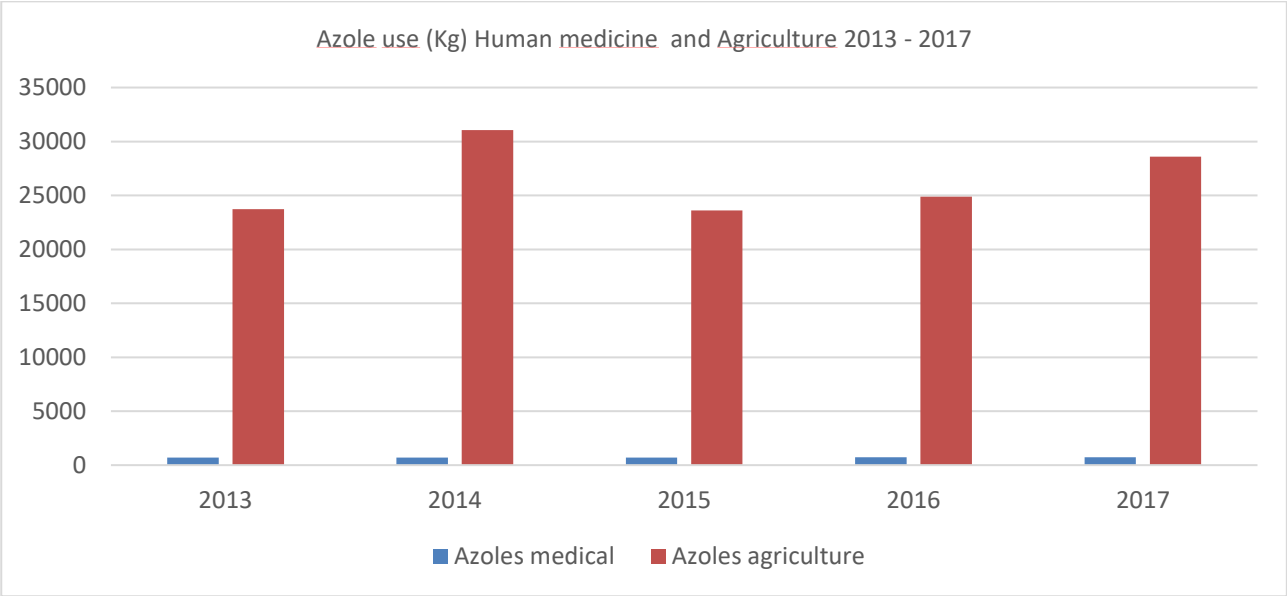

Figure 3. Azole use in human medicine (systemic) and agriculture 2013 – 2017 [19, 20].

Azoles in industry

Application of a broad spectrum of fungicides is used in industry. Concerns about the health and environmental impacts of metallic wood preservatives has opened a marked for non-metallic wood preservatives such as the azoles propiconazole and tebuconazole. Azoles are now widely used both in industrially pressure-treated wood and in painting and preservatives. We lack an overview of the extent of azole use in various types of industry. This must be investigated further.

Antifungal resistance

Some species of fungi are naturally (intrinsic) resistant to treatment with certain types of antifungal medications and the use of antifungals may select for infection with such species. A clear shift in species distribution in candidaemia towards fluconazole resistant species, has already been observed within the Nordic countries, especially in Denmark. The development of acquired resistance is ascribed to antifungal use and overuse. Resistance development may happen in the infecting fungus during repeated and/or longstanding patient treatment [5, 21]. However, as the main reservoir of moulds is the environment (outside the human body), antifungals used as pesticides in agriculture may drive resistance development when they possess activity against human pathogenic fungi. It then becomes a risk for patients to acquire an infection with an antifungal resistant strain.

Resistance mechanisms to azoles

Several fungal species can develop resistance towards the azoles through various mechanisms, some of which are still unknown [22].

The most investigated resistance mechanisms involve mutations in the *Cyp51A*-gene, rendering the *CYP51A* protein resistant to azole binding. The fungus is therefore not susceptible to azole treatment and can continue to produce ergosterol and thrive [23-27]. *Cyp51A* mutations are the dominant cause of azole resistance in clinical strains of *Aspergillus*. Another mechanism involving the Cyp51A gene is usually found in strains originating from the environment. This involves mutations in the gene in combination with

insertion of tandem repeats in the promoter, making the fungus overproduce the CYP51A protein. Examples of these resistance mechanisms are TR_{34}/L98H and TR_{46}/Y121F/T289A [9, 28, 29].

Generally, two routes of resistance selection are recognized; the patient route and the environmental route. The patient route involves patients who primarily suffer from CPA or cystic fibrosis and receive long-term azole therapy. Most patients have a pulmonary cavity, which is colonized with *A. fumigatus*. The fungus is able to undergo asexual reproduction in the cavity thus producing millions of conidia (spores). These spores may contain spontaneous mutations some of which might confer azole resistance. Due to azole selection pressure, the azole resistant spores will thrive and outcompete the wildtype strains.

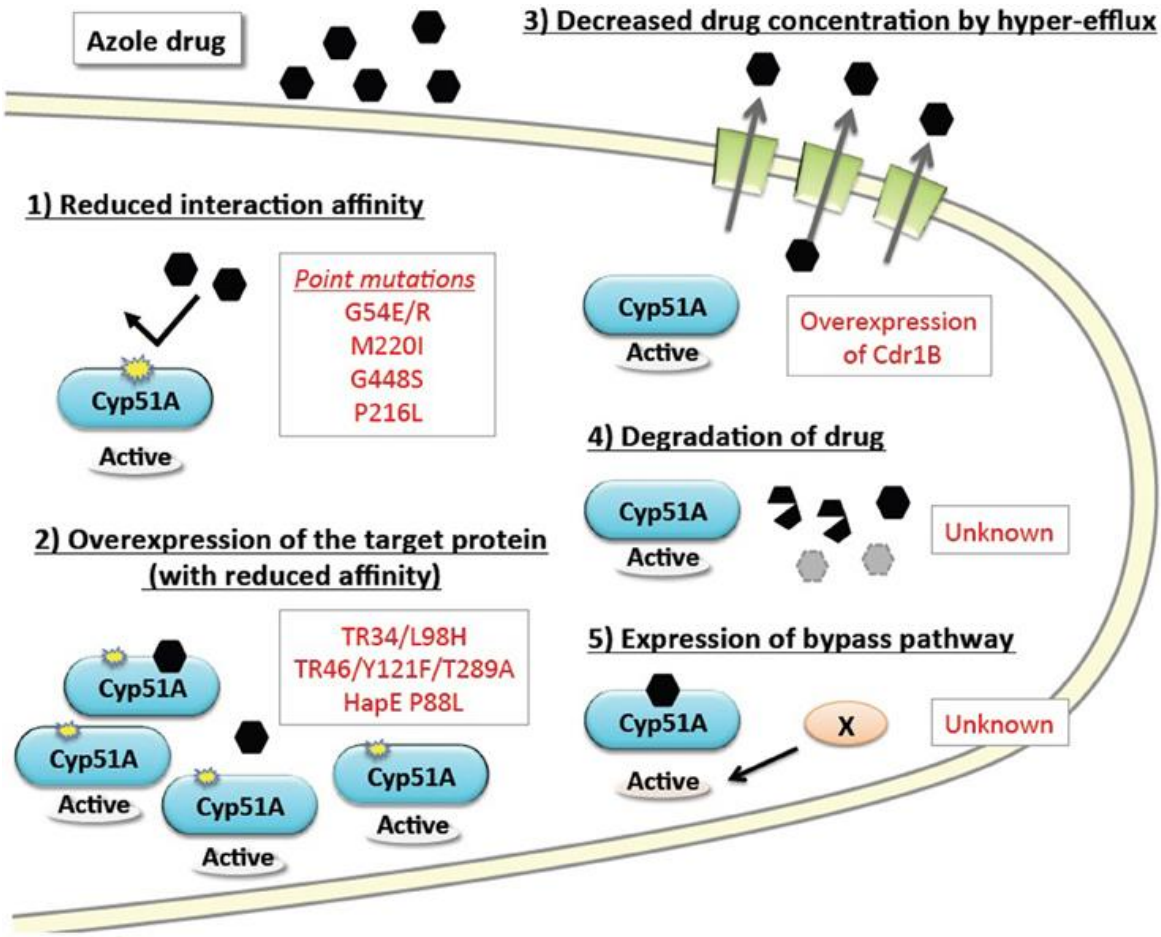

Figure 4a. Summary of possible resistance mechanisms. [30]

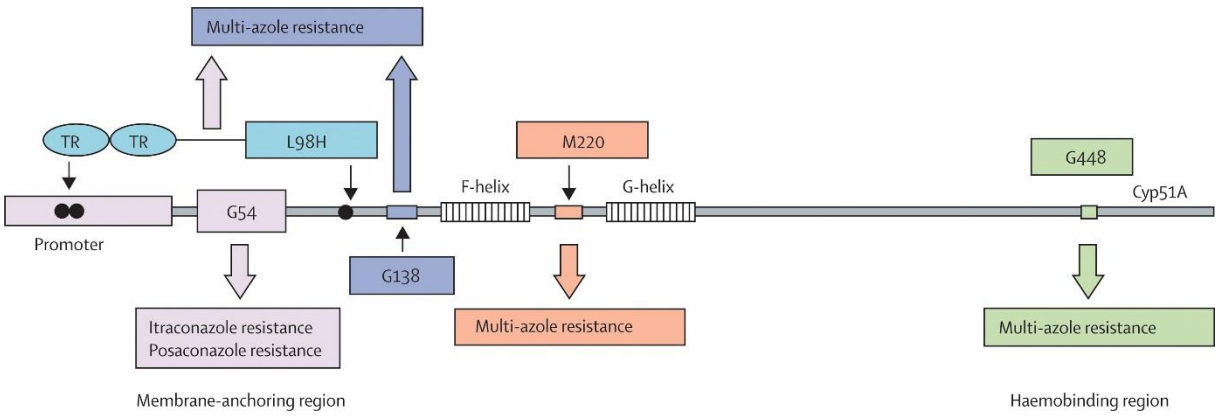

Figure 4b. Summary of the most prominent Cyp51A mutations [31].

The environmental route is due to the use of azole fungicides in the environment commonly used for crop protection. Many of these azole compounds exhibit activity against *A. fumigatus*, which is not the target pathogen as *A. fumigatus* is a saprophyte and not a plantpathogen. It has been shown that through exposure of *A. fumigatus* to azole fungicides cross-resistance to medical triazoles can be obtained [9, 28]. Isolates harboring TR_{34}/L98H or TR_{46}/Y121F/T289A have been recovered from patients with azole-resistant aspergillosis as well as from the environment. Unlike in the patient route, two-thirds of patients with azole-resistant infection originating from the environment had no previous history of azole therapy. Environmental resistance mutations are almost predominantly found in patients with IA, but can be recovered from other aspergillus diseases as well.

A considerable part of fungal strains identified as azole resistant do not harbor any mutations in the *Cyp51A*-gene region suggesting there are other resistance mechanisms by which azole resistance is achieved. These are commonly called non-Cyp51A mutations. Some of these mutations are known, e.g. those that involve overproduction of membrane transporters that are able to pump azoles out of the fungal cell to prevent it from inhibiting ergosterol biosynthesis [32]. Nevertheless, we still do not have a good overview of all the resistance mechanisms.

Hot spots for resistance development

Recently the Ministries of Health and Agriculture in the Netherlands supported research to understand resistance selection in the environment [33]. The presence of *A. fumigatus* and of azole fungicide residues was investigated in a number of agricultural environments. Three environments were found to harbor high numbers of azole-resistant *A. fumigatus*; flower bulb waste, green waste and waste containing wood chippings. Samples taken from these environments also contained residues of azole fungicides. It was postulated that environments that support the growth and reproduction of *A. fumigatus* AND harbor azole fungicide residues present a risk for resistance selection. These environments are referred to as “hotspots”.

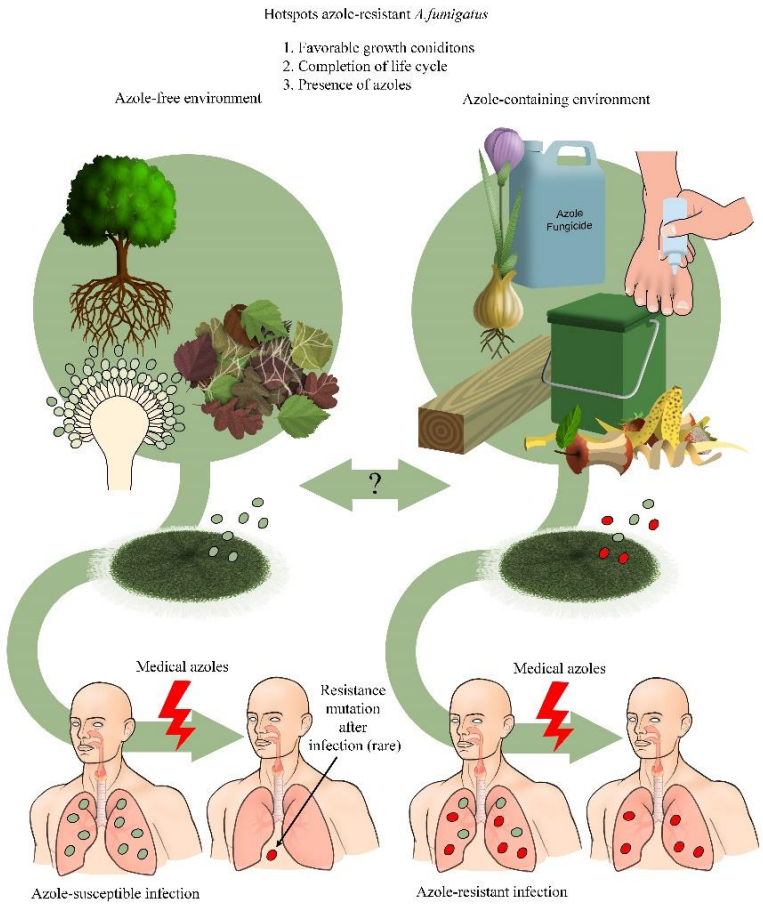

Figure 5. Development of resistance in the environment.
Illustration: © Marc Maas, Wageningen University and Research

The hotspot concept may be broadly applicable and thus any environment that supports growth of *A. fumigatus* and contains azole fungicide residues may contribute to the environmental burden of azole resistance.

Biofilm

It has also been shown that fungi living inside biofilms are able to tolerate higher levels of azoles [34]. The biofilm is recalcitrant to many antimicrobial treatments providing a protective environment for the organisms that live in it. Increased survival and tolerance to antifungal drugs may provide the time needed for the development of resistance mutations and may allow for emergence of novel resistance mechanisms of the non-Cyp51A kind. Biofilms are found on almost any surface where there is humidity, from cellular linings in the human body to the drains in common sinks. Understanding their potential as hot spots for azole

resistance development is therefore important. The research from the Netherlands furthermore showed that hotspots primarily involved storage and stockpiling of organic waste, and that during active composting of organic waste the number of *A. fumigatus* colonies was reduced. During active composting the temperature probably becomes too high for the conidia to survive. The azole-resistant *A. fumigatus* isolates recovered from the hotspots harbored TR_{34}/L98H and TR_{46}/Y121F/T289A mutations, identical to those recovered from patient samples. Another concern is that new resistance mutations continue to emerge in the environment. Recently *A. fumigatus* isolates harboring three and four copies of TR_{46} were recovered from the environment, indicating that our current use of azole fungicides is not durable. Increasing diversity of resistance mutations in *A. fumigatus* further threatens the use of the medical azoles and presents increasing challenges for resistance diagnosis.

Fungal plant diseases

The azole class of fungicides has been the most important class to control fungal plant diseases over the last 40 years. Among fungal plant pathogens wide spread resistance to azoles has not yet occurred. One exception is *Zymoseptoria tritici* (Septoria leaf blotch, aka *Mycosphaerella graminicola*) in wheat [35]. In 2013, 34 CYP51 mutations were described to confer some type of reduced sensitivity to various azoles in *Z. tritici* [36].

The risk of fungicide resistance developing is related to the genetic variation in the population, the degree of sporulation, the population size and the ability of a particular mutation to spread through the fungal population at high rates. Fungicide applications constitute a strong selective pressure for resistance development. However, resistance could be lost from the population, if they were associated with fitness costs and confer a disadvantage to the harboring strains, when no azole containing fungicides are applied (no selection pressure). At this stage, however, we do not know to what extent fitness costs are associated with azole resistance in fungal plant pathogens. In Norway, we have not seen large epidemics of *Z. tritici* in our wheat fields, as the leaf blotch complex is dominated by *Parastagonospora nodorum*, a fungal pathogen not known to have developed resistance to any of the azoles yet. However, in 2016, 7 of the most common mutations were tested in Norwegian *Z. tritici* isolates using PCR markers. This preliminary study showed that the presence of the different CYP51 mutations were at the same level as in Southern Sweden or Denmark [37], where the EC50 values for azoles have degraded continuously over the last years in the national *Z. tritici* populations. The large variation in CYP51 mutations in the Norwegian *Z. tritici* population could lead to failure of the azole class, if this pathogen becomes a major threat in wheat production. It is crucial that fungicide application strategies are carefully designed to minimize the risk of development of azole resistance.

Antifungal resistance and public health concern

In 2013 the European Centre for Disease Prevention and Control (ECDC) published a risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species [7]. This has been recuperated lately as the Netherlands has reported resistance rates of almost 30% in *A. fumigatus* from hospitalized patients with IA, which has forced a change of treatment guidelines for aspergillosis. The risk of losing the most important class of antifungals for the treatment of aspergillosis seems imminent [6, 38, 39], and alternative treatment options with similar efficacy to the azoles are currently lacking. Since the risk assessment in 2013 [7], increasing prevalence of azole resistance in *A. fumigatus* is reported worldwide (Fig 4) [40]. The role of azoles and azole residues as environmental factors that drive resistance is under investigation by the ECDC European Environment and Epidemiology (E3) Network.

The incidence of invasive fungal infections has increased significantly over the last decades and despite new antifungal drugs the lethality remains unacceptably high (40-50%). Many studies indicate that delayed and inaccurate diagnosis and treatment still are the major causes of poor outcomes in patients with invasive infections. It is an unfortunate dilemma, as lack of routine diagnostic testing for fungal diseases and uncertainties of diagnosis, exacerbates the problem of antimicrobial drug empiricism and overuse. Increased antifungal use, both for prophylaxis and treatment, impact the species-distribution, antifungal resistance and healthcare costs. We fear that the development of antifungal resistance may hinder further progress in the treatment of these otherwise lethal infections.

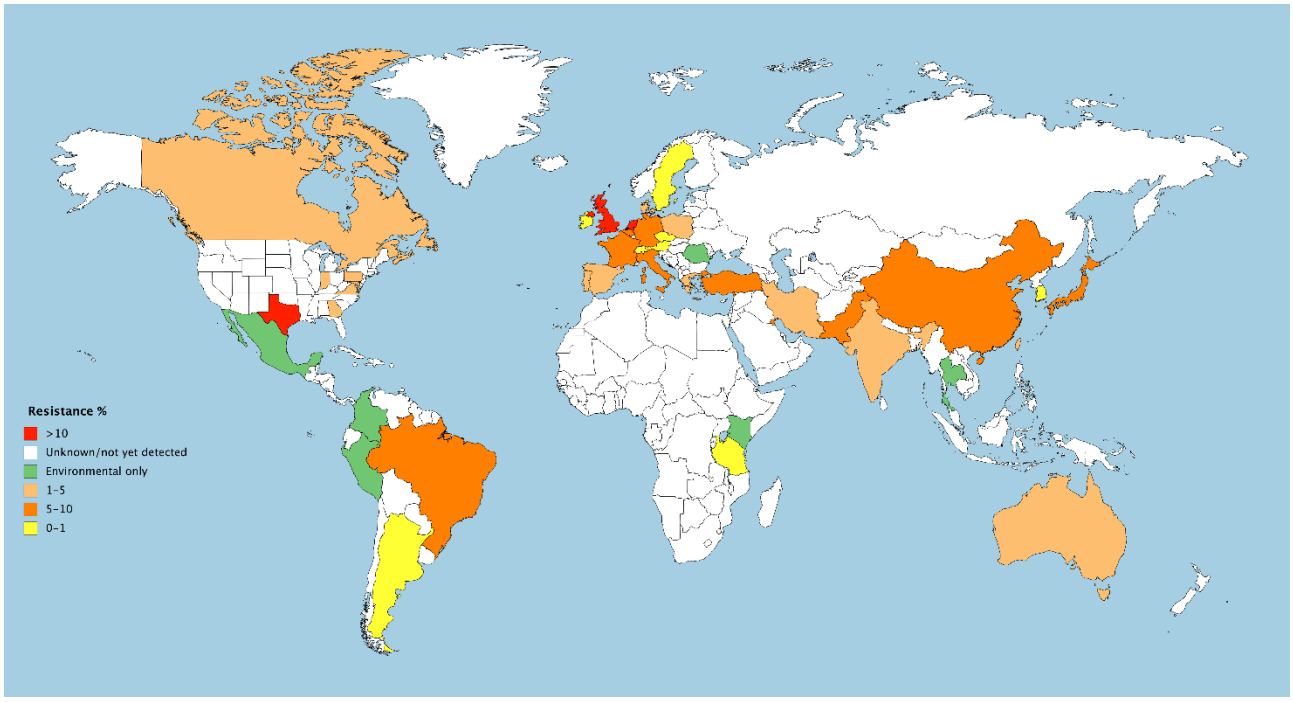

Figure 6. Global epidemiology of azole resistance frequencies in clinical and environmental *A. fumigatus* isolates. [40]. Resistance prevalence was classified for clinical isolates. If only cases were reported they were classified as 0-1%.

Clinical implications of resistance

Azole resistance and the lack of alternative drugs leads to treatment failures in patients with CPA and IA. A recent retrospective cohort study showed that the day-42 mortality was 21% higher in patients with voriconazole-resistant IA compared with voriconazole-susceptible infection. Furthermore, adequate initial therapy proved critical for patient outcome; patients who started therapy on voriconazole, but were switched to adequate therapy after voriconazole-resistance was diagnosed, showed a 23% higher day-42 mortality compared with patients with voriconazole-susceptible infection. Voriconazole resistance was diagnosed through intensive resistance screening of positive cultures and MIC-testing, but the median time to change of antifungal therapy was 10 days. These observations illustrate the diagnostic challenges as early adequate therapy is critical, but a culture-based strategy is too slow to prevent excess mortality. Furthermore, the majority of patients with IA remain culture negative. PCR-based detection of resistance mutations directly in clinical samples, such as bronchoalveolar lavage might be an alternative strategy, but the number of mutations that can be detected is limited and the sensitivity of the assays low. The urgence of better diagnostics is exacerbated by resistance development and might involve next generation sequencing.

As the number of alternative treatment options are limited, some aspergillus diseases may become untreatable. For instance, treatment of central nervous system (CNS) aspergillosis relies on voriconazole as this is the only azole that achieves high concentrations in the cerebral spinal fluid. In patients with voriconazole-resistant CNS aspergillosis there are currently no alternative drugs available with similar efficacy to voriconazole.

Managing fungicide resistance in plant pathogenic fungi

Minimizing fungicide resistance is most effectively achieved by mixing fungicides with different mode of actions with the lowest dose that is still sufficiently controlling the pathogen. In addition, reducing the population size by using resistant plant cultivars and reducing inoculum sources by tillage and rotation are important strategies in reducing the risk of resistance development. However, mixing fungicides with different mode of actions requires availability of fungicides that have different modes of actions and are effective against the target organisms. In Norway, cereal production depends heavily on a few active ingredients, clearly dominated by protioconazole. Registration of new products with different modes of actions, such as succinate dehydrogenase inhibitors (SDHIs) has increased, but usually they are sold in

mixtures already, which makes evaluation of their own effectiveness to control plant diseases more difficult. Further, the risk developing resistance in medically important fungi needs to be taken into account when developing new strategies to control plant pathogens.

What do we know about Norway?

Azole resistant fungal strains are in several countries widespread in nature, but the prevalence in Norway has not been studied.

One environmental isolate of *A. fumigatus* sampled retrospectively in 2001 was identified to harbour the resistance mutation TR_{34}/L98H [39]. No resistant clinical isolates were found in Norway during a prospective multicentre international surveillance of azole resistance in clinical *A. fumigatus* isolates, the SCARE study performed in 2009 and 2010 [41]. However, since then no systematic surveillance has been undertaken. In the years 2013-2018, 980 clinical *A. fumigatus* isolates have been identified at the mycology reference laboratory, of which 561 have been tested for azole susceptibility. Four isolates with the TR_{34}/L98H mutation and one isolate harbouring TR_{46}/Y121F/T289A have been isolated from patient samples. To our knowledge pan-azole-resistant isolates due to long-term treatment has only been isolated from one patient.

The Norwegian Veterinary Institute has started to perform antifungal susceptibility testing. A total of 125 *A. fumigatus* isolates, collected during the period 1997–2018, have been tested, and azole resistant *A. fumigatus* strains have been detected from cattle, dogs, horses and cats. However, molecular analyses are pending.

Conclusions and future perspectives

Norway has a long tradition of sustainable use of antimicrobials. Antibacterial resistance is regarded the biggest challenge as it affects most people. Without effective antimicrobials for prevention and treatment of infections the improvements gained in modern medicine is lost. The awareness of antifungal resistance is less addressed, as is awareness of fungal infections as a global health problem in general [5, 42-44]. In Norway this awareness is almost lacking or regarded negligible as the authorities primarily have focused on antibacterial resistance in their action plans [45].

As medicine, agriculture and industry heavily rely on triazole use, azole resistance in *Aspergillus* is a true One Health challenge. It is important to identify the potential hot spots for resistance development in Norway in order to protect our patients, as well as to collaborate internationally; as the spores of *Aspergillus* know no borders a united international initiative is warranted.

Weather patterns are predicted to become more extreme, warmer and wetter conditions dominating the growing seasons in the future and most likely favoring fungal disease development in many crops grown in Norway. Higher disease pressure will demand more effective disease control to reduce yield loss and close the yield gap needed to feed the growing population until 2050. A multi-pronged approach combining resistant plant varieties, tillage, rotation and careful design of fungicide application strategies with an array of multiple active ingredients for effective control will be necessary to reduce the risk of losing the most important class of fungicides in plant production, the azoles. Monitoring resistance development in the current pathogen population and preparing for shifts in fungal diseases as seen in other countries will be necessary to adjust our management strategies and determine our choices of fungicide compounds. However, whether we will be able to permanently delay or prevent the resistance development to azoles is not known.

By understanding how azole resistance develops and persists in the environment, effective measures can be designed and implemented preventing resistance development. This will contribute to inform and incentivise a global initiative for *"revised regulations of the usage of azoles"* in therapeutic treatments, agriculture and for other purposes.

Knowledge gaps

- The fungal resistance epidemiology in Norway is largely unknown.
- We do not have knowledge of all resistance mechanisms. Development of molecular diagnostics depend on such knowledge.
- We do not fully understand the potential driving forces for azole resistance from industry, agriculture, horticulture, veterinary and human medicine.
- Understanding and identifying potential hotspots for development of azole resistance in Norway is urgently needed.

Recommendations

- We recommend that the Norwegian health, veterinary and agriculture authorities start systematic surveillance of azole resistance in pathogenic fungi from humans, animals, plants and the environment in order to inform our control strategies.
- We must explore, in Norway and internationally, how fungi develop resistance to fungicides. Research on fungal resistance in the environment is performed in the Netherlands and United Kingdom, but both climatic conditions and the structure of agriculture in these countries are different from Norwegian conditions. Therefore, it is important to identify and understand Norwegian environmental conditions where the risk of resistance development is particularly high.
- Globally, we need azoles for medical use, but we also need azoles in agriculture to ensure sufficient and safe food for a growing population. A global initiative for revised regulations for azole use in therapeutic treatments, agriculture and other purposes is needed to meet the future.
- A broad approach that combines resistant plant varieties, soil cultivation, growth shifts and carefully designed strategies for fungicide use is needed to reduce the risk of losing the major class of fungicides in plant production, the azoles.
- The work to prevent azole resistance in plant production is of utmost importance to prevent azole resistant infections in human medicine, and incentives to develop new agricultural antifungals should thus be given.
- Antifungal stewardship to prevent the development of azole resistance should be emphasised.
- When new medical antifungal targets are developed, they should be regulated for medical use only.

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