Using Evolution to Acquire a Better Understanding of the Mechanisms that Constitute Antifungal Resistance

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Abstract

Pathogenic fungi are at the cause of global problems of massive proportions. More than a million lives are lost every year due to fungal infections. Additionally, around a third of our agricultural produce is lost to spoilage annually because of fungi. Furthermore, fungal pathogens are also responsible for significant loss of biodiversity in animal- and plant species in the wild. Our antifungal drugs are increasingly failing to combat these infections caused by pathogenic fungi, because of the evolution of resistance. In this essay, the problem of fungal infections, combined with the increasing occurrence of resistance against antifungal drugs, will be elaborated upon. Subsequently, an argument will be made for the widespread implementation of experimental evolution, as a means of elucidating the mechanisms of antifungal resistance. This argument will be made on the basis of the successful use of this technique in the study of antibiotic resistance, which shares several striking similarities to the study of antifungal resistance. The principle of experimental evolution could provide us with the necessary opportunities to unravel the mechanisms that constitute the evolution of antifungal resistance. Thus helping us stay ahead in the arms race against evolution of antifungal resistance in pathogenic fungi.

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Introduction

Fungal pathogens are a serious problem worldwide, they affect healthcare, agriculture and biodiversity in a detrimental fashion. Fungal infections cause disease in millions of people, resulting in thousands of deaths every year. Furthermore, because of fungi a substantial portion of our food supplies are lost to spoilage annually, causing additional hardships. Fungal pathogens also cause mass mortality in animal populations, leading to massive loss of biodiversity. These factors make fungal infections a global problem of significant proportions. To make matters worse, contemporary treatments for fungal infections are increasingly failing. Pathogenic fungi have developed resistance to most antifungal treatments which are currently used in clinical- and agricultural settings. There have even been accounts of fungal species that are resistant to combinations of fungicides. In order to stay ahead in the evolutionary arms race against antifungal resistance, it is of vital importance that we acquire a better understanding of the mechanisms that constitute this resistance.

In this essay an argument will be made for the widespread implementation of experimental evolution, in order to acquire a more complete understanding of the mechanisms behind antifungal resistance. Firstly, the problem of fungal infections will be elaborated upon. Subsequently, current methods of fungal treatments will be denominated, including known mechanisms of resistance against them. The concept of experimental evolution will then be introduced, followed by examples of its successful implementation in the elucidation of mechanisms of antibiotic resistance. The successful implementation of this technique in the field of antibiotic resistance will be used to validate the argument for its widespread implementation in the study of antifungal resistance. Additionally, two examples of the, sporadic, use of experimental evolution in antifungal resistance-studies will be given, further solidifying the usefulness of the technique.

Fungal Infections

Despite their serious impact on public health, agriculture and biodiversity, pathogenic fungi are rarely the topic of discussion. In comparison to subjects such as bacterial- and viral pathogens and antibiotic resistance, fungal pathogens and fungicide resistance are underrepresented in both media and scientific literature. However, if one looks at the figures concerning fungal infections, this underestimation of fungi is unjustified. Worldwide, over 300 million people suffer from diseases related to fungal pathogens. As a result of this, approximately 1.6 million people die every year. This is a comparable death count to a disease such as malaria [1]. The number of people at risk of fungal infections is steadily growing. Factors such as old age, medical interventions and compromised immune systems all increase the likelihood of a serious fungal infection. Advances in healthcare are allowing people to live to an older age, and increase the likelihood of survival for patients undergoing chemotherapy or organ transplantations. Subsequently, these people are now prime targets for pathogenic fungi [2]. While healthy people have a lower chance of being infected by pathogenic fungi, some studies suggest this might change in the future. Two factors give mammals a relatively high resistance against fungal infections: a complex immune system and high body temperatures. However, the protection granted by high body temperatures might be nullified, to a degree, by global warming. This is due to the fact that global warming forces pathogenic fungi to adapt to higher temperatures, aside from increasing their geographic range [3]. Thus, it is expected that the incidences of healthy people suffering from serious fungal infections will rise in the future.

However, healthcare is not the only sector that is threatened by pathogenic fungi. Fungal infections of crops cause a yearly yield loss of ~20% worldwide. Thereafter, they account for an additional loss of ~10% postharvest. These numbers add up to around a third of all crops worldwide being lost to fungal pathogens every year. This amount of food could be used to feed around 600 million individuals [1]. Two factors are responsible for these alarming numbers of spoilage due to fungal infections. Firstly, fungal infections are often only spotted when the fungi start sporulating. Which often happens after the plant has started dying. Additionally, there are many different species of invasive fungi. Most of these species are still unknown to us. There are an estimated 1.5 million different species of fungi on earth, of which we have only identified around 150,000. Due to our limited knowledge regarding this vast amount of different fungal species, and problems in identification of fungal infections, it is difficult to predict and prevent the arrival of new invasive fungal species. It is estimated that crop loss due to fungal infections costs the agricultural sector ~21 billion dollars every year, in the United States alone [4]. Fungal pathogens are also at the cause of mass mortality of numerous animal- and plant species. One amphibian disease, Chytridiomycosis, has contributed to the decline of at least 501 amphibian species, this accounts for ~6.5% of all amphibian species. Of these 501 species, at least 90 have been declared extinct in the wild [5]. Chytridiomycosis, which is caused by fungal *Batrachochytrium* species, has caused the greatest loss of biodiversity ascribed to a pathogen.

Pathogenic fungal species are a serious threat to not only our own healthcare, but also our food supply and the biodiversity of wildlife. In this essay existing treatments against fungal infections, antifungal drugs, are investigated. Furthermore, the growing problem of resistance against these antifungal drugs in pathogenic fungal species will be elaborated upon. Subsequently, a novel way of elucidating the mechanisms underlying antifungal resistance will be introduced, namely experimental evolution.

Antifungal Treatments

Development of novel fungicides is no easy task because the target cells, the pathogenic fungi, are eukaryotic, just like the host cells. Thus, it is important that a potential drug inhibits the target cells while steering clear of the patient cells. This consideration severely limits the range of targets for novel fungicides. Fungicide drugs are restricted to four major classes of antifungal compounds: the polyenes, the azoles, the echinocandins and the pyrimidine analogs. The polyenes were introduced in the 1950s and target ergosterol, a component of the fungal cell membrane. Ergosterol is the fungal alternative for cholesterol, and serves many of the same functions in fungal cells as cholesterol does in mammalian cells [6]. Polyenes bind ergosterol in the fungal membrane to form pores, which results in death of the fungal cell. One mechanism of resistance that fungi can apply against treatment with polyenes is altering the ergosterol content of the cell membrane. This mechanism of resistance against treatment with Amphotericin B, a type of polyene, was detected in resistant *Candida* isolates from a cancer center [7].

Another class of fungicides involved with ergosterol are the azoles. The azoles are the class of antifungals that are most often approved for clinical use, and are therefore the most prevalent form of clinical antifungal treatment. The azoles do not target ergosterol itself, but instead inhibit the biosynthetic pathway of ergosterol. The azoles target the enzyme lanosterol 14α-demethylase, which functions in an early phase of the ergosterol biosynthesis pathway. Through inhibiting the function of lanosterol 14α-demethylase, the azoles cause an accumulation of ergosterol intermediates in the target cells. This accumulation of intermediates leads to toxic stress in the cell and make it vulnerable to membrane damage [8]. There are several mechanisms of resistance that fungi apply against treatment with azoles. Such as alterations in the target site, lanosterol 14α-demethylase, due to mutations in the ERG11 gene, which encodes the enzyme. Other mechanisms of resistance against azoles are the overexpression of the ERG11 gene, leading to increased amounts of enzyme, and overexpression of efflux pumps, leading to lower concentrations of antifungal compounds within the cells [9].

The third class of antifungal compounds are the echinocandins, which block cell wall synthesis in fungal cells. The echinocandins interfere with synthesis of the cell wall through noncompetitive inhibition of β(1, 3)-D-glucan synthase [10]. This enzyme is involved in the biosynthesis of β-glucan, which is a component of the fungal cell wall. A mechanism of resistance against treatment with echinocandins is the mutation of FKS genes, which encode for the catalytic subunits of β(1, 3)-D-glucan synthase [11]. The fourth class of antifungal drugs are the pyrimidine analogs, of which flucytosine is the most common variant. Flucytosine acts as a noncompetitive inhibitor of thymidylate synthase, disrupting DNA synthesis and blocking protein synthesis. Due to its limited clinical spectrum, flucytosine is often administered in combination with azoles. The multiple targets of this combination limit resistance development, as is often prevalent during monotherapy with flucytosine. Resistance mechanisms to flucytosine include diminished uptake due to mutations of cytosine permease, or mutations in pathways involved with cytosine metabolism [12]. Resistance of pathogenic fungi to all systemic fungicides belonging to the abovementioned classes has been reported [2]. This highlights the importance of finding novel antifungal compounds, or adapting our strategies of antifungal usage.

The Problem of Antifungal Resistance

A pathogen is said to be resistant to a certain drug when its growth is no longer affected by treatment, with that specific drug, at normal therapeutic concentrations. In clinics, a shift from fungal strains that are sensitive to antifungal treatment towards resistant strains has been reported [13]. This shift is likely, in part, caused by overuse of antifungal drugs. With the arrival of the azoles as a treatment for fungal infections, the prescription of antifungal treatments has increased significantly. The pre-emptive use of antifungal drugs is one reason for this. One study, performed at an American hospital, found that 62% of all fluconazole treatments were prescribed pre-emptively [14]. Another study found that, of all 180 patients that received an antifungal treatment, only 5% actually developed a fungal infection [15]. This unnecessary use of antifungal treatments can be partly blamed on poor guidelines. However, a lack of compliance with these guidelines is also to blame. One study, performed in Thailand, found that inappropriate antifungal treatment reached up to 70% [16]. Unnecessary overuse of antifungals has contributed to the growing problem of antifungal resistance in hospitals around the world.

Candida auris is a prime example of the problems that antifungal resistant fungi can pose in clinical settings. *C. auris* is a multidrug resistant pathogenic yeast, which is associated with a high mortality rate and can readily spread in healthcare settings. Since its discovery in 2009, this yeast has been reported in hospitals all over the world. Research showed that *C. auris*, as a human pathogen, only emerged somewhere in the past decade [17]. Since then, a remarkable shift in the distribution of *Candida* species in clinics has been reported. In South Africa *C. auris* is now the leading cause of fungal infections caused by *Candida* species, with similar shifts having been reported worldwide. Whole-genome sequencing was performed on isolates from around the world, to help determine what caused this rapid worldwide emergence of *C. auris*. The results indicated that the various isolates could be grouped into four distinct clades: South Asia, South Africa, South America and East Asia [18]. These results indicate that *C. auris* evolved independently, and around the same time, in four geographic regions. An exact reason for this strange emergence has not yet been determined, however it is hypothesized that it might have been caused by an increase in the global use of antifungal drugs. Analysis of *C. auris* isolates from around the world has shown that this pathogenic yeast is generally resistant against fluconazole, and a substantial proportion of isolates showed resistance against amphotericin B and echinocandins. Treatment of infections caused by *C. auris* is challenging due to these antifungal resistances. Fluconazole is the most widely available antifungal treatment against *Candida* infections, and amphotericin B and echinocandins are more expensive and not readily available in less developed countries.

Another contribution to the development of resistance against antifungal treatment is the dual use of antifungals in agriculture and healthcare. A good example of this is the emergence of azole resistant *Aspergillus fumigatus* strains. Azoles are the preferred treatment of crop infections caused by fungi, due to their effectiveness against a wide spectrum of fungal pathogens. Additionally, azoles are also the main treatment of *Aspergillus* infections. Long term azole treatment of *A. fumigatus* infections in clinics has been demonstrated to lead to the development of azole resistant strains. However, azole resistance has also been reported in *A. fumigatus* isolates from patients who had not received prior azole treatment. It was hypothesized that these resistant strains could have been acquired from the environment [19]. According to this hypothesis, azole resistant *A. fumigatus* spores could have been inhaled by the patients, which resulted in development of azole resistant fungal infections. This hypothesis of development of cross-resistance to medical azoles due to agricultural use of azoles, was validated by comparing azole resistant strains from agricultural- and clinical settings [20]. It was found that several isolates from both settings showed similar mechanisms of resistance. These findings substantiated the hypothesis that dual use of antifungals in agriculture and healthcare contributes to development of antifungal resistance.

One possible way of countering the problem of antifungal resistance in clinical settings would be the treatment of patients with combinations of antifungal drugs. However, due to high risks of development of multidrug-resistance, toxicity and possible antagonistic interactions, this is rarely done. Combinations of antifungal drugs are usually only used in the treatment of very severe cases of fungal infections. This stresses the importance of developing novel antifungal treatments. Several promising candidates for novel antifungal drugs are currently in preclinical- and clinical trials. These include compounds with similar modes of action as those already in use, but also compounds with entirely novel targets [21]. However, continued evaluations and studies are needed to determine whether these compounds are safe and if they are successful in combating fungal infections. Additionally, development of resistance against novel antifungal treatments remains a big risk. In order to stay ahead in this arms race against antifungal resistance, a more complete understanding of the mechanism that underlie antifungal resistance is vital. In this essay a very promising method is introduced that might enable us to accomplish just that, experimental evolution.

Experimental Evolution

In order to retain our ability to effectively combat fungal infections, we need to stay ahead in the arms race against antifungal resistance. This requires us to develop, or adapt, antifungal treatments at a rate that matches the evolution of resistance. For this purpose we need a more complete understanding of the mechanisms that underlie the development of resistance. Experimental evolution is a promising technique, that may help us acquire this more complete understanding. Experimental evolution is the study of the evolution of experimental populations, as a response to conditions imposed by the researcher. While theories of evolution are generally tested by studying the past, e.g. through the use of phylogeny, experimental evolution allows us to research evolutionary theories in real time. While experimental evolution has not seen much use in the study of antifungal resistance, it has been used extensively in other branches of evolutionary biology. Continuous laboratory evolution has been used to study evolutionary topics such as organismal adaptation, phylogenetic reconstruction and has seen increased use in the biotechnological sector.

Long term experimental evolution has been used to decipher the relationship between genotype and phenotype, during adaptation to a new environment. Understanding adaptation requires us to identify the target gene of natural selection, which can be very difficult in environments where multiple traits may increase fitness. Therefore, one study was performed where twelve *Escherichia coli* colonies were taken from a single clonal ancestor and grown on a glucose-restricted medium for 20,000 generations [22]. Global protein profiles of these *E. coli* populations were compared, and remarkable parallelism was found. At a higher level, high parallelism was found in the changes of global regulatory networks. And at a lower level, changes in identical gene sets were found. These results showed a remarkably similar genetic response to an environmental change, across separated populations, granting us real-time insight into the evolutionary mechanisms of natural selection.

Phylogenetic research has also seen the employment of experimental evolution. It was for instance used in research focusing on the evolutionary mechanisms behind multicellularity, one of the most significant contributing factors to life on earth as we know it. Using this technique, the initial evolution of multicellularity was studied [23]. Understanding the shift from unicellular ancestors towards complex multicellular organisms is quite difficult, largely because this shift occurred millions of years in the past. However, using the principle of experimental evolution we were able to mimic this historical event in real time. Researchers used *Saccharomyces cerevisiae* cells, which they subjected to an environment which would promote multicellularity. They discovered the rapid evolution of genotypes that promoted clustering of cells, aside from evolution of multicellular traits and division of labor. These results showed that the evolution of multicellularity might have been quite a rapid process, and less constrained than previously thought. In this case, the technique of experimental evolution allowed for the simulation, and study, of ancient processes inside a modern lab.

Apart from fundamental research, experimental evolution has also contributed to advancements in the bioindustries. Advances in both continuous microbial culturing and selection design have enabled the development of techniques utilizing directed evolution. Directed evolution is a form of experimental evolution, where a specific gene or protein is randomized and subjected to selective pressures. Through the use of directed evolution, the biotechnological sector is able to evolve tailormade biomolecules in an very quick fashion. This is especially useful in the development, or adaptation, of commercially used enzymes. The applicability of directed evolution has been widely reported for the improvement of protein solubility, stability and catalytic efficiency. Because directed evolution provides a simple and effective method for enzyme improvement, it has become a key technology for protein modification.

Thus, the principle of experimental evolution has been successfully applied in multiple aspects of evolutionary biology. It has been used to elucidate the evolutionary mechanisms of natural selection. It has helped grant us insights into the origin of prehistoric processes like multicellularity, in real time. And it has successfully been used by the biotechnological sector in a directed fashion, for the generation and adaptation of biomolecules. However, the link between the usefulness of experimental evolution in these aspects of evolutionary biology, and the proposed usefulness of this technique in the understanding of the mechanisms behind antifungal resistance, might not immediately be apparent. In the following segment, the manifold use of experimental evolution in the elucidation of antibiotic resistance mechanisms will be reviewed. This is a much more closely related subject to our field of interest, antifungal resistance, and might therefore prove to be more convincing.

The Link to Antibiotic Resistance

The history of antibiotic use and antibiotic resistance has some striking similarities with that of antifungals. However, as mentioned at the beginning of this essay, the subject of antibiotic resistance has seen much more coverage in both contemporary media and scientific literature. This larger share of attention is very likely the reason for the fact that the research area of antibiotic resistance has seen significant use of experimental evolution, while the field of antifungal resistance has not. By drawing parallels between the two research areas, and by elaborating on the utility of the use of experimental evolution in the field of antibiotic resistance, an argument for the widespread implementation of this technique in the research of antifungal resistance will be made.

In 1929 Fleming published his now famous discovery of one of the first antibiotics, penicillin [24]. The discovery of the first three antibiotics, penicillin, salvarsan and prontosil, set up the paradigm for further antimicrobial drug research. Their discoveries marked the start of the golden era for discovery of novel classes of antimicrobial compounds. This golden era spanned from 1950 until 1970, with no new classes of compounds being discovered since then. This decline in the discovery of antimicrobial compounds has meant that the main mode of novel antibiotic development has been modification of existing compounds. Many researchers of the golden era did not see antibiotic resistance as a big concern. Strikingly, one of the first people to warn about the problem of resistance was one of the pioneers of antibiotic drug discovery, Fleming himself [25]. Since the golden era, antibiotic resistance has become one of the biggest health concerns worldwide. Multidrug resistant bacteria have become a grave risk to healthcare around the world, and every year thousands of people die because of illnesses related to them. Overuse of antibiotics in clinical- and agricultural settings has been a major cause of this problem of resistance.

Antifungal research went through a similar golden era of discovery as antibiotic research, at a much smaller scale however. As with antibiotics, the twentieth century saw a big increase in the amount of novel antifungal compounds on the market. However, it also saw a big increase in the problem of resistance as well. Now, as will become apparent in the following segments of this essay, antibiotic resistance mechanisms are increasingly being studied using techniques of experimental evolution. In this essay, it is proposed that similar techniques should be applied to the research of antifungal resistance, and the mechanisms that constitute it.

The Use of Experimental Evolution in the Research of Antibiotic Resistance

The field of antibiotics is under very high pressures by the increasing occurrence of antibiotic resistant pathogens. There is an urgent need for novel solutions to combat the spread of resistance. Often, the first response to this problem has been the development, or adaptation, of more antibiotics. However, ironically the use of antibiotic treatments aimed at killing pathogenic bacteria inadvertently provide selective pressures for the development of resistance. Ultimately providing the pathogens with the mechanisms required for their survival. This is due to the fact that resistance is an evolutionary process. This means that strategies aimed at minimizing the problem of resistance should take evolution into account, or better yet use it for their own purposes. The field of antibiotics realized that, in order to combat the problem of antibiotic resistance, a more complete understanding of the mechanisms that constitute the development of resistance was required.

The switch from sensitivity towards resistance against antibiotic treatment is usually studied using comparative genomics on clinical isolates. This method, however, has its limitations. Firstly, clinical strains can only be isolated after discovery. This implies that they have already acquired the mechanisms necessary for resistance. Secondly, reconstruction of the evolutionary events that constitute resistance is often hampered by a lack of information about past selection pressures. Furthermore, it is quite difficult to identify intermediate adaptive mutations which were essential to the development of resistance [26]. Thus, an *a posteriori* approach using comparative genomics grants us only an incomplete picture of the development of resistance. Experimental evolution can be used to grant us a more complete understanding of the mechanisms of this development, in real time and under controlled conditions. In the following segment of this essay, a concise account of the use of experimental evolution, in the elucidation of resistance mechanisms, in the field of antibiotics will be given.

Single Antibiotic Resistance

Several studies have been performed where experimental evolution was utilized in the examination of resistance mechanisms, following treatment with a single antibiotic. One study was performed on the opportunistic pathogen *Pseudomonas aeruginosa*, where the bacterium was exposed to cystic fibrosis-like conditions in the presence and absence of fluoroquinolone antibiotics [27]. Whole genome sequencing of the experimentally evolved cultures revealed parallel evolution of various previously known resistance genes. These resistance genes were deterministic for the level of antibiotic resistance of the various *P. aeruginosa* cultures. However, various novel mutations were also discovered that were specific to individual experimental isolates, and were responsible for the cost of resistance. In evolutionary biology it is generally accepted that there are trade-offs between traits, where a trait can be advantageous in one situation and detrimental in another. This holds true for resistance traits as well. One notable find was that typical quinolone antibiotic resistance mutations often occurred alongside other mutations that, together, conferred high resistance but minimal cost of resistance. The use of experimental evolution in this study helped elucidate the interplay between multiple mutations, in the development of efficient antibiotic resistance of a human pathogen.

Another study was performed on bacteria belonging to the genus *Pseudomonas*, where the effect of genetic diversity on resistance development was investigated [28]. Eight strains of *Pseudomonas* were selected for resistance against the antibiotic rifampicin, using a short-term experimental evolution setup. Comparative analysis was carried out, to analyse the phenotypic- and genotypic adaptation of the eight strains to the rifampicin treatment. Resistance was acquired through 47 possible mutations in the target site of rifampicin, constituting the genotypic adaptation. Due to this high amount of possible mutation sites, the probability of parallel evolution within- and between the different strains was quite low. However, it was found that over 30% of the variation in growth rate, constituting the phenotypic adaptation, could be attributed to between-strain differences. This was likely due to the fact that similar mutations in the target site had different effects on growth in different strains. Thus, the researchers found that genetic diversity does constrain parallel phenotypic adaptation, while it barely effects parallel genotypic adaptation. These findings give us a better understanding of the possible effects of antibiotic treatments of genetically diverse bacterial populations.

In another study, researchers used *E. coli* populations to examine the mechanisms of gradual resistance development [29]. For this purpose they developed a device, called the Morbidostat, which continually measures bacterial growth and regulates antibiotic concentrations, in such a manner that the evolving population is constantly being pressured. The evolution of resistance against three antibiotics was measured, chloramphenicol, doxycycline and trimethoprim. Over a period of twenty days, resistance to all three antibiotics increased immensely. Whole-genome sequencing was carried out and both drug-specific and general mutations were found. It was also found that chloramphenicol- and doxycycline resistance evolved through various combinations of mutations in genes involved with translation, transcription and transport. Strikingly, resistance to trimethoprim only evolved with mutations to the target enzyme, and in a step-wise manner. Sequencing of this target enzyme over time revealed that the parallel populations not only evolved similar mutations, but also in a similar order. These results, in the case of trimethoprim, grant us insights into the chronological order of antibiotic resistance development.

Multidrug Antibiotic Resistance

Experimental evolution has also been used to elucidate the mechanisms of resistance development following multidrug antibiotic treatment. It has long been known that certain drug combinations are more effective in treating infections than single drugs. However, the precise effects of combinations of antibiotics on resistance development is still rather unclear. In one study, researchers tried to elucidate the effects of different kinds of combinations, antagonistic or synergistic, on the evolution of resistance [30]. They tested this by monitoring the parallel evolution of hundreds of *E. coli* cultures, which were subjected to different drug combinations and concentrations. They found a correlation between the synergy of drug combinations and the rate of adaptation to these treatments. Evolution of resistance against synergetic antibiotic combinations developed faster than resistance against antagonistic antibiotic combinations. They even discovered that resistance to some synergetic combinations evolved faster than resistance to single antibiotic treatment. They hypothesized that these findings may be due to the fact that synergetic antibiotic combinations provided stronger selection pressures for resistance mutations. These findings directly contradict the common use of antibiotic treatments, where synergetic drug combinations are quite often the preferred treatment for bacterial infections.

Another experiment was performed that questioned the efficacy of synergetic antibiotic drug treatment [31]. *E. coli* colonies were allowed to evolve over a five day period under treatment of sixteen different combinations of erythromycin- and doxycycline antibiotics. These different combinations of

the two antibiotics produced varying degrees of synergy. The researchers found that the combination with the strongest synergy actually produced the worst results, in terms of long term efficacy. For all combinations, resistance had evolved within the population on day one. By day two, the treatment with the highest synergy showed the least inhibitory power of all the combinations. And by day five, it had lost almost all of its antibacterial activity. The researchers concluded from these results that combinations of antibiotics with high synergy are only effective if super-inhibitory doses are applied, and maintained until all the pathogens are cleared. Otherwise, strong synergetic antibiotics combinations supply the pathogenic bacteria with strong selection pressure for evolution of resistance.

Validity of Experimental Evolution

A study was performed that investigated the validity of experimental evolution as a prediction tool for clinically relevant resistance mutations [32]. The researchers experimentally evolved an antibiotic sensitive strain of *P. aeruginosa* to develop resistance to three antibiotics: ciprofloxacin, meropenem and tobramycin. They found that resistant mutants could tolerate up to an 2,048-times increase in concentrations of antibiotics, compared to sensitive strains. For each antibiotic, the genomes of thirteen resistant mutants were sequenced. It was found that each mutant contained between two and eight mutations. And for each antibiotic at least eight mutated genes were identified. This illustrated the genetic complexity of antibiotic resistance. For all three antibiotics, mutations were identified which were previously linked with resistance, aside from mutations which had previously not been known to infer resistance. To validate the clinical relevance of these experimental findings, 558 clinical *P. aeruginosa* isolates and 172 environmental isolates were analysed. Many of the experimentally identified mutations were also found in clinical isolates, but not in the environmental samples. This showed that experimentally evolved mutations can successfully predict those that occur in clinical settings. These findings simultaneously increase our understanding of the evolutionary mechanisms of antibiotic resistance in *P. aeruginosa*, and show the validity of experimental evolution as an identification tool for clinically relevant mutations.

Thus, experimental evolution has been successfully used to gain a better understanding of the mechanisms behind the evolution of antibiotic resistance. In the case of both single antibiotic treatment and multidrug antibiotic treatment. Furthermore, the validity of this technique as a prediction tool for clinically relevant resistance mutations has been shown in regards to antibiotic resistance. In the following segment, two examples will be given of studies where experimental evolution has already been used in the study of antifungal resistance. These examples will function as evidence that the implementation of this technique is viable in fungi, and in the case of antifungal resistance studies. Strengthening the argument for widescale implementation of experimental evolution, in the elucidation of the mechanisms behind antifungal resistance.

The Use of Experimental Evolution in the Research of Antifungal Resistance

While the use of experimental evolution in the research of antifungal resistance is not completely unprecedented, it is highly sporadic. In the following segment of this essay, two examples of its application will be summarised. These two experiments will show the potential of this technique in the elucidation of antifungal resistance mechanisms.

In the first study, researchers used *S. cerevisiae* colonies to test the effect of different forms of antifungal treatment on the evolution of mechanisms of resistance [33]. The *S. cerevisiae* colonies were exposed to one of two treatments. The first form of treatment was a stepwise increase in fluconazole concentrations over 400 generations, from low to high concentrations. The second form was comprised of a high fluconazole concentration from the outset. They found that the two forms of fluconazole treatment resulted in completely different mechanisms of resistance in their test populations. Under the stepwise form of treatment, two successive mutations were found to have evolved parallel in multiple independent populations. In contrast, the second form of treatment had yielded the parallel evolution of a single mutation in multiple independent populations. Furthermore, additional experiments showed that both of these mechanisms of resistance did not confer any significant fitness costs in the absence of antifungal treatment. These findings, that different forms of treatment with the same antifungal compound can result in wholly different mechanisms of resistance, is very much of clinical relevance. Because the potential for variations in selection to occur would very likely be greater in a complex host body than in a tightly controlled experiment.

In another antifungal resistance-study, strains of *S. cerevisiae* and *C. albicans*, with erg3-loss of function, were used [34]. This mutation made the strains azole resistant in a manner which is dependent on stress responses through hsp90 and calcineurin. Targeting these stress responses is a method used in clinical settings to circumvent azole resistance in fungal pathogens. This research was carried out to investigate the evolution of mechanisms of resistance against the combinational treatment with azoles

and hsp90/calcineurin inhibitors. Of the 290 populations that were studied, all but fourteen went extinct. Drug target mutations that granted resistance to hsp90- and calcineurin inhibitors, were identified in five of these evolved strains. Whole genome sequencing was used to identify mutations in genes which caused upregulation of efflux pumps, and mutations in one gene that caused the resistance against azoles to switch from a calcineurin-dependant manner towards an independent manner. Thus, the researchers identified mechanisms which enabled a switch from calcineurin-dependant azole resistance towards calcineurin-independent azole resistance. Furthermore, multiple mechanisms were identified which constitute resistance against antifungal drug combinations.

These studies show the successful implementation of experimental evolution in the elucidation of mechanisms of resistance against antifungal treatment. In cases of single antifungal treatments and multidrug antifungal treatment. In both examples, experimental evolution enabled us to acquire knowledge about development of resistance against clinically used antifungal treatments.

Discussion

Pathogenic fungi affect society in a multitude of detrimental ways, from causing serious illness in millions of people worldwide to costing us around a third of our food supplies every year. Aside from the impact that fungal infections have on our society, they are also at the cause of massive loss of biodiversity in animal- and plant species in the wild. Our supply of defences against pathogenic fungi is severely limited, with only four major classes of antifungal compounds being used. To make matters worse, resistance against this limited supply of antifungal drugs is on the rise. Overuse of fungicides in both the agricultural- and medical sector have resulted in the onset of widespread evolution of resistance. Alarmingly, resistance to all major systemic antifungal drugs has been reported in clinical isolates of pathogenic fungi. While there are several novel antifungal treatments under development, it will only be a matter of time before resistance against those treatments evolves in pathogens as well. In order to stay ahead in the arms race against the evolution of antifungal resistance, we need to acquire a better understanding of the mechanisms that constitute this resistance. Most of our current knowledge about antifungal resistance mechanisms comes from the analysis of clinical isolates. However, this *a posteriori* approach only grants us an incomplete picture of the development of antifungal resistance. In order to acquire a more complete understanding of the vital mechanisms that constitute resistance against antifungals, we should apply the principle of evolution for our own purposes.

Experimental evolution has been widely utilised in the field of evolutionary biology, as well as in the biotechnological sector. However, its use in the elucidation of resistance mechanisms against antibiotic treatment, a more closely related subject, can provide us with insights into the utility of this technique in the study of antifungal resistance. Studying the evolution of experimental populations, under specific selective pressures, can provide us with a complete picture of the various steps that constitute their evolution. This principle has successfully been applied in the research of antibiotic resistance. Its implementation has helped us understand the mechanisms of resistance against both single- and multidrug antibiotic treatment. Additionally, it has been demonstrated that experimental evolution can successfully predict resistance mechanisms of clinical relevance. Furthermore, experimental evolution has seen sporadic use in the study of antifungal resistance as well. In these studies, the application of this technique provided us with valuable insights into the development of antifungal resistance against clinical antifungal treatments. The widespread implementation of this very promising technique could provide us with a more complete understanding of the mechanisms behind antifungal resistance, which is vital for our ability to stay ahead in the arms race against antifungal resistance.

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