cyp51A-based mechanisms of *Aspergillus fumigatus* azole

drug resistance present in clinical samples from Germany

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Abstract

Since the mid-1990s, a steady increase in the occurrence of itraconazole resistant *Aspergillus fumigatus* isolates has been observed in clinical contexts leading to therapeutic failure in the treatment of aspergillosis. This increase has been predominantly linked to a single allele of the *cyp51A* gene, termed ‘TR/L98H’ which is thought to have arisen through the use of agricultural azoles.

Here, we investigated the current epidemiology of triazole resistant *A. fumigatus* and underlying *cyp51A*-mutations in clinical samples in Germany. From a total of 527 samples, 17 (3.2%) showed elevated MIC values for at least one of the three substances (itraconazole, voriconazole, and posaconazole) tested. The highest prevalence of resistant isolates was observed in cystic fibrosis patients (5.2%). Among resistant isolates, the TR/L98H mutation in *cyp51A* was most prevalent, but also isolates with the G54W, M220I, and the novel F219C were found. The isolate with the G54W substitution was highly resistant to both itraconazole and posaconazole, while all others showed high level resistance only to itraconazole. For the remaining six isolates no mutations in *cyp51A* were found, indicating the presence of other mechanisms. With the exception of the strains carrying the F219C and M220I substitutions, many itraconazole resistant strains also showed cross resistance to voriconazole and posaconazole with moderately increased MIC values.

In conclusion, the prevalence of azole resistant *A. fumigatus* is lower in our clinical test set than previously reported for other countries. Although the TR/L98H mutation frequently occurs among triazole resistant strains in Germany, it is not the only resistance mechanism present.
**Introduction**

Clinical manifestations of aspergillosis range from pulmonary colonization and deep invasive mycoses of the lung and other tissues to fatal sepsis in immunocompromised patients. A steady increase in the occurrence of itraconazole resistant *Aspergillus fumigatus* isolates has been observed in clinical contexts since the mid-1990s (1-2) and has been linked to therapeutic failure in the treatment of aspergillosis (2-3).

Conidia of this soil-dwelling fungus are ubiquitously found in the environment. Its habitats include those with elevated temperatures, e.g. compost heaps, giving this species the intrinsic ability to also survive at elevated mammalian body temperatures. In contrast to endogenous infections with *Candida albicans*, there is no reservoir of *A. fumigatus* in healthy hosts: infections with *A. fumigatus* are therefore generally thought to be acquired exogenously from the environment.

Only a limited number of antifungal drugs are available for the therapy of such life threatening mycoses, among which azoles are competitive inhibitors of the Cyp51A protein, a central enzyme with lanosterol-14α-demethylase activity in the ergosterol biosynthesis pathway of fungi. Several steric mutations are known that affect inhibition constants of azoles towards this enzyme and lead to *in vitro* decreased drug susceptibility (4-5). Such mutations have been thought to arise under prolonged antifungal therapy or prophylaxis in individual patients and genetically independent fungal strains.

The recent increase in itraconazole resistance, however, has been linked to a single allele of *cyp51A*, termed ‘TR/L98H’ and typing studies showed a close genetic relationship between early isolates, indicating a common ancestor (1, 6). The allele contains a tandem repeat in the *cyp51A* promoter region combined with a single amino acid exchange of Leucine to Histidine and is thought to have arisen in the 1990s, possibly through the use of agricultural azoles which are structurally similar to clinically used drugs (6-7). Apparently, this allele is now spreading through the *A. fumigatus* population, since over the past years the TR/L98H allele has been reported to occur world-wide in patients as well as the environment (e.g. (2, 8-9)). This includes two German patients for which case reports were published independently during our study period (10-11).
In this study, we investigated the epidemiology of triazole resistant *A. fumigatus* and underlying *cyp51A*-mutations in viable clinical isolates obtained over an 18 month period in Germany during 2011/12.

**Materials and Methods**

**Acquisition and processing of isolates.** Clinical isolates were obtained during routine diagnostic procedures in the respective laboratories of the MykoLabNet-D network. They were isolated from various body locations and irrespectively of clinical relevance of the material collected for further processing. Where available pseudonymized anamnesis data including the patient age and gender, underlying disease, previous and current antifungal drug treatment, as well as outcome of treatment was obtained. For all isolates the species was confirmed and the antifungal drug susceptibility pattern tested as outlined below. Conidia were archived at -70°C in Cryobank tubes (Mast Diagnostica, Reinfeld, Germany).

**Species determination.** The species of all isolates in this study was confirmed by MALDI-TOF MS (Biotyper, Bruker Daltonics, Bremen, Germany) on extracted cells harvested from over-night shaking cultures in Sabouraud’s medium (Oxoid, Wesel, Germany) using the ‘Fungi Library’ database.

**Susceptibility testing.** Antifungal drug susceptibility towards itraconazole, voriconazole (both from Discovery Fine Chemicals, Bournemouth, UK), and posaconazole (MSD Sharp & Dohme, Haar, Germany) was tested by broth micro dilution according to the EUCAST reference method (10). Plates were incubated at 37°C for 48 h. The MICₐ values of all drugs were determined visually as the lowest concentrations with no visible growth. To establish the tests, drug resistant control isolates CR019, CR055, CR059, CR060, and CR061 (kindly provided by E. Mellado, ISCII Madrid, Spain) and drug susceptible isolates DSM819 and ATCC46645 were used (Table1). All isolates with elevated MICₐ values were re-tested additionally at least three times in parallel with the control strains. Across the entire study drug resistant control isolates grew over the full itraconazole concentration range while susceptible controls produced MICₐ values ≤0.250 mg·l⁻¹. Additionally, at one study site (Hamburg) isolates were pre-screened by E-Test and only the resistant ones submitted for further testing by broth dilution. For quality control purposes, eight isolates (six susceptible,
Sequence analysis. From isolates with MIC\textsubscript{0} values above EUCAST breakpoints (11) the cyp51A coding region and its promoter was amplified by PCR in two overlapping fragments (primers used: CYP51A-5: 5’-ataatcgcagcaccacattcaga-3’, CYP51A-7: 5’-cccttgctaccgtaagacgg-3’ and CYP51A-6: 5’-tggtggttttttcgaccgct-3’, CYP51A-8 5’-cggatcggacgtggtatg-3’) and each fragment sequenced from both ends. Sequences from all isolates were assembled using the CAP contig assembly program and manually inspected for nucleotide changes. For isolates other than TR/L98H two independent sequences for cyp51A were obtained. For control purposes the cyp51A sequences of twelve additional random isolates of the upper susceptible itraconazole range were determined initially, but showed no amino acid substitutions (data not shown).

Results

Epidemiology of reduced A. fumigatus azole drug susceptibility in Germany. Over a period of 18 months in 2011/12, a total of N=527 clinical isolates were processed. The vast majority of isolates received were obtained from pulmonary/oropharyngeal specimen (N=353) out of which at least N=163 were derived from cystic fibrosis patients. Other isolates were either from skin (N=30) or from invasive/wound infections (N=39).

MIC\textsubscript{0} values in the susceptible range determined by EUCAST broth microdilution for all three substances followed a Gaussian normal distribution (Figure 1A). Posaconazole MIC\textsubscript{0} values were lowest on average, itraconazole intermediate and voriconazole highest. Distributions of itraconazole and voriconazole were shifted apart approximately one 2-fold dilution. This was comparable to data obtained by the CLSI methodology (12), although differences to posaconazole were not as pronounced in our test set. This, however, may reflect differences between EUCAST and CLSI methodologies.

A total of N=17 (3.2\%) strains showed MIC\textsubscript{0} values above clinical breakpoints against at least one of the antifungal agents tested (Figure 1A and Table1). Out of these, 14 were highly resistant to itraconazole (MIC\textsubscript{0}>32) and one (strain 237) additionally to posaconazole (MIC\textsubscript{0}>32). Two other strains had a moderately reduced susceptibility to posaconazole (strains 31 and 279), or (strain 273) to all three substances (Figure 1C, D, and E, Table 1).
No geographical hotspots with isolates of a particular resistance mechanism could be identified (Figure 1B). For specimen subgroups prevalence of resistant isolates was 2.4% (non-CF pulmonary) 2.6% (invasive/wound) and 0% (skin). In isolates from cystic fibrosis patients the resistance rate was 5.2% (9/163).

Mutations underlying decreased azole drug susceptibility. Among the cyp51A mutations found in the set of isolates with decreased drug susceptibility, the TR/L98H variant was most prominent, but also isolates with G54W, M220I and the novel F219C substitution were found. Interestingly, the G54W isolate had apparently undergone a gene duplication, since sequencing reactions of PCR products consistently showed double signals specifically at this position. Most importantly, a similar number of isolates with the wild type allele was present among those with decreased susceptibility (Figure 1 C-E, Table 1). The MIC$_0$ values obtained from isolates carrying the M220I, G54W, and TR/L98H substitutions were within previously reported ranges (12-15).

Discussion
Prevalence of azole resistant A. fumigatus isolates in our cohort was 3.2% including isolates pre-screened by E-test at one study site. This rate is lower than what has been found in other studies. Prevalences ranging from 4.5% in Denmark (12), 8% in French cystic fibrosis patients, and 17% in the UK (2) have been described. In cystic fibrosis patients known to have received itraconazole prophylaxis, a prevalence of itraconazole resistant isolates was found up to 20% (16). The prevalence of resistant isolates in cystic fibrosis patients within our study was only 5.5%, however the prophylaxis status for our patients was unknown. The rate of PCR-detectable DNA from resistant strains in specimen from patients with chronic (CPA) or allergic (ABPA) pulmonary aspergillosis is again significantly higher (up to 50-75% (17) in small cohort sizes) than what is observed in viable A. fumigatus isolates. How this relates to disease and therapeutic outcome is currently unknown, but it indicates that current prevalence rates may still be underestimated.

Our epidemiological survey shows that, among other resistance phenotypes, the TR/L98H allele of cyp51A is also present in isolates from German patients. Isolates of this type were highly resistant to itraconazole and showed cross resistance to both voriconazole and
posaconazole. Nevertheless, although it constitutes a significant proportion ($N=6/17, 35.3\%$), it is not the only azole resistance-conferring \textit{cyp51A} mutation occurring: in addition to the isolates with the previously described G54W and M220I substitutions, we also found one isolate carrying the yet unobserved F219C substitution. The substitution F219I at this position has previously been implicated in azole resistance (18) indicating that F219C may also be causative for decreased azole susceptibility in this particular isolate; however this still needs to be confirmed on a molecular level. Furthermore, one isolate carrying the G54W substitution was resistant against both itraconazole and posaconazole (13). This isolate also had a potential duplication of \textit{cyp51A}.

As in this study, most other epidemiological studies found a significant proportion of isolates for which no mutation in \textit{cyp51A} can be identified (here $N=8/17, 47.1\%$). In such isolates other, unrelated mechanisms must be at work. Potentially, this may include increased production of the drug target protein Cyp51A (19-21) or increased drug efflux (22-23).

Currently, voriconazole is still the first-line treatment of choice for pulmonary aspergillosis (24). The major high level resistance observed within our set of isolates was directed solely against itraconazole; high level cross resistance was observed only in a single isolate between itraconazole and posaconazole. Nevertheless, all TR/L98H isolates showed increased MIC\textsubscript{50} values against the other two substances tested, at least partially above the clinical breakpoint. The same was true for itraconazole resistant isolates with the wildtype \textit{cyp51A} allele.

The TR/L98H allele is assumed to have derived through use of agricultural azoles which are structurally similar to clinically used ones (6-7). To what degree the different mutations are present in the German environment is unknown; this will be investigated in a future study. Also whether such resistant strains are propagated within the community or hospital settings is still unclear. Sometimes, conidiation is observed within tissues where the fungus is in contact with air (25); one can hypothesize that this could allow the distribution of isolates with resistance mutations from patients, however, this has not been described yet.

Where clinically relevant, azole susceptibility testing of \textit{A. fumigatus} should be implemented, including those patients receiving azole prophylaxis.
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Figure legends and Tables

Figure 1: Epidemiology. (A) Distribution of MIC<sub>0</sub> values for itraconazole, voriconazole and posaconazole in clinical isolates. (B) Geographical origin of clinical isolates. Numbers in grey fields are total number of isolates tested from a single city (if applicable including multiple laboratories); colored dots represent color-coded cyp51 mutations of sequenced isolates (see legend) contained in total. (C, D, E) Cross-resistances between itraconazole, voriconazole and posaconazole. Grey boxes in background are zones of intermediate susceptibility (EUCAST (11)). Grey ball sizes are relative to number of isolates with that particular MIC combination; color coded balls represent single, individual isolates.

a Isolates from Hamburg were pre-screened on-site by E-Test and only drug resistant isolates (N=1) were submitted to the broth microdilution procedure as outlined. Susceptible isolates by this definition are omitted from (A) and (C), see text.

b The non-normal distribution for posaconazole and voriconazole at the lower end is explained by the fact that this category probably contains isolates from multiple MIC values: 0.063 was the lowest drug concentration tested.

c Table 1: characteristics of isolates with decreased drug susceptibility

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<sup>a</sup> numbers in bold face are MIC<sub>0</sub> values above EUCAST clinical breakpoints (11) used for itraconazole (ITZ) and voriconazole (VRZ): 2 mg l<sup>-1</sup> (intermediate) and >2 mg l<sup>-1</sup> (resistant); for posaconazole (PSZ), 0.25 (intermediate) and >0.25 mg l<sup>-1</sup> (resistant).

<sup>b</sup> empty fields indicate “unknown”.

<sup>c</sup> albino variant with sparse conidiation only.
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