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# *cyp51A*-Based Mechanisms of *Aspergillus fumigatus* Azole Drug Resistance Present in Clinical Samples from Germany

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**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 $MIC_0$ values (the lowest concentrations with no visible growth) 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 the most prevalent, but isolates with the G54W and M220I substitutions and the novel F219C substitution were also 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 $MIC_0$ values. In conclusion, the prevalence of azole-resistant *A. fumigatus* in our clinical test set is lower than that 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.**

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 the increase 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 that affect the inhibition constants of azoles toward this enzyme and lead to decreased drug susceptibility *in vitro* are known (4, 5). Such mutations have been thought to arise during 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 $leucine_{98}$ 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 in the past few years the TR/L98H allele has been reported to occur worldwide in patients as well as the environment (e.g., see references 2, 8, and 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 and 2012.

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TABLE 1 Characteristics of isolates with decreased drug susceptibility

| Control type or geographical origin | Strain | $MIC_0$ (mg·liter$^{-1}$)[a] | | | Cyp51 amino acid substitution | Previous antifungal therapy[b] | Specimen type[b] | Cystic fibrosis[b] | Outcome[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | ITZ | VRZ | PSZ | | | | | |
| Negative control | DSM819 | 0.25 | 0.5 | 0.063 | None | | | | |
| Negative control | ATCC 46645 | 0.125 | 0.5 | 0.063 | None | | | | |
| Positive control | CR019 | **>32** | **8** | 0.5 | TR/L98H | | | | |
| Positive control | CR055 | **>32** | 1 | **2** | G54E | | | | |
| Positive control | CR059 | **>32** | **4** | **2** | M220V | | | | |
| Positive control | CR060 | **32** | **4** | 0.063 | M220T | | | | |
| Positive control | CR061 | **>32** | 2 | **2** | G54R | | | | |
| Augsburg | 273 | **8** | **8** | **1** | None | | Sputum | Yes | |
| Düsseldorf | 168 | **>32** | 1 | 0.125 | TR/L98H | Caspofungin | Alveolar lavage fluid | | Deceased |
| Freiburg | 231 | **>32** | 2 | 0.25 | None | | Bronchial secretion | | |
| Hamburg | 164 | **>32** | 2 | 0.25 | TR/L98H | | Tracheal secretion | | |
| Hannover | 158 | **>32** | 2 | 0.125 | None | None | Bronchus biopsy specimen | | |
| Hannover | 237 | **>32** | 0.25 | **>32** | Duplication + G54W | None | Alveolar lavage fluid | | Deceased |
| Koblenz | 243 | **>32** | **4** | 0.5 | None | | Nasal swab | | |
| Munich | 251 | **>32** | 2 | **1** | TR/L98H | | Sputum | Yes | |
| Munich | 262 | **>32** | 0.25 | 0.5 | M220I | | Oral swab | Yes | |
| Munich | 270 | **>32** | **4** | **1** | TR/L98H | | Sputum | Yes | |
| Munich | 281 | **>32** | **8** | **1** | TR/L98H | | Sputum | Yes | |
| Munich | 279[c] | 1 | 0.25 | **0.5** | None | | Sputum | Yes | |
| Tübingen | 34-1 | **32** | 1 | **1** | None | | Sputum | | |
| Tübingen | 31 | 0.125 | 0.5 | **0.5** | None | | Oral swab | Yes | |
| Tübingen | 170 | **>32** | 0.25 | 0.125 | F219C | | Sputum | Yes | |
| Tübingen | 248 | **>32** | 2 | 0.5 | None | | Sputum | Yes | |
| Würzburg | 267 | **>32** | **4** | 0.5 | TR/L98H | | Sputum | | |

[a] Numbers in boldface are $MIC_0$ values above the EUCAST clinical breakpoints (11) used for itraconazole (ITZ) and voriconazole (VRZ), which were 2 mg·liter$^{-1}$ for intermediate and >2 mg·liter$^{-1}$ for resistant, and for posaconazole (PSZ), which were 0.25 mg·liter$^{-1}$ for intermediate and >0.25 mg·liter$^{-1}$ for resistant.

[b] Empty fields indicate unknown.

[c] Albino variant with sparse conidiation only.

## 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 irrespective of the clinical relevance of the material collected for further processing. Where available, pseudonymized anamnesis data, including patient age and gender, underlying disease, previous and current antifungal drug treatment, as well as outcome of treatment, were obtained. For all isolates, the species was confirmed and the antifungal drug susceptibility pattern was 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 were confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Biotyper; Bruker Daltonics, Bremen, Germany) on extracted cells harvested from overnight shaking cultures in Sabouraud's medium (Oxoid, Wesel, Germany) using the Fungi Library database.

**Susceptibility testing.** Susceptibility to the antifungal drugs itraconazole and voriconazole (both from Discovery Fine Chemicals, Bournemouth, United Kingdom) and posaconazole (MSD Sharp & Dohme, Haar, Germany) was tested by broth microdilution according to the EUCAST reference method (10). Plates were incubated at 37°C for 48 h. The $MIC_0$ 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 ATCC 46645 were used (Table 1). All isolates with elevated $MIC_0$ values were additionally retested 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 $MIC_0$ values of ≤0.250 mg·liter$^{-1}$. Additionally, at one study site (Hamburg, Germany), isolates were prescreened by Etest, and only the resistant ones were submitted for further testing by broth dilution. For quality control purposes, eight isolates (six susceptible, two resistant) were blindly retested at a separate institution (Robert Koch-Institut, Berlin, Germany). The categorical agreement between those and our tests was 100% (data not shown).

**Sequence analysis.** From isolates with $MIC_0$ values above EUCAST breakpoints (11), the *cyp51A* coding region and its promoter were amplified by PCR in two overlapping fragments (the primers used were CYP51A-5 [5′-ATAATCGCAGCACCACTTCAGA-3′], CYP51A-7 [5′-CCTTGTCACCGTCAAGACGG-3′], CYP51A-6 [5′-TGGATGTGTTTTTCGACCGCTT-3′], and CYP51A-8 [5′-CGGATCGGACGTGGTGTATG-3′]), and each fragment was 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 those with the TR/L98H mutation, two independent sequences for *cyp51A* were obtained. For control purposes, the *cyp51A* sequences of 12 additional random isolates with itraconazole susceptibility in the upper susceptible range were initially determined 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 and 2012, a total of 527 clinical isolates were processed. The vast majority of isolates received were obtained from pulmonary/oropharyngeal specimens ($n = 353$), and out of these, at least 163 were derived from cystic fibrosis patients. Other isolates were either from skin ($n = 30$) or from invasive/wound infections ($n = 39$).

$MIC_0$ values in the susceptible range determined by the EUCAST broth microdilution procedure for all three substances followed a Gaussian normal distribution (Fig. 1A). Posaconazole $MIC_0$ values were the lowest, on average, itraconazole $MIC_0$ values were intermediate, and voriconazole $MIC_0$ values were the highest. The distributions of the itraconazole and voriconazole $MIC_0$ values were shifted apart approximately one 2-fold dilution. These data were comparable to data obtained by the CLSI methodology (12), although differences from the data for posaconazole were not as pronounced in our test set. This, however, may reflect differences between the EUCAST and CLSI methodologies.

A total of 17 (3.2%) strains showed $MIC_0$ values above the clinical breakpoints for at least one of the antifungal agents tested (Fig. 1A and Table 1). Out of these, 14 were highly resistant to itraconazole ($MIC_0 > 32$ mg·liter$^{-1}$) and 1 (strain 237) was addi-

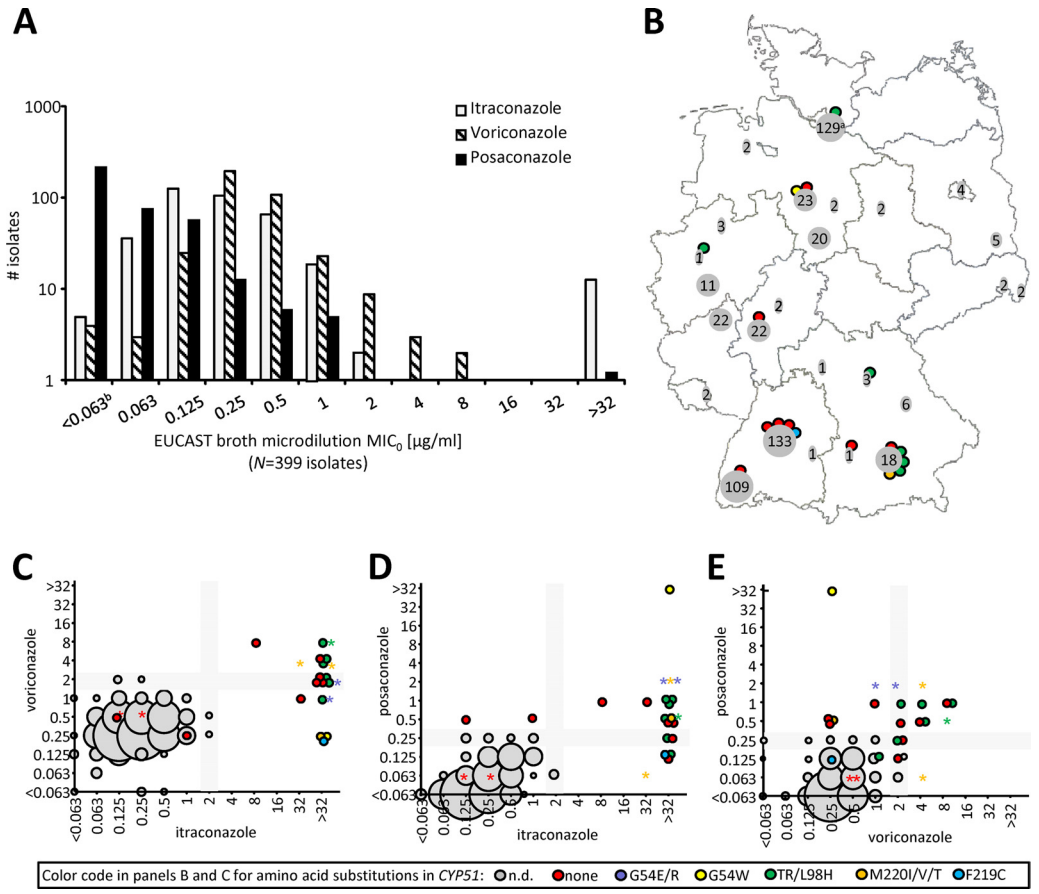


**FIG 1** Epidemiology. (A) Distribution of $MIC_0$ values for itraconazole, voriconazole, and posaconazole in clinical isolates. (B) Geographical origin of clinical isolates. Numbers in gray fields are the total number of isolates tested from a single city (if applicable, including multiple laboratories); colored dots represent color-coded *cyp51* mutations that the sequenced isolates (see the key at the bottom) contained in total. (C to E) Cross-resistance between itraconazole, voriconazole, and posaconazole. Gray boxes in the background are zones of intermediate susceptibility according to EUCAST breakpoints (11). The sizes of the gray balls are relative to the number of isolates with that particular MIC combination; color-coded balls represent single, individual isolates. [a], isolates from Hamburg were prescreened on-site by Etest, and only drug-resistant isolates ($n = 1$) were submitted to the broth microdilution procedure, as outlined in the text; the data for isolates susceptible by this definition are omitted from panels A and C (see the text); [b], the nonnormal distribution for posaconazole and voriconazole at the lower end is explained by the fact that this category probably contains isolates with multiple MIC values; 0.063 mg·liter$^{-1}$ was the lowest drug concentration tested. Asterisks, control isolates; balls, clinical isolates; n.d., not determined.

tionally highly resistant to posaconazole ($MIC_0 > 32$ mg·liter$^{-1}$). Two other strains had moderately reduced susceptibility to posaconazole (strains 31 and 279) or to all three substances (strain 273) (Fig. 1C to E; Table 1).

No geographical hot spots of isolates with a particular resistance mechanism could be identified (Fig. 1B). For specimen subgroups, the prevalences of resistant isolates were 2.4% for non-cystic fibrosis pulmonary isolates, 2.6% for invasive/wound isolates, and 0% for skin isolates. Among the 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 the most prominent, but isolates with the G54W and M220I substitutions and the novel F219C substitution were also 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 (Fig. 1C to 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

The prevalence of azole-resistant *A. fumigatus* isolates in our cohort, including isolates prescreened by Etest at one study site, was 3.2%. This rate is lower than what has been found in other studies. Prevalences ranging from 4.5% in Denmark (12) to 8% in French cystic fibrosis patients and 17% in the United Kingdom (2) have been described. In cystic fibrosis patients known to have received itraconazole prophylaxis, a prevalence of itraconazole-resistant isolates of up to 20% was found (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 specimens from patients with chronic pulmonary aspergillosis (CPA) or allergic bronchopulmonary aspergillosis (ABPA) was again significantly higher (up to 50 to 75% [17] in small cohorts) 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 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 constituted a significant proportion of isolates ($n = 6/17$, 35.3%), TR/L98H was not the only azole resistance-conferring *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 F-to-I substitution at position 219 has previously been implicated in azole resistance (18), indicating that F219C may also be the cause of 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 to both itraconazole and posaconazole. This isolate also had a potential duplication of *cyp51A*.

As in this study, most other epidemiological studies found a significant proportion of isolates for which no mutation in *cyp51A* could be identified (here, $n = 8/17$, 47.1%). In such isolates, other, unrelated mechanisms must be at work. This may potentially 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 and was observed between itraconazole and posaconazole. Nevertheless, all TR/L98H isolates showed increased $MIC_0$ values at least partially above the clinical breakpoint for the other two substances tested. The same was true for itraconazole-resistant isolates with the wild-type *cyp51A* allele.

The TR/L98H allele is assumed to have been derived through the 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. Whether such resistant strains are propagated within the community or hospital settings is also still unclear. Conidiation is sometimes 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 *A. fumigatus* isolates, including isolates from patients receiving azole prophylaxis, should be implemented.

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