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Investigation of Azole Resistance Involving *cyp*51A and *cyp*51B Genes in Clinical *Aspergillus flavus* Isolates

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Abstract

This study aimed to investigate azole resistance mechanisms in *Aspergillus flavus,* which involve *cyp*51A and *cyp*51B genes. Real-time Reverse Transcriptase qPCR method was applied to determine the overexpression of *cyp*51A and *cyp*51B genes for 34 *A. flavus* isolates. PCR sequencing of these two genes was used to detect the presence of gene mutations. Susceptibility test found sensitivity to voriconazole (VOR) in all strains. 14.7% and 8.8% of isolates were resistant to itraconazole (IT) and posaconazole (POS), respectively, with a cross-resistance in 5.8%. For the double resistant isolates (IT/POS), the expression of *cyp*51A was up to 17-fold higher. PCR sequencing showed the presence of 2 mutations in *cyp*51A: a synonymous point mutation (P61P) in eight isolates, which did not affect the structure of CYP51A protein, and another non synonymous mutation (G206L) for only the TN-33 strain (cross IT/POS resistance) causing an amino acid change in the protein sequence. However, we noted in *cyp*51B the presence of the only non-synonymous mutation (L177G) causing a change in amino acids in the protein sequence for the TN-31 strain, which exhibits IT/POS cross-resistance. A short single intron of 67 bp was identified in the *cyp*51A gene, whereas three short introns of 54, 53, and 160 bp were identified in the *cyp*51B gene. According to the models provided by PatchDock software, the presence of non-synonymous mutations did not affect the interaction of CYP51A and CYP51B proteins with antifungals. In our study, the overexpression of the *cyp*51A and *cyp*51B genes is the primary mechanism responsible for resistance in *A. flavus* collection. Nevertheless, other resistance mechanisms can be involved.

K e y w o r d s: *Aspergillus flavus*, antifungal susceptibility, mechanisms of resistance, azole resistance, DNA mutation, *cyp*51A, *cyp*51B

Introduction

Aspergillus flavus is a filamentous fungus, with worldwide distribution (Hedayati et al. 2007). After *Aspergillus fumigatus* this species is the second most important causative agent of invasive and non-invasive aspergillosis in patients (Baranyi et al. 2017). Three classes of antifungal agents form current treatments of invasive aspergillosis (IA): polyenes (amphotericin B (AMB)), azoles (voriconazole (VOR), itraconazole (IT), posaconazole (POS)) and more recently semi-synthetic echinocandins (caspofungin, micafungin, rezafungin and anidulafungin). Among these, azoles are the first choice drugs in managing and prophylaxis of aspergillosis (Patterson et al. 2016). The resistance to azoles has increased, especially in *Aspergillus* species. The overall azole resistance rate of *A. fumigatus* could reach 27.8%, according to Vermeulen et al. (2013). Prevalence rate variation between studies could be due to regional and disease differences (Vermeulen et al. 2013). Recently, Rhodes et al. (2022) explored 218 isolates collected from patients with different aspergillosis manifestations and from the environment. These *A. fumigatus* isolates were collected from different hospital centers (England, Wales, Scotland, and Ireland). They found that 49% showed resistance to at least one of the tested antifungal drugs. For specific azoles, 52% (n = 104) exceeded minimal inhibitory concentration (MIC) breakpoints to itraconazole (≥ 2 mg/l), 31% (n = 64) to voriconazole (≥ 2 mg/l), and 25% (n = 44) to posaconazole (≥ 0.5 mg/l). They also

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found that 26 isolates (12%) exceeded MIC breakpoints to two or more azole drugs from both clinical (n = 23) and environmental (n = 3) sources (Rhodes et al. 2022). Shelton et al. (2023) explored 1,894 air samples collected from England, Wales, Scotland, and Northern Ireland. They identified 99 TEB-resistant *A. fumigatus* isolates. These isolates were resistant to itraconazole (86%), to voriconazole (64%), to posaconazole (18%) and to isavuconazole (83%). Thus, there was clear cross-resistance between the agrochemical TEB and the medical azole antifungals (Shelton et al. 2023).

Knowing that antifungal susceptibility testing is not routine testing in many centers around the world, the azole resistance in *A. flavus* still needs to be fully explored. In addition, the molecular mechanism of azole resistance concerning this species of filamentous mold has yet to be fully explained.

Several studies have shown that azoles inhibit the ergosterol biosynthetic pathway by interacting with the two CYP51 proteins (CYP51A and CYP51B) and alter the metabolism of sterols in cells (Alcazar-Fuoli et al. 2008). However, a risk of decisive infection may occur if resistance is acquired (Howard and Arendrup 2011; Hadrich et al. 2012a). Various studies focus on azole-resistant *Aspergillus* species, with a particular interest in *A. fumigatus* isolates (Verweij et al. 2016; Chowdhary et al. 2017). Studying molecular mechanisms of resistance is an essential step and complementary to the in vitro identification of antifungal resistance (Buil et al. 2018). Over the last decade, there has been some progress in identifying genes responsible for antifungal resistance in *A. fumigatus*, but studies have been very rare in *A. flavus* (Hagiwara et al. 2016). A duplication of several genes (*cyp*51A and *cyp*51B) involved in the sterol biosynthesis pathway was noted in *A. fumigatus* (Mellado et al. 2001; Da Silva Ferreira et al. 2005; Nierman et al. 2005). In *A. flavus*, there are three CYP51 proteins: CYP51A, CYP51B, and CYP51C. Recently, rare studies have analyzed genetic alterations and/or overexpression of azole target genes and efflux pump genes (EPs) that reduce intracellular drug concentrations in azole-resistant isolates (Krishnan-Natesan et al. 2008; Liu et al. 2012; Paul et al. 2015). Furukawa et al. (2020) constructed a 484-member transcription factor null mutant library to provide a systematic evaluation of regulators that contribute to azole resistance in *A. fumigatus*. They identified 12 regulators that have a demonstrable role in itraconazole susceptibility. The loss of the negative cofactor 2 complex leads to resistance to itraconazole, amphotericin B, and terbinafine.

This study evaluated the mRNA expression levels of *cyp*51 (A and B) genes in clinical *Aspergillus flavus* isolates determined by RT-qPCR. A PCR sequencing of *cyp*51A and *cyp*51B genes was used to detect the presence of gene mutations.

Experimental

Materials and Methods

Patients and isolates. The 34 molecularly confirmed *A. flavus* sensu stricto clinical isolates included in the present study were collected from 14 patients hospitalized in the hematology unit at Hedi-Chaker Hospital, Sfax, Tunisia. Weekly samples (septum and nasal) were collected from immunocompromised patients with invasive aspergillosis. Eighteen isolates were cultured from sputum, ten from nasal swabs, five from bronchoalveolar lavage, and one from a pulmonary biopsy. All *A. flavus* strains were cultured on Sabouraud dextrose agar plates (SDA; AES™, bioMérieux, France). These *A. flavus* strains were identified based on morphological features and DNA sequences of the rRNA gene internal transcribed spaced regions ITS (de Hoog et al. 2007).

Antifungal resistance susceptibility by ETEST® method. The ETEST® strips (AES™, bioMérieux, France) were used as previously described to determine antifungal susceptibility profiles after 48 h of incubation (Hadrich et al. 2012b). Epidemiological cutoff values (ECVs) for POS (0.25 µg/ml), IT, and VOR (1 µg/ml) were previously described by five laboratories, according to the CLSI M38-A2 microdilution method (Espinel-Ingroff and Turnidge 2016).

Mechanisms of azole resistance. *DNA* and *RNA extractions.* DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Germany), as indicated by the manufacturer's instructions.

The RNeasy® Mini Kit (QIAGEN, Germany) was used according to the manufacturer's instructions to extract total RNA from the cell lysates. To avoid DNA contamination, RNA extracts were treated with RNase-free DNase (Promega, UK). The concentration and purity of the RNA were determined using a UV spectrophotometer by measuring the absorbance at 230 (A230), 260 (A260), and 280 nm (A280). The A260 nm/A280 nm ratio of the samples, reflecting the purity ranged from 2.06 to 2.21. The A260 nm/A230 nm ratio ranged from 1.90 to 2.50.

First strand cDNA synthesis. The transformation of the RNA extract from each isolate into cDNA was done by reverse transcription method (RT), according to the manufacturer's instructions of the PrimeScript™ RT Reagent Kit (Perfect Real Time) from Takara Bio Inc. (Japan).

Quantitative real-time PCR (qPCR). Specific primers and probes for gene expression analysis were designed using Primer3 software (https://bioinfo.ut.ee/primer3-0.4.0) (Koressaar and Remm 2007; Untergasser et al. 2012) and verified by sequence manipulation suite (https://www.bioinformatics.org/sms2) (Stothard 2000) (Table I).

Table I
The sequences of primers and probes used in RT-qPCR.

Gene	Primers and probes	
*cyp*51A	F	5'-GCGCGCATGAGGGAGAT-3'
	R	5'-CAATGCATGAGGTTCCAGATCA-3'
	Probe	HEX-TCATTAACGAGCGCCGCAAGAACC-MGB
*cyp*51B	F	5'-ATTCGACTCGACATTTGCTGAA-3'
	R	5'-GCATCACGCTTGCGGTTAT-3'
	Probe	FAM-CATGATCTCGACATGGGTTTTGCCC-MGB
ANXC4	F	5'-CCAACCCATAAACGCTCTGT-3'
	R	5'-TGGTGGGAATCTTGGAGAAC-3'
	Probe	CY5-ATCGAAGCAGCCTGTCTCAT-MGB

The measurement of the expression level and the number of copies of each of the two genes *cyp*51 A and *cyp*51 B was performed by qPCR quantitative time PCR. In this work, the normalization was done in relation to housekeeping gene Annexin (ANXC4).

Gene expression was analyzed using 10 µl TaqMan™ Universal PCR Master Mix (Applied Biosystems™; Thermo Fisher Scientific, Inc., USA) and 1 µl template (DNA or cDNA) with 20 pmol forward and reverse primers, 7 pmol hydrolysis probe. The PCR amplification conditions were as follows. The first step is 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, and the final step is 54°C for 1 minute. All reactions were performed in triplicate using the StepOne™ Real-Time PCR instrument (Applied Biosystems™; Thermo Fisher Scientific, Inc., USA). StepOne™ software version 2.1 was used to collect Cq data and calculate relative quantification (RQ). We then used the published comparative $2^{-\Delta\Delta Cq}$ method to normalize the folding changes in target gene expression to the reference gene according to the following formula:

$$RQ = 2 - (CqTarget - Cq\ Reference)\ Tested - (Cq\ Target - Cq\ Reference)\ Control$$

A 2.5-fold change was considered gene overexpression or an increase in gene copy number (Livak and Schmittgen 2001; VanGuilder et al. 2008).

Sequencing f PCR products. The *A. flavus cyp*51A gene, namely, *cyp*51A (NCBI accession number XM_002375082.1) and *cyp*51B (NCBI accession number XM_002379089.1) were obtained from the nucleotide databank NCBI BLAST (http://www.ncbi.nlm.nih.gov). Thus, we divided the gene of *cyp*51A into three overlapping fragments (*cyp*51AP1, *cyp*51AP2, and *cyp*51AP3) and the same for *cyp*51B (*cyp*51BP1, *cyp*51BP2, and *cyp*51BP3). According to these sequences, the primers for PCR sequencing assays were designed using Primer (version 3) software (https://frodo.wi.mit.edu) (Table II).

Amplification reactions were performed with a final volume of 50 µl containing 10 µl 5× reaction buffer (pH 8.5), 25 mM $MgCl_2$, 0.2 mM (each) dATP, dCTP, dGTP and dTTP (Promega, UK), 20 pmol of each primer, 2.5 U of GOTaq® DNA Polymerase (Promega, UK) and 400 ng of genomic DNA. PCR was performed in a thermocycler (Eppendorf, Germany), with the following amplification conditions: 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C and a final extension at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis and purified using the Purification PCR Wizard® Kit (Promega, UK). Both sequences of the triplicate PCR product were directly sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™; Thermo Fisher Scientific, Inc., USA) with primers and automated sequencer (ABI 3730; Applied Biosystems™; Thermo Fisher Scientific, Inc., USA), according to the manufacturer's instructions. Product sequences were compared with those of wild-type *A. flavus cyp*51A and *cyp*51B sequences using the NCBI Align Sequence Nucleotide

Table II
The sequences of primers used in PCR sequencing.

Gene Primers		
*cyp*51A	AP1F	5'-ATGGCATCCTTCACTCTCGT-3'
	AP1R	5'-CGATCAACTTCATGCTTCCG-3'
	AP2F	5'-TCTGGAACCTCATGCATTGT-3'
	AP2R	5'-TCCCTCGAAACCAGCAATTA-3'
	AP3F	5'-GGCAGGGTCGAAATCACGGA-3'
	AP3R	5'-GCCCGGATGGAAGAACCCTT-3'
*cyp*51B	BP1F	5'-CTTTTATCGGAAGTACCATC-3'
	BP1R	5'-CTTTATCAGGAACAACTTCG-3'
	BP2F	5'-ACTCTTCGCATACATGCACC-3'
	BP2R	5'-CCAACGTGCATGATATTGCC-3'
	BP3F	5'-CGACTCGACATTTGCTGAAC-3'
	BP3R	5'-CTCTTCGCATACATGCACCA-3'

BLAST and ClustalW V2 tools (https://www.genome.jp/tools-bin/clustalw).

Homology modeling. The DNA sequences of *cyp*51A and *cyp*51 B genes for 11 strains of *A. flavus* (six resistant and five sensitive to azoles) were submitted to the NCBI GenBank database with the access number in Table III. Then, they were translated into the equivalent amino acid sequences by using the Geniegene software (http://acces.ens-lyon.fr/acces/logiciels/applications/geniegen). The BLAST server (NCBI, USA) was used for Protein Data Bank (PDB) similarity searches to find homologous sequences (models) that matched known experimental 3D structures (Altschul et al. 1997). To align the protein sequences, the Clustal Omega program server (EMBL-EBI, UK) was used (Sievers et al. 2011). The automated protein structure homology-modeling server SWISS-MODEL (SIB, Switzerland) has generated a homology model for each target protein (Biasini et al. 2014).

The crystal structures of sterol 14-alpha demethylase (CYP51B) from a pathogenic filamentous fungus *A. fumigatus* in complex with different triazole deposited in the PDB under accession number 4UYL, 5FRB, and 6CR2 were used as the template.

The molecular docking studies were performed using open-source software (PatchDock) (http://bioinfo3d.cs.tau.ac.il/PatchDock) (Thenmozhi and Kannabiran 2013). For this, the input is two molecules of any type: proteins, DNA, peptides, and drugs. The output is a list of potential complexes sorted by shape complementarity criteria and a redirection of the results for refinement and scoring by the FireDock web server (http://bioinfo3d.cs.tau.ac.il/FireDock). A set of candidate complexes (consisting of receptors and ligands) is the input to the FireDock algorithm. All candidates are refined and ranked based on binding energies. The 3D visualization for observation and comparison of refined complexes is provided in the output (Mashiach et al. 2008).

Results

Antifungal susceptibility of *A. flavus*. The antifungal drug sensitivity profiles of the *A. flavus* strains were established by ETEST® strips. In all isolates, the MIC levels varied from 0.032 µg/ml to 1.5 µg/ml for IT and from 0.064 to 1 µg/ml for POS. According to the triazole ECVs established for *A. flavus* by the CLSI M38-A2 microdilution method, we noted the presence of 5 out of 34 *A. flavus* strains (14.71%) and 3 (8.82%) strains having IT and POS-resistant profiles, respectively. Only two strains (5.88%) could be considered cross-resistant to IT and POS (Table III). All isolates were sensitive to VOR.

Mechanisms of azole resistance. *Levels of cyp51A and cyp51B expression by A. flavus isolates.* Thirty-four clinical *A. flavus* isolates were analyzed by RT-qPCR to quantify expression and number of copies of the two genes (*cyp*51A and *cyp*51B) implicated in the molecular mechanisms associated with azole resistance.

So, the expression of *cyp*51A and B genes were tested on three IT resistant strains, two IT and POS resistant strains, one POS resistant strains, 28 susceptible strains compared to the ANXC4 gene, and the susceptible strain TN-855 (MIC IT: 0,032 µg/ml; MIC VOR: 0,025 µg/ml; MIC POS: 0,012 µg/ml).

The RT-qPCR results are presented in Fig. 1 and 2 as well as in Table III. In the 28 IT/POS sensitive isolates, *cyp*51A was overexpressed in four isolates and two isolates concerning *cyp*51B. When examining the 2 IT/POS resistant isolates, overexpression was observed in these two isolates for *cyp*51 A. The strain (TN-33) showed the highest level (16.89-fold), followed by the strain (TN-31), which had an overproduction of 12.99-fold (Table III). Furthermore, the overexpression was observed only for strain (TN-31) in the *cyp*51B gene (11.32 fold). We noted that three strains of resistant IT phenotype had no overexpression for the two genes *cyp*51A and *cyp*51B. However, the TN-32 strain of the resistant

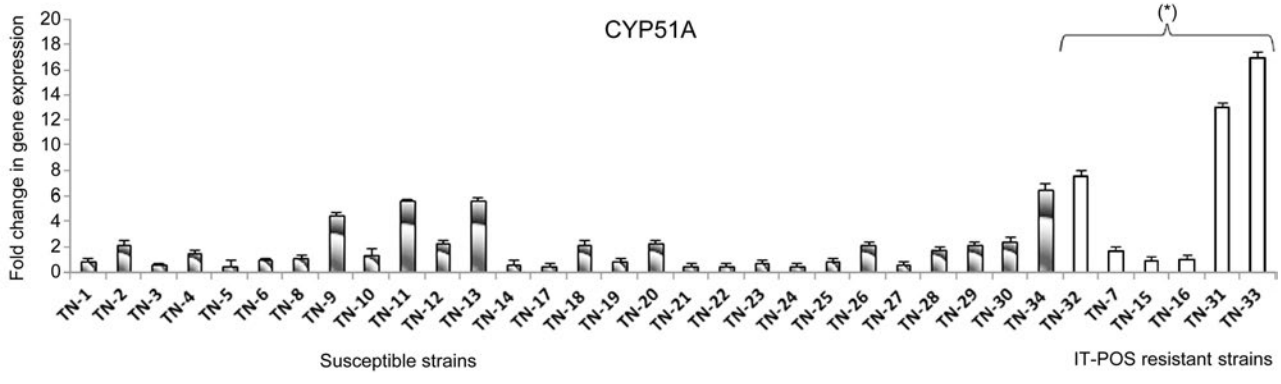


Fig. 1. Study of the level of expression of *cyp*51A genes by relative quantification with RT-qPCR in *Aspergillus flavus*: 28 susceptible strains (striped bars) and 6 IT-POS resistant strains (white bars). Gene expression values are represented as bar plots with mean + SD. *p*-Values were calculated using the Mann-Whitney *U* test, *p*-values of statistical tests are shown within the graphs.

$^{*}p < 0.005$

Table III
Antifungal susceptibility to the azoles, relative quantification of gene expression, gene copy number of *cyp*51A and *cyp*51B genes, and mutation in *Aspergillus flavus*.

		Strain	GenBank accession	Isolation site	Isolation date	Itraconazole		Posaconazole		RNA relative quantification				DNA relative quantification				*Cyp*51A		*Cyp*51B	
						MIC (µg/ml)	R/S	MIC (µg/ml)	R/S	*cyp*51A	± SD	*cyp*51B	± SD	*cyp*51A	± SD	*cyp*51B	± SD	GenBank accession	Punctual mutation	GenBank accession	Punctual mutation
Patient 1	Invasive aspergillosis	TN-1	MN964040	nasal	15/10/2018	0.125	S	0.125	S	0.90	± 0,02	1.16	± 0,04	0.69	± 0,05	0.57	± 0,02	ND	ND	ND	ND
		TN-2	MN964041	sputum	15/10/2018	0.125	S	0.125	S	2.15	± 0,03	0.73	± 0,02	0.89	± 0,02	1.98	± 0,04	ND	ND	ND	ND
		TN-3	MN964042	sputum	22/10/2018	0.125	S	0.125	S	0.58	± 0,01	0.90	± 0,02	1.37	± 0,02	0.97	± 0,02	ND	ND	ND	ND
Patient 2	Invasive aspergillosis	TN-4	MN964043	nasal	15/10/2018	0.125	S	0.19	S	1.53	± 0,02	1.05	± 0,04	1.27	± 0,03	1.93	± 0,02	ND	ND	ND	ND
		TN-5	MN964044	sputum	15/10/2018	0.125	S	0.064	S	0.51	± 0,04	0.05	± 0,02	1.05	± 0,02	1.92	± 0,06	ND	ND	ND	ND
		TN-6	MN964045	nasal	23/10/2018	0.032	S	0.094	S	0.95	± 0,01	0.89	± 0,02	1.69	± 0,03	0.93	± 0,02	ND	ND	ND	ND
Patient 3	Invasive aspergillosis	TN-7	MN964046	nasal	30/10/2018	1.5	R	0.125	S	1.65	± 0,02	0.73	± 0,03	2.51	± 0,02	1.98	± 0,02	MN964023	C-T*(183)	MN964034	No mutation
		TN-8	MN964047	sputum	30/10/2018	0.5	S	0.094	S	1.12	± 0,02	0.73	± 0,02	1.13	± 0,07	5.97	± 0,04	ND	ND	ND	ND
Patient 4	Invasive aspergillosis	TN-9	MN964048	sputum	30/10/2018	0.75	S	0.125	S	4.48	± 0,02	3.89	± 0,02	2.71	± 0,02	2.67	± 0,02	ND	ND	ND	ND
		TN-10	MN964049	nasal	30/10/2018	0.38	S	0.064	S	1.42	± 0,02	1.97	± 0,04	2.49	± 0,03	2.01	± 0,03	ND	ND	ND	ND
Patient 5	Invasive aspergillosis	TN-11	MN964050	sputum	10/11/2018	0.5	S	0.125	S	5.65	± 0,01	2.31	± 0,02	1.87	± 0,02	1.02	± 0,02	MN964018	No mutation	MN964029	No mutation
		TN-12	MN964051	BAL	17/11/2018	0.125	S	0.125	S	2.35	± 0,02	2.31	± 0,02	1.87	± 0,02	1.02	± 0,02	MN964019	C-T*(183)	MN964030	No mutation
Patient 6	Invasive aspergillosis	TN-13	MN964052	BAL	03/12/2018	0.75	S	0.19	S	5.68	± 0,02	2.35	± 0,05	2.83	± 0,05	3.95	± 0,02	ND	ND	ND	ND
		TN-14	MN964053	sputum	26/11/2018	0.5	S	0.125	S	0.63	± 0,03	0.88	± 0,06	6.95	± 0,07	1.06	± 0,02	ND	ND	ND	ND
		TN-15	MN964054	nasal	26/11/2018	1	R	0.19	S	0.90	± 0,02	0.76	± 0,02	0.53	± 0,02	1.65	± 0,05	MN964024	C-T*(183)	MN964035	No mutation
		TN-16	MN964055	sputum	10/11/2018	1	R	0.19	S	1.02	± 0,02	1.22	± 0,05	2.34	± 0,04	2.84	± 0,02	MN964025	C-T*(183)	MN964036	No mutation
Patient 7	Invasive aspergillosis	TN-17	MN964056	sputum	01/03/2017	0.75	S	0.125	S	0.48	± 0,02	1.10	± 0,02	0.77	± 0,02	2.07	± 0,06	ND	ND	ND	ND
		TN-18	MN964057	BAL	15/03/2017	0.5	S	0.125	S	2.18	± 0,03	1.81	± 0,02	2.11	± 0,02	2.17	± 0,02	ND	ND	ND	ND
		TN-19	MN964058	sputum	08/03/2017	0.75	S	0.125	S	0.92	± 0,02	0.76	± 0,02	0.57	± 0,02	1.11	± 0,02	ND	ND	ND	ND
		TN-20	MN964059	sputum	15/03/2017	0.38	S	0.064	S	2.27	± 0,02	2.88	± 0,05	0.64	± 0,04	1.09	± 0,02	ND	ND	ND	ND
Patient 8	Invasive aspergillosis	TN-21	MN964060	sputum	08/03/2017	0.38	S	0.125	S	0.45	± 0,02	0.88	± 0,02	6.87	± 0,02	1.82	± 0,02	MN964020	C-T*(183)	MN964031	No mutation
Patient 9	Invasive aspergillosis	TN-22	MN964061	sputum	01/03/2017	0.25	S	0.125	S	0.49	± 0,02	0.55	± 0,02	1.84	± 0,02	1.68	± 0,04	ND	ND	ND	ND
Patient 10	Invasive aspergillosis	TN-23	MN964062	sputum	15/03/2017	0.38	S	0.125	S	0.69	± 0,02	0.95	± 0,03	1.73	± 0,03	1.59	± 0,02	MN964021	C-T*(183)	MN964032	No mutation
Patient 11	Invasive aspergillosis	TN-24	MN964063	sputum	01/03/2017	0.38	S	0.125	S	0.51	± 0,02	0.87	± 0,02	1.62	± 0,02	1.66	± 0,09	ND	ND	ND	ND
		TN-25	MN964064	sputum	08/03/2017	0.75	S	0.125	S	0.81	± 0,02	0.75	± 0,01	1.62	± 0,01	1.48	± 0,02	ND	ND	ND	ND
		TN-26	MN964065	BAL	15/03/2017	0.75	S	0.125	S	2.21	± 0,02	2.51	± 0,02	0.67	± 0,02	1.42	± 0,02	ND	ND	ND	ND
Patient 12	Invasive aspergillosis	TN-27	MN964066	BAL	03/12/2018	0.25	S	0.125	S	0.65	± 0,02	0.78	± 0,04	5.95	± 0,03	1.03	± 0,08	ND	ND	ND	ND
		TN-28	MN964067	nasal	26/11/2018	0.25	S	0.125	S	1.73	± 0,02	0.88	± 0,02	1.95	± 0,02	1.06	± 0,02	ND	ND	ND	ND
		TN-29	MN964068	sputum	26/11/2018	0.5	S	0.094	S	2.11	± 0,02	0.48	± 0,02	4.39	± 0,02	1.95	± 0,02	MN964022	No mutation	MN964033	No mutation
Patient 13	Invasive aspergillosis	TN-30	MN964069	sputum	01/03/2017	0.5	S	0.094	S	2.37	± 0,02	2.27	± 0,08	0.68	± 0,07	1.44	± 0,03	ND	ND	ND	ND
		TN-31	MN964070	lung biopsy	17/04/2017	1.5	R	0.75	R	12.99	± 0,02	11.32	± 0,02	3.07	± 0,04	2.71	± 0,02	MN964026	C-T*(183)	MN964037	A-G*(529)
		TN-32	MN964071	nasal	01/03/2017	0.5	S	1	R	7.49	± 0,02	3.51	± 0,08	1.97	± 0,02	0.77	± 0,05	MN964027	C-T*(183)	MN964038	No mutation
Patient 14	Invasive aspergillosis	TN-33	MN964072	nasal	21/03/2018	1	R	0.75	R	16.89	± 0,02	2.48	± 0,02	2.57	± 0,06	1.71	± 0,02	MN964028	G-A*(616)	MN964039	No mutation
		TN-34	MN964073	nasal	28/03/2018	0.38	S	0.064	S	6.49	± 0,02	1.52	± 0,08	0.97	± 0,02	0.77	± 0,06	ND	ND	ND	ND

* – Punctual mutation, S – susceptible, R – resistant, VOR – voriconazole, AMB – amphotericin B, ND – not done

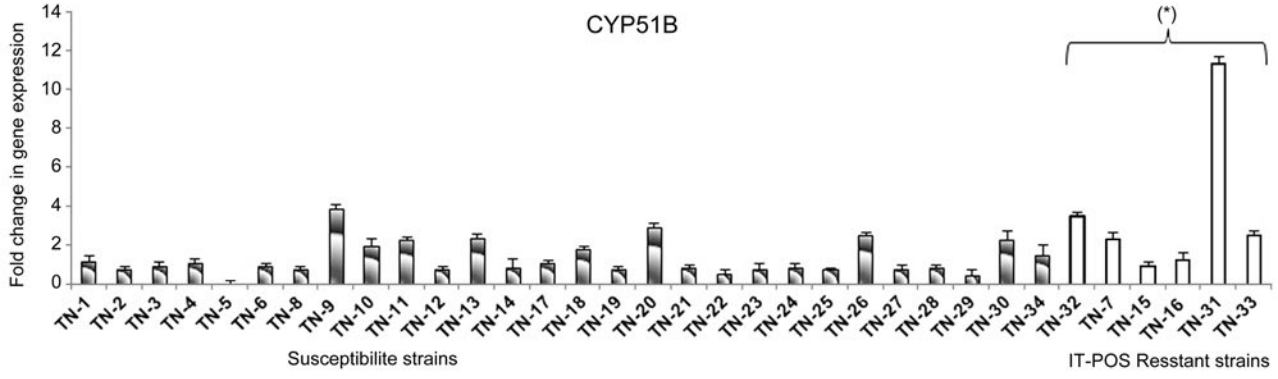


Fig. 2. Study of the level of expression of *cyp*51B genes by relative quantification with RT-qPCR in *Aspergillus flavus*: 28 susceptible strains (striped bars) and 6 IT-POS resistant strains (white bars). Gene expression values are represented as bar plots with mean + SD. *p*-Values were calculated using the Mann-Whitney *U* test, *p*-values of statistical tests are shown within the graphs.
$^{*}p < 0.005$

phenotype showed an overexpression of 7.49 and 3.51-fold for these two genes, respectively.

The overexpression of the *cyp*51A genes was associated with an increased copy number of genes in *A. flavus* strains TN-31 and TN-33. Additionally, the presence of *cyp*51B in multiple copies in the genome has been associated with overexpression in the TN-31 strain.

Sequencing of PCR products. The PCR sequencing showed the presence of three mutations for *cyp*51A and *cyp*51B.

The protein-coding regions of the *cyp*51A and *cyp*51B genes consist of 1,606 bp and 1,533 bp, respectively. A single short 67 bp intron was identified in the *cyp*51A gene, while three short 54, 53, and 160 bp introns were identified in the *cyp*51B gene. The protein coding sequence (1,539 bp) of *cyp*51A encodes a polypeptide of 513 amino acids, while the coding sequence of the *cyp*51B gene (1,266 bp) encodes a polypeptide of 422 amino acids.

The sequence of the *cyp*51A gene in 11 *A. flavus* isolates was compared with the sequence of wild-type *A. flavus* strain (NCBI access number XM_002375082.1) (Fig. 3). A nucleotide transition from "C" to "T" at codon 183 in 8 isolates (23.52%) was shown. However, this mutation was synonymous (P61P) and does not affect the structure of the CYP51A protein. This synonymous punctual mutation was also detected in 2 sensitive strains (TN-21, TN-23). We also showed another nonsynonymous mutation caused by the nucleotide transition from "G" to "A" at codon 616 (G206L)

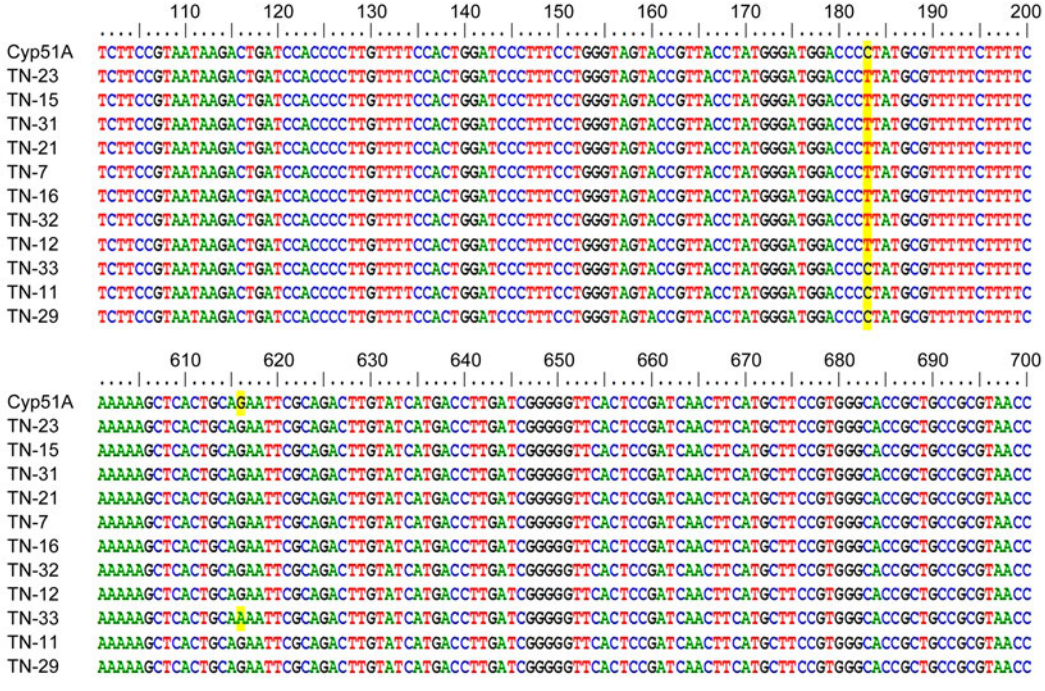


Fig.3. Alignment of the DNA sequences of the *cyp*51A gene fragments from *Aspergillus flavus*, was compared with wild type *A. flavus* strain (Genbank ID: XM_002375082.1).

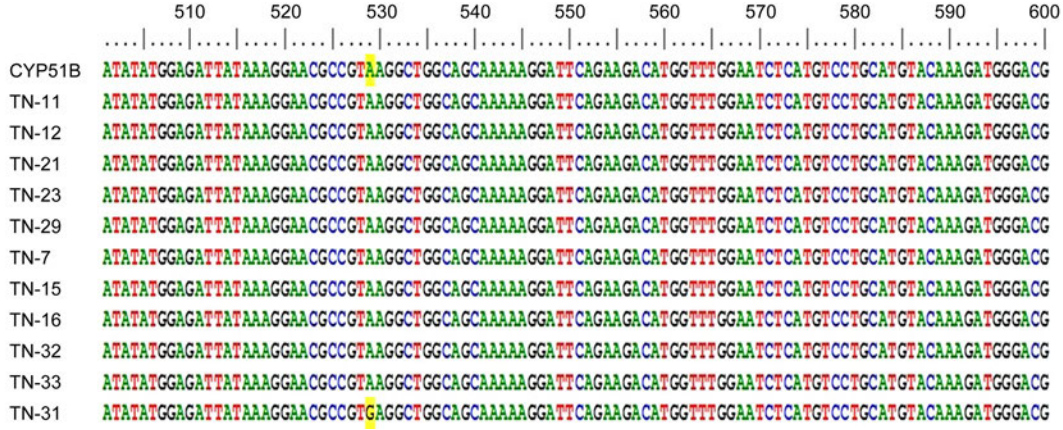


Fig. 4. DNA sequences of the *cyp*51B gene fragments from *Aspergillus flavus* were compared with wild type *A. flavus* strain (Genbank ID: XM_002379089.1).

for the TN-33 strain with cross IT/POS resistance, causing an amino acid change in the protein sequence (Table III and Fig. 3).

For *cyp*51B, Fig. 4 illustrates the alignment of our sequences *A. flavus* isolates of the *cyp*51B gene with wild type *A. flavus* strain (NCBI access number XM_002379089.1). Our results showed the presence of a nucleotide transition from "A" to "G" at codon 529 (L177G) for only the TN-31 strain, which in fact exhibits IT-POS cross resistance causing a change in amino acids in the protein sequence.

Homology modeling. Sequence alignment was performed using the Clustal Omega program for homology modeling, and open-source software (PatchDock) was used to generate the interaction between the structure of the protein and the drug automatically.

In addition, for all *A. flavus* isolates, the wild or modified structure of the protein CYP51A and CYP51B showed the interaction with VOR, IT and POS (Fig. 5 and 6), and these could be explained by the fact that these modifications did not affect the protein and antifungal interaction site.

Discussion

Several antifungal drugs can be used against *Aspergillus* spp. such as amphotericin B, triazoles and echinocandins. In patients with aspergillosis who require long-term treatment, triazoles become the most effective because they are essential therapeutic options and are of particular significance due to the availability of oral formulations (Denning and Perlin 2011; Arikan-Akdagli et al. 2018).

The triazole antifungals present a broad spectrum for the management of invasive aspergillosis. The treatment of *A. flavus* infection is similar to that caused by other species of *Aspergillus* (Meena et al. 2021).

Over the past two decades, many researchers have studied and identified that resistance mechanisms to azoles vary depending on the country and the studies of clinical and environmental strains of *A. flavus* (Sharma et al. 2018). However, most recently published studies refer to alterations in the structure of *cyp*51A, *cyp*51B, or *cyp*51C, or of these expression levels, as the primary resistance mechanisms of this fungus, which leads to the failure of the treatment in patients with aspergillosis infected with azole-resistant strains. It should be noted that there are two reported pathways for developing azole resistance: the first environmental, corresponding to the use of fungicides to protect crops, and a second medicine due to long-term azole therapies (Gonzalez-Jimenez et al. 2020).

A. flavus has three different enzymes, which are successfully inhibited by azole drugs, suggesting that these enzymes can play a role in azole resistance (Mellado et al. 2001; Howard et al. 2011). However, other *Aspergillus* spp. and most filamentous fungi possess two CYP51 proteins, CYP51A and CYP51B, although some species have only one (Hawkins et al. 2014). A CYP51A, A2, and B paralog pattern was observed in the azole-resistant species *Aspergillus fumigatiaffinis*. A new CYP51 paralog, CYP51D, was recently described in fungi, which predominantly occurs in genomes of intrinsically azole-resistant Eurotiomycetes (Van Rhijn et al. 2021). Celia-Sanchez et al. (2022) found four major CYP51 groups: CYP51, CYP51A, CYP51B, and CYP51C. They confirm that all filamentous Ascomycota had a CYP51B paralog, while only 50% had a CYP51A paralog. They also suggest that CYP51 is present in all fungi, including CYP51C, which appears to have diverged from the progenitor of the CYP51A and CYP51B groups. Several synonymous and non-synonymous point mutations were found in *cyp*51C gene of *A. flavus* among both susceptible and non-susceptible strains such as M54T, S240A, D254N, I285V, and N423D (Liu et al. 2012; Paul et al. 2015; Sharma et al.

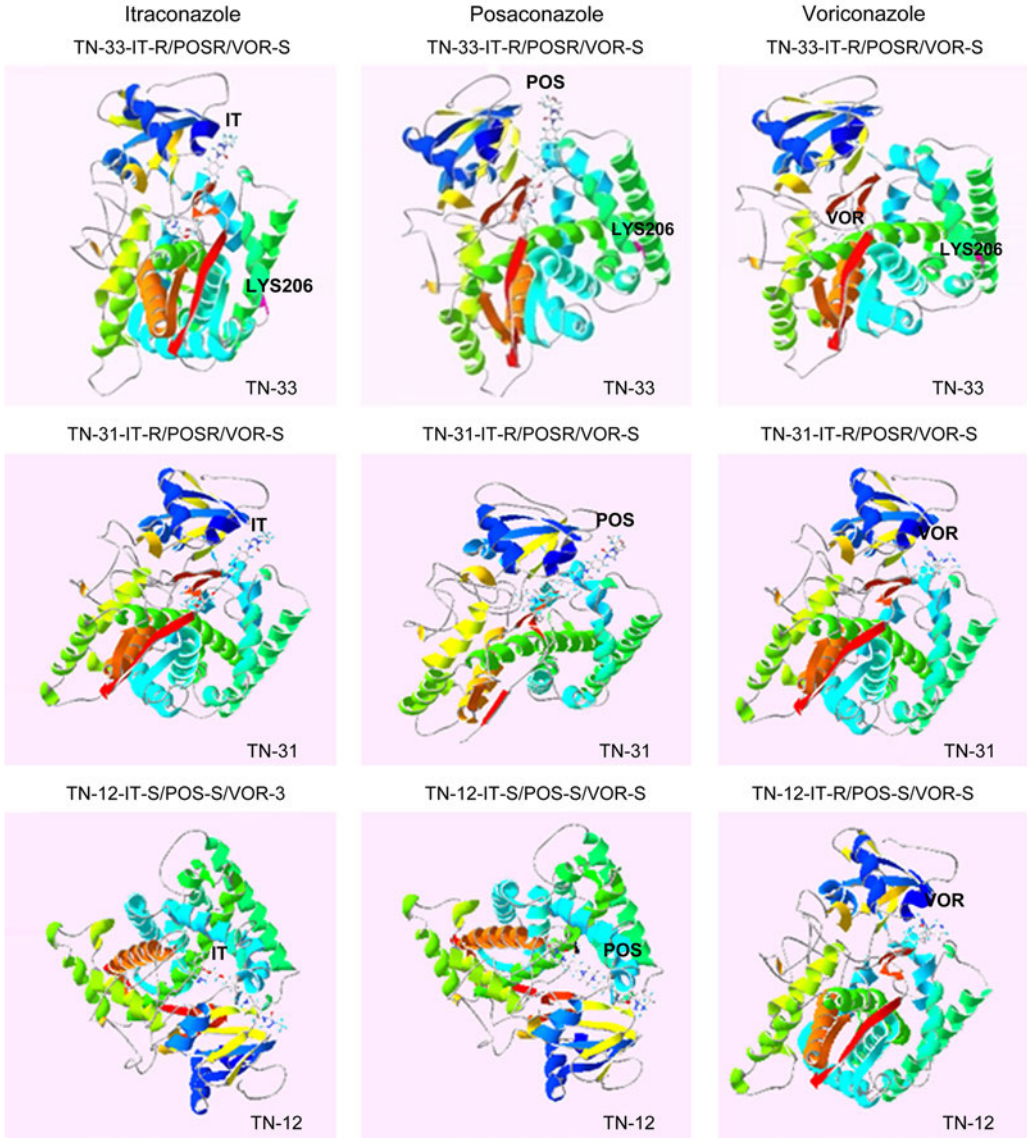


Fig. 5. Overall view of the 3D model of the *Aspergillus flavus* CYP51A protein in complex with three antifungal (IT, POS and VOR). Only TN33 strain presented the structure of CYP51A with the substitution of lysine for a glycine in the position 206 of the protein.

2018), D254G, P276T, S399I, and a mutation responsible for a stop codon (R250ST) (Lucio et al. 2020). The point mutation (Y319H) in *A. flavus cyp*51C suggests a relation to azole resistance although its role still needs further confirmation (Paul et al. 2015; Sharma et al. 2018).

However, there is minimal data on *A. flavus* isolates in Tunisia, and less is known of their resistance mechanisms, despite being the most commonly isolated mold species from patients and their environment (Hadrich et al. 2010). Therefore, our study evaluated the quantification of expression and number of copies for the two genes (*cyp*51A and *cyp*51B) in clinical *A. flavus* isolates IT-resistant and POS-resistant.

Our findings showed overexpression in two IT/POS resistant *A. flavus* isolates for *cyp*51A with the highest level of 16.8-fold and in one IT/POS resistant isolate (TN-31) for *cyp*51B, suggesting that these two genes contribute to resistance to azoles. According to Fattahi et al. (2015) a significant up-regulation of *cyp*51A (6.89 to 7.93-folds) ($p < 0.001$) in four *A. flavus* VOR-resistant isolates was found. Several authors have agreed that the up-regulation of the *cyp*51A gene is most commonly associated with azole resistance in *A. fumigatus* and *Aspergillus lentulus* (Howard et al. 2010; Mellado et al. 2011). In addition, CYP51B has been shown to be a functional biosynthetic enzyme that is inhibited by azoles. However, Liu et al. indicated that the expression levels of the three genes *cyp*51A, *cyp*51B, and *cyp*51C were not associated with azole resistance in *A. flavus* (Liu et al. 2012).

However, Sharma et al. (2018), found that *cyp*51A and *cyp*51B were upregulated (>3-fold) for a single

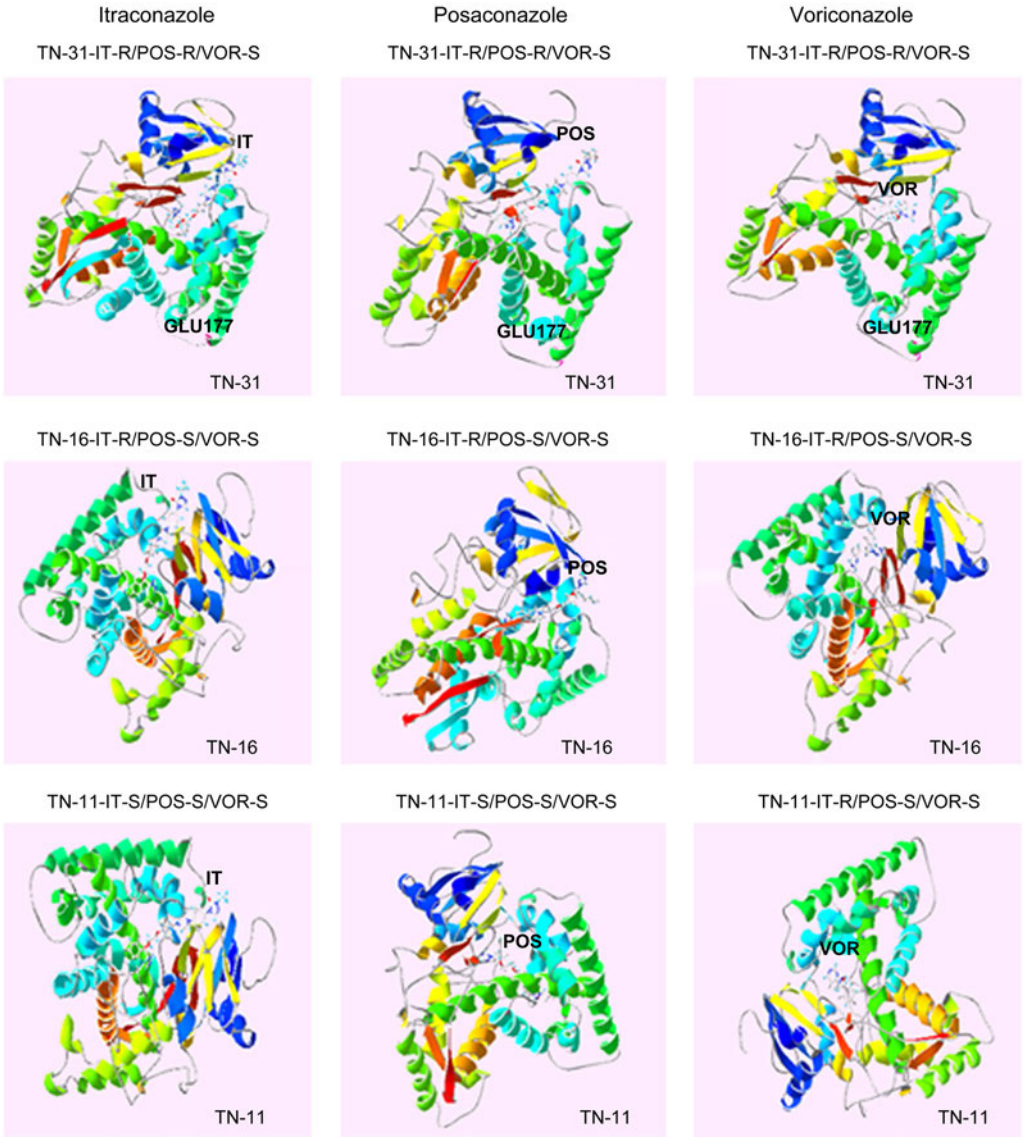


Fig. 6. Overall view of the 3D model of the *Aspergillus flavus* CYP51B protein in complex with three antifungal (IT, POS and VOR). Only TN31 strain presented the structure of CYP51B with the substitution of glycine for a lysine in the position 177 of the protein.

VOR-resistant strain (VPCI195/P/10). Contrary to what was expected, the only strain with cross-resistance to antifungals did not show up-regulation of any target genes analyzed.

In this study, the option of overexpression in sensitive profile strains may be explained by the vast clinical use of this antifungal in our country, particularly in patients with chronic diseases. This suggests that the *cyp*51A and B gene overexpression does not necessarily result in azole resistance in this fungus.

Furthermore, the mechanism of azole resistance in most of the previously published studies is associated with changes that are often corresponding to mutations in the *cyp*51A gene (Nascimento et al. 2003; Chen et al. 2005; Garcia-Effron et al. 2005; Buil et al. 2018). Several publications have clearly described single amino acid substitutions at G54, P216, F219, M220, and G448 in the CYP51A protein to confer azole resistance in *A. fumigatus* (Mann et al. 2003; Mellado et al. 2004; Camps et al. 2012; Krishnan Natesan et al. 2012). Over time, besides these 'hot spots', amino acid changes at other sites (Y121, G138, and Y431) have also been found in the CYP51A protein of resistant strains (Albarrag et al. 2001; Lescar et al. 2014). Prolonged azole treatment in chronic aspergillosis is a significant cause of these resistance mutations (Howard et al. 2009), as is environmental exposure to azole fungicides (Snelders et al. 2008). Typically, combinations of tandem repeats and point mutations of the *cyp*51A gene (TR34/L98H and TR46/Y121F/T289A) have been observed in azole-resistant *A. fumigatus* isolated from patients regardless of their azole treatment history (Snelders et al. 2008).

Here, we described for *A. flavus*, three new point mutations observed in *Cyp*51A (a synonymous substitution (P61P) and a non-synonymous (G206L)) and *Cyp*51B (a non-synonymous mutation in *cyp*51B (L177G). To our knowledge, these point mutations have not been described before. In a recent report, susceptible and non-susceptible *A. flavus* strains presented several synonymous and nonsynonymous point mutations in *cyp*51A and *cyp*51C genes. However, two amino acid mutations were noted in only two resistant strains: one strain contained a P214L substitution in *cyp*51A, and another was H349R in *cyp*51C (Gonzalez-Jimenez et al. 2020).

As stated previously, Krishnan-Natesan et al. (2008) were among the first to identify different mutations of *cyp*51A and *cyp*51B in spontaneous mutant strains obtained after exposure to voriconazole. These mutations were found in both *cyp*51A (K197N, D282E, M288L, Y132N, and T469S) and *cyp*51B (H399P, D411N, T454P, and T486P). However, these substitutions were recently revealed in azoles-sensitive *A. flavus* strains, making their implication in drug resistance highly unlikely (Hagiwara et al. 2016; Lucio et al. 2020). Similarly, Lucio et al. (2020) found that *cyp*51B showed no amino acid changes in *A. flavus* strains, except for some polymorphisms responsible for synonymous mutations. In our study, synonym and nonsynonymous mutations of *cyp*51A (P61P and G206L) were detected in azole-sensitive and non-sensitive *A. flavus* strains, excluding the possibility that they are associated with azole resistance. Similarly, Lucio et al. (2020) reported in susceptible and resistant strains the presence of three synonymous and nonsynonymous point mutations.

In previous studies, the crystallographic structures of the CYP51 proteins of *Saccharomyces cerevisiae* complexed with the substrates lanosterol, itraconazole, voriconazole, and fluconazole have been described (Monk et al. 2014; Sagatova et al. 2015). So also, the structure of *Candida albicans* CYP51 complexed with posaconazole (5FSA) and the tetrazole-based antifungal drug candidate VT1161 was analyzed (Hargrove et al. 2017a). Hargrove et al. (2017b) have described the structure of CYP51B in complex with the NIV derivative and the tetrazole-based VT-1598 inhibitor for *A. fumigatus*. Upon the recently resolved crystal structure of the CYP51B protein of *A. fumigatus*, a new 3D structural model of *A. flavus* CYP51A and CYP51B was built. Docking analysis of three azole molecules to their target proteins, CYP51A and CYP51B was performed based on this structural model.

According to the models provided by the PatchDock software, three new mutations, a synonym and a non-synonym in *cyp*51A (P61P and G206L) and a nonsynonymous mutation in *cyp*51B (L177G) do not affect the interaction between the drug and protein CYP51A or CYP51B. The resistance of our strains could be mainly due to the overexpression of the *cyp*51A and *cyp*51B genes. However, an in-depth study of azole resistance-related genes in a large population of clinical isolates will expand our knowledge.

Therefore, further examination of other resistance mechanisms is necessary, like the CCAAT-binding domain complex CBC (a heterotrimer comprising HapB, HapC, and HapE, which is a negative regulator of sterol biosynthesis directly binding the promoters of 14 ergosterol biosynthetic genes), overexpression of MFS (major facilitator superfamily) transporters or overexpression of ABC transporters (ATP binding cassette superfamily). A deeper understanding of azole resistance mechanisms will facilitate the development of new therapeutic drugs against azole-resistant strains of *Aspergillus*.

Ethical statement

This study protocol was reviewed and the need for approval was waived by the Ministry of Education and Scientific Research.

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Author contributions

Conceived and designed the experiments: Dhoha Ghorbel, Imen Amouri, Ines Hadrich. Performed the experiments: Dhoha Ghorbel, Ines Hadrich. Analyzed the data: Dhoha Ghorbel, Ines Hadrich, Imen Amouri, Sourour Neji. Contributed reagents/materials/analysis tools: Sourour Neji, Houaida Trabelsi, Nahed Khemekem, Hayet Sellami, Fattouma Makni, Ali Ayadi. Wrote the paper: Dhoha Ghorbel, Ines Hadrich, Sourour Neji. Involved in clinical management and provided clinical details: Sourour Neji, Nahed Khemekem, Houaida Trabelsi, Moez Elloumi, Hayet Sellami, Fattouma Makni, Ali Ayadi.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Alcazar-Fuoli L, Mellado E, Garcia-Effron G, Lopez JF, Grimalt JO, Cuenca-Estrella JM, Rodriguez-Tudela JL. Ergosterol biosynthesis pathway in *Aspergillus fumigatus*. Steroids. 2008 Mar;73(3):339–347. https://doi.org/10.1016/j.steroids.2007.11.005

Albarrag AM, Anderson MJ, Howard SJ, Robson GD, Warn PA, Sanglard D, Denning DW. Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in *cyp51A*, upregulation of *cyp51A*, and integration and activation of transposon *Atf1* in the *cyp51A* promoter. Antimicrob Agents Chemother. 2011 Nov; 55(11):5113–5121. https://doi.org/10.1128/AAC.00517-11

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 1997 Sep; 25(17):3389–3402. https://doi.org/10.1093/nar/25.17.3389

Arikan-Akdagli S, Ghannoum M, Meis JF. Antifungal resistance: Specific focus on multidrug resistance in *Candida auris* and secondary azole resistance in *Aspergillus fumigatus*. J Fungi. 2018 Dec; 4(4):129. https://doi.org/10.3390/jof4040129

Baranyi N, Kocsubé S, Jakšić Despot D, Šegvić Klarić M, Szekeres A, Bencsik O, Kecskeméti A, Manikandan P, et al. Combined genotyping strategy reveals structural differences between *Aspergillus flavus* lineages from different habitats impacting human health. J Basic Microbiol. 2017 Nov;57(11):899–909. https://doi.org/10.1002/jobm.201700243

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassarino T, Bertoni M, Bordoli L, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic Acids Res. 2014 Jul;42(W1):W252–W258. https://doi.org/10.1093/nar/gku340

Buil JB, Zoll J, Verweij PE, Melchers WJG. Molecular detection of azole-resistant *Aspergillus fumigatus* in clinical samples. Front Microbiol. 2018 Mar;9:515. https://doi.org/10.3389/fmicb.2018.00515

Camps SM, van der Linden JW, Li Y, Kuijper EJ, van Dissel JT, Verweij PE, Melchers WJ. Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: A case study and review of the literature. Antimicrob Agents Chemother. 2012 Jan;56(1):10–16. https://doi.org/10.1128/AAC.05088-11

Celia-Sanchez BN, Mangum B, Brewer M, Momany M. Analysis of Cyp51 protein sequences shows 4 major Cyp51 gene family groups across fungi. G3 Genes Genomes Genet. 2022 Nov;12(11):jkac249. https://doi.org/10.1093/g3journal/jkac249

Chen J, Li H, Li R, Bu D, Wan Z. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. J Antimicrob Chemother. 2005 Jan;55(1):31–37. https://doi.org/10.1093/jac/dkh507

Chowdhary A, Sharma C, Meis JF. Azole-resistant aspergillosis: Epidemiology, molecular mechanisms, and treatment. J Infect Dis. 2017 Aug;216(suppl_3):S436–S444. https://doi.org/10.1093/infdis/jix210

Da Silva Ferreira ME, Colombo AL, Paulsen I, Ren Q, Wortman J, Huang J, Goldman MHS, Goldman GH. The ergosterol biosynthesis pathway, transporter genes, and azole resistance in *Aspergillus fumigatus*. Med Mycol. 2005 Jan;43(Suppl_1):S313–S319. https://doi.org/10.1080/13693780400029114

de Hoog GS, Nishikaku AS, Fernandez-Zeppenfeldt G, Padín-González C, Burger E, Badali H, Richard-Yegres N, van den Ende AH. Molecular analysis and pathogenicity of the *Cladophialophora carrionii* complex, with the description of a novel species. Stud Mycol. 2007;58:219–34. https://doi.org/10.3114/sim.2007.58.08

Denning DW, Perlin DS. Azole resistance in *Aspergillus*: A growing public health menace. Future Microbiol. 2011 Nov;6(11):1229–32. https://doi.org/10.2217/fmb.11.118

Espinel-Ingroff A, Turnidge J. The role of epidemiological cutoff values (ECVs/ECOFFs) in antifungal susceptibility testing and interpretation for uncommon yeasts and moulds. Rev Iberoam Micol. 2016 Apr-Jun;33(2):63–75. https://doi.org/10.1016/j.riam.2016.04.001

Fattahi A, Zaini F, Kordbacheh P, Rezaie S, Safara M, Fateh R, Farahyar S, Kanani A, Heidari M. Evaluation of mRNA expression levels of *cyp51A* and *mdr1*, candidate genes for voriconazole resistance in *Aspergillus flavus*. Jundishapur J Microbiol. 2015; 8(12): e26990. https://doi.org/10.5812/jjm.26990

Ferreira ME, Colombo AL, Paulsen I, Ren Q, Wortman J, Huang J, Goldman MH, Goldman GH. The ergosterol biosynthesis pathway, transporter genes, and azole resistance in *Aspergillus fumigatus.* Med Mycol. 2005 May;43(Suppl_1):S313–S319. https://doi.org/10.1080/13693780400029114

Fuhren J, Voskuil WS, Boel CH, Haas PJ, Hagen F, Meis JF, Kusters JG. High prevalence of azole resistance in *Aspergillus fumigatus* isolates from high-risk patients. J Antimicrob Chemother. 2015 Oct;70(10):2894–2898. https://doi.org/10.1093/jac/dkv177

Furukawa T, van Rhijn N, Fraczek M, Gsaller F, Davies E, Carr P, Gago S, Fortune-Grant R, Rahman S, Gilsenan JM, et al. The negative cofactor 2 complex is a key regulator of drug resistance in *Aspergillus fumigatus*. Nat Commun. 2020 Jan;11(1):427. https://doi.org/10.1038/s41467-019-14191-1

Garcia-Effron G, Mellado E, Gomez-Lopez A, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Differences in interactions between azole drugs related to modifications in the 14-alpha sterol demethylase gene (*cyp51A*) of *Aspergillus fumigatus*. Antimicrob Agents Chemother. 2005 May;49(5):2119–2121. https://doi.org/10.1128/AAC.49.5.2119-2121.2005

Gonzalez-Jimenez I, Lucio J, Amich J, Cuesta I, Sanchez Arroyo R, Alcazar-Fuoli L, Mellado E. A Cyp51B mutation contributes to azole resistance in *Aspergillus fumigatus*. J Fungi. 2020 Nov;6(4):315. https://doi.org/10.3390/jof6040315

Hadrich I, Makni F, Neji S, Abbes S, Cheikhrouhou F, Trabelsi H, Sellami H, Ayadi A. Invasive aspergillosis: Resistance to antifungal drugs. Mycopathologia. 2012a Aug;174(2):131–141. https://doi.org/10.1007/s11046-012-9526-y

Hadrich I, Makni F, Neji S, Cheikhrouhou F, Bellaaj H, Elloumi M, Ayadi A, Ranque S. Amphotericin B *in vitro* resistance is associated with fatal *Aspergillus flavus* infection. Med Mycol. 2012b Nov; 50(8):829–834. https://doi.org/10.3109/13693786.2012.684154

Hadrich I, Makni F, Sellami H, Cheikhrouhou F, Sellami A, Bouaziz H, Hdiji S, Elloumi M, Ayadi A. Invasive aspergillosis: Epidemiology and environmental study in haematology patients (Sfax, Tunisia). Mycoses. 2010 Sep;53(5):443–447. https://doi.org/10.1111/j.1439-0507.2009.01710.x

Hagiwara D, Watanabe A, Kamei K, Goldman GH. Epidemiological and genomic landscape of azole resistance mechanisms in *Aspergillus* Fungi. Front Microbiol. 2016 Sep;7:1382. https://doi.org/10.3389/fmicb.2016.01382

Hargrove TY, Friggeri L, Wawrzak Z, Qi A, Hoekstra WJ, Schotzinger RJ, York JD, Guengerich FP, Lepesheva GI. Structural analyses of *Candida albicans* sterol 14α-demethylase complexed with azole drugs address the molecular basis of azole-mediated inhibition of fungal sterol biosynthesis. J Biol Chem. 2017a Apr;292(16): 6728–6743. https://doi.org/10.1074/jbc.M117.778308

Hargrove TY, Garvey EP, Hoekstra WJ, Yates CM, Wawrzak Z, Rachakonda G, Villalta F, Lepesheva GI. Crystal structure of the new investigational drug candidate VT-1598 in complex with *Aspergillus fumigatus* sterol 14α-demethylase provides insights into its broad-spectrum antifungal activity. Antimicrob Agents Chemother. 2017b Jun;61(7):e00570-17. https://doi.org/10.1128/AAC.00570-17

Hawkins NJ, Cools HJ, Sierotzki H, Shaw MW, Knogge W, Kelly SL, Kelly DE, Fraaije BA. Paralog re-emergence: A novel, historically contingent mechanism in the evolution of antimicrobial resistance. Mol Biol Evol. 2014 Jul;31(7):1793–1802. https://doi.org/10.1093/molbev/msu134

Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW. *Aspergillus flavus*: Human pathogen, allergen and mycotoxin producer. Microbiology. 2007 Jun;153(6):1677–1692. https://doi.org/10.1099/mic.0.2007/007641-0

Howard SJ, Arendrup MC. Acquired antifungal drug resistance in *Aspergillus fumigatus*: Epidemiology and detection. Med Mycol. 2011 Apr;49(Suppl_1):S90–S95. https://doi.org/10.3109/13693786.2010.508469

Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW. Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis. 2009 Jul;15(7):1068–1076. https://doi.org/10.3201/eid1507.090043

Howard SJ, Pasqualotto AC, Denning DW. Azole resistance in allergic bronchopulmonary aspergillosis and *Aspergillus bronchitis*. Clin Microbiol Infect. 2010 Jun;16(6):683–688. https://doi.org/10.1111/j.1469-0691.2009.02911.x

Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007 May;23(10): 1289–1291. https://doi.org/10.1093/bioinformatics/btm091

Krishnan-Natesan S, Chandrasekar PH, Alangaden GJ, Manavathu EK. Molecular characterisation of *cyp51A* and *cyp51B* genes coding for P450 14alpha-lanosterol demethylases A (CYP51Ap) and B (CYP51Bp) from voriconazole-resistant laboratory isolates of Aspergillus flavus. Int J Antimicrob Agents. 2008 Dec; 32(6): 519–524. https://doi.org/10.1016/j.ijantimicag.2008.06.018

Krishnan Natesan S, Wu W, Cutright JL, Chandrasekar PH. In vitro-in vivo correlation of voriconazole resistance due to G448S mutation (*cyp51A* gene) in *Aspergillus fumigatus*. Diagn Microbiol Infect Dis. 2012 Nov;74(3):272–277. https://doi.org/10.1016/j.diagmicrobio.2012.06.030

Lescar J, Meyer I, Akshita K, Srinivasaraghavan K, Verma C, Palous M, Mazier D, Datry A, Fekkar A. *Aspergillus fumigatus* harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. J Antimicrob Chemother. 2014 Dec;69(12):3244–3247. https://doi.org/10.1093/jac/dku316

Liu W, Sun Y, Chen W, Liu W, Wan Z, Bu D, Li R. The T788G mutation in the *cyp51C* gene confers voriconazole resistance in *Aspergillus flavus* causing aspergillosis. Antimicrob Agents Chemother. 2012 May;56(5):2598–603. https://doi.org/10.1128/AAC.05477-11

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 2001 Dec;25(4):402–408. https://doi.org/10.1006/meth.2001.1262

Lucio J, Gonzalez-Jimenez I, Rivero-Menendez O, Alastruey-Izquierdo A, Pelaez T, Alcazar-Fuoli L, Mellado E. Point mutations in the 14-α sterol demethylase Cyp51A or Cyp51C could contribute to azole resistance in *Aspergillus flavus*. Genes. 2020 Oct; 11(10):1217. https://doi.org/10.3390/genes11101217

Mann PA, Parmegiani RM, Wei SQ, Mendrick CA, Li X, Loebenberg D, DiDomenico B, Hare RS, Walker SS, McNicholas PM. Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase. Antimicrob Agents Chemother. 2003 Feb;47(2):577-81. https://doi.org/10.1128/AAC.47.2.577-581.2003

Mashiach E, Schneidman-Duhovny D, Andrusier N, Nussinov R, Wolfson HJ. FireDock: A web server for fast interaction refinement in molecular docking. Nucleic Acids Res. 2008 Jul;36(suppl_2): W229–W232. https://doi.org/10.1093/nar/gkn186

Meena DS, Kumar D, Bohra GK, Kumar G. Clinical manifestations, diagnosis, and treatment outcome of CNS aspergillosis: A systematic review of 235 cases. Infect Dis Now. 2021 Nov;51(8): 654–660. https://doi.org/10.1016/j.idnow.2021.04.002

Mellado E, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Role of *Aspergillus lentulus* 14-α sterol demethylase (Cyp51A) in azole drug susceptibility. Antimicrob Agents Chemother. 2011 Dec;55(12):5459–5468. https://doi.org/10.1128/AAC.05178-11

Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol. 2001 Jul;39(7):2431–2438. https://doi.org/10.1128/JCM.39.7.2431-2438.2001

Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. Antimicrob Agents Chemother. 2004 Jul;48(7):2747–2750. https://doi.org/10.1128/AAC.48.7.2747-2750.2004

Monk BC, Tomasiak TM, Keniya MV, Huschmann FU, Tyndall JD, O'Connell JD 3rd, Cannon RD, McDonald JG, Rodriguez A, Finer-Moore JS, et al. Architecture of a single membrane spanning cytochrome P450 suggests constraints that orient the catalytic domain relative to a bilayer. Proc Natl Acad Sci USA. 2014 Mar;111(10):3865–3870. https://doi.org/10.1073/pnas.1324245111

Nascimento AM, Goldman GH, Park S, Marras SA, Delmas G, Oza U, Lolans K, Dudley MN, Mann PA, Perlin DS. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob Agents Chemother. 2003 May;47(5):1719–1726. https://doi.org/10.1128/AAC.47.5.1719-1726.2003

Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, Berriman M, Abe K, Archer DB, Bermejo C, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature. 2005 Dec;438(7071):1151–6. https://doi.org/10.1038/nature04332

Patterson TF, Thompson GR 3rd, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis. 2016 Aug;63(4):e1–e60. https://doi.org/10.1093/cid/ciw326

Paul RA, Rudramurthy SM, Meis JF, Mouton JW, Chakrabarti A. A novel Y319H substitution in CYP51C associated with azole resistance in *Aspergillus flavus*. Antimicrob Agents Chemother. 2015 Oct; 59(10):6615–6619. https://doi.org/10.1128/AAC.00637-15

Rhodes J, Abdolrasouli A, Dunne K, Sewell TR, Zhang Y, Ballard E, Brackin AP, van Rhijn N, Chown H, Tsitsopoulou A, et al. Population genomics confirms acquisition of drug-resistant *Aspergillus fumigatus* infection by humans from the environment. Nat Microbiol. 2022 May;7(5):663–674. https://doi.org/10.1038/s41564-022-01091-2

Sagatova AA, Keniya MV, Wilson RK, Monk BC, Tyndall JD. Structural insights into binding of the antifungal drug fluconazole to *Saccharomyces cerevisiae* lanosterol 14α-demethylase. Antimicrob Agents Chemother. 2015 Aug;59(8):4982–9. https://doi.org/10.1128/AAC.00925-15

Sharma C, Kumar R, Kumar N, Masih A, Gupta D, Chowdhary A. Investigation of multiple resistance mechanisms in voriconazole-resistant *Aspergillus flavus* clinical isolates from a chest hospital surveillance in Delhi, India. Antimicrob Agents Chemother. 2018 Feb;62(3):e01928–17. https://doi.org/10.1128/AAC.01928-17

Shelton JMG, Rhodes J, Uzzell CB, Hemmings S, Brackin AP, Sewell TR, Alghamdi A, Dyer PS, Fraser M, Borman AM, et al. Citizen science reveals landscape-scale exposures to multiazole-resistant *Aspergillus fumigatus* bioaerosols. Sci Adv. 2023 Jul;9(29): eadh8839. https://doi.org/10.1126/sciadv.adh8839

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol. 2011 Oct;7:539. https://doi.org/10.1038/msb.2011.75

Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, Mellado E, Donders AR, Melchers WJ, Verweij PE. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS Med. 2008 Nov;5(11):e219. https://doi.org/10.1371/journal.pmed.0050219

Stothard P. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques. 2000 Jun;28(6):1102–1104. https://doi.org/10.2144/00286ir01

Thenmozhi M, Kannabiran K. Interaction of *Streptomyces* sp. VITSTK7 compounds with selected antifungal drug target enzymes by *in silico* molecular docking studies. Interdiscip Sci. 2013 Jun;5(2): 145–149. https://doi.org/10.1007/s12539-013-0163-z

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3 – new capabilities and interfaces. Nucleic Acids Res. 2012 Aug;40(15):e115. https://doi.org/10.1093/nar/gks596

Van Rhijn N, Bromley M, Richardson M, Bowyer P. CYP51 paralogue structure is associated with intrinsic azole resistance in fungi. mBio. 2021 Oct 26;12(5):e0194521. https://doi.org/10.1128/mBio.01945-21

VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques. 2008 Apr;44(5):619–626. https://doi.org/10.2144/000112776

Vermeulen E, Lagrou K, Verweij PE. Azole resistance in *Aspergillus fumigatus*: A growing public health concern. Curr Opin Infect Dis. 2013 Dec;26(6):493–500. https://doi.org/10.1097/QCO.0000000000000005

Verweij PE, Chowdhary A, Melchers WJ, Meis JF. Azole resistance in *Aspergillus fumigatus*: Can we retain the clinical use of mold-active antifungal azoles? Clin Infect Dis. 2016 Feb;62(3):362–368. https://doi.org/10.1093/cid/civ885