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Determination of azole antifungal drug resistance mechanisms involving *Cyp51A* gene in clinical isolates of *Aspergillus fumigatus* and *Aspergillus niger*

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ABSTRACT

Aims: The main aim of this research is to investigate azole resistance mechanisms in *Aspergillus fumigatus* and *Aspergillus niger* which involve *Cyp51A* gene that encodes 14-α *sterol demethylase* enzyme.
Methodology and results: Itraconazole susceptibility was determined through E-test method. A conventional PCR method was used to amplify and sequence *Cyp51A* gene in fungal DNA, to detect the presence of gene mutations. Real-time PCR method was applied to determine overexpression of *Cyp51A* gene in *A. fumigatus* and *A. niger* isolates. Susceptibility test found that 3/13 (23.1%) *A. fumigatus* and 7/23 (30.4%) *A. niger* isolates were resistant to itraconazole, with minimum inhibitory concentrations (MICs) of 2.5 µg/mL to 3.0 µg/mL. Sequencing of *A. fumigatus* DNA showed presence of L98H mutation in 7/13 (53.8%) and M220 mutation in 3/13 (23%) isolates. Whereas, sequencing of *A. niger* DNA detected the presence of G427S mutation in 3/23 (13%) isolates. Tandem Repeat mutation was not detected in all *A. fumigatus* and *A. niger* isolates. Only M220 mutation showed significant correlation ($r(13)=0.041038$, $p< 0.05$) with itraconazole antifungal resistance in *A. fumigatus* isolates while L98H mutation was not involved. G427S mutation also showed correlation ($r(15)=0.038434$, $p< 0.05$) with itraconazole antifungal resistance in *A. niger* isolates. A higher level of *Cyp51A* gene expression was detected in 4/8 (50%) *A. fumigatus* isolates and 7/10 (70%) *A. niger* isolates. Resistant isolates more often showed higher level of *Cyp51A* gene expression compared to susceptible isolates, however the difference in level of expression between resistant isolates and susceptible isolates is not significant.
Conclusion, significance and impact of study: In conclusion the level of azole resistance in *A. fumigatus* and *A. niger* isolates in Malaysia is low and mutations in *Cyp51A* gene may contribute towards itraconazole antifungal resistance, however other factors may also be involved.

Keywords: Aspergillus fumigatus, Aspergillus niger, itraconazole*, Cyp51A,* resistance

INTRODUCTION

Pathogenic fungi often cause serious infections to humans and other organisms. Although there are more than 1.5 million fungal species, only 72,000 species have been identified (Hawksworth, 2001) and fewer than 500 species are known to cause disease in humans (Warnock and Richardson, 2008). Many cases are caused by filamentous fungi such as *A. fumigatus* and *A. niger*. *Aspergillus* species are widespread in the environment, growing in the soil, on plants and on decomposing organic matter (Bennett, 2010). These moulds are often found in outdoor and indoor air, in water, on food items and dust. Infections with *A. fumigatus* and *A. niger* cause invasive aspergillosis worldwide, resulting in high rates of mortality and morbidity in immunocompromised patients (Warnock and Richardson, 2008).

Various antifungal drugs are used as treatment for fungal infections including azole compounds. An increase in infections due to azole-resistant *Aspergillus* species has been observed leading to a higher case fatality rate among patients with azole-resistant invasive aspergillosis. Increasing incidence of resistance including cross-resistance towards itraconazole, voriconazole and posaconazole has been reported in *A. fumigatus* and *A. niger* (Verweij *et al.*, 2011). In filamentous fungi, azole drugs inhibit ergosterol biosynthesis by targeting the enzyme 14-α sterol demethylase which is encoded by the gene *Cyp51A* (Brookman and Denning, 2000). By confirming that *Cyp51A* protein is the target of these antifungal agents, three molecular mechanisms of

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resistance to azole drugs have been described (a) azole drug resistance in *A. fumigatus* and *A*. *niger* seems to be mostly related to point mutations in *Cyp51A* gene, (b) overexpression of *Cyp51A* gene and (c) upregulation of efflux pumps (Snelders *et al.*, 2008; Howard and Arendrup, 2011). Point mutations result in a change of amino acids in the *Cyp51A* protein that prevents binding of the azole drug to the protein (Snelders *et al*., 2010), while the gene overexpression results in an increased amount of the target protein enzyme, preventing complete inhibition of ergosterol synthesis by the azole drug (Ghannoum and Rice, 1999). The upregulation of the efflux pumps causes the azole drugs to pumped out of the fungal cell, preventing their action (Rajendran *et al.*, 2011).

Although these factors influence antifungal drug resistance but other possible mechanisms have yet to be determined. Regarding the modification of *A. fumigatus* and *A*. *niger Cyp51A* gene, specific mutations have been associated with susceptibility profiles whereby cross-resistance to itraconazole has been associated with amino acid substitutions at leucine 98 (L98H) and methionine 220 (M220) of the target protein (Howard *et al.*, 2009). It has been determined that a base change causing an amino acid substitution in *Cyp51A* (L98H) in combination with the duplication in tandem of a 34 bp sequence in the *Cyp51A* promoter, is responsible for the increased level of *Cyp51A* gene expression, which accounted for resistance (Denning *et al.*, 1996).

There is minimal data on antifungal susceptibility of filamentous fungi in Malaysia and less is known of their resistance mechanisms, while *Aspergillus* is the most commonly isolated mold species (Tzar *et al.*, 2013). Therefore, the aim of this study was to determine the in vitro suseptibility of *A*. *fumigatus* and *A. niger* clinical isolates in Malaysia towards itraconazole and also to detect mutation or overexpression in *Cyp51A* gene that may contribute to antifungal drug resistance in both *Aspergillus* species.

MATERIALS AND METHODS

Fungal strains and growth conditions

The fungal strain used in the study were (i) *A*. *fumigatus* strains ATCC 22019, ATCC 204305, reference strain F/19029; (ii) *A*. *niger* strains ATCC 6275, ATCC 90028, reference strain F/13295 (iii) Quality control strains ATCC 90028 and ATCC 22019. The reference strains were kindly provided by Dr. David W. Denning (University of Manchester, United Kingdom). A total of 13 clinical isolates of *A. fumigatus* and 23 clinical isolates of *A. niger* isolated from specimens such as sputum, pus, skin and nail were collected from the Mycology Unit, Universiti Kebangsaan Malaysia Medical Centre (UKMMC) and evaluated for susceptibility towards itraconazole. The fungi were grown at room temperature on Sabouraud Dextrose Agar (SDA), (Merck, Germany) and the fungus stocks were preserved in Potato Dextrose Agar slants (PDA), (Merck, Germany) at 4 °C.

Antifungal susceptibility testing

The clinical isolates were cultured on Potato Dextrose Agar at room temperature for 48 h to prepare the fungal inoculum at the recommended colony forming unit (CFU) concentration. The inoculum suspension of the fungal conidia was prepared at 0.4×10^3 CFU/mL to 0.5×10^3 CFU/mL using a spectrophotometer (530 nm; OD 0.09 - 0.13) as described in the CLS document M38-A (Espinel-Ingroff *et al*., 2010). Susceptibility assay was performed by the E-test method according to the manufacturer's instructions (AB Biodisk, Sweden). *A*. *fumigatus* and *A*. *niger* conidia were plated onto RPMI 1640 agar supplemented with 2% glucose, buffered with 0.165 M MOPS (3-(N-morpholinopropanesulfonic acid) containing L-glutamine and sodium bicarbonate and the plate was allowed to dry. E-test strips containing itraconazole were applied, and the MIC was determined after 48 h. The MIC was considered to be the drug concentration at which dense colony growth intersected the strip, but sparse subsurface hyphal growth at the margins was ignored (see Figure 1). *Candida albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were used as quality control strains and all results were within the target range (MIC: 0.0064 µg/mL - 0.25 µg/mL).

Figure 1: E-test result showing zone of inhibition of fungal growth intersecting the drug strip.

Fungal DNA extraction

Extraction of DNA from fungal cultures on PDA was performed using Dneasy Plant Mini kit, (Qiagen, Germany) according to the manufacturer's instructions.

Fungal RNA extraction

All selected fungal isolates were processed in liquid nitrogen for hyphal disruption. Extraction of RNA was performed using RNeasy Plant Mini kit, (Qiagen, Germany) according to the manufacturer's instructions.

PCR and sequencing

Four different primer sets for amplification of four *Cyp51A* gene mutations were synthesized by 1st Base, Seri Kembangan, Malaysia (Table 1). Primers were selected based on previous studies (Mellado *et al*., 2001; Howard

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et al., 2011) and for *A. niger*, additional primer sequences were provided by Dr. Susan Howard (personal communication). Wild type strains *A. fumigatus Cyp51A* gene (GenBank accession number AF338659.1) and *A. niger Cyp51A* gene (GenBank accession number JF450900) were used as reference. PCRs were performed in 20 µL, with 2 µM primers, 2 µg DNA template, 0.5× *Hotstar Taq Plus* Master Mix (Qiagen, Germany) and 1 µL free RNase water. Thermal cycling profiles for PCR amplification were as follows: 5 min at 94 °C, 45 sec at 58 °C and 2 min at 72 °C for first cycle, followed by 30 cycles of 30 sec at 94 °C, 45 min at 98 °C, and extension at 72 °C for 2 min. The final extension step was at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and purified using QIAquick PCR Purification kit, (Qiagen, Germany) according to the manufacturer's instructions, for sequencing. Nucleotide sequencing analysis was performed by automated DNA sequencing. The sequence of the products was compared to the sequence of the *A. fumigatus Cyp51A* wild type sequence using the nucleotide alignment service BLAST and *ClustalW* tools.

Table 1: *A. fumigatus* and *A. niger Cyp51A* gene primer sets.

Primers	Primer Sequence	Amplicon size (bp)
(*A. fumigatus*)		
CypA-L98H-S_A (F)	5'AAAAAACCACAGTCTACCTGG 3'	512
CypA-M220-AS_A (R)	5'CTGATTGATGATGTCAACGTA 3'	
(*A. fumigatus*)		
CypA-TR-S_A (F)	5'AGCACCACTTCAGAGTTGTCTA 3'	100
CypA-TR-AS_A (R)	5'TGTATGGTATGCGGAACTACACCTT 3'	
(*A.niger*)		
Ancyp51A1 (F)	5'AACAATCTTTCTCATCAACTGGTCC 3'	190
Ancyp51A5 (R)	5'GATGCTTATTACAAGGTACTAGTTGG 3'	

PCR and sequencing primers: F, forward strand; R, reverse strand.

Real-time PCR

Two primer sets for both *Aspergillus* species and housekeeping gene (β-Actin) were used (Table 2). The cDNA was synthesized from the isolated mRNA using RT-PCR QuantiFast SYBR Green Master Mix Real-Time PCR reaction kit (Qiagen, Germany). Real-time PCR reactions were performed in 25 µL, with 2 µM primers, 1 µg cDNA template (approximately 70 - 90 mg), 2× QuantiFast SYBR Green PCR Master Mix (Qiagen, Germany) and 1 µL free RNase water. Thermal cycling profiles for real-time PCR amplification were as follows: 3 min at 95 °C, 10 sec at 95 °C followed by 40 cycles and with a final extension step at 55 °C for 30 sec.

Table 2: Real-time PCR primer sets.

Primers	Primer Sequnce
CypA_ F (F)	5′-TCCTGCTCCTTAGTAGCCTGGTT -3′
CypA_R (R)	5′-GTGCTCCTTGCTTCACCTG -3′
β-Actin_F (F)	5′-ATTGCTCCTCCTGAGCGTAAATAC-3′
β-Actin_R (R)	5′-GAAGGACCGCTCTCGTCGTAC-3′

F, forward strand; R, reverse strand.

Data analysis

The significance of the different mutations in *A. fumigatus* and *A. niger* isolates was determined by Chi-Square after logarithmic conversion of the values (unpaired, unequal variance). For statistical evaluation of the crossing point and relative expression variations, the data were analyzed by analysis of variance for significant differences. Statistical analysis was done with the SPSS package (version 14.0; SPSS S.L., Madrid, Spain). A *p* value of < 0.05 was considered significant.

RESULTS

Antifungal susceptibility testing

Antifungal susceptibility data showed only three *A. fumigatus* and seven *A. niger* isolates with low level resistance to itraconazole antifungal agent (MIC: 2.5 ug/mL - 3.0 ug/mL) (Table 3).

PCR amplification and sequence analysis of *Cyp51A* gene

The PCR amplification using CypA-L98H-S_A and CypA-M220-AS_A primers in *A. fumigatus* isolates and sequencing of the amplicons, showed the presence of

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Table 3: *In vitro* susceptibilities, mutation and level of *Cyp51A* gene expression of 13 *A. fumigatus* isolates and 17 *A. niger* isolates towards itraconazole antifungal agent.

a) *A. fumigatus* isolates

Isolates	MIC(µg/mL) E-Test ITR	Mutation			*CYP51A* gene expression	
		L98H	M220	TR	High	Low
C21	2.0 (S)	+	-	-	1.9	
C53	3.0 (R)	+	-	-	2.0	
M310	2.0 (S)	+	-	-		0.4
M965	0.75 (S)	-	-	-		N.T
M976	1.0 (S)	-	-	-		N.T
M1420	1.5 (S)	-	-	-		N.T
M1663	1.5 (S)	-	-	-		N.T
M2470	2.0 (S)	+	-	-	1	
UZ23	3.0 (R)	+	+	-	1.0	
UZ59	2.0 (S)	-	-	-		N.T
UZ165	2.0 (S)	-	+	-		0.6
UZ291	2.0 (S)	+	-	-		0.9
UZ685	3.0 (R)	+	+	-	1	

ITR, itraconazole; MIC (EUCAST) > 2.0 µg/mL, Resistant (R); MIC (EUCAST) ≤ 2.0 µg/mL, Susceptible (S); N.D, Not Detected; N.T, Not Tested; Expression level ≥ 1.0, High; Expression level < 1.0, Low; Mutation present, +; Mutation absent, -

b) *A. niger* isolates

Isolates	MIC (µg/mL) E-Test ITR	Mutation	*Cyp51A* gene Expression	
		G427S	High	Low
M309/12	3.0 (R)	N.D	3.3	
M2502/12	1.5 (S)	N.D		0.3
M2463/11	1.5 (S)	N.D		0.1
M1587/12	1.0 (S)	N.D		N.T
M1459/12	0.75 (S)	N.D		N.T
M407	3.0 (R)	+	2.2	
M254	2.5 (R)	N.D	1.6	
M854	2.0 (S)	N.D	3.3	
M701/1	2.5 (R)	N.D	3.4	
M701/2	2.5 (R)	N.D	6.9	
M046/12	2.5 (R)	+	1	
M1772	3.0 (R)	+	6.6	
M1483	1.0 (S)	N.D		N.T
M0200	2.0 (S)	N.D		N.T
M167	2.0 (S)	N.D		N.T
M054	1.5 (S)	N.D		N.T
M166	2.0 (S)	N.D		N.T
MM1008	2.0 (S)	N.D		N.T
MM695	2.0 (S)	N.D		N.T
M895	2.0 (S)	N.D		N.T
MM1769	2.0 (S)	N.D		N.T
M1769	2.0 (S)	N.D		N.T
MM046/12	2.0 (S)	N.D		N.T

ITR, itraconazole; MIC (EUCAST) > 2.0 µg/mL, Resistant (R); MIC (EUCAST) ≤ 2.0 µg/mL, Susceptible (S); N.D, Not Detected; N.T, Not Tested; Expression Level ≥ 1.0, High; Expression Level < 1.0, Low; Mutation present, +; Mutation absent, -

L98H and M220 mutation. The tandem repeat (TR) mutation was not found in both *A. niger* and *A. fumigatus* isolates using CypA-TR-S_A and CypA-TR-AS_A primers as seen by the amplicon size of 100 bp, indicating the 34-bp repeat sequence was not present. The G427S mutation was only found in three *A. niger isolates* which were amplifiable using Ancyp51A1 and Ancyp51A5 primers. Azole-resistant *A. fumigatus* isolate F/19029 and

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A. niger isolate F/13295 served as the positive control for the detection of the L98H, M220, TR and G427S alterations in the *Cyp51A* gene via PCR and consecutive DNA sequence analysis. The *Cyp51A* gene sequence of 13 *A*. *fumigatus* isolates compared with wild type *A*. *fumigatus* (Genbank ID: AF338659.1) showed the nucleotide change from 'T' to 'A' at codon 364 (L98H mutation) in seven isolates (53.8%). The nucleotide change from 'G' to 'C' at codon 731 (M220 mutation) was found in three (23%) *A*. *fumigatus* isolates (Table 3a). The DNA sequences of 3 *A*. *niger* isolates compared with wild type *A*. *niger* (Genbank ID: JF450900) showed the nucleotide change from 'G' to 'C' at codon 427 indicating G427S mutation (Table 3b).

Cyp51A gene expression

A total of four *A. fumigatus* and seven *A. niger* isolates showed increased expression of *Cyp51A* gene (Table 3). Comparison of level of *Cyp51A* gene expression between resistant and susceptible isolates of *A. fumigatus* and *A. niger* respectively, was analyzed statistically, however a significant difference was not found. The experiment was repeated in triplicates for both species and *β*-Actin gene was used as housekeeping gene. Isolate UZ685 *A*. *fumigatus* and M046 *A*. *niger* was used as calibrator.

DISCUSSION

In this study, both *A. fumigatus* and *A*. *niger* showed low level of resistance to itraconazole with MIC values between 2.5 µg/mL - 3.0 µg/mL, unlike studies in United Kingdom that found 50% -70% of isolates with high level of azole resistance (MIC > 8 µg/mL) (Denning *et al.*, 2011). The susceptibility test results clearly showed that isolates of *A*. *fumigatus* and *A*. *niger* were mostly susceptible towards itraconazole with 72% of isolates (n=36) with MIC < 2 µg/mL. The E-test method was used and interpreted based on the EUCAST method guidelines. According to Denning *et al*. (1996) itraconazole antifungal drug can be used as treatment for invasive aspergillosis and gives less side effects compared to amphotericin B.

In determining the resistance mechanism towards itraconazole, sequence analysis and level of Cyp51A gene expression were studied. Primer sets were selected for *Cyp51A* gene amplification based on previous studies. Based on sequence analysis *A*. *fumigatus* isolates C21, C53, M310, M2470 and UZ291 showed the presence of L98H mutation. Meanwhile M220 mutation was detected in UZ165 isolate and both L98H and M220 mutations were detected in UZ23 and UZ685 isolates. The tandem repeat (TR) mutation was determined based on the amplicon size (100 bp) and this mutation was not detected as there was no increase of 34 bp in the amplicons. Some *A. fumigatus* susceptible isolates also showed point mutation therefore this may not be the sole factor contributing to resistance in the isolates tested. Previous reports have related point mutation in susceptible isolates to high level of *Cyp51A* and *Cyp51B* gene expression which includes gene or chromosome duplication and promoter modification as well as changes in amino acids (Mellado *et al*., 2007).

Based on previous research (Bueid et *al*., 2010) the cross resistance towards azole drugs is related to the expression level of *Cyp51A* gene which is due to a 34 bp Tandem Repeat in the promoter region and amino acid changes at location leucine 98 (TR-L98H). The L98H, M220 and Tandem Repeat mutations were not detected at all in *A*. *niger* isolates, therefore resistance in *A*. *niger* may be due to other factors.

While the level of *Cyp51A* gene expression is higher especially in resistant *A*. *niger* isolates this increase is not significant. The presence of M220 mutation correlated significantly with resistance in *A. fumigatus*, however the number of resistant isolates tested were very few (3/13). A limitation in this study was the low number of *A. fumigatus* isolates obtained during the study period. Isolates were mostly obtained from non-systemic sites and *A. niger* isolates were predominant, which accounted for the larger number of *A. niger* isolates evaluated.

The G427S mutation was detected in M046, M407 and M1772 isolates of *A. niger* which may indicate a significant correlation with itraconazole antifungal drug resistance. However it was not possible to amplify the other isolates with the primer pair employed. This is because *A. niger* is a species complex consisting of numerous different strains (Howard *et al.*, 2011) and several other primer pairs would have to be used in order to amplify the other isolates.

Furthermore, resistance in isolates which do not show any increased gene expression or L98H, M220 and TR mutation could be due to the presence of other mutations such as F46Y, G89G, M172V, N248 T, D255E, L358L, E427K, C454C, L358L or efflux pump mechanism (Howard *et al.*, 2011).

In conclusion, a very low level of resistance towards itraconazole antifungal drug was detected in *A. fumigatus* and *A. niger* in Malaysia, which may be due to mutations in the *Cyp51A* gene.

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