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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 *A. fumigatus* and *A. 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 7/12 (58.3%) *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. This may be due to similar MIC values in resistant and susceptible isolates.

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 500 fungal species, fewer than 100 cause disease in humans. Many cases are caused by filamentous fungi such as *Aspergillus fumigatus* and *Aspergillus niger*. *Aspergillus* species are widespread in the environment, growing in the soil, on plants and on decomposing organic matter [1]. 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 [2].

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. In filamentous fungi, azole drugs inhibit ergosterol biosynthesis by targeting the enzyme 14-*α sterol demethylase* which is encoded by the gene *Cyp51A* [3]. By confirming that *Cyp51A* protein is the target of these antifungal agents, two molecular mechanisms of 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 [4-6].

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. 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, which is responsible for the increased level of *Cyp51A* gene expression, accounted for resistance [7].

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 [8]. Therefore, the aim of this study is to determine the in vitro suseptibility of *Aspergillus fumigatus* and *Aspergillus 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 *Aspergillus fumigatus* and 23 clinical isolates of *Aspergillus niger* from various source were collected from the Mycology Unit, Universiti Kebangsaan Malaysia, Medical Centre and evaluated for susceptibility towards Itraconazole. The fungi were grown at room temperature on Sabouraud dextrose agar (Merck, German) and the fungus stocks were preserved in potato dextrose agar slants (Merck, German) at 4°C.

Culture conditions and antifungal susceptibility testing

The clinical isolates fungals were cultured on PDA agar at room temperature for 48 hours to obtain the fungal inoculum at the desired colony forming unit (CFU) size. The inoculum suspension of the fungal conidia was prepared at 0.4 × 10^3 CFU/ml to 0.5 ×10^3 CFU/ml using spectrophotometer (530 nm) (OD; 0.09-0.13) as described in CLSI (formerly NCCLS) document M38-A (33) [9]. 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 hour. 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 (Figure 1). *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 22019 were used as quality control strains and all results were within the target range.

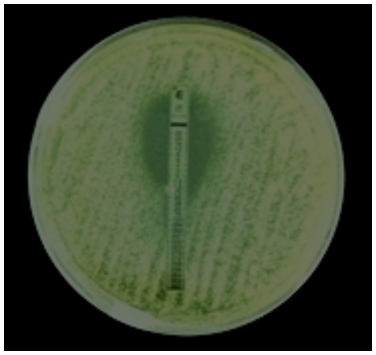

Figure 1: Figure shows that at MIC dense colony growth was found near the strip, but no hyphal growth was found at the margins.

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

Primers for PCR assays and sequencing

Four different primer sets for amplification of four *Cyp51A* gene mutations were synthesized by 1st Base, Seri Kembangan, Malaysia (Table 1). All the primers were selected based on pervious study [10]. 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, 0.5x 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, extension at 72°C for 2 min and within cubation at 37°C. The final extension step is 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 NCBI alignment service Align Sequence Nucleotide Blast and ClustalW tool (http://www.ncbi.nlm.nih.gov/).

Primers for Real Time PCR and gene expression

Two primer sets for both *Aspergillus* species and housekeeping gene (β-Actin) were used (Table 2). The cDNA was synthesized from the isolated mRNA to DNA complementary using RT-PCR Quanti Fast SYBR Green Master Mix Real Time PCR reaction (Qiagen, Germany).

Real Time PCRs were performed in 25 µl, with 2 µm primers, 1 µg cDNA template (approximately70-90 mg), 2x 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.

Data analysis

The significance of the different mutation in *Aspergillus fumigatus* and *Aspergillus 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 SL., Madrid, Spain). A P value of <0.05 was considered significant.

Table 1: *A. fumigatus* and *A. niger Cyp51A* gene primer sets. [a]PCR and sequencing primers: F, forward strand; R, reverse strand.

	Primers	Primer Sequnce	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'	

Table 2: Real Time PCR primer sets. [a]Real Time PCR primers: F, forward strand; R, reverse strand.

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'

Results

Antifungal susceptibility testing

Antifungal susceptibility data showed only 3 *Aspergillus fumigatus* and 7 *Aspergillus 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 showed the presence of L98H and M220 mutation using *CypA-L98H-S_A* and *CypA-M220-AS_A* set primers in *Aspergillus fumigatus* isolates based on the amplicon size (500 bp) and the presence of TR mutation using *CypA-TR-S_A* and *CypA-TR-AS_A* set primers were not found in both *Aspergillus niger* and *Aspergillus* fumigtus isolates (Figure 2b). The G427S mutations were only found in 3 *Aspergillus niger* isolates using An *cyp51A*1 and An *cyp51A*5 set primers. Meanwhile the sequencing data showed 53.8% (7 isolates) contains L98H mutation, M220 in 23% (3 isolates) in both *Aspergillus* fumigtus and *Aspergillus niger* isolates. Azole-resistant *A. fumigatus* isolate F/19029 and *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 sequence analysis of *CYP51A* gene in 13 *A. fumigatus* which has different susceptibilty (MICs) range towards Itraconazole is compared with wild type *A. fumigatus* (Genbank ID: AF338659.1) and showed the nucleotide changes from 'T' to 'A' at codon 364 (L98H mutation) in 7 isolates (53.8%). The nucleotide changes from 'G' to 'C' at codon 731 (M220 mutation) was found in 3 (23%) *A. fumigatus* isolates (Table 3). The DNA sequences of 3 *A. niger* isolates compared with wild type *A. niger* (Genbank ID: JF450900) showed the nucleotide changes from 'G' to 'C' at codon 427 indicating G427S mutation (Table 3).

Levels of *Cyp51A* expression by *A. fumigatus* and *A. niger* azole-resistant strains

Total of 5 *Aspergillus fumigatus* and 8 *Aspergillus niger* isolates showed overexpression of *Cyp51A* gene. Comparison of level of *Cyp51A* gene expression between resistant and susceptible isolates of *Aspergillus fumigatus* and *Aspergillus niger* respectively were analyzed statistically, however a significant differences 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.

Table 3: In vitro susceptibilities, mutation and level of *Cyp51A* gene expression of 12 *Aspergillus niger* and 13 *Aspergillus fumigatus* isolates towards Itraconazole antifungal agent. a ITR; Itraconazole b MIC (EUCAST): >2.0 µg/mL; Resistant (R) ≤ 2.0 µg/mL; Susceptible (S)C N.D; Not Detected d Expression Level (High); ≥ 1.0 e Expression Level (Low); <1.0 f+Mutation present g-Mutation absent.

	Isolates	MIC(µg/mL) E-Test ITR	Mutation				*CYP51A*gene Expression	
			A. fumigatus			*A. niger*	High	Low
			L98H	M220	TR	G427S		
A. Fumigatus	C21	2.0 (S)	+	-	-	-	1.9	-
	C53	3.0 (R)	+	-	-	-	2	-
	M310	2.0 (S)	+	-	-	-	-	0.4
	M965	0.75 (S)	-	-	-	-	-	N.D
	M976	1.0 (S)	-	-	-	-	-	N.D
	M1420	1.5 (S)	-	-	-	-	-	N.D
	M1663	1.5 (S)	-	-	-	-	-	N.D
	M2470	2.0 (S)	+	-	-	-	1	-
	UZ23	3.0 (R)	+	+	-	-	1	-
	UZ59	2.0 (S)	-	-	-	-	-	N.D
	UZ165	2.0 (S)	-	+	-	-	-	0.6
	UZ291	2.0 (S)	+	-	-	-	-	0.9
	UZ685	3.0 (R)	+	+	-	-	1	-
A. Niger	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.D
	M1459/12	0.75 (S)	-	-	-	N.D	-	N.D
	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.D
	M0200	2.0 (S)	-	-	-	N.D	-	N.D
	M167	2.0 (S)	-	-	-	N.D	-	N.D

	M054	1.5 (S)	-	-	-	N.D	-	N.D
	M166	2.0 (S)	-	-	-	N.D	-	N.D
	MM1008	2.0 (S)	-	-	-	N.D	-	N.D
	MM695	2.0 (S)	-	-	-	N.D	-	N.D
	M895	2.0 (S)	-	-	-	N.D	-	N.D
	MM1769	2.0 (S)	-	-	-	N.D	-	N.D
	M1769	2.0 (S)	-	-	-	N.D	-	N.D
	MM046/12	2.0 (S)	-	-	-	N.D	-	N.D

Discussion

In this study, both *Aspergillus fumigatus* and *Aspergillus niger* showed low level of resistance to Itraconazole with MIC values between 2.5 ug/ml-3.0 ug/ml, unlike studies in United Kingdom that found 50%-70% of isolates with high level of azole resistance (MIC>8 ug/ml) [11]. The susceptibility test results clearly showed that isolates of *Aspergillus fumigatus* and *Aspergillus niger* were mostly susceptible towards Itraconazole with 72% of isolates, (n=36) with MIC<2 ug/ml. The E-test method was used based on the EUCAST method guidelines and according to Denning et al (1996) Itraconazole antifungal drug can be used as treatment for invasive Aspergillosis and gives less gives side effects compared to amphotericin B.

In determination of the resistance mechanism in *Cyp51A* gene towards Itraconazole, sequence analysis and level *Cyp51A* gene expression were studied. Primer sets were selected for *Cyp51A* gene amplification based on previous studies. Based on sequence analysis *Aspergillus 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 mutation 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 [12].

Based on previous research [13] the cross resistance towards azole drugs is related to expression level of *Cyp51A* gene which is due to a Tandem Repeat 34 bp in the promoter region and amino acid changes at location 98 leucine (TR-L98H). The L98H, M220 and Tandem Repeat were not detected at all in *Aspergillus niger* isolates and the resistance in *A. niger* may be due to other factors [6]. Meanwhile the resistance isolates which do not show any gene expression or L98H, M220 and TR mutation could be due to presence of other mutations such as F46Y, G89G, M172V, N248 T, D255E, L358L, E427K, C454C, L358L or efflux pump mechanism [14].

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). 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 [14] and several other primer pairs would have to be used in order to amplify the other isolates [15-29].

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

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