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ORIGINAL RESEARCH

Antifungal Susceptibility and Genotypic Analysis of *cyp51A* Mutations in *Aspergillus fumigatus* Isolates in Malaysia

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Purpose: Azole resistance in *Aspergillus fumigatus* poses a significant challenge in the management of invasive aspergillosis. This study aimed to investigate the antifungal susceptibility and *cyp51A* mutation profiles of *A. fumigatus* isolates in Malaysia.

Patients and Methods: Sixty clinical *A. fumigatus* isolates were collected and subjected to antifungal susceptibility testing (AFST) and molecular analysis. The antifungal susceptibility testing was performed according to CLSI M38 guideline. The geometric mean (GM) minimum inhibitory concentration (MIC), MIC_{50}/MIC_{90} for voriconazole, itraconazole, posaconazole, amphotericin B, and isavuconazole against *A. fumigatus* in non-invasive cases and invasive cases were calculated. In addition, the presence of *cyp51A* mutations was also identified.

Results: The present study revealed an overall resistance rate of 6.7% among the isolates. In non-invasive cases, isavuconazole and posaconazole demonstrated the lowest GM MIC of 0.08 µg/mL. Following them were itraconazole, voriconazole, and amphotericin B with concentrations of 0.15µg/mL, 0.16µg/mL and 0.90µg/mL, respectively. Similarly, in invasive cases, isavuconazole and posaconazole exhibited the lowest GM MIC of 0.09µg/mL. Following them were itraconazole, voriconazole, and amphotericin B with concentrations of 0.14µg/mL, 0.17µg/mL and 0.80µg/mL, respectively. Genotypic analysis revealed various *cyp51A* mutations, including F46Y, M172V, N248K, R34L, V244A, V244S, and E427K. However, not all mutations corresponded to antifungal resistance.

Conclusion: The majority of clinical *Aspergillus fumigatus* isolates demonstrated susceptibility to the antifungal agents tested, with isavuconazole and posaconazole demonstrating the lowest MIC values. However, *cyp51A* mutations were discovered without a consistent correlation to antifungal resistance, emphasising the need for additional research.

Keywords: invasive aspergillosis, minimum inhibitory concentration, voriconazole, isavuconazole, posaconazole

Introduction

Aspergillus is a saprophytic fungus that is widely distributed throughout our living environment.[1] The infectious propagule of *Aspergillus* is conidia[2] that can be carried by air currents and inhaled into the lungs through the mouth and nose.[1,2] Daily exposure to the conidia can result in several clinical conditions, including Allergic Bronchopulmonary Aspergillosis (ABPA), Chronic Pulmonary Aspergillosis (CPA), allergic sinusitis, aspergilloma, and invasive aspergillosis (IA).[1,2]

IA is a significant opportunistic infection with high morbidity and mortality rates.[3,4] This severe and life-threatening form of the disease always affect immunocompromised people who receiving chemotherapy or organ-transplant patients or those infected with acquired immunodeficiency syndrome (AIDS). IA has a high death rate and can affect several organs, such as kidneys, liver, brain, and lungs. However, the confirmation of IA cases is suggested to have microscopic analysis, molecular diagnosis or culture detection of the organism from sterile material.[5–7] Every year, more than 300,000 people was affected by IA, with 58–99% mortality rate.[8–10] In Malaysia, the rate of aspergillosis was estimated 3.3% per

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100,000 population.[11] Among many species of *Aspergillus*, the most common aetiologic agent of IA is *Aspergillus fumigatus*.[12–15]

Triazole antifungals are strongly recommended for the treatment of aspergillosis.[16,17] However, the emergence of azole resistance in *A. fumigatus* poses a significant threat to the treatment outcomes.[18] Mutations in the *cyp51A* gene, which codes for the target enzyme lanosterol 14-demethylase, are the main cause of azole resistance in *A. fumigatus*.[19,20] These mutations can differ in frequency and geographic distribution, which emphasizes the importance of regional research in determining the resistance landscape in certain places. For example, G54W, M220I/V/T and F219C were reported in Germany;[21] while F46Y, M172V, N248T, D255E and E427K were reported in Taiwan.[22] Additionally, F46Y, G54E/R/V, G138C, H147Y, M172V, P216L, M220K/T, N248T, D255E, E427G/K, Y431C, G434C, G448S were reported in United Kingdom.[23] The mutations including G54, M220, and G448S have been linked to azole resistance in *A. fumigatus* in several studies.[24–27] In Malaysia, limited data are available on the susceptibility pattern of *A. fumigatus* and its *cyp51A* mutation profile. This data is important as it is crucial to antifungal stewardship programs and contributes to a better understanding of local resistance mechanisms. Therefore, our study aimed to be the first to determine the antifungal susceptibility patterns of voriconazole, itraconazole, posaconazole, amphotericin B and isavuconazole and the mutations in *cyp51A* gene of clinical *A. fumigatus* in Malaysia.

Materials and Methods

Ethics

Ethical review was conducted and approved by the Medical Research and Ethics Committee, Ministry of Health of Malaysia, Malaysia (NMRR-20-207-53067). This study also complies with the Declaration of Helsinki. The informed consent had been obtained from the study participants prior to study commencement.

Isolates

In this study, 60 clinical *A. fumigatus* was isolated from various hospitals in Malaysia between 2019 and 2023 (Figure 1). In detail, one isolate was collected from Sabah (Duchess of Kent Sandakan Hospital), Perak (Raja Permaisuri Bainun

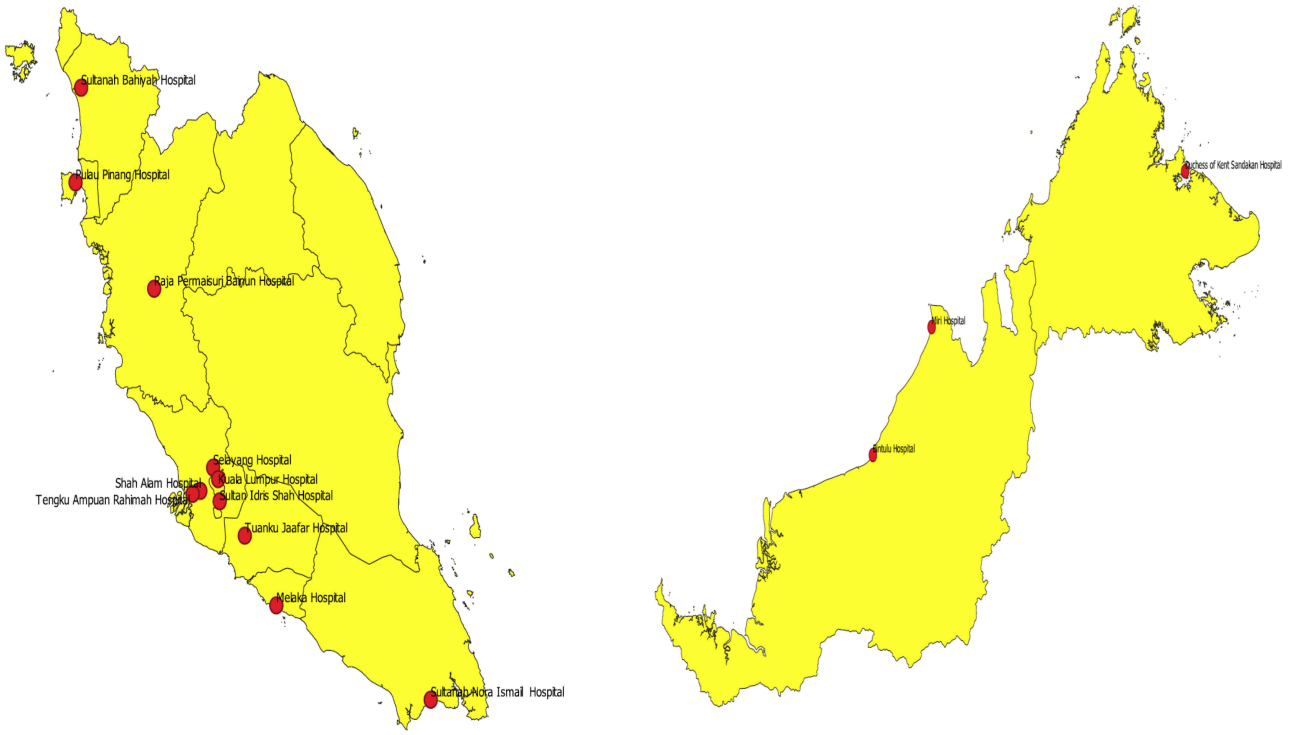

Figure 1 Geographical locations of the hospitals in Malaysia from which *A. fumigatus* was isolated and provided to this study.

Hospital), Selangor (Sultan Idris Shah Hospital), Melaka (Melaka Hospital), Negeri Sembilan (Tuanku Jaafar Hospital), and Sarawak (Miri Hospital). Meanwhile, two isolates each were collected from hospitals located in Johor (Sultanah Nora Ismail Hospital) and Pulau Pinang (Pulau Pinang Hospital). In hospitals in Kedah (Sultanah Bahiyah Hospital), and Sarawak (Bintulu Hospital), three isolates were collected. Notably, five isolates were obtained from Shah Alam Hospital in Selangor. Additionally, seven isolates were collected from Kuala Lumpur Hospital in Wilayah Persekutuan Kuala Lumpur. Furthermore, fourteen isolates were collected from Selayang Hospital in Selangor, with an even greater count of 18 isolates from Tengku Ampuan Rahimah Hospital, also in Selangor.

The categorization of IA in the laboratory was according to Thornton,[2] Peter Donnelly et al[6] and Ascioglu et al.[7] Briefly, invasive cases are classified as positive cultures obtained from samples through a sterile method from a clinically or radiologically abnormal site that is indicative of infection.[6]

Out of the 60 clinical specimens, 47 were obtained from patients with invasive cases, while 13 were from patients with non-invasive cases. Among the invasive cases, different types of clinical specimens were collected. This included one blood sample; two endotracheal fluid, two corneal scrapings, two pleural fluid samples; three tracheal aspirates and 37 bronchoalveolar lavages. On the other hand, 13 non-invasive cases were collected. This included one maxillary sinus sample, two nail clippings, and ten sputum samples.

In this study, the identification was determined using a combination of microscopic and macroscopic observations, as well as molecular methods. The amplification of internal transcribed spacers of ribosomal DNA (*ITS*) and calmodulin gene (*CAL*) was performed according to Tam et al.[28]

Antifungal Susceptibility Testing

The minimum inhibitory concentration (MIC) was determined according to the broth microdilution method as mentioned in M38.[29] The antifungals, namely voriconazole, itraconazole, posaconazole, amphotericin B, and isavuconazole, were selected based on the recommendations for the treatment for IA.[16] In brief, the conidia suspension of 0.4×10^4 to 5×10^4 CFU/mL was prepared. Next, each antifungal was prepared with a final concentration ranging from 0.0313 µg/mL to 16 µg/mL. Subsequently, 100µL of each conidia suspension and antifungal were transferred to the well and incubated for 48 hours at 35°C. The MIC was read as the lowest drug concentration that 100% inhibits the growth of the isolate. To ensure that the MIC achieved falls within the reference range, two reference strains, namely *A. fumigatus* ATCC 204305 and *A. flavus* ATCC 204304 were included in each test.

Mutation Analysis

The fungal DNA was isolated according to the instructions of the Zymoresearch Quick-DNA™ Fungal/Bacterial Miniprep kit (Murphy Ave, Irvine, United States).

To detect the resistant *cyp51A* gene of *A. fumigatus*, primers cyp51A_1 and cyp51A_2 were designed, and the presence of mutations in *cyp51A* was identified by Sanger sequencing. The sequence of the products was compared to the reference sequence of the *A. fumigatus cyp51A* (GenBank accession number AF338659.1) (http://www.ncbi.nlm.nih.gov/) using MEGA 11 tool.[30]

The primer cyp51A_1 was designed to amplify the 3th-723th nucleotide of *cyp51A* gene. The sequences of cyp51A_1F and cyp51A_1R were 5'AATCGCAGCACCACTTCAGA3' and 5'ATCCTTGAGCTTGCCGTTGA3', respectively. PCR was performed in 25µL, with 1µL of both primers (20µM), 2µL DNA template, 12.5µL MyTaq™ HS Mix (Meridian Bioscience, Ohio, United States) and 8.5µL RNase free water. Thermal cycling profiles for PCR amplifications were as follows: 1 min at 95°C, followed by 30 cycles of 15 sec at 95°C, 15 sec at 60°C; then 10 sec at 72°C and hold at 10°C. The amplification size is 721bp.

The primer cyp51A_2 was designed to amplify the 666th-1955th nucleotide of *cyp51A* gene. The sequences of primers cyp51A_2F and cyp51A_2R were 5'CACAGTCTACCTGGGCGTTC3' and 5'TTCGACCGCTTCTCCCAG3', respectively. PCR was performed in 25µL, with 0.5µL for both primers (10µM), 2µL DNA template, 12.5µL OneTaq® Hot Start 2X Master Mix (New England Biolabs, Massachusetts, United States) and 9.5 µL RNase free water. Thermal cycling profiles for PCR amplifications were as follows: 30 sec at 94°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 55°C, and 1 min at 68°C; then 5 min at 68°C and hold at 10°C. The amplification size is 1290bp.

Data Analysis

The range and geometric mean (GM) MIC, MIC_{50} and MIC_{90} were calculated using Microsoft Excel 2019 software. The calculations were performed separately for invasive and non-invasive cases. MIC_{50} and MIC_{90} values were defined as the lowest concentration of the antifungal at which 50% and 90% of the isolates were inhibited, respectively.[31]

The clinical breakpoint for *A. fumigatus* against voriconazole had been determined by M61.[32] According to M61, MIC of ≤0.5 was categorized as susceptible, 1.0 was categorized as intermediate and ≥2.0 was categorized as resistant. For other antifungal agents, the interpretation of the MIC was categorised according to Li et al[33] as their breakpoints are not available in the M61. Therefore, *A. fumigatus* isolates were considered resistant if the MIC for amphotericin B was >2 μg/mL, and itraconazole was >2 μg/mL, while MIC for posaconazole was >1μg/mL. In addition, this study also utilized the epidemiological cut-off values (ECVs) established for itraconazole (1 μg/mL), amphotericin B (2 μg/mL), and isavuconazole (1 μg/mL) as determined by CLSI M59.[34] These ECVs were employed to analyze the susceptibility patterns of the isolates against these antifungal agents in the absence of specific breakpoints.

Furthermore, boxplots illustrating the MIC of each antifungal against the invasiveness of aspergillosis have been generated. The significant differences between the MIC of each antifungal and the invasiveness of aspergillosis were evaluated using Mann–Whitney Wilcoxon test. Both boxplots and the Mann–Whitney Wilcoxon test were conducted using Statistical Package for Social Sciences (SPSS), version 20.0 (IBM®, Armonk, NY). Statistical significance was set at $p<0.05$.

Results

Antifungal Susceptibility Testing

The MIC pattern of *A. fumigatus* against antifungals in non-invasive and invasive cases is shown in Table 1 and Figure 2a-e. Overall, the MIC of each antifungal between invasive and non-invasive aspergillosis has no significant difference (Figure 2a-e).

The GMs of all *A. fumigatus* isolates against tested antifungals were less than 2μg/mL. In non-invasive cases, the lowest GM MIC was achieved by posaconazole and isavuconazole, followed by itraconazole, voriconazole and amphotericin B, namely 0.08μg/mL, 0.08μg/mL, 0.15μg/mL, 0.16μg/mL and 0.90μg/mL, respectively.

The MIC_{50} of *A. fumigatus* against antifungals was also less than 2μg/mL, with posaconazole and isavuconazole having the lowest MIC_{50} of 0.06μg/mL, followed by itraconazole, voriconazole, and amphotericin B with MIC_{50} values of 0.13μg/mL, 0.25μg/mL, and 1μg/mL, respectively. Similarly, the MIC_{90} of *A. fumigatus* against antifungals followed

Table 1 MIC of A. Fumigatus Against Voriconazole, Itraconazole, Posaconazole, Amphotericin B and Isavuconazole for Non-Invasive and Invasive Cases

Aspergillosis invasiveness (no. of isolates tested)	Variables	MIC (μg/mL)				
		VCZ	ITZ	PSZ	AMB	ISV
Non-invasive (n=13)	Range	0.03–1.00	0.03–1.00	0.03–0.25	0.50–2.00	0.03–0.50
	GM	0.16	0.15	0.08	0.90	0.08
	MIC_{50}	0.25	0.13	0.06	1.00	0.06
	MIC_{90}	0.50	0.50	0.25	1.00	0.25
Invasive (n=47)	Range	0.03–0.50	0.03–2.0	0.03–0.50	0.03–16.00	0.03–0.50
	GM	0.17	0.14	0.09	0.80	0.09
	MIC_{50}	0.25	0.13	0.06	1.00	0.13
	MIC_{90}	0.50	0.50	0.25	1.00	0.25

Abbreviations: MIC, minimum inhibitory concentration; GM, geometric mean; VCZ, voriconazole; ITZ, itraconazole; PSZ, posaconazole; AMB, amphotericin B; ISV, isavuconazole.

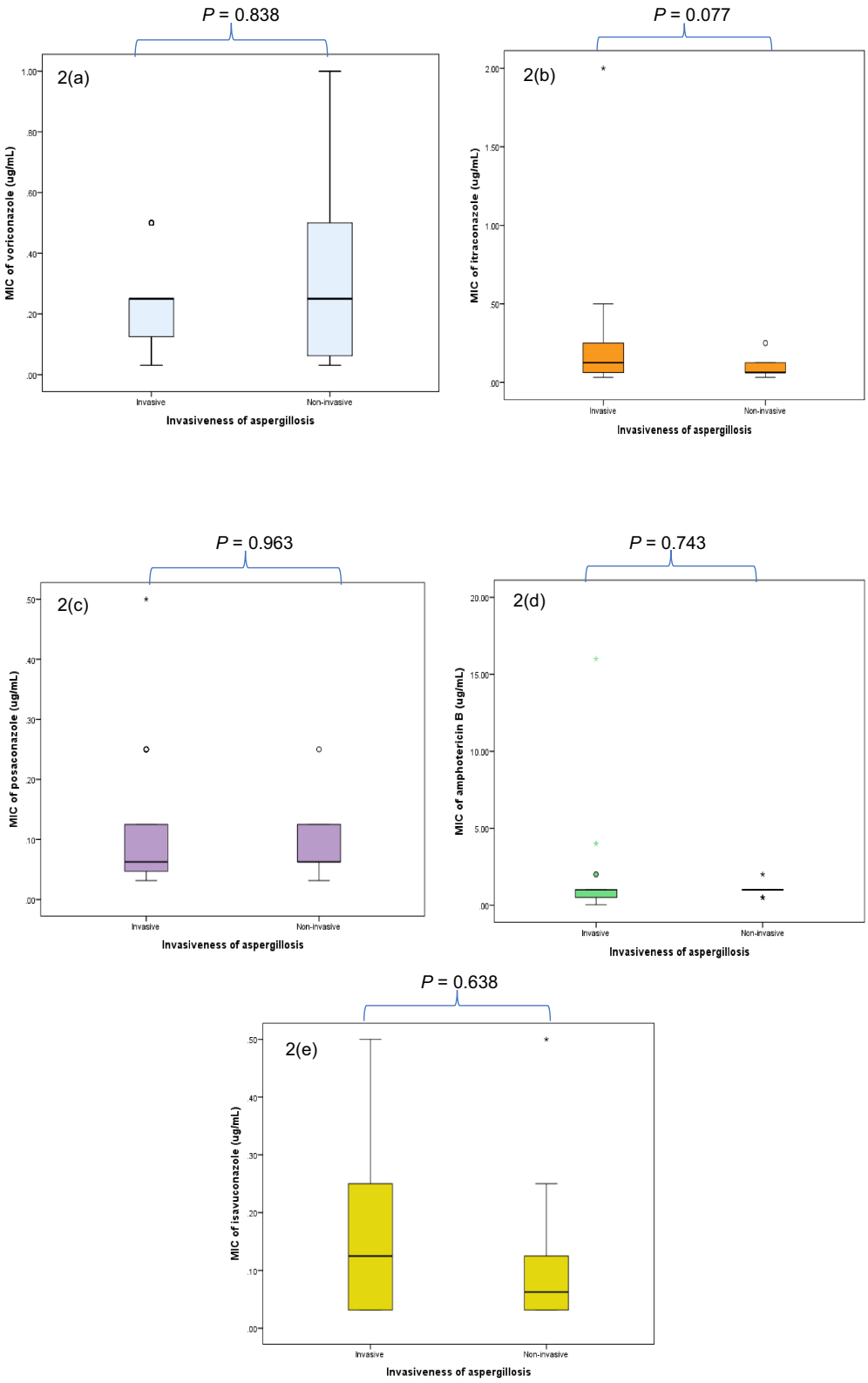

Figure 2 (**a-e**) Boxplot comparing MIC of voriconazole (**a**), itraconazole (**b**), posaconazole (**c**), amphotericin B (**d**) and isavuconazole (**e**) against invasiveness of aspergillosis. The symbols (* and °) represents extreme values of MIC. The significant differences were calculated using Mann–Whitney Wilcoxon test.

the same trend as MIC_{50}, with posaconazole and isavuconazole having the lowest MIC_{90} of 0.25 μg/mL, followed by voriconazole, itraconazole, and amphotericin B with MIC_{90} values of 0.50 μg/mL, 0.50 μg/mL, and 1 μg/mL, respectively.

In comparison, the lowest GM MIC of the invasive cases was achieved by posaconazole, and isavuconazole, followed by itraconazole, voriconazole and amphotericin B, namely 0.09μg/mL, 0.09μg/mL, 0.14μg/mL, 0.17μg/mL and 0.80μg/mL, respectively. The lowest MIC_{50} of *A. fumigatus* against antifungals in invasive cases was achieved by posaconazole and isavuconazole with the value of 0.06μg/mL, followed by isavuconazole, itraconazole, voriconazole, and amphotericin B with MIC_{50} values of 0.13μg/mL, 0.13μg/mL, 0.25μg/mL, and 1μg/mL, respectively. On the other hand, the MIC_{90} of *A. fumigatus* against antifungals in both non-invasive and invasive cases was consistent.

In contrast to most of the tested isolates, four isolates (A, B, C and D) showed resistance or decreased susceptibility to one or more antifungal agents (Table 2). A and B were resistant to amphotericin B with a MIC of 4μg/mL, whereas C was intermediately resistant to voriconazole with a MIC of 1μg/mL. Next, D was resistant to amphotericin B with a MIC of 16μg/mL and had a high MIC of 2μg/mL against itraconazole. Overall, all tested isolates, excluding the mentioned isolates, demonstrated MIC values below the resistant breakpoints of voriconazole and the established ECV of other antifungals.

Sequence Analysis of *cyp51A* Gene for A. fumigatus

The multiple sequence alignment analysis showed seven types of amino acid substitutions existed among clinical *A. fumigatus*. Out of sixty isolates, thirteen isolates were designated as isolates "A to M", possessing mutations in *cyp51A* (Table 2 and Table S1).

E427K was found in three isolates (A, D and F); F46Y was present in another three isolates (E, F and G); N248K was present in another four isolates (C, D, H and I); R34L and V244S were present in isolate J and K, respectively. In addition, V244A was found in isolate B, and M172V was found in three isolates (F, L and M).

Table 2 Antifungal Susceptibility and Amino Acid Substitutions in Cyp51A of Some Aspergillus Fumigatus

Isolate	Cases	MIC (μg/mL)					Cyp51A Substitutions
		VOR	ITZ	PSZ	AMB	ISV	
A	I	0.03	0.03	0.03	**4**	0.06	E427K
B	I	0.03	0.13	0.06	**4**	0.06	V244A
C	NI	**1**	1	0.06	2	0.03	N248K
D	I	0.13	**2**	0.25	**16**	0.25	N248K, E427K
E	I	0.5	0.25	0.25	0.5	0.25	F46Y
F	NI	0.5	0.25	0.13	0.5	0.13	F46Y, M172V, E427K
G	I	0.13	0.25	0.25	1	0.25	F46Y
H	I	0.25	0.5	0.25	2	0.25	N248K
I	I	0.13	0.03	0.25	0.5	0.03	N248K
J	I	0.13	0.13	0.06	1	0.03	R34L
K	NI	0.06	0.03	0.03	0.5	0.06	V244S
L	NI	0.25	0.25	0.13	0.5	0.03	M172V
M	I	0.25	0.13	0.06	0.5	0.03	M172V

Notes: Resistant and intermediately resistant MICs are highlighted in bold.
Abbreviations: VOR, Voriconazole; ITZ, Itraconazole; PSZ, Posaconazole; AMB, Amphotericin B; ISV, Isavuconazole; I, invasive case; NI, non-invasive case.

The mutation was observed in four non-invasive cases (C, F, K and L) and nine invasive cases (A, B, D, E, G, H, I, J and M). Unlike isolates A-D, isolates E to M did not show decreased susceptibility to any of the antifungal agents tested despite their *cyp51A* mutations.

Discussion

The present study revealed an overall resistance or decreased susceptibility rate of 6.7% (4/60) among the isolates. Specifically, this comprised of 3.3% (2/60) resistance solely to amphotericin B, 1.7% to voriconazole, and 1.7% exhibited resistance to both itraconazole and amphotericin B. This azole-resistance rate is consistent with previous studies in Asia, including China,[35–37] Japan,[38] Pakistan,[39] Korea,[40] where the azole-resistance was less than 10%. However, azole resistance rates were significantly higher in other regions. For instance, Sudan reported 44% resistance to itraconazole and 11% resistance to voriconazole;[41] while in Netherlands,[42] 19% voriconazole-resistance was reported and 4.1% voriconazole-resistance and 14.5% itraconazole-resistance in France.[43]

In addition, our study also demonstrated that isavuconazole and posaconazole possessed the highest antifungal activity against *A. fumigatus*. Both antifungals exhibited the lowest GM MIC, MIC_{50} and MIC_{90} compared to the other tested antifungals. This finding supports the inclusion of both isavuconazole and posaconazole in the management of IA.[44,45] In addition, these findings were consistent with the previous study,[46–48] where the isavuconazole was shown to possess fungicidal activity and potentially become first-line therapy for IA. Similarly, posaconazole has also been suggested as the first-line treatment for IA due to the high success rate after treatment.[49–51] Additionally, it may serve as an alternative for patients who cannot tolerate voriconazole and amphotericin B treatment.[50,52]

Both isavuconazole and posaconazole are among the newest triazole antifungals available for clinical use.[44,53] However, voriconazole and amphotericin B were suggested by the Malaysian Ministry of Health as preemptive and directed therapy for IA, while more extensive clinical data on the efficacy and side effects of these antifungals was being gathered.[17] Interestingly, most *A. fumigatus* isolates in this study exhibited MIC values below the resistant breakpoints of all tested antifungal agents. This finding is significant because it suggests that the current recommended treatment for IA in Malaysia remains relevant.[17]

Despite our study demonstrated a 6.7% resistance rate, the PCR method revealed an increase in the mutation rate to 21.7% (13/60). All four isolates that exhibited phenotype profiles possessed alterations in their mutation profile. However, the remaining isolates showed silent mutations as they did not present phenotypical resistance or decreased susceptibility. This observation was consistent with the findings of other researchers including Escribano et al,[54] Won et al,[55] and Snelders et al.[56]

The N248K mutation in the Cyp51A has been frequently observed in *A. fumigatus* isolates from China.[57,58] The presence of this mutation was proven to reduce the efficacy of azole therapy by Mandal et al.[59] In this study, isolate C possessed N248K and was intermediately resistant to voriconazole, and this phenomenon was also reported by Won et al.[55] Furthermore, isolate D, which also carried the N248K mutation, showed a higher MIC than the ECV of itraconazole. Notably, the patient with isolate D presented with severe conditions, including septic shock, was intubated and admitted to the intensive care unit (ICU). However, the finding from our study was only partially consistent with the previous reports. Isolates H and I also possessed N248K but were not resistant to any azole agents. This finding was consistent with the observations by Liu et al[58] who reported that many azole-susceptible *A. fumigatus* strains that harboured N248K were unrelated to drug resistance.

Since isolate D also possessed E427K, therefore, the relationship between this amino acid substitution and azole resistance was examined. When we compared it to isolate A, which also contained the same point mutation, we only observed resistance to amphotericin B and not resistance to azole. Therefore, we suggest that individual N248K or E427K might not be solely responsible for the development of azole resistance. However, the coexistence of E248K and E427K may have influenced the alteration in azole resistance. The combination of the two mutations may increase the azole-*cyp51* enzyme binding affinity and therefore make the fungus resistant to the treatment. So far, no prior reports have directly supported this hypothesis. Thus, further investigation is required to elucidate the underlying mechanisms that cause the resistance.

The F46Y in this study was also not associated with azole resistance. This finding was consistent with the findings reported by Liu et al.[58] Interestingly, the F46Y mutation was frequently observed in combination with other mutations, such as M172V, E427K, N248T, and D25E, as reported by Garcia-Rubio et al and Escribano et al.[54,60] However, their phenotypic profiles were inconsistent with the genotype profile. For instance, isolate F in our study harbored the combination of F46Y, M172V, and E427K, but it did not exhibit any signs of azole resistance. This suggests that azole resistance may not always result from the presence of F46Y alone or when combined with other mutations.

In this study, we did not observe any of the common mutations, namely G54, M220, and G448.[24–27] This indicates that the distribution and genetic diversity of *cyp51A* mutations in *A. fumigatus* may vary depending on the geographic location and population. Interestingly, despite the absence of the commonly reported mutations, we found two novel mutations in this investigation which did not contribute to resistance to any antifungals. The isolates J and K had the R34L mutation and the V244S mutation, respectively. Additionally, we identified another novel mutation, V244A in isolate B, which may contribute to amphotericin B-resistance. Further research is necessary to clarify the precise processes by which these novel mutations lead to antifungal resistance and determine their clinical implications.

The inconsistency between genotype and phenotype profile could be due to several factors, including host immune response, environmental conditions, or exposure to antifungal drugs that can influence the development of resistance.[61] These factors may affect the genotype and phenotypic profile, thus contributing to variations in the resistance phenotype. The investigation of alternate resistance mechanisms, such as efflux pumps or alterations in the composition of cell walls, may aid in explaining the relationship between mutations and resistance.

In addition, the present study also demonstrated no significant difference in MIC of each antifungal between invasive and non-invasive cases. It can be a sign of a persistent and widespread *A. fumigatus* population in the community or at the healthcare facility, which increases the risk of infection. To establish a direct link between the susceptibility patterns and the specific environmental or clinical strains of *A. fumigatus*, future research including genetic analysis would be necessary.

Furthermore, the present study offers antifungal susceptibility data and the occurrence of genetic mutation in the clinical isolates of *A. fumigatus* in Malaysia. However, due to the lack of comprehensive clinical data for the patients, it is challenging to establish a direct correlation between specific mutations and treatment outcomes.

Conclusion

In conclusion, we found 6.7% of clinical isolates causing invasive aspergillosis in Malaysia to be resistant to one or more antifungal. Furthermore, both isavuconazole and posaconazole showed in vitro good activity against clinical isolates of *Aspergillus fumigatus*. Most of the *A. fumigatus* isolates remained susceptible to the tested antifungal agents. However, the presence of point mutations emphasizes the importance of ongoing research to enhance our understanding and management of antifungal resistance in clinical settings.

Ethics Approval

An ethical review was conducted and approved by the Medical Research and Ethics Committee, Ministry of Health of Malaysia, Malaysia (NMRR-20-207-53067).

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Disclosure

The authors report no conflicts of interest in this work.

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