Malays J Pathol 2024; 46(1) : 71 – 78

ORIGINAL ARTICLE

Antifungal susceptibility of molecularly confirmed *Aspergillus* species from clinical samples

Mohd Nizam TZAR[1]*, Sahlawati MUSTAKIM[2], Hamidah YUSOFF[1], Ratna Mohd TAP[3]

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, 56000 Cheras, Kuala Lumpur Malaysia; [2]Microbiology Unit, Pathology Department, Hospital Tengku Ampuan Rahimah, Klang, Selangor, Malaysia; [3]Bacteriology Unit, Infectious Disease Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia.

Abstract

Invasive aspergillosis is the second most common invasive human mycosis but susceptibility data of *Aspergillus* species is limited. Antifungal treatment of aspergillosis is often done empirically without knowing the true susceptibility. Therefore, we aimed to determine antifungal susceptibility of *Aspergillus* species isolated from various clinical specimens over a 1-year period. We identified 28 *Aspergillus* isolates by sequencing the internal transcribed spacer (ITS) and β-tubulin genes and performed antifungal susceptibility testing on these isolates using Sensititre YeastOne. The isolates were identified as *Aspergillus niger* (60.7%), *A. fumigatus* (21.4%), *A. flavus* (10.7%), *A. chevalieri* (3.6%) and *A. tubingensis* (3.6%). Based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Antifungal Clinical Breakpoint for *Aspergillus* spp., 16/17 (94.1%) *A. niger* isolates were susceptible to amphotericin B, all six isolates (100%) of *A. fumigatus* were susceptible to amphotericin B, itraconazole and voriconazole, but only 5/6 (83.3%) *A. fumigatus* were susceptible to posaconazole. Meanwhile, all three (100%) *A. flavus* isolates were susceptible to itraconazole. There are no other breakpoints established by the EUCAST for other antifungal-species combinations. In conclusions, *Aspergillus niger* remains the most commonly isolated species from clinical specimens and *Aspergillus* isolates at our centre are still largely susceptible to amphotericin B, echinocandins and most azoles. This information is valuable in guiding antifungal therapy in the treatment of aspergillosis.

Keywords: Aspergillus, aspergillosis, antifungal, susceptibility, resistance

INTRODUCTION

Aspergillus species are ubiquitous in our environment and can be found especially in soil and decaying plant materials. Although most *Aspergillus* species are harmless, some of them are harmful especially to immunocompromised patients by causing allergic reactions and invasive diseases. They are one of the most common pathogenic moulds isolated from many hospitals around the world. Globally, there were approximately three million cases of chronic pulmonary aspergillosis and 2.5 million cases of invasive aspergillosis that occurred each year.[1] Further, the aetiological agents of invasive aspergillosis in Asia are no longer predominated by *A. fumigatus* and *A. flavus*.[2,3] Emergence of rare *Aspergillus* species including *Aspergillus* species complexes and cryptic species has been reported in tropical and subtropical regions of Asia.[2,4] In general, laboratory support for mycology is weak in Asia, particularly for galactomannan detection and therapeutic drug monitoring. Although the treatment algorithm of invasive aspergillosis in Asia is similar to the ones in the west, some clinicians find it financially challenging to acquire some of the antifungal drugs for therapy.[5] In view of these, there is a pressing need to know the susceptibility patterns of *Aspergillus* isolates so that a more targeted approach to therapy can be instituted. Targeted therapy

**Address for correspondence:* Dr. Mohd Nizam TZAR, Department of Medical Microbiology and Immunology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, 56000 Cheras, Kuala Lumpur Malaysia. Tel: +603 9145 9534. Email: *tzar@ppukm.ukm.edu.my*

could minimize unnecessary cost and adverse effects related to the use of antifungal agents. In addition, reports on emergence of antifungal resistance among *Aspergillus* species have made antifungal susceptibility testing more desirable.[6] Despite this, antifungal susceptibility testing of *Aspergillus* is not routinely done and hence, susceptibility profiles are not usually available in many hospitals. Therefore, we conducted this study to gain more insights into the susceptibility profiles of *Aspergillus* species.

MATERIALS AND METHODS

Ethics Committee Permission

Since this study only examined fungal isolates and did not involve humans or animals or any treatment intervention, no ethical issues were brought up and this study was approved by the institutional Medical Research and Ethics Committee on 16 March 2017 (No. 5/2017).

Study site and design

We conducted a prospective cross-sectional study at a tertiary-level teaching hospital in Kuala Lumpur, Malaysia. This is a 1000-bedded medical centre with various specialities including general intensive care, haematology and bone marrow transplant unit.

Aspergillus isolates

We collected all isolates of *Aspergillus* species that were cultured on Sabouraud dextrose agar from various clinical specimens that were sent to the microbiology laboratory from October 2016 to September 2017. The isolates were examined macroscopically and microscopically by scotch tape and lactophenol cotton blue mounts, but the final genus and species identities were confirmed by molecular methods (ITS regions and β-tubulin gene sequencing).

Molecular identification

DNA was extracted from various species of *Aspergillus* using ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, California, USA), which utilizes the bead beating system during the lysis step. The internal transcribed spacer (ITS) and β-tubulin gene were amplified according to previous reports using a pair of universal primers of ITS5/ITS4 and Bt2a/Bt2b, respectively.[7,8] Amplifications were performed separately using MyTaq HS mix (Bioline, London, UK) and were accomplished in a total volume of 25 μl in the presence of 0.2 μM of each primer set. The polymerase chain reaction (PCR) was performed for 35 cycles with an initial denaturation step of 1 min at 95 °C in a Mastercycler Gradient (Eppendorf, Hamburg, Germany). Each cycle consisted of 15 seconds at 95 °C for denaturation, followed by 15 seconds at 56 °C for annealing and 10 seconds at 72 °C for the extension step. PCR products were purified using QIAquick PCR Purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purified products were concentrated to a final volume of 30 μl and sequenced. The sequence analysis was performed using MEGA software (Version 6.0, Arizona, USA). The sequences were compared pairwise using the BLASTN search program and were aligned with the sequences of related species retrieved from GenBank.

Susceptibility testing

We performed antifungal susceptibility testing on each *Aspergillus* isolate by using Sensititre YeastOne YO10 (Trek Diagnostic, UK) broth microdilution method according to the manufacturer's instructions. Briefly, conidia from freshly cultured colonies were mixed in sterile saline and vortexed to get a homogenised suspension. We adjusted the turbidity to 0.5 McFarland standards with a Sensititre Nephelometer, to give an approximate inoculum density of 0.6 – 5 x 10^6 cfu/mL. Sensititre plates were inoculated within 5 hours of removal from the pouch and incubated at 35°C in a non-CO_2 incubator. Sensititre YeastOne is a well-described colorimetric microdilution panel that contains dried antifungal agents in a 96-well microplate format and a colorimetric indicator with serial two-fold dilutions of nine antifungal in individual well. Results were read manually after 48-72 hours (depending on colour change in the control well). The minimal inhibitory concentration (MIC) was taken as the lowest antifungal concentration that inhibited fungal growth (the first blue well) while the minimum effective concentration (MEC) was taken as the lowest echinocandin concentration that produced stunted hyphal growth. Antifungal susceptibility was interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Antifungal Clinical Breakpoint for *Aspergillus* spp. version 10.0, 2020.[9]

Data analysis

Data were analysed and reported descriptively. Species identification using the β-tubulin method was taken as the final species identification in reporting antifungal susceptibility profiles and

MIC/MEC analyses. MIC/MEC_{50} and MIC/MEC_{90} were defined as the concentrations of antifungal agents that inhibited 50% and 90% of the isolates, respectively. The geometric mean was defined as mean or average, which indicates the central tendency or typical value of a set of numbers by using the product of their values. The geometric mean MIC/MECs were calculated by finding the n^{th} root of the product of n numbers, i.e., geometric mean $MIC = \sqrt[n]{MIC1 \times MIC2 \times MIC3 \times ...MICn}$. If an MIC/MEC was very high and reported using 'more than' (>) sign, the value of the next higher double dilution was used in the calculation, e.g. if the MIC was >256 μg/mL, 512 was used in the calculation of geometric mean MIC.

RESULTS

Twenty-eight *Aspergillus* species were isolated from various clinical specimens including sputum, tissue, bronchoalveolar lavage and dermatology specimens. Based on β-tubulin identification, the most common species was *A. niger* (17/28, 60.7%), followed by *A. fumigatus* (6/28, 21.4%), *A. flavus* (3/28, 10.7%), *A. chevalieri* (1/28, 3.6%) and *A. tubingensis* (1/28, 3.6%).

Antifungal susceptibility testing was performed using Sensititre YeastOne against nine antifungal agents, including amphotericin B, anidulafungin, caspofungin, micafungin, fluconazole, itraconazole, voriconazole, posaconazole and flucytosine. The MIC distributions of individual isolates are shown in Table 1. Further analyses were performed to look at antifungal MIC/MEC ranges, MIC/MEC_{50}, MIC/MEC_{90} and geometric mean MIC/MECs to *A. niger* and all *Aspergillus* species (Table 2). Analyses of other *Aspergillus* species were not performed due to the small number of isolates present in each species (less than ten isolates), as the results would not be reliable. For amphotericin B, MIC_{50} and MIC_{90} values against *A. niger* and all *Aspergillus* spp. ranged from 1 to 2 μg/mL, respectively. Meanwhile for the echinocandins, the MEC_{50} and MEC_{90} values for both *A. niger* and all *Aspergillus* spp. ranged from 0.008-0.06 μg/mL and 0.015-0.06 μg/mL, respectively. Among the azoles, fluconazole exhibited markedly elevated MIC_{50} and MIC_{90} against all *Aspergillus* species, with values of 256 and >256 μg/mL, respectively. For the other azoles, viz. itraconazole, voriconazole and posaconazole, the MIC_{50} and MIC_{90} against *A. niger* and all *Aspergillus* spp. were much lower, ranging from 0.25-0.5 μg/mL and 0.12-0.5 μg/mL, respectively. Finally, flucytosine MIC_{50} and MIC_{90} were 0.5 and 1 μg/mL, respectively for *A. niger*; and 1 and 4 μg/mL respectively for all *Aspergillus* spp. (Table 2).

Based on the EUCAST Antifungal Clinical Breakpoint for *Aspergillus* spp.,[9] 16/17 (94.1%) *A. niger* isolates were susceptible to amphotericin B. One isolate (5.9%) of *A. niger* was noted to be resistant to amphotericin B, with a MIC of 2 μg/mL. There are no breakpoints for other antifungal agents against *A. niger* established by the EUCAST. As for *A. fumigatus*, all six isolates were susceptible to amphotericin B, itraconazole and voriconazole, but only 5/6 isolates (83.3%) were susceptible to posaconazole. The remaining isolate of *A. fumigatus* had a posaconazole MIC of 0.25 μg/mL, which according to the EUCAST breakpoints, falls between the susceptible and resistant categories called the 'Area of Technical Uncertainties or ATU'. Meanwhile, all three (100%) *A. flavus* isolates were susceptible to itraconazole. No breakpoints are available for the other antifungal agents.[9] The overall susceptibility pattern is depicted in Figure 1.

DISCUSSION

Aspergillus susceptibility testing is not routinely performed in many hospitals. However, in view of the increasing population at risk of invasive aspergillosis, availability of several antifungal agents, and emerging azole resistance, it is desirable to know the susceptibility of these pathogens to guide a more targeted antifungal therapy.[10] This in turn may potentially reduce morbidity and mortality from aspergillosis, as well as the unnecessary costs and adverse effects of antifungal agents.[11]

Analyses of MIC ranges, MIC_{50}, MIC_{90} and geometric mean MICs are important in determining trends in antimicrobial MICs against pathogens. They are also useful when comparing susceptibility data with other institutions or other geographical regions. Subtle increases in MICs (MIC creep) may not be noticeable if regular surveillance is not in place. As this was our first-time performing *Aspergillus* susceptibility testing, we could not really determine any trends of antifungal MIC at our centre. When researchers in Spain compared the MICs of voriconazole against 400 clinical strains of *Aspergillus* from the pre-voriconazole (1999 to 2002) and post-voriconazole (2003 to 2007) periods, they found that the mean MICs

TABLE 1: Minimum inhibitory concentrations (MICs) or minimum effective concentrations (MECs) of various antifungal agents against Aspergillus species as determined by the Sensititre YeastOne broth microdilution method

No	*Aspergillus* species	Antifungal MICs/MECs (μg/mL)								
		AMB	**AND**	**CAS**	**MIC**	**FLU**	**ITR**	**VOR**	**POS**	**5FC**
1.	*A. chevalieri*	1	0.015	0.03	0.008	32	0.03	0.06	0.015	16
2.	*A. flavus*	2	0.015	0.015	0.008	128	0.25	0.25	0.12	1
3.	*A. flavus*	1	0.015	0.008	0.06	64	0.25	0.25	0.25	0.25
4.	*A. flavus*	2	0.015	0.03	0.008	64	0.12	0.12	0.12	2
5.	*A. fumigatus*	1	0.03	0.03	0.008	>256	0.5	0.5	0.12	4
6.	A. fumigatus	1	0.015	0.03	0.008	128	0.25	0.12	0.06	4
7.	*A. fumigatus*	0.5	0.03	0.03	0.008	>256	0.5	0.5	0.25	4
8.	*A. fumigatus*	1	0.015	0.015	0.008	256	0.25	0.12	0.12	1
9.	*A. fumigatus*	0.5	0.015	0.015	0.008	256	0.25	0.25	0.12	1
10.	*A. fumigatus*	1	0.015	0.015	0.008	256	0.25	0.12	0.12	1
11.	*A. niger*	1	0.06	0.12	0.03	256	0.5	0.12	0.12	0.5
12.	*A. niger*	2	0.03	0.06	0.008	256	0.5	0.25	0.25	1
13.	*A. niger*	1	0.015	0.06	0.008	256	0.5	0.25	0.25	0.5
14.	*A. niger*	1	0.015	0.015	0.008	256	0.12	0.12	0.06	0.25
15.	*A. niger*	1	0.015	0.03	0.008	256	0.5	0.12	0.12	1
16.	*A. niger*	1	0.015	0.06	0.015	256	0.25	0.25	0.25	0.5
17.	*A. niger*	1	0.015	0.03	0.008	128	0.25	0.12	0.03	4
18.	*A. niger*	1	0.015	0.008	0.008	64	0.06	0.06	0.015	1
19.	*A. niger*	1	0.015	0.06	0.008	256	0.5	0.25	0.25	0.25
20.	*A. niger*	1	0.03	0.06	0.015	256	0.25	0.25	0.12	0.5
21.	*A. niger*	0.5	0.015	0.03	0.008	256	0.5	0.25	0.25	1
22.	*A. niger*	1	0.015	0.03	0.008	256	0.5	0.25	0.25	1
23.	*A. niger*	1	0.015	0.06	0.008	>256	0.5	0.12	0.25	0.5
24.	*A. niger*	1	0.015	0.06	0.008	256	0.5	0.25	0.25	0.5
25.	*A. niger*	1	0.03	0.03	0.008	256	0.5	0.25	0.25	1
26.	A. niger	1	0.015	0.03	0.008	256	0.25	0.12	0.12	0.5
27.	*A. niger*	1	0.06	0.12	0.015	128	0.25	0.12	0.25	0.5
28.	*A. tubingensis*	1	0.015	0.06	0.015	128	0.5	0.25	0.25	0.5

AMB, amphotericin B; AND, anidulafungin; CAS, caspofungin; MIC, micafungin; FLU, fluconazole; ITR, itraconazole; VOR, voriconazole; POS, posaconazole; 5FC, flucytosine

TABLE 2: Antifungal MIC/MEC ranges, MIC/MEC_{50}, MIC/MEC_{90} and geometric mean MIC/MECs (μg/mL) to Aspergillus niger (n=17) and all Aspergillus species (n=28)

Antifungal agent	Aspergillus spp.*	MIC/MEC range	MIC/MEC_{50}	MIC/MEC_{90}	Geometric mean †
Amphotericin B	*A. niger*	0.5-2	1	1	1
	All *Aspergillus*	0.5-2	1	2	1
Anidulafungin	*A. niger*	0.015-0.06	0.015	0.06	0.02
	All *Aspergillus*	0.015-0.06	0.015	0.03	0.02
Caspofungin	*A. niger*	0.008-0.12	0.06	0.12	0.04
	All *Aspergillus*	0.008-0.12	0.03	0.06	0.03
Micafungin	*A. niger*	0.008-0.03	0.008	0.015	0.01
	All *Aspergillus*	0.008-0.06	0.008	0.015	0.01
Fluconazole	*A. niger*	64->256	256	256	227
	All *Aspergillus*	32->256	256	>256	195
Itraconazole	*A. niger*	0.06-0.5	0.5	0.5	0.33
	All *Aspergillus*	0.03-0.5	0.25	0.5	0.29
Voriconazole	*A. niger*	0.06-0.25	0.25	0.25	0.17
	All *Aspergillus*	0.06-0.5	0.25	0.25	0.18
Posaconazole	*A. niger*	0.015-0.25	0.25	0.25	0.14
	All *Aspergillus*	0.015-0.25	0.12	0.25	0.13
Flucytosine	*A. niger*	0.25-4	0.5	1	0.67
	All *Aspergillus*	0.25-16	1	4	0.95

MEC, minimum effective concentration; MIC, minimum inhibitory concentration
* *Aspergillus* spp. of more than nine isolates only were included in these analyses.
† If the MIC/MEC was preceded by 'more than' sign (>), the next higher MIC/MEC dilution was taken to calculate the geometric mean MIC/MECs (MIC/MEC_{GEO}), e.g. 512 was used in the calculation when the MIC/MEC was >256.

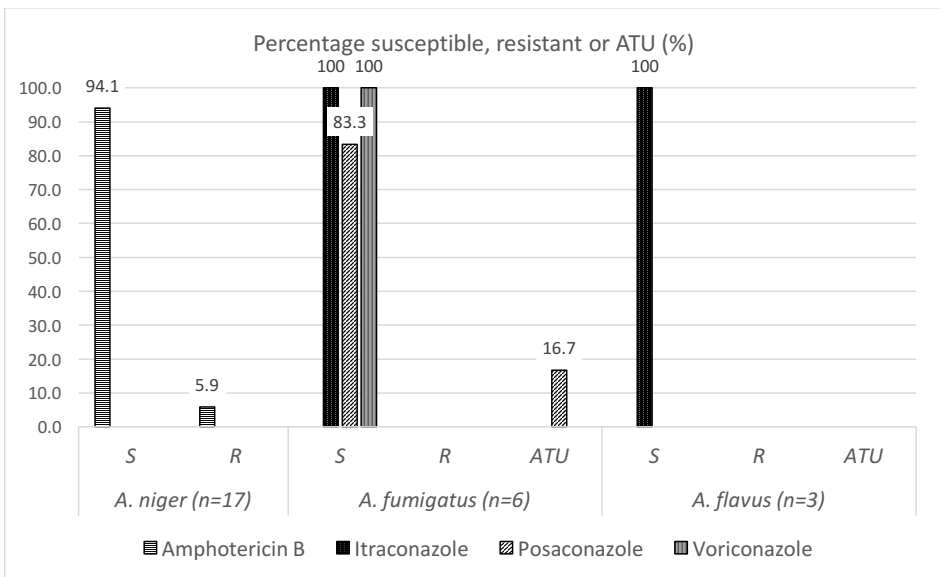

FIG. 1: Antifungal susceptibility profiles of *Aspergillus* species according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Clinical Breakpoints (S, susceptible; R, resistant; ATU, area of technical uncertainty)

of strains from the post-voriconazole period were slightly higher (0.39 versus 0.57g/ml; P < 0.001).[12] Although in this case, all strains remained susceptible to voriconazole, detection of an MIC creep may provide an early warning sign of an emerging resistance.

Although the Sensititre method was developed according to the Clinical and Laboratory Standards Institute (CLSI) reference methods, we have opted to use the breakpoints produced by the EUCAST to interpret the MICs/MECs because the CLSI has not largely determined the antifungal breakpoints for *Aspergillus* species. In general, *Aspergillus* isolates at our centre are still susceptible to amphotericin B and voriconazole. This is important because these two antifungal agents are used commonly at our centre for treating invasive aspergillosis. Emergence of resistance to these antifungal agents may force us to use echinocandins, which are more expensive and may cause financial strains to our institution and patients. We rarely had to resort to echinocandins as salvage therapies of invasive aspergillosis. This lack of exposure probably explains the low echinocandins MEC_{50} and MEC_{90} values against our *Aspergillus* isolates. The EUCAST has stated that fluconazole testing is not recommended for *Aspergillus* spp. and some *Candida* isolates due to fluconazole intrinsic resistance.[9] However, since fluconazole is already present in the Sensititre panel, our study confirmed this recommendation when the fluconazole MICs ranged from 32 to >256 μg/mL against all our *Aspergillus* isolates. Other azoles, in general, showed relatively low MICs to all our isolates. However, one particular isolate of *A. fumigatus* had a posaconazole MIC of 0.25 μg/mL, which is categorised under 'Area of Technical Uncertainties' or 'ATU'. According to the EUCAST description for posaconazole susceptibility that falls under the ATU category, the decision to report posaconazole depends on the isolate susceptibility to itraconazole. If the isolate is susceptible to itraconazole, then report posaconazole as susceptible and add the following comment: "The MIC is 0.25 mg/L and thus one dilution above the susceptible breakpoint due to overlapping wild type and non-wild type populations". If the isolate is not susceptible to itraconazole, then report posaconazole as resistant and refer to reference laboratory for CYP51A sequencing and confirmation of MICs.[9] The ATUs serve as warnings to laboratory staff that there is an uncertainty that needs to be addressed before reporting antifungal susceptibility results to clinical colleagues. The ATU is not to be conveyed to clinical colleagues except under special circumstances and only as part of a discussion about therapeutic alternatives in difficult cases.[9] As for flucytosine, the MICs ranged from 0.25 to 4 μg/mL for all isolates except for a single isolate of *A. chevalieri*, with flucytosine MIC of 16 μg/mL. *Aspergillus chevalieri* is a cryptic *Aspergillus* species that has been associated with opportunistic cutaneous aspergillosis.[13] Some cryptic *Aspergillus* species have been reported to be multidrug resistant.[14] However, similar to our findings, a few studies reported low antifungal MIC ranges against *A. chevalieri* such as amphotericin B (0.03-0.5 μg/mL), echinocandins (0.015-0.125 μg/mL), itraconazole (0.03-0.5 μg/mL), posaconazole (0.015-0.03 μg/mL), voriconazole (0.12-0.5 μg/mL), isavuconazole (0.125 μg/mL) and terbinafine (0.03-0.12 μg/mL). However, there was no result on flucytosine in these studies.[15,16] Another cryptic species identified in our study was *A. tubingensis*. This isolate is morphologically indistinguishable from *A. niger*. Excluding fluconazole, our isolate showed low MICs across the board (amphotericin B 1 μg/mL, echinocandins <0.06 μg/mL, azoles <0.5 μg/mL and flucytosine 0.5 μg/mL), which is similar to another study in China.[17] Meanwhile, other studies reported elevated itraconazole and voriconazole MICs of >1 μg/mL.[18,19,20]

Despite several shortcomings in performance and interpretation of antifungal susceptibility testing for moulds, continuous data collection is vital in building a large enough antifungal database to develop reliable susceptibility patterns, epidemiological cut-off values and clinical breakpoints, to better correlate with clinical response.[10] Findings from our study could add to the data collection of *Aspergillus* antifungal susceptibility. Another strength of this study is that it could provide a baseline of antifungal susceptibility for detecting emergence of antifungal resistance particularly in the Southeast Asian region. This study also noted occurrence of cryptic *Aspergillus* species in clinical samples that could have different susceptibility patterns which in turn, could affect treatment outcomes. Even though our study is limited in being single-centred and having a small sample size, data gained from this study provides important insights into antifungal susceptibility of *Aspergillus* species, particularly for Malaysia. Another limitation was the inability to study only isolates from sterile

sites. In other words, some of these isolates could potentially be contaminants, especially those that were cultured from dermatological specimens. However, *Aspergillus* cultivation from normally sterile specimens such as blood and cerebrospinal fluid is very rare. Therefore, susceptibility data from any *Aspergillus* isolates would be extremely valuable.

In conclusion, *Aspergillus niger* remains the most commonly isolated *Aspergillus* species from clinical specimens. *Aspergillus* isolates at our centre are still largely susceptible to amphotericin B and have low minimum inhibitory concentrations to echinocandins and most azoles. Data obtained from this study may be useful in guiding antifungal therapy in the treatment of aspergillosis.

Acknowledgements: The authors would like to thank Universiti Kebangsaan Malaysia for funding this study (grant no. FF-2017-148).

Authors' contributions: TMN and SM contributed to the concept and design of the study. TMN, SM, RMT and HY did literature search, experimental studies and data acquisition. TMN and SM performed data analysis and statistical analysis. TMN, SM, HY and RMT did the manuscript preparation. TMN edited the manuscript. All authors have reviewed the manuscript and approved it for publication. TMN and SM take responsibility for the integrity of the work as a whole and are designated as 'guarantors'.

Conflict of interest: The authors declare no conflicts of interest.

REFERENCES

1. Bongomin F, Asio LG, Baluku JB, Kwizera R, Denning DW. Chronic pulmonary aspergillosis: notes for a clinician in a resource-limited setting where there is no mycologist. J Fungi. 2020; 6: 75.
2. Zanganeh E, Zarrinfar H, Rezaeetalab F, *et al*. Predominance of non-fumigatus Aspergillus species among patients suspected to pulmonary aspergillosis in a tropical and subtropical region of the Middle East. 2018. Microb Pathog. 2018; 116: 296-300.
3. Rudramurthy SM, Paul RA, Chakrabarti A, Mouton JW, Meis JF. Invasive aspergillosis by Aspergillus flavus: epidemiology, diagnosis, antifungal resistance, and management. J Fungi 2019; 5: 55.
4. Salah H, Lackner M, Houbraken J, *et al*. The emergence of rare clinical Aspergillus species in Qatar: Molecular characterization and antifungal susceptibility profiles. Front Microbiol. 2019; 10: 1677.
5. Tan BH. Invasive Aspergillosis in Asia. In: Chakrabarti A, editor. Clinical Practice of Medical Mycology in Asia. Singapore: Springer; 2020. p. 257-70.
6. Zakaria A, Osman M, Dabboussi F, *et al*. Recent trends in the epidemiology, diagnosis, treatment, and mechanisms of resistance in clinical Aspergillus species: a general review with a special focus on the Middle Eastern and North African region. J Infect Public Health. 2020; 13: 1-10.
7. White TJ, Bruns T, Lee SJ, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. 1990; 18: 315-22.
8. Glass NL, Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol. 1995; 61:1323-30.
9. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs for antifungal agents, version 10.0, 2020 [cited 2021 Aug 30]. Available from http://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/.
10. Lamoth F, Lewis RE, Kontoyiannis DP. Role and interpretation of antifungal susceptibility testing for the management of invasive fungal infections. J Fungi. 2021; 7: 17.
11. Zaoutis TE, Heydon K, Chu JH, Walsh TJ, Steinbach WJ. Epidemiology, outcomes, and costs of invasive aspergillosis in immunocompromised children in the United States, 2000. Pediatrics. 2006; 117: e711-6.
12. Guinea J, Recio S, Peláez T, Torres-Narbona M, Bouza E. Clinical isolates of Aspergillus species remain fully susceptible to voriconazole in the post-voriconazole era. Antimicrob Agents Chemother. 2008; 52: 3444-6.
13. Naidu J, Singh SM. Aspergillus chevalieri (Mangin) Thom and Church: a new opportunistic pathogen of human cutaneous aspergillosis: Aspergillus chevalieri (Mangin) Thorn und Church: Ein neuer opportunistischer Erreger von kutaner Aspergillose beim Menschen. Mycoses. 1994; 37: 271-4.
14. Howard SJ. Multi-resistant aspergillosis due to cryptic species. Mycopathologia. 2014. 178: 435-9.
15. Masih A, Singh PK, Kathuria S, Agarwal K, Meis JF, Chowdhary A. Identification by molecular methods and matrix-assisted laser desorption ionization–time of flight mass spectrometry and antifungal susceptibility profiles of clinically significant rare Aspergillus species in a referral chest hospital in Delhi, India. J Clin Microbiol. 2016; 54: 2354-64.
16. Siqueira JP, Sutton DA, Gené J, García D, Wiederhold N, Guarro J. Species of Aspergillus section Aspergillus from clinical samples in the United States. Med Mycol. 2018; 56: 541-50.
17. Li Y, Wan Z, Liu W, Li R. Identification and susceptibility of Aspergillus section Nigri in China: prevalence of species and paradoxical growth in response to echinocandins. J Clin Microbiol. 2015; 53: 702-5.
18. Bathoorn E, Salazar NE, Sepehrkhouy S, Meijer M, de Cock H, Haas PJ. Involvement of the opportunistic pathogen Aspergillus tubingensis in osteomyelitis of the maxillary bone: a case report. BMC Infect Dis. 2013; 13:1-4.

19. Hashimoto A, Hagiwara D, Watanabe A, Yahiro M, Yikelamu A, Yaguchi T. *et al*. Drug sensitivity and resistance mechanism in Aspergillus section Nigri strains from Japan. Antimicrob Agents Chemother. 2017; 61: e02583-16.
20. Hendrickx M, Beguin H, Detandt M. Genetic re-identification and antifungal susceptibility testing of Aspergillus section Nigri strains of the BCCM/IHEM collection. Mycoses. 2012; 55: 148-55.