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Susceptibility Testing of Clinical Mould Isolates in Malaysia with the E-test and M38-A Methods

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Susceptibility testing of filamentous fungal isolates was carried out with the aim of obtaining susceptibility data for Malaysian clinical isolates and to determine the agreement between two methods of susceptibility testing. Fifteen fungal isolates comprising of *Aspergillus* sp., *Fusarium* sp. and *Cladosporium* sp. were evaluated for susceptibility to amphotericin B with E-test and M38-A broth microdilution methods. Susceptibility to itraconazole and voriconazole was also determined with E-test method. With the M38-A method, nine *Aspergillus* sp. and two *Cladosporium* sp. isolates were found to be sensitive (MIC 2 μg mL^{-1}), while three *Fusarium* sp. isolates appeared resistant (MIC≥8 μg mL^{-1}) to Amphotericin B. The E-test showed MIC values which were much lower (0.002-0.5 μg mL^{-1}) for six *Aspergillus* sp. isolates and the two *Cladosporium* sp. isolates, while for *Fusarium* sp., MIC was ≥2 μg mL^{-1}. Almost all isolates, other than *Fusarium* sp., were uniformly more susceptible to voriconazole (MIC: 0.008-0.5 μg mL^{-1}) than itraconazole (MIC: 0.006-6 μg mL^{-1}). In conclusion, *Fusarium* sp. showed least susceptibility to all antifungals tested. E-test and M38-A methods generated low agreement in determining MIC of amphotericin B and the agreement between the two test results was highest when results were read at 72 h incubation.

Key words: Susceptibility, fungi, E-test, M38-A, agreement, Malaysia

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Opportunistic infections due to filamentous fungi are increasingly emerging due to the rising numbers of immunocompromised patients in hospital and in the community (Clark and Hajjeh, 2002). Despite the increasing incidence and high mortality rates of these infections (Marr *et al*., 2001) effective treatment options are lacking and the problem of antifungal resistance may have contributed to therapeutic failure (Maschmeyer, 2002).

Therefore, it is important to carry out susceptibility testing of clinical mould isolates for the purpose of selecting and monitoring antifungal therapy. Susceptibility testing of clinical mould isolates in Malaysia is not routinely carried out and antifungal susceptibility data is lacking. This data, if available would be of value to clinicians and would contribute to effective treatment for the patient.

The Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS; National Committee for Clinical Laboratory Standards) has developed methods for fungal susceptibility testing which are the M28-A method for yeasts and the M38-A for filamentous fungi (NCCLS, 2002a, b). These standardized broth microdilution methods are tedious and time-consuming to perform. Commercially available test methods such as the agar-based E-test method (AB Biodisk, Sweden) utilize plastic test strips impregnated with a continuous concentration gradient of antifungal agent to determine the Minimum Inhibitory Concentration (MIC). This test is therefore simpler to perform compared to the CLSI/NCCLS reference method. The agreement between susceptibility test results for filamentous fungi with the CLSI/NCCLS proposed M38-A method and the E-test method is variable and differs according to antifungal agent and fungal species tested (Espinel-Ingroff, 2001; Martín-Mazuelos *et al*., 2003; Pfaller *et al*., 2003).

We carried out a study to obtain susceptibility data for Malaysian clinical mould isolates and to assess the E-test as an alternative method for *in vitro* susceptibility testing. In order to determine the agreement between the M38-A broth microdilution method and E-test, both methods were used for susceptibility testing of fungal isolates with Amphotericin B. In addition, the E-test was also used to determine susceptibility to the newer azole drugs, itraconazole and voriconazole.

Fungal isolates were recovered from clinical specimens (oral swabs, pus, tracheal aspirate, lung biopsy, corneal scraping, skin and nail) taken from patients in hospitals across Malaysia and from zoonotic sources (cats). The 15 mould isolates consisted of *Aspergillus niger* (7 isolates), *A. flavus* (2 isolates), *A. fumigatus* (1 isolate), *Fusarium* sp. (3 isolates) and *Cladosporium* sp. (2 isolates). The quality control strain used was *Candida parapsilosis* ATCC 22019. All fungal isolates and reference strain were cultured on Sabouraud dextrose agar to ensure viability and purity prior to testing.

Susceptibility testing of fungal isolates was performed using the reference broth microdilution method (M38-A) and the commercial E-test method. The broth microdilution method was carried out adhering stringently to the CLSI/NCCLS guidelines (NCCLS, 2002b). The fungal inoculum concentration was determined according to the method of Espinel-Ingroff and Kerkering (1991). Amphotericin B was used for susceptibility testing of the fungal isolates with the M38-A method, which were tested in duplicates. The E-test was performed exactly according to the manufacturer's protocols (AB Biodisk, 2007) protocols. The media used was RPMI agar with 2% glucose. The antifungals tested were Amphotericin B, itraconazole and voriconazole. Quality control was ensured by testing the reference strain *Candida parapsilosis* ATCC 22019 each time the susceptibility test was performed with either the M38-A method or the E-test.

The Minimum Inhibitory Concentration (MIC) of antifungals tested was determined at 24, 48 and 72 h incubation. For the M38-A method the MIC was the concentration at which 100% growth inhibition was seen. For the E-test, the MIC for Amphotericin B was determined for 100% growth inhibition whereas for the azole drugs the MIC value was read at 80% fungal growth inhibition, i.e., only microcolonies are present. Discrepancies between MICs of no more than 2 dilutions were used to calculate the percent agreement of the two methods.

The quality control test with the reference strain was performed a total of 7 times on different occasions. The reference range should be 0.25-1.0 μg mL^{-1} for amphotericin B, 0.016-0.064 μg mL^{-1} for voriconazole and 0.064-0.25 μg mL^{-1} for itraconazole (NCCLS, 2002b). Quality control testing of the E-test revealed that the MICs were consistently low for amphotericin B at 24 h and even at 72 h MIC values were within acceptable limits only on 4 occasions (Table 1). Variable results were obtained for itraconazole, mostly low MICs at 24 h, but at 48 h both low and high MICs were obtained. For voriconazole the MIC values were acceptable at 24 h 6 out of 7 times and fell within range at 48 h (all 7 times). The E-test quality control results for Amphotericin B and itraconazole which were highly variable, with a more than 3 dilution difference in the lowest and highest MICs for the same reference strain on different occasions indicate that further standardization of either the incubation period

Table 1: Quality control test results for E-test with the reference strain *C. parapsilosis* ATCC 22019

	MIC (μg mL^{-1})								
	Amphotericin B			Itraconazole			Voriconazole		
Test No.	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
1	0.016**	0.032**	0.047**	0.008**	0.064	0.094	0.008**	0.016	0.094*
2	0.008**	0.38	0.50	0.012**	0.032**	0.047**	0.016	0.032	0.032
3	0.032**	0.38	0.75	0.023**	0.064	0.25	0.032	0.047	0.064
4	0.047**	0.25	0.50	0.032**	0.19	0.38*	0.032	0.064	0.064
5	0.064**	0.094**	0.25	0.19	0.25	0.50*	0.023	0.032	0.047
6	0.032**	0.047**	0.064**	0.047**	0.064	0.094	0.032	0.064	0.094*
7	0.016**	0.125**	0.19**	0.023**	1.00*	1.00*	0.047	0.064	0.125*

*: MIC higher than quality control limit, **: MIC lower than quality control limit, Quality control limits : Ampotericin B (0.25-1 μg mL^{-1}); Itraconazole (0.064-0.25 μg mL^{-1}); Voriconazole (0.016-0.064 μg mL^{-1})

Table 2: Susceptibility results for isolates with the M38-A method

	MIC Amphotericin B (μg mL^{-1})		
Isolate (source)	24 h	48 h	72 h
A. niger			
UZ 792 (oral swab)	2	2	2
UZ 822 (pus)	2	2	2
UZ 832 (aspirate)	1	2	2
UZ 964 (oral swab)	2	2	2
UZ 1083 (cornea)	2	2	2
UZ 1416 (skin)	2	2	2
UZ 1579 (lung)	0.38	2	2
A. flavus			
UZ 972 (zoonotic)	2	2	2
UZ 973 (zoonotic)	4	4	8
A. famigatus			
UZ 836 (nail)	2	2	2
***Fusarium* sp.**			
UZ 1070 (nail)	4	8	8
UZ 1627 (nail)	>16	>16	>16
UZ 1650 (nail)	>16	>16	>16
***Cladosporium* sp.**	2	2	8
UZ 895 (skin)			
UZ 1283 (skin)	2	2	8

or fungal inoculum size may be necessary. The experimental procedures for this study was carried out by the same researcher on all occasions. The recommended incubation period for yeasts is 24-48 h. With the M38-A method, quality control results were consistent; the MIC for amphotericin B with the reference strain was 2 μg mL^{-1} (acceptable range is 0.5-4 μg mL^{-1}), each time the test was carried out.

MICs for the clinical isolates read at 48 h, which is the recommended incubation period for filamentous fungi, showed that nine *Aspergillus* sp. isolates and two *Cladosporium* sp. isolates were sensitive (MIC 2 μg mL^{-1}) with the M38-A method, while all three *Fusarium* sp. isolates appeared resistant (MIC $\geq$8 μg mL^{-1}) to Amphotericin B (Table 2). Fungal isolates with MIC>2 μg mL^{-1} for amphotericin B or >8 μg mL^{-1} for itraconazole are considered resistant to these antifungals, however no data is available for newer triazoles such as voriconazole (NCCLS, 2002b). The E-test showed MIC values which were much lower (0.002-0.5 μg mL^{-1}) for six *Aspergillus* sp. isolates and the two *Cladosporium* sp. isolates, while for *Fusarium* sp., MIC was $\geq$ 2 μg mL^{-1} (Table 3). This is consistent with results obtained with the reference strain which also showed lower MICs. Almost all isolates, other than *Fusarium* sp., were uniformly more susceptible to voriconazole (MIC: 0.008-0.5 μg mL^{-1}) than itraconazole (MIC: 0.006-6 μg mL^{-1}). *Fusarium* sp. showed least susceptibility to all antifungals tested.

Comparison of results obtained with E-test and M38-A for amphotericin B revealed low agreement between the two methods for the 15 isolates tested. The percent agreement was 20% at 24 h, 26.7% at 48 and 66.7% at 72 h. Although a higher agreement was noted at 72 h, but it was still at a low level compared to other researchers (Espinel-Ingroff, 2001; Martín-Mazuelos *et al*., 2003). In this research only 15 isolates were tested. Evaluation with a larger number of isolates may provide differing results. The incubation time most often used was 24 to 48 h (Pfaller *et al*., 2003; Espinel-Ingroff, 2001, 2006).

In conclusion, among the Malaysian clinical mould isolates tested, *Fusarium* sp. showed least susceptibility to all antifungals tested. E-test and M38-A methods generated low agreement in determining MIC of amphotericin B and the agreement between the two test results was highest when results were read at 72 h

Table 3: Susceptibility results for isolates with the E-test method

	MIC (µg mL^{-1})								
	Amphotericin B			Itraconazole			Voriconazole		
Isolate	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
A. niger									
UZ 792	0.125	0.25	0.50	1.50	4.00	4.00	0.064	0.25	0.38
UZ 822	0.25	0.25	2.00	0.50	1.00	8.00	0.047	0.125	0.25
UZ 832	-	0.002	0.003	-	0.006	0.016	-	0.008	0.12
UZ 964	0.094	0.38	1.00	0.75	6.00	8.00	0.125	0.50	1.00
UZ 1083	0.125	0.50	4.00	0.012	2.00	6.00	0.075	0.094	0.125
UZ 1416	-	>32.00	>32.00	-	2.00	4.00	-	0.25	0.38
UZ 1579	0.094	0.25	1.50	0.50	2.00	4.00	0.064	0.19	0.38
A. flavus									
UZ 972	1.50	32.00	>32.00	0.023	0.25	0.50	0.25	0.38	0.50
UZ 973	0.50	3.00	6.00	0.75	2.00	3.00	0.38	0.38	3.00
A. fumigatus									
UZ 836	1.50	2.00	2.00	3.00	3.00	4.00	0.38	0.50	2.00
***Fusarium* sp.**									
UZ 1070	-	8.00	12.00	-	16.00	24.00	-	0.25	0.50
UZ 1627	1.50	6.00	8.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00
UZ 1650	0.19	2.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00	>32.00
***Cladosporium* sp.**									
UZ 895	-	0.064	0.25	-	0.25	0.75	-	0.023	0.047
UZ 1283	0.016	0.047	0.38	0.008	0.50	>32.00	0.094	0.25	2.00

incubation. Our quality control results indicate that for the E-test, results are variable and parameters such as incubation time and inoculum size may need to be further standardized.

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