AUSTRALIAN ANTIFUNGAL SUSCEPTIBILITY DATA 2008-2011: PART 2 – THE MOULDS ASPERGILLUS, SCEDOSPORIUM AND FUSARIUM.

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In this article, an overview of recent changes to the CLSI antifungal standards for susceptibility testing of moulds is presented. We also summarise current Australian antifungal susceptibility data for *Aspergillus, Scedosporium* and *Fusarium species*.

CLSI M38-A2 standard for moulds:1

The M38-A2 standard describes a microbroth dilution method for testing the susceptibility of filamentous fungi (moulds) that cause invasive infections (including *Aspergillus spp*., *Fusarium spp*., *Scedosporium spp*., *Sporothrix schenckii*, *Rhizopus oryzae*, zygomycetes and other pathogenic moulds) and cutaneous infections (the dermatophytes *Trichophyton*, *Microsporum* and *Epidermophyton*) to antifungal agents.

Recent changes to the M38-A2 standard include:

1. Additional sections on reading MIC and MEC results for the echinocandins and methodology for testing dermatophytes.

2. For non-dermatophyte species the inoculum preparation of conidial or sporangiospore suspensions must be adjusted using a spectrophotometer, with a test inoculum in the range $0.4x10^4$ – $5x10^4$ CFU/mL providing the most reproducible MIC data. Inoculum density (OD) is dependent on the size of the conidia/sporangiospores of the mould being tested; i.e. for *Aspergillus*, *Paecilomyces*, *Exophiala* and *Sporothrix species* the OD = 0.09-0.13; for *Fusarium*, *Scedosporium, Ochroconis*, *Cladophialophora*, *Rhizopus* and other zygomycete species the OD = 0.15-0.17; for *Bipolaris* and *Alternaria* species the OD = 0.25-0.3. Note the addition of a very small drop of Tween 20 as a wetting agent will facilitate the preparation of conidial or sporangiospore suspensions.

3. For amphotericin B, itraconazole, voriconazole and posaconazole an MIC is read at 100% inhibition. The MICs for 5-fluorocytosine and fluconazole are read at 50% growth reduction. For the echinocandins, an MEC (minimum effective concentration) is read at the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms as compared to hyphal growth seen in the growth control well; this requires a reading mirror and the eye of faith!

4. Breakpoints have not been established for mould testing. However, working breakpoints were assigned for analytical purposes for moulds against amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin.(1,2) Isolates were grouped as susceptible (MIC or MEC <1 µg/mL), intermediate (MIC or MEC 2 µg/mL), and resistant (MIC or MEC >4 µg/mL). It must be emphasized that these are working breakpoints for analytical purposes only. The clinical relevance of testing pathogenic moulds remains uncertain, and breakpoints with proven relevance have yet to be identified or approved by CLSI or any regulatory agency (M38-A2).

Australian antifungal susceptibility data: *Aspergillus*, *Scedosporium* and *Fusarium* species (2008-2011)

Isolates recovered from patients with invasive fungal infections were tested using the CLSI M38-A2 microbroth dilution susceptibility standard for moulds. The range of MICs for *Aspergillus, Scedosporium* and *Fusarium* species against the common mould active antifungal agents are summarised below.

Aspergillus species *(see table 1)*.

A total of 248 *Aspergillus* isolates were received for antifungal susceptibility testing from 2008-2011. The most common species was *A. fumigatus* (62.5%), followed by *A. niger* (10.1%), *A. terreus* (9.7%), *A. flavus* (8.9%) and *A. nidulans* (3.2%). However a number of emerging *Aspergillus* species were also identified, notably *Neosartorya fischeri* (3.2%), *A. lentulus* (0.8%), *A. ustus* (0.8%), *A. sydowii* (0.4%), and *A. glaucus* (0.4%).

The clinical emergence of unusual *Aspergillus* species has led to a revision of the taxonomy of the aspergilli. The *A. fumigatus* morpho-species is now known to comprise a number of species that can only be reliably distinguished by molecular methods; this group is now referred to as the '*Aspergillus fumigatus species complex*'. Of particular clinical importance in this group is *A.lentulus*, which frequently has high MICs to amphotericin B, itraconazole, voriconazole and caspofungin;(3) *Neosartorya pseudofischeri* and *A. udagawae* have also been reported to have high in vitro MICs.(4) While ITS sequencing can be used to identify aspergilli to species complex level, β-tubulin (*benA*) gene sequencing is required to determine the exact species. *A. flavus, A. terreus, A. niger, A. nidulans*, and *A. ustus* species complexes are also recognised.(5)

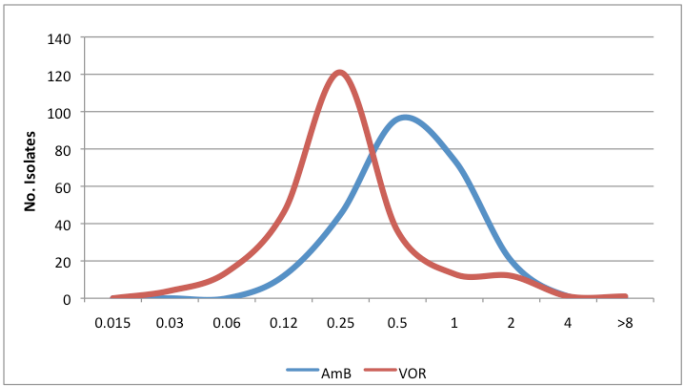
Table 1. *Aspergillus species*

Species (No. isolates tested)	Antifungal agent	No. of isolates at MIC (µg/mL)									
		<0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	>8
Aspergillus fumigatus species complex											
A. fumigatus (155)	AmB				7	27	82	32	7		
	ITR	4	8	15	26	54	42	4	1		
	VOR		1	10	35	95	7	2	4		1
	POS	22	25	30	32	38	7				1
A. lentulus (2)	AmB								2		
	ITR					1					1
	VOR							1	1		
	POS				1		1				
N. fischeri (8)	AmB					1		5	2		
	ITR				1	2	2	3			
	VOR						1	3	3	1	
	POS				4	1	1	2			
Aspergillus flavus species complex											
A. flavus (22)	AmB						2	14	5	1	
	ITR		1		3	15	3				
	VOR				2	9	9	2			
	POS	1		3	5	9	4				
Aspergillus terreus species complex											
A. terreus (24)	AmB					1	4	15	4		
	ITR	4	1	2		13	4				
	VOR			1	4	12	4	1	2		
	POS	1	5	1	5	9	2	1			
Aspergillus niger species complex											
A. niger (25)	AmB				3	11	7	4			
	ITR			1	3	4	10	7			
	VOR		2		1	4	14	4			
	POS		3	2	8	5	7				
A. glaucus (1)	AmB							1			
	ITR				1						
	VOR						1				
	POS					1					
Aspergillus nidulans species complex											
A. nidulans (8)	AmB				2	4		2			
	ITR	1	1	3	2	1					
	VOR		1	3	4						
	POS	1	3	2	1	1					
A. sydowii (1)	AmB							1			
	ITR		1								
	VOR					1					
	POS			1							
Aspergillus ustus species complex											
A. ustus (2)	AmB					1	1				
	ITR					2					
	VOR								2		
	POS						1	1			

For *A. fumigatus* (n=155), only one isolate was resistant to voriconazole (MIC=8 µg/mL), but was susceptible to posaconazole (MIC=0.5 µg/mL). Conversely, another isolate was resistant to posaconazole (MIC=8 µg/mL), but susceptible to voriconazole (MIC=1 µg/mL). Overall, only 5 isolates of *Aspergillus* had MICs >4µg/mL, indicating a theoretical resistance rate of 2%. No cross-resistance was detected.

The MIC distributions observed for all *Aspergillus spp.* isolates against voriconazole and amphotericin B are shown in Figure 1.

Figure 1. MIC distributions for all *Aspergillus species* against Voriconazole and Amphotericin B



The emergence of invasive infection due to triazole-resistant, including cross-resistant, *A. fumigatus* isolates is of increasing concern in Europe, where 3-6% of isolates have been reported resistant at different centres.(6) While a number of mutations in the *cyp51A* gene have been associated with triazole resistance, the dominant mechanism for cross-resistance is a L98H substitution accompanied by a 34 bp tandem repeat in the promoter region of this gene.(7) Triazole-resistant isolates harbouring this mutation have been cultured from environmental sources in The Netherlands, and molecular epidemiology studies indicate that colonisation and infection with these resistant isolates may be environmentally acquired.(8)

To date, a similar emergence of triazole- and/or cross-resistant *A. fumigatus* has not been observed in the Australian setting. A recent surveillance study of all *A. fumigatus* isolates at The Alfred Hospital, Melbourne, identified no triazole-resistant *A. fumigatus* isolates over a one year period (May 2009 - April 2010) (S. Kidd, unpublished data).

Scedosporium species *(see Tables 2 and 3)*

A total of 131 *Scedosporium* isolates were received for antifungal susceptibility testing from 2008-2011. *S. prolificans* accounted for 54%, while *S. apiospermum* and *S. aurantiacum* accounted for 41% and 5%, respectively. While most *S. aurantiacum* and *S. apiospermum* isolates were resistant to amphotericin B, all were susceptible to voriconazole. In contrast, *S. prolificans*, not unexpectedly, demonstrated multidrug resistance with high MICs to amphotericin B, itraconazole, voriconazole and posaconazole. Given the resistance observed for *S. prolificans*, it is worthwhile testing for synergistic action of terbinafine with the triazoles.

Table 2. *Scedosporium species*

Species (No. isolates tested)	Antifungal agent	No. of isolates at MIC (µg/mL)									
		<0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	>8
S. apiospermum (54)	AmB					1	2	2	13	24	12
	ITR			1	2	7	22	16			6
	VOR		2	7	25	13	6				1
	POS				4	8	25	15	1		1
S. aurantiacum (6)	AmB								1	3	2
	ITR						1	3			2
	VOR				2	4					
	POS						2	2	2		
S. prolificans (71)	AmB										46
	ITR										71
	VOR										30
	POS										70

Synergy testing: Since 2001, a total of 109 isolates of *S. prolificans* from patients with invasive infection have undergone synergy testing for a combination of voriconazole and terbinafine (Table 3). Synergy studies were performed using a two-dimensional two-agent micro-dilution checkerboard method.(9) *Paecilomyces variotii* (ATCC 22319) was used as a control strain to monitor reproducibility. Growth and sterility control wells were included for each isolate tested for both compounds. MICs were read at 48 and 72 hours and the end points taken at 50% or greater inhibition than the growth control.

Table 3. *Scedosporium prolificans* Synergy testing

Drug combination	∑FIC < 0.5 (S)	∑FIC > 0.5-4 (NS)	∑FIC > 4 (A)
Voriconazole/Terbinafine (n=109)	94 (86.2%)	15 (13.8%)	0

The summation of the fractional inhibitory concentration (ΣFIC) was calculated as follows: (MIC agent A in combination/MIC agent A alone) + (MIC agent B in combination/MIC agent B alone). Synergy was defined as a ΣFIC of ≤ 0.5; No synergy >0.5 - 4;Antagonism > 4.

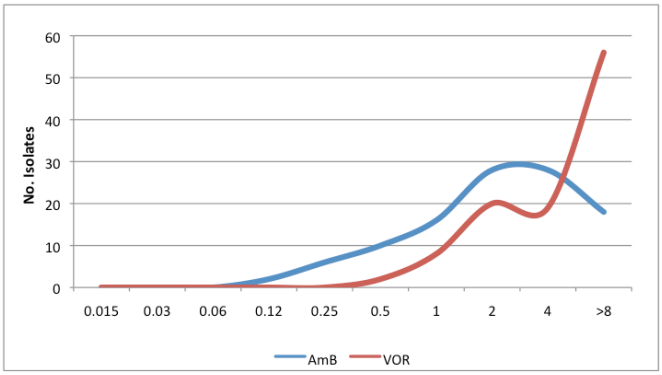
Terbinafine demonstrated synergy with voriconazole against 94 of 109 (86%) *S. prolificans* isolates. No antagonism was observed. There was no correlation between individual MICs and the synergy results. The MICs of the voriconazole and terbinafine combinations appear to be within achievable plasma ranges. Although *in vitro* results have not yet been directly correlated with therapeutic outcome, there have been some case reports of successful combination therapy.(10,11,12)

Fusarium species (see Table 4).

Like *Aspergillus*, the taxonomy of *Fusarium* is undergoing revision, and species complexes are now recognised. Accurate species identification can only be achieved by multilocus sequence typing (http://www.cbs.knaw.nl/fusarium/). Among 108 *Fusarium* isolates submitted for susceptibility testing, the majority (56%) belonged to the *F. solani species complex*, followed by the *F. oxysporum species complex* (19.4%), *F. dimerum species complex* (12.9%), *Giberella fujikuroi species complex* (10.2%), *F. incarnatum-equiseti species complex* (0.9%). No isolates from the *F. chlamydosporum species complex* were observed in this dataset.

The susceptibility profiles of the fusaria are quite variable, although *F. solani* isolates tend to have consistently higher MIC values than those of other species. This variability in MIC, coupled with frequently inaccurate species identification by morphology, means that individual isolate susceptibility testing is recommended.

Figure 2. MIC distributions for all *Fusarium species* against Voriconazole and Amphotericin B



The MIC distributions for all *Fusarium* species against voriconazole and amphotericin B are shown in Figure 2.

Conclusion: For *Aspergillus* and *Scedosporium* species identification provides predictable antifungal susceptibility information. For *Fusarium* the poor discrimination of species and high MICs to both amphotericin B and the triazoles make it essential to have individual isolates thoroughly investigated.

Table 4. Fusarium species

Species Complex (No. isolates tested)	Antifungal agent	No. of isolates at MIC (µg/mL)								
		0.03	0.06	0.12	0.25	0.5	1	2	4	>8
F. solani species complex(61)	AmB					7	9	18	17	10
	ITR				1					24
	VOR						1	10	11	39
	POS				1			1		58
F. oxysporum species complex(21)	AmB			1	1	2	4	5	6	2
	ITR					2				9
	VOR						4	4	4	6
	POS					2	3	4		12
F. dimerum species complex(14)	AmB			1	5	1	1	2	3	1
	ITR						1			7
	VOR					2	3	3	2	4
	POS					1	2	2		9
F. incarnatum-equiseti species complex (1)	AmB									1
	VOR									1
	POS									1
***Giberella fujikuroi species complex* (11)**										
F. proliferatum (9)	AmB									4
	ITR									1
	VOR									6
	POS									6
F. subglutinans (2)	AmB							2		
	VOR								2	
	POS								1	1

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