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Evaluation of a Modified EUCAST Fragmented-Mycelium Inoculum Method for *In Vitro* Susceptibility Testing of Dermatophytes and the Activity of Novel Antifungal Agents

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For antifungal susceptibility testing of nonsporulating or poorly sporulating dermatophytes, a fragmented-mycelium inoculum preparation method was established and compared to broth microdilution testing according to CLSI and EUCAST guidelines. Moreover, the *in vitro* activity of new antifungal agents against dermatophytes was evaluated. Agreement between the mycelial inoculum method and the CLSI broth microdilution method was high (93% to 100%). Echinocandins (minimal effective concentration [MEC], ≤0.5 mg/liter) and posaconazole (MIC, ≤3.00 mg/liter) showed good activity against all tested dermatophytes.

Antifungal susceptibility testing (AST) remains an open research field since the development of the first antifungal agent (AFA). Dermatomycosis and onychomycosis are infectious diseases which are associated with long-term therapy (1) and high relapse rates (2, 3). As treatment responses become evident only weeks or months after initiation of antifungal therapy, the choice of an effective antifungal therapy is essential; antifungal susceptibility testing simplifies decision making.

Several inoculum preparation methods exist, but they are all based on conidia, which are difficult to gain from poorly or slowly sporulating dermatophytes within a reasonable time. To overcome this limitation, fragmented mycelium could be used instead. The usefulness of this approach for susceptibility testing of dermatophytes was evaluated in a previous study (4, 5), but the comparison with the current gold standard of the Clinical and Laboratory Standards Institute (CLSI) (6) remains to be performed. So far, mycelium inoculum was compared for only *Aspergillus* spp. with European Committee on Antifungal Susceptibility Testing (EUCAST) broth microdilution (5).

The aim of this study was 2-fold: (a) to compare fragmented inoculum preparation as a modified EUCAST approach with the standard CLSI (6) and EUCAST (7) methods for antifungal susceptibility testing of dermatophytes and (b) to investigate the *in vitro* activity of new AFAs against common dermatophytes.

The following five strains from the German culture collection (DSMZ, Braunschweig, Germany) were used for quality control (QC) as they deliver stable and reproducible results: *Microsporum canis* ATCC 28327, *Trichophyton mentagrophytes* ATCC 18748 and ATCC 9533, *Trichophyton verrucosum* ATCC 38485, and *Trichophyton rubrum* DSM 4167. For mycelium inoculum preparation, all strains were cultured on Sabouraud (SAB)-2% glucose agar for 2 to 6 days at 30°C, while for conidial suspension preparation, strains had to be grown on average for 21 days (±7 days).

Susceptibility testing was performed according to CLSI (6) or EUCAST (7) guidelines for broth microdilution testing of molds. The fragmented inoculum preparation method was performed as previously published (4, 5). In short, NaCl was dropped on the colony surface and the colony was rubbed with a sterile scalpel to release hyphae for inoculum preparation. Hyphae were homogenized, checked microscopically, and diluted with 0.85% NaCl to a final concentration of 1.2×10^5 to 5×10^5 viable units (VU)/ml (viable units are the hyphal segment framed by two intact septa) using a Neubauer counting chamber.

Inoculum viability concentration was verified by plating 100 μl on SAB-2% glucose agar plates in duplicate and incubating it at 30°C for 72 h. The deviation between counted VU and grown colonies was approximately 5%, which is in line with the conidial inoculum. The ready-to-use microdilution panels based on RPMI 1640 manufactured by Merlin GmbH (Berlin, Germany) consisting of voriconazole (VRC), posaconazole (PSC), fluconazole (FLC), anidulafungin (ANI), caspofungin (CAS), micafungin (MCA), and amphotericin B (AMB) were used as described by Czaika (4) and Schmalreck et al. (5). *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus flavus* (ATCC 204304) were used for quality control of AST (7). The results obtained for amphotericin B and echinocandins were also included in the study for the possible benefit they may provide for isolates refractory to standard treatment choices for dermatophyte infections. Microdilution plates were incubated at 30°C ± 1°C, until optimal growth in the control well was achieved. The MIC and minimal effective concentration (MEC) were determined visually with a magnification mirror and microscope, respectively, after 2 to 9 days and 1 day after the first reading, respectively. All calculations and statistical analyses were performed with $\log_2$ MIC values using SAS software (SAS Institute, North Carolina, USA). Nonparametric parameters (MIC values) were compared using the Mann-Whitney U test and the Wilcoxon signed-rank test. A P value of <0.05 was considered statistically significant.

In general, the fragmented inoculum preparation method was

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TABLE 1 Comparison of conidial versus fragmented-mycelium inoculum for CLSI method using a set of clinical isolates

Clinical strain	AFA[a]	n[b]	Concn range (mg/liter) for method:			
			Conidium[c]		Fragmented-mycelium inoculum prepn method[d]	
			MIC	MEC	MIC	MEC
Trichophyton mentagrophytes	AMP	4	0.25–1.0		1.0	
	TER	4	0.03		0.03–0.06	
	FLC	4	16.0–32.0		16.0–32.0	
	POS	4	0.5–1.0		0.25–2.0	
	VRC	4	0.125–0.5		0.5	
	ITR	4	0.25–2.0		1.0	
	MCA	4		0.016		0.016–16.0
	ANI	4		0.016–0.03		0.016–16.0
	CAS	4		0.25–1.0		0.06–0.5
Trichophyton tonsurans	AMP	3	0.06–0.5		0.25–1.0	
	TER	3	0.03		0.03	
	FLC	3	0.5–32.0		8.0–32.0	
	POS	3	0.5–1.0		0.25–0.5	
	VRC	3	0.06–0.5		0.03–0.25	
	ITR	3	0.125–1.0		0.125–2.0	
	MCA	3		0.016–0.125		0.016–0.03
	ANI	3		0.016–0.03		0.016–0.03
	CAS	3		0.25–2.0		0.06–1.0
Trichophyton rubrum	AMP	7	0.125–0.5		0.5	
	TER	7	0.03		0.03	
	FLC	7	0.125–32.0		0.25–4.0	
	POS	7	0.06–1.0		0.06–0.5	
	VRC	7	0.03–2.0		0.03–0.06	
	ITR	7	0.06–1.0		0.06–1.0	
	MCA	7		0.016		0.016
	ANI	7		0.016		0.016
	CAS	7		0.125–1.0		0.5–1.0
Microsporum canis	AMP	4	0.06–0.25		0.25	
	TER	4	0.06		0.03–0.06	
	FLC	4	4.0–16.0		2.0–32.0	
	POS	4	1.0		0.5	
	VRC	4	0.06–0.125		0.03–0.125	
	ITR	4	0.25–1.0		0.125–1.0	
	MCA	4		0.016		0.016
	ANI	4		0.016		0.016
	CAS	4		0.25–0.5		0.125–0.5

[a] Tested antifungal agents (AFAs): amphotericin B (AMP), terbinafine (TER), fluconazole (FLC), posaconazole (POS), voriconazole (VRC), itraconazole (ITR), micafungin (MCA), anidulafungin (ANI), and caspofungin (CAS).
[b] Number of isolates; each isolate was tested once.
[c] Microdilution testing according to Clinical and Laboratory Standards Institute (CLSI) method (6) using conidia for inoculum preparation.
[d] Microdilution testing according to Clinical and Laboratory Standards Institute (CLSI) method using fragmented mycelium for inoculum preparation.

TABLE 2 Comparison of conidial versus fragmented-mycelium inoculum for EUCAST method using a set of clinical isolates

Clinical strain	AFA[a]	n[b]	Concn range (mg/liter) for method:			
			Conidium[c]		Fragmented-mycelium inoculum prepn method[d]	
			MIC	MEC	MIC	MEC
Trichophyton mentagrophytes	AMP	4	0.5–1.0		0.5–1.0	
	TER	4	0.03–0.06		0.03–0.06	
	FLC	4	16.0–64.0		64.0	
	POS	4	0.25–1.0		0.25–1.0	
	VRC	4	0.125–0.5		0.25–0.5	
	ITR	4	0.25–1.0		0.5–1.0	
	MCA	4		0.03		0.03–0.125
	ANI	4		0.016		0.016–0.25
	CAS	4		0.25		0.06–0.5
Trichophyton tonsurans	AMP	3	0.5–1.0		0.5–1.0	
	TER	3	0.03–0.06		0.03	
	FLC	3	4.0–64.0		64.0	
	POS	3	0.125–16.0		0.06–0.5	
	VRC	3	0.06–16.0		0.06–0.125	
	ITR	3	0.125–8.0		0.5	
	MCA	3		0.03–1.0		0.03
	ANI	3		0.016–1.0		0.016–0.03
	CAS	3		0.125–0.5		0.03–0.25
Trichophyton rubrum	AMP	7	0.5–2.0		0.25–2.0	
	TER	7	0.03–0.06		0.03	
	FLC	7	1.0–64.0		0.06–8.0	
	POS	7	0.5–1.0		0.25–1.0	
	VRC	7	0.03–0.5		0.03–0.06	
	ITR	7	0.25–0.5		0.25–1.0	
	MCA	7		0.03		0.03
	ANI	7		0.016–0.03		0.016–0.03
	CAS	7		0.06–0.5		0.03–0.25
Microsporum canis	AMP	4	0.25–0.5		0.125–0.25	
	TER	4	0.06–0.125		0.03–0.125	
	FLC	4	32.0		16.0–32.0	
	POS	4	0.25–0.5		0.03–1.0	
	VRC	4	0.06–0.125		0.03–0.125	
	ITR	4	0.25–0.5		0.03–1.0	
	MCA	4		0.03		0.03
	ANI	4		0.016–0.03		0.003
	CAS	4		0.06–0.25		0.06–0.25

[a] Tested antifungal agents (AFAs): amphotericin B (AMP), terbinafine (TER), fluconazole (FLC), posaconazole (POS), voriconazole (VRC), itraconazole (ITR), micafungin (MCA), anidulafungin (ANI), and caspofungin (CAS).
[b] Number of isolates; each isolate was tested once.
[c] Microdilution testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) method using conidia for inoculum preparation (7).
[d] Microdilution testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) method using fragmented mycelium for inoculum preparation.

TABLE 3 Statistical comparisons between the conidial (standard) and fragmented-mycelium inoculum using CLSI and EUCAST guidelines[a]

Guideline	AFA[b]	*n*[c]	Concn range (mg/liter) for method:				*P* value
			Conidium		Fragmented mycelium		
			MIC	MEC	MIC	MEC	
CLSI[d]	AMP	18	0.06–1.0		0.25–1.0		0.011
	TER	18	0.03–0.06		0.03–0.06		1.000
	FLC	18	0.125–32.0		0.25–32.0		1.000
	POS	18	0.06–1.0		0.06–2.0		0.929
	VRC	18	0.03–2.0		0.03–0.5		0.404
	ITR	18	0.06–2.0		0.06–2.0		0.674
	MCA	18		0.016–0.125		0.016–16.0	0.472
	ANI	18		0.016–0.03		0.016–16.0	0.461
	CAS	18		0.125–2.0		0.06–1.0	0.660
EUCAST[e]	AMP	18	0.25–2.0		0.125–2.0		0.149
	TER	18	0.03–0.125		0.03–0.125		0.180
	FLC	18	1.0–64.0		0.06–64.0		0.674
	POS	18	0.25–2.0		0.03–1.0		0.440
	VRC	18	0.03–2.0		0.03–0.5		0.141
	ITR	18	0.25–8.0		0.03–1.0		0.306
	MCA	18		0.03–1.0		0.03–0.125	1.000
	ANI	18		0.016–1.0		0.016–0.25	0.483
	CAS	18		0.06–0.5		0.03–0.5	0.905

[a] Tested clinical strains included *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton tonsurans*, and *Microsporum canis*.
[b] Tested antifungal agents (AFAs): amphotericin B (AMP), terbinafine (TER), fluconazole (FLC), posaconazole (POS), voriconazole (VRC), itraconazole (ITR), micafungin (MCA), anidulafungin (ANI), and caspofungin (CAS).
[c] Number of isolates; each isolate was tested once.
[d] Microdilution testing according to Clinical and Laboratory Standards Institute (CLSI) guidelines (6).
[e] Microdilution testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (7).

TABLE 4 Comparison of conidial and fragmented-mycelium inoculum methods for CLSI using a set of quality control strains

Control strain	AFA[a]	n[b]	Concn range (mg/liter) for method:				P value
			CLSI[c]		Fragmented-mycelium inoculum prepn method[d]		
			MIC	MEC	MIC	MEC	
Trichophyton mentagrophytes ATCC 9533	VRC	23	0.032–0.5		0.064–0.5		0.3
	POS	3	0.125–0.5		0.125–0.5		NT[e]
	FLC	23	8.0–125.0		2.0–32.0		0.1
	ANI	3		0.001–0.016		0.004–0.008	NT
	CAS	3		0.008–0.125		0.032–0.064	NT
	MCA	3		0.001–0.016		0.004–0.008	NT
Trichophyton mentagrophytes ATCC 18748	VRC	18	0.032–0.5		0.032–0.25		0.8
	POS	18	0.064–1.0		0.064–0.5		0.3
	FLC	18	16.0–256.0		8.0–64.0		0.2
	ANI	3		0.004–0.016		0.004–0.032	NT
	CAS	3		0.032–0.125		0.064–0.125	NT
	MCA	3		0.004–0.016		0.004–0.025	NT
Trichophyton tonsurans DSM 12285	VRC	12	0.016–0.25		0.064–0.25		0.4
	POS	3	0.25–1.0		0.25–0.25		NT
	FLC	12	16.0–256.0		8.0–64.0		0.3
	ANI	3		0.002–0.008		0.004–0.004	NT
	CAS	3		0.004–0.016		0.008–0.008	NT
	MCA	3		0.002–0.008		0.004–0.004	NT
Trichophyton rubrum DSM 4167	VRC	18	0.064–0.25		0.064–0.25		0.2
	POS	18	0.064–0.25		0.25–0.5		0.1
	FLC	18	4.0–64.0		2.0–64.0		0.3
	ANI	4		0.016–0.064		0.032–0.064	NT
	CAS	4		0.064–0.25		0.125–0.125	NT
	MCA	4		0.016–0.064		0.032–0.064	NT
Microsporum canis ATCC 28327	VRC	9	0.032–0.5		0.125–0.5		NT
	POS	3	0.25–1.0		0.5–0.5		NT
	FLC	9	8.0–128.0		8.0–32.0		NT
	ANI	3		0.064–0.125		0.008–0.008	NT
	CAS	3		0.008–0.032		0.016–0.016	NT
	MCA	3		0.004–0.016		0.008–0.008	NT

[a] Tested antifungal agents (AFAs): voriconazole (VRC), posaconazole (POS), fluconazole (FLC), anidulafungin (ANI), caspofungin (CAS), and micafungin (MCA).
[b] Number of replicates tested with the same quality control strain.
[c] Microdilution testing according to Clinical and Laboratory Standards Institute (CLSI) guidelines (6).
[d] Microdilution testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (7) using fragmented mycelium for inoculum preparation.
[e] NT, insufficient sample size for statistical analysis.

comparable to CLSI (6) and EUCAST (7) methods ($P > 0.05$) (Tables 1 to 3).

For the QC strains, no significant difference between the CLSI method (Table 3) and modified EUCAST method (=fragmented-mycelium inoculum) was observed. The best results were gained for *T. mentagrophytes* ($P = 0.8$) (Table 4). The agreement between the modified EUCAST method and CLSI method (6) was 88.9 to 100% (Table 5). Eighty clinical isolates and seven AFAs (Table 6) were tested with the modified EUCAST method, finding echinocandins *in vitro* as the most effective agents against dermatophytes (MEC at which 90% of the isolates tested are inhibited [MEC_{90}], ≤0.5 mg/liter). Echinocandins (MEC_{90}, ≤0.031 mg/liter) and AMB (MIC at which 90% of the isolates tested are inhibited [MIC_{90}], ≤0.4 mg/liter) were the only compounds active against *M. canis*, while azoles had only limited activity. All tested *Trichophyton* spp. were susceptible against a wide range of AFAs, including AMB (MIC_{90} of ≤0.5 mg/liter), VRC, and PSC.

The major finding of this study was that the modified EUCAST method is highly comparable to the well-established EUCAST (7) and CLSI (6) methods. Moreover, susceptibility testing of poorly sporulating dermatophytes was faster and could be conducted even with sterile dermatophytes. The limitation of the modified EUCAST method was a delayed growth as a consequence of slower proliferation of hyphae than conidia. Our *in vitro* data suggest that AMB, PSC, and all echinocandins are AFAs that merit further evaluation in respect to their activity against dermatophytes. The activity of echinocandins against dermatophytes was previously reported (1, 2). Only *M. canis* (ATCC 28327) showed a high degree of variability between modified EUCAST and CLSI methods (6) for anidulafungin (−4 dilution steps compared to CLSI method). The modified EUCAST method was compared with the conidium method and already applied for susceptibility testing of *Penicillium notatum* and *Penicillium chrysogenum* (8). The MICs and MECs gen-

TABLE 5 MICs of quality control strains and cumulative percentages of agreement within $\log_2$ dilutions for the standard CLSI and modified EUCAST fragmented-mycelium inoculum methods

Organism	AFA[a]	n[b]	% of isolates with MIC and MEC difference:							Agreement[c] (%)	
			>+2	+2	+1	0	−1	−2	>−2	±1	±2
T. mentagrophytes ATCC 9533	VRC	23		8.7	13.0	78.3				91.3	100.0
	POS	3			33.3	66.6				100.0	100.0
	FLC	23		13.0	21.7	26.1	17.4	17.4	4.3	65.2	95.7
	ANI	3		33.3			66.6			66.6	100.0
	CAS	3		33.3			66.6			66.6	100.0
	MCA	3		33.3		33.3	33.3			66.6	100.0
T. mentagrophytes ATCC 18748	VRC	18			5.6	66.7	27.8			100.0	100.0
	POS	18		5.6	11.1	55.6	27.8			94.4	100.0
	FLC	18				77.8	11.1		11.1	88.9	88.9
	ANI	3		33.3		66.6				66.6	100.0
	CAS	3			33.3	66.6				100.0	100.0
	MCA	3			33.3	33.3		33.3		66.6	100.0
Trichophyton tonsurans DSM 12285	VRC	12		8.3	16.7	66.7	8.3			91.7	100.0
	POS	3				33.3	33.3	33.3		66.7	100.0
	FLC	12		8.3			41.7	50.0		50.0	100.0
	ANI	3			33.3		66.6			100.0	100.0
	CAS	3			66.6		33.3			100.0	100.0
	MCA	3			33.3		66.6			100.0	100.0
Microsporum canis ATCC 28327	VRC	9		66.7	11.1	22.2				33.3	100.0
	POS	3			33.3	33.3	33.3			100.0	100.0
	FLC	9				11.1		88.9		11.1	100.0
	ANI	3							100.0	0.0	0.0
	CAS	3			33.3		66.6			100.0	100.0
	MCA	3			33.3		66.6			100.0	100.0
T. rubrum DSM 4167	VRC	18			27.8	72.2				100.0	100.0
	POS	18	5.6	61.1	27.8	5.6				33.3	94.4
	FLC	18			5.6	94.4				100.0	100.0
	ANI	4			25.0	75.0				100.0	100.0
	CAS	4			25.0	25.0	50.0			100.0	100.0
	MCA	4			25.0	75.0				100.0	100.0

[a] Tested antifungal agents (AFAs): voriconazole (VRC), posaconazole (POS), fluconazole (FLC), anidulafungin (ANI), caspofungin (CAS), and micafungin (MCA).

[b] Number of replicates tested with the same quality control strain.

[c] Cumulative percentage of agreement between the results is defined as the proportion of CLSI (6) MIC results that were within ±1 $\log_2$ and ±2 $\log_2$ dilutions of the modified EUCAST fragmented-mycelium inoculum method MIC results.

TABLE 6 Antifungal activities of antifungal agents against clinical isolates of *Microsporum canis*, *T. mentagrophytes*, *T. rubrum*, and *Trichophyton tonsurans*[c]

Organism ($n = 20$[b])	AFA[a]	Concn range (mg/liter)		Characteristic MIC or MEC value (mg/liter)			
		MIC	MEC	MIC_{50}	MEC_{50}	MIC_{90}	MEC_{90}
Microsporum canis	VRC	0.06–32.00		0.25		32.00	
	PSC	0.13–3.00		1.00		2.00	
	FLC	2.00–256.00		32.00		256.00	
	AMB	0.06–0.50		0.25		0.40	
	ANI		0.00–0.03		0.01		0.03
	CAS		0.00–0.02		0.02		0.02
	MCA		0.00–0.03		0.01		0.03
Trichophyton mentagrophytes	VRC	0.01–1.00		0.06		0.25	
	PSC	0.02–1.00		0.06		0.25	
	FLC	2.00–128.00		16.00		64.00	
	AMB	0.13–1.00		0.25		0.50	
	ANI		0.00–0.06		0.01		0.02
	CAS		0.00–0.25		0.03		0.06
	MCA		0.00–1.00		0.13		0.50
Trichophyton rubrum	VRC	0.01–32.00		0.06		0.13	
	PSC	0.02–0.50		0.05		0.38	
	FLC	1.00–256.00		16.00		32.00	
	AMB	0.13–0.25		0.13		0.25	
	ANI		0.00–0.06		0.01		0.06
	CAS		0.03–0.13		0.06		0.13
	MCA		0.00–0.06		0.01		0.50
Trichophyton tonsurans	VRC	0.02–1.00		0.25		0.55	
	PSC	0.03–0.25		0.06		0.25	
	FLC	2.00–64.00		24.00		64.00	
	AMB	0.13–0.25		0.13		0.25	
	ANI		0.00–0.03		0.02		0.06
	CAS		0.01–0.02		0.02		0.06
	MCA		0.00–0.03		0.00		0.06

[a] Tested antifungal agents (AFAs): voriconazole (VRC), posaconazole (PSC), fluconazole (FLC), amphotericin B (AMB), anidulafungin (ANI), caspofungin (CAS), and micafungin (MCA).
[b] Number of replicates tested with the same quality control strain.
[c] Activity was evaluated using the fragmented-mycelium inoculum preparation (modified EUCAST) method.

erated with the modified EUCAST method and classical EUCAST method (7) were in line with previous studies by Bezjak (9). Even though MICs and MECs generated with the modified EUCAST method tended to be lower (due to the lower growth rate) than those with conidial inoculum, overall agreement was sufficient to make the mycelium preparation method a potential alternative for AST of dermatophytes.

The suitability of mycelium inoculum was previously found sufficient for broth micro-and macrodilution and for agar-based (e.g., disc, tablet, or strip test) susceptibility assays of filamentous fungi (5) and dermatophytes (4).

In conclusion, fragmented-mycelium inoculum preparation is a reliable modification of the EUCAST broth microdilution method and enables AST within a reasonable time. The role of echinocandins as possible effective AFAs against dermatophytes deserves further investigation.

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