Comparing Etest and Broth Microdilution for Antifungal Susceptibility Testing of the Most-Relevant Pathogenic Molds

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Invasive mold infections are life-threatening diseases for which appropriate antifungal therapy is crucial. Their epidemiology is evolving, with the emergence of triazole-resistant *Aspergillus* spp. and multidrug-resistant non-*Aspergillus* molds. Despite the lack of interpretive criteria, antifungal susceptibility testing of molds may be useful in guiding antifungal therapy. The standard broth microdilution method (BMD) is demanding and requires expertise. We assessed the performance of a commercialized gradient diffusion method (Etest method) as an alternative to BMD. The MICs or minimal effective concentrations (MECs) of amphotericin B, voriconazole, posaconazole, caspofungin, and micafungin were assessed for 290 clinical isolates of the most representative pathogenic molds (154 *Aspergillus* and 136 non-*Aspergillus* isolates) with the BMD and Etest methods. Essential agreements (EAs) within ± 2 dilutions of $\geq 90\%$ between the two methods were considered acceptable. EAs for amphotericin B and voriconazole were $> 90\%$ for most potentially susceptible species. For posaconazole, the correlation was acceptable for *Mucoromycotina* but Etest MIC values were consistently lower for *Aspergillus* spp. (EAs of $< 90\%$). Excellent EAs were found for echinocandins with highly susceptible (MECs of $< 0.015 \mu\text{g/ml}$) or intrinsically resistant (MECs of $> 16 \mu\text{g/ml}$) strains. However, MEC determinations lacked consistency between methods for strains exhibiting mid-range MECs for echinocandins. We concluded that the Etest method is an appropriate alternative to BMD for antifungal susceptibility testing of molds under specific circumstances, including testing with amphotericin B or triazoles for non-*Aspergillus* molds (*Mucoromycotina* and *Fusarium* spp.). Additional study of molecularly characterized triazole-resistant *Aspergillus* isolates is required to confirm the ability of the Etest method to detect voriconazole and posaconazole resistance among *Aspergillus* spp.

Filamentous fungi account for about 50% to 75% of invasive fungal infections (IFIs) in severely immunocompromised patients, such as bone marrow and solid organ transplant recipients, and are associated with high morbidity and mortality rates (1, 2). Identification of the fungal species and targeted antifungal therapy are key for successful management of these infections. Invasive aspergillosis (IA) is the most common invasive mold infection, and voriconazole, considered to be first-line therapy for IA, has historically exhibited excellent *in vitro* activity against the most common *Aspergillus* species that cause invasive disease (3). However, triazole resistance among *Aspergillus* species is an emerging worldwide problem (4). Furthermore, non-*Aspergillus* molds, for which antifungal agents often have variable and unpredictable *in vitro* activities, account for increasing proportions of IFIs (1, 2, 5). Thus, clinicians are increasingly seeking guidance from clinical laboratories regarding antifungal drug activities against clinically significant mold isolates (6, 7).

The Clinical and Laboratory Standards Institute (CLSI) has developed a reference broth microdilution (BMD) method for antifungal susceptibility testing of filamentous fungi (8). Unfortunately, the CLSI BMD method is labor intensive (8). Commercialized Etest strips (bioMérieux, Durham, NC) containing defined gradients of antifungal drugs represent a more convenient approach for antifungal susceptibility testing. Various studies have compared the Etest and CLSI BMD methods for testing molds, but they were restricted to selected antifungal agents or a limited subset of mold species (9–21). In particular, there is a paucity of data regarding Etest performance for non-*Aspergillus* molds, especially for the newer antifungal agents. Furthermore, studies have suggested that additional validation is required be-

fore the Etest method can be recommended for testing of molds. In this study, we assessed the agreement between Etest and CLSI BMD testing methods for commonly used antifungal drugs against a collection of 290 clinical mold isolates, including 154 *Aspergillus* and 136 non-*Aspergillus* mold isolates.

MATERIALS AND METHODS

Specimens. All isolates originated from clinical (respiratory or deep tissue) samples collected between 2009 and 2013 and stored at Duke University Hospital (Durham, NC). A total of 290 isolates, representing the most-relevant pathogenic *Aspergillus* and non-*Aspergillus* molds, were selected, as follows: *Aspergillus fumigatus*, 74 isolates; *Aspergillus flavus*, 29 isolates; *Aspergillus terreus*, 25 isolates; *Aspergillus niger*, 12 isolates; *Aspergillus ustus*, 8 isolates; *Aspergillus versicolor*, 6 isolates; *Rhizopus* spp., 18 isolates; *Mucor* spp., 9 isolates; *Lichtheimia* (formerly *Absidia*) spp., 4 isolates; *Cunninghamella* spp., 4 isolates; *Fusarium* spp., 34 isolates; *Pur-*

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pureocillium lilacinum, 30 isolates; *Paecilomyces variotii*, 4 isolates; *Scedosporium apiospermum*/*Pseudallescheria boydii*, 20 isolates; *Scedosporium prolificans*, 5 isolates; *Scopulariopsis* spp., 8 isolates. Identification was performed according to standard methods based on macroscopic and microscopic morphological examinations. For 141 isolates (48%), identification of the fungal species was confirmed by PCR sequencing of the internal transcribed spacer (ITS) region (ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT). Two reference strains (*A. fumigatus* ATCC MYA-3626 and *P. variotii* ATCC MYA-3630) were used for quality control. All isolates were grown on potato dextrose agar for 2 to 7 days. Conidia were harvested and suspended in sterile 0.85% saline solution.

Antifungal drugs. Standard powders of amphotericin B, voriconazole, posaconazole, and caspofungin (Sigma-Aldrich, St. Louis, MO) were solubilized in dimethyl sulfoxide (DMSO) or water, according to the manufacturer's recommendations, and stored at -20°C . Plates (96 well) containing gradient concentrations (2-fold dilutions) of each drug in 0.1 ml RPMI 1640 medium (with glutamine, without bicarbonate sodium, and with phenol red as a pH indicator; Sigma-Aldrich, St. Louis, MO) were prepared and stored at -80°C until use. Customized 96-well plates with gradient concentrations of micafungin were provided by the manufacturer (Thermo Fisher Scientific, Waltham, MA). The range of concentrations tested for the BMD method was 0.015 to 16 $\mu\text{g}/\text{ml}$ for all drugs. Etest gradient strips with amphotericin B, voriconazole, posaconazole, caspofungin, and micafungin (range, 0.004 to 32 $\mu\text{g}/\text{ml}$) were obtained from the manufacturer (bioMérieux, Durham, NC).

Antifungal susceptibility testing. Conidial suspensions were adjusted to an optical density corresponding to approximately 10^6 CFU/ml (8). For the Etest method, this suspension was used to create a confluent lawn of mold via direct inoculation with a swab onto the surface of RPMI 1640 agar supplemented with MOPS (morpholinepropanesulfonic acid) and 2% glucose (Remel, Lenexa, KS). For BMD testing, a further 1:50 dilution of the stock solution was made in RPMI 1640 medium to a final concentration of 0.4×10^4 to 5×10^4 CFU/ml, as confirmed by the colony counts after inoculation on Sabouraud dextrose agar control plates (8). A volume of 0.1 ml of this final solution was inoculated in each well of a 96-well plate, containing 0.1 ml of the drug solution (at 2 times the final desired concentration), with an automated inoculation system (Sensititre AIM; Thermo Fisher Scientific, Waltham, MA). Plates were incubated at 35°C .

Interpretation of results. For most fungal species, final readings were performed for both BMD and Etest methods at 24 h for echinocandins (caspofungin and micafungin) and at 48 h for other drugs. For *Mucoromycotina*, readings were performed at 24 h for all drugs. For *Scedosporium* spp., readings were performed at 48 h for echinocandins and at 72 h for other drugs. For strains exhibiting slow or insufficient growth, additional time was allowed until sufficient growth (i.e., confluent growth filling the entire well) was achieved in the control well. For BMD, examinations were performed with the aid of an inverted reading mirror.

The MIC, defined as the lowest concentration preventing any discernible growth, was assessed for amphotericin B and triazoles (voriconazole and posaconazole). The minimal effective concentration (MEC), defined as the lowest concentration achieving small rounded compact hyphal forms, was assessed for echinocandins (caspofungin and micafungin) (8). Etest MICs were recorded as the lowest concentrations of antifungal drug for which the elliptical zone of growth inhibition intersected the Etest strip. For the fungistatic echinocandin drugs, MECs were read at the intersection between the lawn of confluent colonies and the presence of distinct microcolonies.

For comparison with MIC/MEC results obtained with the BMD method, values obtained with the Etest method were adjusted to the next upper concentration matching the 2-fold dilution scheme of the BMD method. As recommended previously, essential agreement (EA) between Etest and BMD results was considered when the MIC/MEC values obtained with the two methods fell within ± 2 dilutions of the 2-fold dilution

scheme (e.g., 0.5, 1, and 2 $\mu\text{g}/\text{ml}$) (11, 14). EA values of $\geq 90\%$ were considered acceptable.

RESULTS

MIC₅₀/MEC₅₀ and MIC₉₀/MEC₉₀ values (i.e., concentrations inhibiting $\geq 50\%$ and $\geq 90\%$ of isolates, respectively) and ranges for the 290 isolates are presented in Table 1. The BMD and Etest methods generated similar MIC₅₀/MEC₅₀ and MIC₉₀/MEC₉₀ values (within ± 2 dilutions) for all species and drugs tested except for echinocandins (caspofungin and/or micafungin) and the following species: *A. ustus* (caspofungin), *A. versicolor* (micafungin), *Scedosporium apiospermum* (caspofungin and micafungin), and *Scopulariopsis* spp. (caspofungin and micafungin). Etest MIC values for amphotericin B were also higher than BMD values for *A. terreus*.

The EA between BMD and Etest results is presented for each species-drug combination in Table 2. EA values were $\geq 90\%$ for the majority of species-drug combinations; however, there were some notable exceptions. EA values were $< 90\%$ for *A. terreus*, *S. apiospermum*, and *Scopulariopsis* spp. for amphotericin B, and Etest MIC values were consistently higher than BMD values. Ninety percent agreement also was not reached for most *Aspergillus* spp. (*A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*) for posaconazole, for which Etest results were lower than BMD results. Although voriconazole MIC values for *Aspergillus* spp. tended to be lower by the Etest method, the differences remained in the acceptable range of ± 2 dilutions, resulting in EA values of $> 90\%$. Of note, one *A. fumigatus* isolate that exhibited particularly high MIC values for voriconazole (i.e., ≥ 4 $\mu\text{g}/\text{ml}$, corresponding to possible resistance) demonstrated similarly high MICs with the two methods. For most *Aspergillus* and non-*Aspergillus* molds, EA values for echinocandins were excellent. Exceptions included *A. ustus* (caspofungin and micafungin), *A. versicolor* (micafungin), *S. apiospermum* (caspofungin and micafungin), *Scopulariopsis* spp. (caspofungin and micafungin), and *P. variotii* (caspofungin).

DISCUSSION

To our knowledge, this study represents one of the largest comparisons of the CLSI standard BMD and Etest methods for MIC/MEC determinations among clinically relevant molds. Our results indicated different degrees of agreement between the two methods, depending on the drug and mold tested. Most molds showed overall good essential agreement for amphotericin B. The exceptions were *A. terreus*, *Scopulariopsis* spp., and *Scedosporium apiospermum*, for which the Etest method demonstrated higher MICs, perhaps more accurately reflecting the intrinsic resistance of these molds to amphotericin B (22–24). For non-*Aspergillus* spp. for which amphotericin B has historically shown variable *in vitro* activities and for which guidance in drug selection is needed (*Mucoromycotina*, *Fusarium* spp., and *P. variotii*), EAs between the two methods were $> 90\%$. Based on these observations, we consider the Etest method to be a suitable method for testing the *in vitro* activity of amphotericin B for *Aspergillus* and non-*Aspergillus* molds (Table 3).

Triazole MIC values for *Aspergillus* spp. tended to be lower with the Etest method versus the BMD method. While the values for EA within ± 2 dilutions remained acceptable ($> 90\%$) for voriconazole, the EA values for posaconazole were lower, ranging between 64% and 89% for the four *Aspergillus* spp. (*A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*) that most commonly cause inva-

TABLE 1 CLSI broth microdilution versus Etest *in vitro* susceptibility results for clinical mold isolates

Species	Method	MIC ₅₀ /MIC ₉₀ (range) ^a (μg/ml) ^a			MEC ₅₀ /MEC ₉₀ (range) (μg/ml)	
		AMB	VCZ	POS	CSP	MCF
<i>Aspergillus fumigatus</i> (n = 74)	BMD	2/4 (1–4)	0.5/2 (0.25–8)	0.5/1 (0.25–2)	<0.015/0.06 (<0.015–0.125)	<0.015/<0.015 (<0.015–0.03)
	Etest	4/4 (1–16)	0.25/0.5 (0.125 to >16)	0.25/0.25 (0.06–1)	<0.015/0.06 (<0.015–0.125)	<0.015/<0.015 (<0.015–0.03)
<i>Aspergillus flavus</i> (n = 29)	BMD	2/2 (2–4)	0.5/1 (0.5–1)	0.5/1 (0.25–2)	<0.015/<0.015 (<0.015)	<0.015/<0.015 (<0.015)
	Etest	4/8 (0.5 to >16)	0.5/0.5 (0.125–1)	0.25/0.25 (0.06–0.5)	<0.015/<0.015 (<0.015)	<0.015/<0.015 (<0.015)
<i>Aspergillus terreus</i> (n = 25)	BMD	4 ^b /4 ^b (2–8)	1/1 (0.25–1)	0.5/1 (0.25–1)	<0.015/0.03 (<0.015–0.03)	<0.015/<0.015 (<0.015–0.03)
	Etest	>16 ^b />16 ^b (4 to >16)	0.5/1 (0.25–2)	0.125/0.25 (0.06–0.25)	<0.015/0.06 (<0.015–0.125)	<0.015/<0.015 (<0.015)
<i>Aspergillus niger</i> (n = 12)	BMD	0.5/1 (0.5–1)	1/2 (0.5–2)	1/2 (1–2)	<0.015/<0.015 (<0.015)	<0.015/<0.015 (<0.015)
	Etest	1/2 (0.5–2)	0.5/0.5 (0.125–1)	0.5/0.5 (0.125–0.5)	<0.015/<0.015 (<0.015)	<0.015/<0.015 (<0.015)
<i>Aspergillus ustus</i> (n = 8)	BMD	2/4 (2–4)	8/16 (4–16)	>16/>16 (8 to >16)	2 ^b /4 ^b (0.03–4)	0.06/0.125 (0.03–0.25)
	Etest	2/2 (0.5–4)	8/>16 (2 to >16)	>16/>16 (>16)	0.03 ^b />16 ^b (<0.015 to >16)	0.03/0.06 (<0.015–0.06)
<i>Aspergillus versicolor</i> (n = 6)	BMD	2/4 (2–4)	1/2 (0.5–2)	1/2 (1–2)	<0.015/0.03 (<0.015–0.03)	0.06 ^b /0.06 (<0.015–0.06)
	Etest	4/4 (2–4)	0.5/1 (0.25–1)	0.5/1 (0.5–1)	<0.015/>16 (<0.015 to >16)	<0.015 ^b /0.03 (<0.015–0.03)
<i>Mucoromycotina</i> (n = 35) ^c	BMD	1/4 (0.125–4)	>16/>16 (8 to >16)	>16/>16 (0.5 to >16)	>16/>16 (>16)	>16/>16 (>16)
	Etest	1/4 (0.125 to >16)	>16/>16 (8 to >16)	>16/>16 (0.5 to >16)	>16/>16 (>16)	>16/>16 (>16)
<i>Fusarium</i> spp. (n = 34)	BMD	2/4 (1 to >16)	>16/>16 (2 to >16)	>16/>16 (2 to >16)	>16/>16 (>16)	>16/>16 (>16)
	Etest	2/16 (1 to >16)	>16/>16 (2 to >16)	>16/>16 (2 to >16)	>16/>16 (>16)	>16/>16 (>16)
<i>Scedosporium apiospermum</i> (n = 20)	BMD	8/>16 (4 to >16)	1/2 (0.5–4)	>16/>16 (2 to >16)	0.5/1 ^b (0.06 to >16)	1/2 ^b (0.25 to >16)
	Etest	>16/>16 (4 to >16)	0.25/1 (0.125 to >16)	>16/>16 (1 to >16)	1/>16 ^b (0.125 to >16)	0.25/>16 ^b (0.06 to >16)
<i>Scedosporium prolificans</i> (n = 5)	BMD	>16/>16 (8 to >16)	>16/>16 (>16)	>16/>16 (>16)	16/16 (16)	>16/>16 (>16)
	Etest	>16/>16 (16 to >16)	>16/>16 (>16)	>16/>16 (>16)	>16/>16 (>16)	>16/>16 (>16)
<i>Purpureocillium lilacinum</i> (n = 30)	BMD	>16/>16 (16 to >16)	0.25/0.5 (0.125–0.5)	1/1 (0.25–2)	>16/>16 (0.25 to >16)	>16/>16 (0.06 to >16)
	Etest	>16/>16 (>16)	0.25/0.25 (0.06–0.5)	0.5/1 (0.125–2)	>16/>16 (0.25 to >16)	>16/>16 (0.125 to >16)
<i>Paeclomyces variotii</i> (n = 4)	BMD	0.25/2 (0.125–2)	>16/>16 (16 to >16)	0.5/>16 (0.25 to >16)	0.03/2 (0.03–2)	<0.015/0.03 (<0.015–0.03)
	Etest	0.25/4 (0.06–4)	>16/>16 (>16)	0.125/>16 (0.06 to >16)	0.03/>16 (0.015 to >16)	<0.015/0.03 (<0.015–0.03)
<i>Scopulariopsis</i> spp. (n = 8)	BMD	8/>16 (2 to >16)	>16/>16 (16 to >16)	>16/>16 (>16)	0.5 ^b /8 (0.25–16)	1 ^b />16 (<0.015 to >16)
	Etest	>16/>16 (2 to >16)	>16/>16 (>16)	>16/>16 (>16)	>16 ^b />16 (0.5 to >16)	>16 ^b />16 (0.125 to >16)

^a MIC₅₀/MIC₉₀ values (MICs inhibiting ≥50% and ≥90% of strains, respectively) are presented for amphotericin B (AMB), voriconazole (VCZ), and posaconazole (POS). MEC₅₀/MEC₉₀ values (minimal effective concentrations for ≥50% and ≥90% of strains, respectively) are presented for caspofungin (CSP) and micafungin (MCF). MIC/MEC values obtained with the Etest method were adjusted to the next upper value matching the 2-fold broth dilution scheme of the BMD method.

^b Difference of >2 dilutions in MIC/MEC values obtained with the Etest and BMD methods.

^c *Mucoromycotina* (*Rhizopus* spp., *Mucor* spp., *Lichtheimia* spp., and *Cunninghamella* spp.) were grouped together because of their small individual numbers and similarities in susceptibility profiles.

TABLE 2 Essential agreement between CLSI broth microdilution and Etest results

Species	Essential agreement (%) ^a				
	AMB	VCZ	POS	CSP	MCF
<i>A. fumigatus</i> (n = 74)	99	95	89 ^b	96	100
<i>A. flavus</i> (n = 29)	97	100	83 ^b	100	100
<i>A. terreus</i> (n = 25)	16 ^b	100	64 ^b	96	100
<i>A. niger</i> (n = 12)	100	100	83 ^b	100	100
<i>A. ustus</i> (n = 8)	100	100	100	14 ^b	57 ^b
<i>A. versicolor</i> (n = 6)	100	100	100	80 ^b	100
<i>Mucoromycotina</i> (n = 35)	91	100	94	100	100
<i>Fusarium</i> spp. (n = 34)	94	100	100	100	100
<i>S. apiospermum</i> (n = 20)	80 ^b	90	90	63 ^b	37 ^b
<i>S. prolificans</i> (n = 5)	100	100	100	100	100
<i>P. lilacinum</i> (n = 30)	100	100	97	100	100
<i>P. variotii</i> (n = 4)	100	100	100	50 ^b	100
<i>Scopulariopsis</i> spp. (n = 8)	75 ^b	100	100	43 ^b	29 ^b

^a Essential agreement was within ± 2 dilutions. AMB, amphotericin B; VCZ, voriconazole; POS, posaconazole; CSP, caspofungin; MCF, micafungin.

^b Essential agreement of <90% for MIC/MEC values obtained with the Etest and BMD methods.

sive disease. Thus, our data suggest caution regarding the use of the Etest method for antifungal susceptibility testing of posaconazole among *Aspergillus* spp. A similar low level of agreement (80 to 90%) between these two methods for posaconazole was reported previously (9, 10, 18). Whether the lower MICs determined with the Etest method might have effects on interpretation and clinical decision-making is unclear in the absence of defined clinical breakpoints. In our study, most *Aspergillus* spp. (with the exception of *A. ustus*) exhibited relatively low MIC values (ranging from 0.125 and 1 $\mu\text{g/ml}$) for triazoles. Only one *A. fumigatus* strain exhibited a high MIC ($\geq 4 \mu\text{g/ml}$ for voriconazole), with consistent results being obtained with the two methods, which suggests that the Etest method is able to detect triazole-resistant isolates. Obviously, because of the small number of triazole-resistant *Aspergillus* isolates in this study and the absence of molecular characterization of the isolates, the ability of the Etest method to detect triazole-resistant isolates should be further investigated.

Agreement between BMD and Etest results for the triazoles was much better for non-*Aspergillus* spp. In particular, agreement was good for *Mucoromycotina* and posaconazole, an important finding given the variable MICs for posaconazole for the different molds in this group. Thus, the Etest method appears to be comparable to the BMD method for *in vitro* testing of voriconazole among *Aspergillus* and non-*Aspergillus* molds and for assessment of posaconazole activity among *Mucoromycotina* and other non-*Aspergillus* molds (Table 3).

For most *Aspergillus* species except *A. ustus*, MEC values for caspofungin and micafungin were consistently low (<0.015 $\mu\text{g/ml}$) and EA between BMD and Etest results was excellent. For the non-*Aspergillus* molds, MEC values tended to be consistently high (>16 $\mu\text{g/ml}$) and, as with *Aspergillus* spp., excellent EA was demonstrated. Discrepancies were observed among fungal species for which the echinocandins tended to show more-variable *in vitro* activity, such as *A. ustus*, *S. apiospermum*, *P. variotii*, and *Scopulariopsis* spp. Because the testing scale is abbreviated at the upper and lower ends, we were perhaps not able to discriminate true differences between the testing meth-

TABLE 3 Proposed recommendations for use of the Etest method for antifungal susceptibility testing of molds

Drug	Recommendation	
	<i>Aspergillus</i> spp.	Non- <i>Aspergillus</i> spp. ^a
Amphotericin B	Yes; exception: <i>A. terreus</i>	Yes; exceptions: <i>S. apiospermum</i> and <i>Scopulariopsis</i> spp.
Voriconazole	Yes; caveat: ability to detect azole-resistant isolates not yet proven	Yes
Posaconazole	No (or use with extreme caution); lower MICs with Etest vs BMD	Yes
Caspofungin/micafungin	Yes; exception: <i>A. ustus</i> (poor agreement with BMD for this species, which demonstrated MICs across the testing range)	Yes; exceptions: <i>S. apiospermum</i> , <i>P. variotii</i> , and <i>Scopulariopsis</i> spp. (poor agreement with BMD for these species, which demonstrated MICs across the testing range)

^a *Mucoromycotina*, *Fusarium* spp., *S. apiospermum*, *S. prolificans*, *P. lilacinum*, *P. variotii*, and *Scopulariopsis* spp.

ods for isolates with MECs at either end of the testing range. Thus, false essential agreement values may be at play in such cases. Indeed, a previous analysis showed poor correlation between BMD and Etest results in the lower ranges of MEC values for *Aspergillus* spp. (15). However, it is doubtful that lack of agreement at the extremes of the testing range would negatively affect clinical decision-making. The low EA values among *Aspergillus* and non-*Aspergillus* molds demonstrating variable activity (with MECs in the middle of the testing range) are concerning, because such conditions represent scenarios in which clinicians are most likely to need guidance in drug selection. These observations suggest that the Etest method should not be used for antifungal susceptibility testing of certain molds with the echinocandins, particularly *A. ustus*, *S. apiospermum*, *P. variotii*, and *Scopulariopsis* spp., owing to poor correlation with BMD test results (Table 3). Moreover, interpretation of MEC cutoff values for both BMD and Etest results is difficult and may vary depending on the observer and the time of the reading. Correlations between *in vitro* MEC values from both BMD and Etest methods and *in vivo* efficacy data should be investigated further.

Some limitations of this study should be noted. Due to the large number of molds tested, sequencing for identification could be performed for only about one-half of the isolates. However, morphological examinations were performed by highly experienced mycologists, as indicated by the 97% agreement with the genus and species determined by sequencing for the isolates that were molecularly characterized (see Table S1 in the supplemental material). Furthermore, molecular identification by conventional sequencing methods was not able to discriminate species within the *Aspergillus* section *Fumigati*, which are known to exhibit variable azole susceptibility (25). Molecular characterization of azole resistance also was not performed for the single *A. fumigatus* isolate with high MICs determined by both methods. With these limitations acknowledged, our approach corresponds to standard practices used in most clinical microbiology laboratories, and the

study was designed to assess the utility of the Etest method in such settings.

In summary, the analysis of this large collection of clinical mold isolates suggests that the Etest method may be an appropriate alternative to BMD testing of molds in specific circumstances. The Etest method may be used for amphotericin B susceptibility testing of *Aspergillus* and non-*Aspergillus* molds, particularly for molds with unpredictable *in vitro* amphotericin B sensitivity (i.e., *Mucoromycotina*, *Fusarium* spp., and *P. variotii*), for which guidance regarding drug selection is needed. The Etest method may be used to test voriconazole activity against *Aspergillus* and non-*Aspergillus* molds and to assess the *in vitro* activity of posaconazole against *Mucoromycotina* and other non-*Aspergillus* molds. However, the validity of using the Etest method for antifungal susceptibility testing of posaconazole against *Aspergillus* spp. is not established, due to the consistently lower MIC values obtained with the Etest method versus the BMD method in this study. Further, additional study of molecularly characterized triazole-resistant *Aspergillus* isolates is required to confirm the ability of the Etest method to detect voriconazole and posaconazole resistance among *Aspergillus* spp. Owing to poor correlation with BMD test results, the Etest method should not be used for antifungal susceptibility testing of *A. ustus*, *S. apiospermum*, *P. variotii*, and *Scopulariopsis* spp. with the echinocandins.

In general, caution is warranted regarding *in vitro* susceptibility testing of molds with drugs whose activity is predictable, in particular, molds for which intrinsic resistance is known to exist for a given drug or mold isolates for which there is no prior antifungal exposure and thus little concern regarding acquired resistance. Finally, there is an urgent need for analyses correlating MIC/MEC values with clinical outcomes for all drugs and the most clinically relevant molds, in order to guide the development of interpretive clinical breakpoints.

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