Original Article

Antifungal Susceptibility of the Aspergillus Species by Etest and CLSI Reference Methods

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Abstract

Background: Because resistance to antifungal drugs is seen in patients, susceptibility testing of these drugs aids in choosing the appropriate drug and respective epidemiology. This study has investigated and compared susceptibility patterns of the *Aspergillus* species isolated from patients by the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution (MD) assay and Etest method.

Methods: The minimum inhibitory concentrations (MICs) of various antifungal agents (amphotericin B, ketoconazole, itraconazole, and voriconazole) for 108 *Aspergillus* species isolated from patients were determined by CLSI M38-A broth MD and Etest. The isolates were obtained from clinical samples that included tissues, sputum, bronchoalveolar lavage, abdominal tap, and cerebrospinal fluid.

Results: As revealed by the MD method, 63.9% of the isolates were sensitive to amphotericin B and 36.1% were resistant.Etest revealed that 61.1% were sensitive to amphotericin B and 38.9% were resistant. As for ketoconazole, 108 isolates (100%) were shown to be sensitive through the MD method; while the Etest revealedan 88.9% sensitivity and 11.1% were resistant. All species were susceptible to voriconazole, according to both methods. The measure of agreement (Kappa Index) for these three drugs was satisfactory (≥0.6). According to the MD method, 69.4% of the species were susceptible to itraconazole, whereas 30.6% were not.For this drug, the Etest showed 86.1% susceptible and 13.9% resistant.

Conclusion: Voriconazole was the most effective agent against isolates. Using RPMI agar, we found the Etest to behelpful, readilya vailable, and easy to use for determining invitro susceptibilities of *Aspergillus* species to voriconazole, amphotericin B, ketoconazole, and itraconazole in the region of this study.

Keywords: Amphotericin B, *Aspergillus,* Etest, itraconazole, voriconazole

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Introduction

Aspergillus species cause many infections especially in immunocompromised patients. Effective surveillance of these patients depends on early, suitable antifungal treatment. Unfortunately, despite advancements in antifungal therapy, the invasive form of aspergillosis is still associated with a high mortality rate. Since resistance to these drugs has been seen in patients, susceptibility testing can then assist with determining the appropriate drug for treatment and respective epidemiology. The availability of a standardized method for in vitro susceptibility testing of filamentous fungi is essential to properly carry outlaboratory comparisons of the minimum inhibitory concentration (MICs) of various antifungal agents.

The Clinical and Laboratory Standards Institute (CLSI) has developed standardized methods for in vitro susceptibility testing of antifungal agents for yeasts and molds.These methods include variables in inoculum preparation size, duration of incubation, medium, temperature, and MIC endpoint determination. However, when utilizing standard methods)there is the chance that laboratory personnel can become contaminated,which leads to errors in inoculating microdilution (MD) plates as well as subjective interpretation of endpoints. The Etest is an agar dilution-diffusion method that has been used to evaluate the susceptibility of fungi to antifungal drugs. This method is faster and simpler than the CLSI method for routine use in laboratories. However, the level of agreement between the two methods for analysis offilamentous fungi is variable.[1,2]

The purpose of this study was to investigate and compare the susceptibility patterns of the *Aspergillus* species, isolated from patients using the CLSI reference MD (document M38-A) assay and the Etest method.

Patients and Methods

MICs of various antifungal agents (amphotericin B, ketoconazole, itraconazole and voriconazole) for the 108 *Aspergillus* species isolated from patients included in this study were determined by CLSI M38-A MD[3] and Etest. The isolates were obtained from clinical samples that included tissues, sputum, bronchoalveolar lavage, abdominal tap, and cerebrospinal fluid. Isolated *Aspergillus* species included 66 cases of *A. flavus*, 30 cases of *A. fumigatus*, and 12 cases of *A. niger.*

In order to obtain adequate sporulation, isolates were cultured twice onto potato dextrose agar (Oxoid, England) for three to five days at 35°C; the conidia of the isolates were obtained from the fresh cultures. The conidia were mixed in sterile saline with 0.05% tween20.The turbidities of the supernatants were adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11

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Table 1. Distribution of MICs (μg/ml) by CLSI and Etest methods.*

Species (no. of isolates)	Antifungal agent	Range	Etest		Range	CLSI	
			50%	90%		50%	90%
****A.flavus** (66)	Amphotericin	0.190–32.000	3.000	8.00	0.250–32.00	1.00	4.00
	Ketoconazole	0.125–1.500	0.500	1.00	0.032–1.00	0.250	0.500
	Itraconazole	0.250–1.500	0.500	1.00	0.250–2.00	1.00	1.00
	Voriconazole	0.064–0.640	0.125	0.190	0.125–0.500	0.250	0.250
A.fumigatus **(30)**	Amphotericin	0.032–8.000	1.00	2.00	0.064–2.00	0.250	1.00
	Ketoconazole	0.125–16.00	4.00	12.00	0.125–4.00	2.00	4.00
	Itraconazole	0.125–1.500	0.750	1.00	0.250–8.00	1.00	2.00
	Voriconazole	0.032–0.190	0.125	0.190	0.064–0.500	0.125	0.250
A.niger**(12)**	Amphotericin	0.125–0.380	0.125	0.380	0.064–1.00	0.125	1.00
	Ketoconazole	0.750–4.00	2.00	4.00	0.500–1.00	0.500	1.00
	Itraconazole	1.500–32.00	2.00	4.00	0.500–16.00	8.00	16.00
	Voriconazole	0.064–0.190	0.125	0.190	0.250–1.00	0.250	1.00
Total (108)	Amphotericin	0.032–32.000	1.500	6.000	0.064–32.00	0.500	4.000
	Ketoconazole	0.125–32.00	0.750	6.000	0.064–4.00	0.250	2.000
	Itraconazole	0.125–32.00	0.500	1.500	0.250–16.00	0.500	4.000
	Voriconazole	0.032–0.640	0.940	0.190	0.064–1.00	0.125	0.250

*Etest read at 24h and MD read at 48h; ** A: *Aspergillus*.

Table 2. Set of mold isolates evaluated and in vitro MIC.*

Species	Antifungal agent	Method	No. of isolates Categorized as [a]			Species	Antifungal agent	Method	No. of isolates Categorized as [a]		
			S	I	R				S	I	R
****A.flavus**(66)	Amphotericin B	Etest	30		36	*A. niger* (12)	Amphotericin B	Etest	12		0
		CLSI	30		36			CLSI	12		0
	Ketoconazole	Etest	60		6		Ketoconazole	Etest	9		3
		CLSI	66		0			CLSI	12		0
	Itraconazole	Etest	36	27	3		Itraconazole	Etest	0	3	9
		CLSI	48	6	12			CLSI	3	3	6
	Voriconazole	Etest	60	6	0		Voriconazole	Etest	12	0	0
		CLSI	66	0	0			CLSI	12	0	0
A.fumigatus(30)	Amphotericin B	Etest	24		6	Total (108)	Amphotericin B	Etest	66		42
		CLSI	27		3			CLSI	69		39
	Ketoconazole	Etest	27		3		Ketoconazole	Etest	96		12
		CLSI	30		0			CLSI	108		0
	Itraconazole	Etest	24	3	3		Itraconazole	Etest	60	33	15
		CLSI	12	3	15			CLSI	63	12	33
	Voriconazole	Etest	30	0	0		Voriconazole	Etest	108		0
		CLSI	30	0	0			CLSI	108		0

*Etest read at 24h and MD read at 48h; **: *Aspergillus.*
[a]*S: Susceptible, I: Intermediate, R: Resistant*

(bioWave, S2100 Diode Array Spectrophotometer) at 530 nm. The inoculum numbers of conidia were confirmed by plating serial dilutions onto Sabouraud dextrose agar plates. Suspensions were diluted at 1:50 in RPMI (HiMedia, India) 1640 broth. Reference MICs were defined as the lowest drug concentrations that showed 100% (amphotericin B, voriconazole, and itraconazole) growth inhibition and 50% growth reduction for ketoconazole, compared to the control. Two CLSI quality control strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were tested in the same manner.[4]

Stock solutions of amphotericin B, ketoconazole (Sigma-Aldrichemie GmbH-Steinheim, Germany), itraconazole (Janssen Pharmaceutical, Beerse, Belgium), and voriconazole (Pfizer, United Kingdom) were prepared in dimethyl sulfoxide (Merck, Germany) according tothe CLSI method and used in the MD assay.

Etest was performed in accordance with the manufacturer's instructions. After culturing the isolates onto potato dextrose agar slants (Remel) at 35°C for seven days, spore suspensions were prepared in sterile saline and adjusted to a concentration of 10^6 spores/ml. Agar formulations used for the Etest were RPMI 1640,

supplemented with 1.5% agar and 2% glucose and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma, St. Louis, MO.). The 90-mm diameter plates contained RPMI at a depth of 4.0 mm. The plates were inoculated by dipping a sterile swab into the suspension and streaking it across the surface of the agar in three directions. The plates were dried at ambient temperature for 15 min before applying the Etest strips. For both methods, MIC endpoints were determined after 24 and 48h of incubation at 35°C. The Etest MIC was read for amphotericin B, as the drug concentration of that zone determined the point of complete inhibition (100%).For ketoconazole, itraconazole, and voriconazole,significant inhibition decreased 80% of the growth. Microcolonies within the eclipse were ignored.

The levels of agreement between the observations were calculated for each strain, drug, MIC endpoint, and incubation time combination. Data were analyzed in SPSS software version 15, using the Wilcoxon signed ranks test and t-test. MIC_{50} (the MIC at which 50% of the isolates are inhibited) and MIC_{90} (the MIC at which 90% of the isolates are inhibited) were also calculated.

Results

The results of MICs for the testing of fungi using the MD method showed that at 24h and 48h they were either 90% similar or increased on 1 to 2 dilutions.The difference was greater in some cases when using the Etest. Thus, all the plates were read at 24 h incubation for Etest plates and 48 h incubation for MD plates (according to CLSI).

Of the 108 *Aspergillus* isolates tested, the MD method showed that 69 (63.9%) were sensitive to amphotericin B, whereas 39 (36.1%) were resistant. The Etest method showed 66 (61.1%) sensitivityand 42 (38.9%) were resistant to amphotericin B. With the MD method,108 isolates (100%) were sensitive to ketoconazole,whereas with the Etest 96 (88.9%) were sensitive and 12 (11.1%) were resistant.In both methods, all species showed susceptibility to voriconazole. The measure of agreement (Kappa Index) for these three drugs was satisfactory (≥ 0.6).According to the MD method, 75 (69.4%) species were susceptible to itraconazoleand 33 (30.6%) were resistant; in the Etest 93 (86.1%) species were susceptible, with 15 (13.9%) resistant. Compared to the MD test as the gold standard, the Etest had the following sensitivities: amphotericin B (70%), ketoconazole (81%), itraconazole (51%), and voriconazole (100%). The distribution of MICs (μg/ml) by MD and Etest methods is presented in Tables 1and 2.

For the 108 isolates tested, after 48 hof incubation the two methods demonstrated the highest agreement for voriconazole (100%), ketoconazole (83.3%), and amphotericin B (75%) in MICs, whereas itraconazole had a less favorable MICs agreement of 58.3%. The agreement between the two methods for the four antifungals was the highest when Etest was read at 24 h and MD at 48h.

Discussion

Due to an increase in the numbers of patients at-risk for fungal infections, such as transplant recipients and cancer patients who receive chemotherapy, the incidence of systemic mycoses has increased. One of the most common fungal pathogens is the species of *Aspergillus.* Since fungal infections in hospitals are much less frequent than microbial infections, susceptibility tests for fungi are not routinely performed. The use of a method that is sensitive, reliable, and easy to usein routine laboratory tests would enable clinicians to appropriately treat their patients.

As recorded in Table 1, both voriconazole (MIC_{90}: ≤ 0.250 μg/ml) and ketoconazole (MIC_{90}: ≤ 2 μg/ml) were highly active against all species of*Aspergillus* and inhibited the growth of 100% of the samples. Amphotericin B inhibited 63.9% and itraconazole inhibited the growth of 69.4% of cases. Pfalleret al. reported that voriconazole inhibited 98% of isolates at an MIC of ≤ 1 μg/ml, followed by amphotericin B (89%), and itraconazole (72%).[5] In Taiwan, Hsueh and coworkers reported that voriconazole was the most potent agent against fungal isolates thatlacked susceptibility to fluconazole and amphotericin B.[6]

In Iran, itraconazole is used for prophylaxis in immunocompromised patients and amphotericin is the drug of choice for patients when clinicians suspect systemic fungal infections. As shownin Table 1, the MIC_{90} for these drugs is 4 μg/ml, with a high resistance to both drugs. The same finding has been noted in isolated yeast from patients in this region.[7,8] Cross-tabulation of voriconazole, fluconazole, and itraconazole MICs indicated that voriconazole MICs increased when fluconazole and itraconazole MICs increased.[8] However, Espinel-Ingr off et al.[9] have noted that cross-resistance between itraconazole and the newer triazoles was not universal and may vary according to the strain of *Aspergillus.*In the present study, only in *A. niger* (which was notably resistant to itraconazole) did the $MIC_{90}$for voriconazole increase.

Amongthe most prevalent *Aspergillus* species in our region, *A. flavus* exhibited the highest resistance to amphotericin B. *A. niger* showed the highest resistance to itraconazole.

In clinical practice, a complete relationship between susceptibility test results and patients' outcomes is not to be expected, thusa wide spectrum of antifungal agents effective against yeasts and molds are used. Johnson et al.[10] in a study on amphotericin B and aspergillosis have maintained that clinical outcomesmay not be entirely predictable because of host factors, including underlying disease, cellular immune function, antibody titers, and complement and pharmacokinetic properties that play critical roles in determining treatment outcomes. In the same study, five patients, whose isolated fungi were found to be sensitive but practically resistant to amphotericin by MD, were switched to voriconazole. To improve the correlation between the in-vitro susceptibility testing and patient outcomes, further studies should be conducted.

The Etest method that uses RPMI agar appears to be a useful method for determining the itraconazole susceptibilities of *Aspergillus* species and other filamentous fungi.[1] For many molds, including *Aspergillus* species, good correlations have been shown between the MICs of amphotericin B and itraconazole by both Etest and CLSI.[1,2,9] In one study on itraconazole, it was reported that the agreement level between Etest MICs read at 24h and reference MD MICs read at 48 h was 100%.[11] Meletiadis reported low levels of agreement between MD and Etest methods using the recommended MIC endpoints for most species, especially after 48h of incubation (< 50%).[12] However in another study, the researchers reported 93.3% to 100% agreement (within three dilutions) between methods independent of the incubation time.[13] In the current study, we read all plates after 24 and 48 h, however after statistical analysis of the data, a close relationship was demonstrated between Etest after 24h and MD after 48 h.

In practice, accuracy in reporting results of fungal susceptibility tests is imperative for patients when the fungi are on the borderline between sensitive and resistant. Reliable and cost-efficient suscep-

tibility tests are vitalfor patients and clinicians' management of the patients.A comparison of the two methods showed was negative ranks between MD and Etest for each of the four antifungals. Although the Etest was higher in one or two wells relative to MD, the Etest appears to be a suitable alternative procedure for testing the susceptibility of *Aspergillus* speciesto antifungal agents. According to the Etest manufacturer, "many factors such as media, buffer, inoculums, incubation time and end point criteria can affect the results significantly." On the other hand, the potential errors in any step of processing in MD (making the concentrations of drugs and the plates) may result in improper choice of drug and treatment.

In conclusion we found that the Etest (using RPMI agar) to be useful, readily available, and easy to use in determining invitro susceptibility of *Aspergillus* species to voriconazole, amphotericin B, ketoconazole, and itraconazole in Iran. Nevertheless, because of the differences between the results of Etest in terms of media, species, and antifungal agents, anotherinternationally accepted method is required. The reference MD method seems to satisfy this need. In this study, voriconazole is shown to be the most effective agent against the isolates.

Conflict of interest: *The authorsreport no conflicts of interest in this study.*

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