

Multicenter Italian Study on “In Vitro Activities” of Isavuconazole, Voriconazole, Amphotericin B, and Caspofungin for *Aspergillus* Species: Comparison between Sensititre™ YeastOne™ and MIC Test Strip

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Abstract: In this study the activity of Isavuconazole, Voriconazole, Amphotericin B, and Caspofungin against 224 clinical isolates of *Aspergillus* spp. originating from seven Italian hospitals, was comparatively evaluated with two commercial antifungal susceptibility tests (AST): Sensititre™ YeastOne™ (SYO) and MIC Test Strip. More attention was focused on Isavuconazole activity, given the new introduction of the drug in widely distributed antifungal susceptibilities methods in the clinical microbiology lab. The minimum inhibitory concentrations of antifungal drug that can inhibit the growth of pathogen by 90% (MIC90) for Isavuconazole detected by SYO were 0.5, 1, 0.25, and 2 µg/mL for *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger*, respectively, whilst they were 0.25, 0.25, 0.5, and 0.75 µg/mL by MIC Test Strip. Essential agreement between the two tested methods for Isavuconazole is 70% for all the species tested, 75.7% for *A. fumigatus*, 45.2% for *A. flavus*, 90.6% for *A. terreus*, and 40% for *A. niger*. Although the tested strains do not express any phenotypic resistance, MIC results were quite different if tested with microdilution broth or gradient agar method. This is the first Italian multicenter report on Isavuconazole MIC obtained employing the widely used Sensititre™ Yeast One™ (SYO) and MIC Test Strip on clinical isolates of *Aspergillus*.

Keywords: antifungal susceptibilities, *Aspergillus* Isavuconazole susceptibilities, Sensititre and MIC Test Strip

Introduction

The number of infections caused by *Aspergillus* species is constantly growing, involving either known immunocompromised patients or immunocompetent subjects, for example, those with the latter Influenza- or COVID-19-associated pulmonary aspergillosis.^{1,2}

Currently, available antifungal drugs for the treatment of invasive fungal infections (IFI) include polyenes, azoles, and echinocandins. Voriconazole and the new mold-active Isavuconazole are the first line drugs for the treatment of aspergillosis.³⁻⁵ The emergence of azole-resistant strains of *Aspergillus* spp. has been increasingly reported in patients undergoing long-term antifungal treatment.⁶ Previous studies have shown the usefulness of commercial antifungal susceptibility testing in comparison with reference methods, such as the Clinical and Laboratory Standard Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) ones for mold azole-resistance surveillance.^{7,8}

Sensititre™ YeastOne™ (TREK Diagnostic Systems, Cleveland OH) (SYO) is a widely used broth microdilution susceptibility testing system, based on the CLSI M27-A3 microdilution reference method. Based on the alamarBlue colorimetric indicator, SYO has been approved by the FDA as a method for susceptibility testing of yeasts; nevertheless, it has also been extensively used for molds.^{9,10}

MIC Test Strip (Liofilchem, Italy) is a gradient diffusion method extensively used in the clinical microbiology laboratory for the evaluation of susceptibility to azoles and echinocandins of both yeasts and filamentous fungi.¹¹

The aim of this study was to evaluate the Minimum Inhibitory Concentration (MIC) distributions of Isavuconazole, Voriconazole, and Amphotericin B, and the Minimal Effective Concentration (MEC) distribution of Caspofungin obtained simultaneously by two commercial methods, SYO and MIC Test Strip, on the most frequently isolated species of *Aspergillus* spp. Isavuconazole activity was deeply evaluated in order to study any difference between the methods, given the new introduction of the drug in widely commercial distributed antifungal susceptibilities methods in the clinical microbiology lab. Furthermore, the study was conducted in order to highlight any differences in the evaluation of Caspofungin activity on the basis of MIC or MEC distribution.

Materials and Methods

Two hundred and twenty-four *Aspergillus* strains isolated from clinical respiratory samples were collected anonymously between November 2018 and January 2019 in seven Italian Clinical Microbiology laboratories. In detail, 51 strains were collected in Azienda Ospedaliera Universitaria Integrata (AOUI) of Verona, 48 in Azienda Ospedaliera Universitaria (AOU) Policlinico Vittorio Emanuele–Catania, 35 in Bergamo, 29 in Milan-Niguarda hospital, 24 in Novara, 31 in Sassari, and six strains in Modena hospital. Species-level morphological identification was confirmed, if necessary, by MALDI-TOF mass spectrometry analysis (VITEK MS system equipped with the v3.2 IVD database or Bruker equipped with Compass 1.4 database). Few cases needed DNA-based identification by sequencing of the internal transcribed spacer (ITS). The collected *Aspergillus* strains included the sections *Fumigati* (*A. fumigatus* complex, n=127, *Aspergillus thermomutatus*, n=1), *Flavi* (*A. flavus*, n= 30, *A. oryzae*, n=1), *Nigri* (n=25), *Terrei* (n=32), *Nidulantes* (n=2), one *A. ochraceus*, one *A. versicolor*, one *A. ustus*, and three *Aspergillus* spp. Approval of the ethics committee was not needed, as the strains were collected retrospectively and anonymously.

SYO and MIC Test Strip were performed according to manufacturer's instructions.^{12,13} Briefly, SYO color endpoints were determined for triazole and Amphotericin B. In detail, the lowest drug concentration corresponding to the first blue or purple well after 48 hours incubation was considered the MIC result. In cases of slow or insufficient growth, the microplates were incubated for an additional 24 hours. Due to a lack of manufacturer indications, the activity of Caspofungin was read looking at the MIC, as described above, in 50% of the laboratory involved in the study. Residual lab read the MEC as the lowest concentration at which short, stubby, and highly branched hyphal clusters were microscopically observed, compared with the growth control well after 48 hours of incubation.

The SYO panel used was Y-010 and the antifungal concentration of this panel ranged from 0.008–8 µg/mL for Isavuconazole and Voriconazole, from 0.015–8 µg/mL for Caspofungin, and from 0.12–8 µg/mL for Amphotericin B. The MIC Test Strip was performed by plating a 0.5 McFarland conidial suspension into RPMI 1640 medium 2% glucose agar plates (Liofilchem, Italy). Strip MICs were read after 48 hours incubation by three independent technicians. The MIC corresponded to the lowest drug concentration at which the pointed end of the inhibition ellipse intercepted the scale on the antifungal strip; small colonies inside the ellipse were not ignored for amphotericin B and triazoles. MEC for Caspofungin was read ignoring small colonies inside the ellipse, as the fungistatic activities of the molecule. Minimum Inhibitory Concentration required to inhibit the growth of 50% or 90% of organisms, MIC₅₀ and MIC₉₀, respectively, was calculated for the species with more than 10 strains represented. *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as Quality Control strains. MIC ranges and MIC₅₀/MIC₉₀ values were calculated for each species and method. The high off-scale MICs were converted to the next highest concentration and the low off-scale MICs were recorded as the lowest tested concentrations. For the comparison with SYO microdilution data, MIC results for MIC Test Strip were rounded up to the next double dilution step value. Essential agreement (EA) was calculated for the two methods as the percentage of isolates with MIC within one 2-fold dilution for each species and each drug.

Results

Sensititre™ YeastOne™ microdilution broth method results are shown in Table 1, which depicts the MIC distribution for Isavuconazole, Voriconazole, Amphotericin B, and MIC/MEC for Caspofungin. Most of the isolates (90%) showed a MIC ≤ 1 $\mu\text{g/mL}$ for Isavuconazole, below the CLSI ECV for all the *Aspergillus* species. Most of the species showed Isavuconazole MICs similar to Voriconazole MICs, the only exception was *A. niger*, which showed the highest Isavuconazole MIC values (MIC₉₀ 2 $\mu\text{g/mL}$), conversely *A. terreus* isolates were the most susceptible (MIC₉₀ 0.25 $\mu\text{g/mL}$). The susceptibility to Voriconazole matches with that recently reported by Espinel-Ingroff in *Aspergillus* not harboring a *cyp51A* mutation (range=0.25–0.5 $\mu\text{g/mL}$).¹⁴ MIC values for Amphotericin B ranged from 1–2 $\mu\text{g/mL}$ for more than 90% of *A. fumigatus* and *A. niger* and 80% of *A. flavus*. Few *A. fumigatus* (seven strains, 5.4%) showed MIC

Table 1 MIC and MEC ($\mu\text{g/mL}$) Distributions for *Aspergillus* Species by SYO

Species	Total	Drug	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
<i>Aspergillus fumigatus</i>	128	Isavuconazole			1	10	58	53	5	1			
		Voriconazole			3	8	93	22	1				
		Amphotericin B							25	96	6	1	
		Caspofungin MIC					1						86
		Caspofungin MEC		3	15	16	1			1	1	4	
<i>Aspergillus flavus</i>	30	Isavuconazole					3	23	3	1			
		Voriconazole					5	19	5	1			
		Amphotericin B							3	22	5		
		Caspofungin MIC											11
		Caspofungin MEC	2	12	5								
<i>Aspergillus terreus</i>	32	Isavuconazole			1	20	8	3					
		Voriconazole				20	10	2					
		Amphotericin B							3	17	12		
		Caspofungin MIC											7
		Caspofungin MEC		4	8	8	3		1			1	
<i>Aspergillus niger</i>	25	Isavuconazole					3	2	13	6	1		
		Voriconazole				1	18	4	2				
		Amphotericin B						2	12	11			
		Caspofungin MIC											13
		Caspofungin MEC	2	6	3	1							
Other <i>Aspergillus</i> spp.	9	Isavuconazole			3	3		1	1	1			
		Voriconazole			1	4	1	2		1			
		Amphotericin B				1			1	2	3	2	
		Caspofungin MIC								1			7
		Caspofungin MEC										2	

Notes: The most frequent MIC/MEC obtained for different species are shown in bold.

over 2 µg/mL. As expected, *A. terreus* shows the highest MIC, as previously reported.¹⁰ Caspofungin MIC values obtained by half of the center with SYO, conforming to manufacturer instructions, were always >8 µg/mL. The MEC results, after 48 hours, were <0.5 µg/mL for 85% of *A. fumigatus* complex, <0.12 µg/mL for 100% *A. flavus*, <0.5 µg/mL for 92% *A. terreus*, and <0.25 µg/mL for 100% *A. niger*. The results obtained from uncommon clinically relevant *Aspergillus* spp. were reported on a single line.

Table 2 shows the MIC Test Strip distribution for all the antifungal drugs tested on clinical isolates. Isavuconazole strip MIC values were lower than SYO MIC for all the *Aspergillus* species, with the lowest result being obtained with *A. fumigatus* (mode 0.19 µg/mL). Unexpectedly few *A. fumigatus* strains (9, 7%) showed high Amphotericin MIC (>4 µg/mL), which is uncommon, and was not confirmed by SYO results. Amphotericin data were higher than SYO for *A. terreus*, in line with those previously reported by other authors.¹⁰ Many strains of *A. flavus* (19, 63%) were not readable (Figure 1) because it is undeniably difficult to establish a real MIC on the intercept ellipse of the strip. MEC for Caspofungin were considerably lower than SYO results, even if it was difficult to establish for a few strains of *A. fumigatus* and *A. terreus*, as is shown in Figure 2.

Voriconazole, Amphotericin B, and Caspofungin were not determinable for five strains of *A. niger* as the high level of plate growth after 48 hours incubation.

The MICs for the quality control and reference strains for both methods were within the expected ranges.

Table 3 illustrates the Essential Agreement (EA) and the MIC₅₀/MIC₉₀ with more than 10 strains represented for each species, compared with both CLSI and EUCAST epidemiological cut-off (ECV).^{11,14,15} No break point criteria are available for triazole susceptibility testing with the widely used Sensititre™ YeastOne™ and MIC Test Strip, but for Voriconazole and Amphotericin B a proposed method dependent ECV was reported.

Essential agreement, considered as ± one 2-fold dilution between SYO results and MIC test strip, turned out to be disappointing (Table 3). Isavuconazole and Voriconazole reached the best performance with an EA of 69.7% and 70%, respectively, instead of 52% for Amphotericin and 63% for Caspofungin MEC. The analysis by species reveals an excellent agreement for Isavuconazole MIC with 90% of EA for *A. terreus*, followed by *A. fumigatus* (EA 75%). Voriconazole showed for *A. fumigatus* and *A. flavus* acceptable results, with an EA of 78.9 and 77.4% MIC agreement.

Discussion

Aspergillus species are the most common causes of invasive fungal infections in immunocompromised patients. Nowadays the COVID-19 pandemic underlines the strict relationship between viral interstitial pneumonia and *aspergillus* opportunistic infections, especially in Intensive Care Units patients.¹⁶ The introduction of new antifungal agents and recent reports of resistance emerging during treatment of *Aspergillus* infections have highlighted the need for in vitro susceptibility testing.¹⁶ Isavuconazole or Voriconazole are considered first line therapy, alternatively liposomal amphotericin B could be used. Echinocandins could be used in combination in suspected azole resistance.¹⁷ Broth microdilution Antifungal susceptibility methods (BDM) have been standardized by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) but are labor-intensive for the clinical microbiology lab. Few commercial methods have been proposed to evaluate the susceptibility to azoles, amphotericin, or echinocandins for molds, among them Sensititre™ YeastOne™ (Thermofisher) and gradient agar test (Etest or MIC test strip) are the most widely distributed. To explore the correlation of the MIC/MEC results obtained with Sensititre™ YeastOne™ and the MIC test strip on *Aspergillus* clinical strains, an Italian multicenter study was done. Moreover, Isavuconazole MIC distribution on Sensititre BMD was never reported. Our study provides range data on the susceptibility of main clinical *Aspergillus* strains derived from many geographically distributed Italian mycology laboratories. Both the assays employed in this study revealed Isavuconazole MIC₉₀ values within the ECV established by CLSI for all the species tested, included *A. niger* which evidenced the highest MIC for Isavuconazole, but in accordance with other authors.^{18,19} SYO has been demonstrated as a suitable method for assessing the susceptibilities of *Aspergillus* common species to Voriconazole and Amphotericin B in comparison with CLSI method (10). MIC₉₀ detected in our study by SYO for Voriconazole and Amphotericin B are in line with results obtained by Wang et al¹⁰ for Wild type isolates, below the CLSI ECV. As Wang et al previously reported, the SYO method showed an increase of Amphotericin B MIC of 1.6-fold higher geometric mean MIC than the CLSI M38-A method.

Table 2 MIC and MEC ($\mu\text{g/mL}$) Distribution for *Aspergillus* Species by MIC Test Strip

Species	Total	Drug	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	32	>32	N. E.
<i>Aspergillus fumigatus</i>	128	Isavuconazole	1	1	1	4	4	19	33	40	19	6													
		Voriconazole	1		2	2	3	12	17	48	24	16	1		1				1						
		Amphotericin B								2	7	4	10	12	11	7	30	9	17	10	1	1		7	
		Caspofungin MIC			1		3	5	11	18	29	12	3										4	1	
		Caspofungin MEC				1	1	3	12	8	6	2												8	
<i>Aspergillus flavus</i>	30	Isavuconazole					2	7	5	7	7	1	1												
		Voriconazole					1		3	6	4	7	6	2											1
		Amphotericin B											1			1	3						3	3	19
		Caspofungin MIC					1	2	5		1								1				1		
		Caspofungin MEC						1	8	4	2	1		1									1	1	
<i>Aspergillus terreus</i>	32	Isavuconazole					1	2	6	5	7	7	1	3											
		Voriconazole				1			1	2	3	4	3	11	3	4									
		Amphotericin B												2	1	1	2	3	4	1	2			16	
		Caspofungin MIC					2	2		2	1														
		Caspofungin MEC				1		1	10	5	4												4		
<i>Aspergillus niger</i>	25	Isavuconazole				1	1	3		2	6	4	2	5		1									
		Voriconazole			1	1		2	4	4	2	3	2			1									
		Amphotericin B							2	1	2		3	2	1	2	3	3		1					5
		Caspofungin MIC					1	2	4	3	1	1	1												

(Continued)

Table 2 (Continued).

Species	Total	Drug	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	32	>32	N. E.
		Caspofungin MEC						3	4																5
Other <i>Aspergillus</i> spp.	9	Isavuconazole	2	1	1	1	1								1		2								
		Voriconazole	1		2	1	1		1			1	1				1								
		Amphotericin B															1		1		4	1	2		
		Caspofungin MIC						1	2		1	2									1				
		Caspofungin MEC											1											1	

Notes: The most frequent MIC/MEC obtained for different species are shown in bold.
Abbreviations: N.E., not evaluable.



Figure 1 Amphotericin MIC test strip with *Aspergillus flavus*.

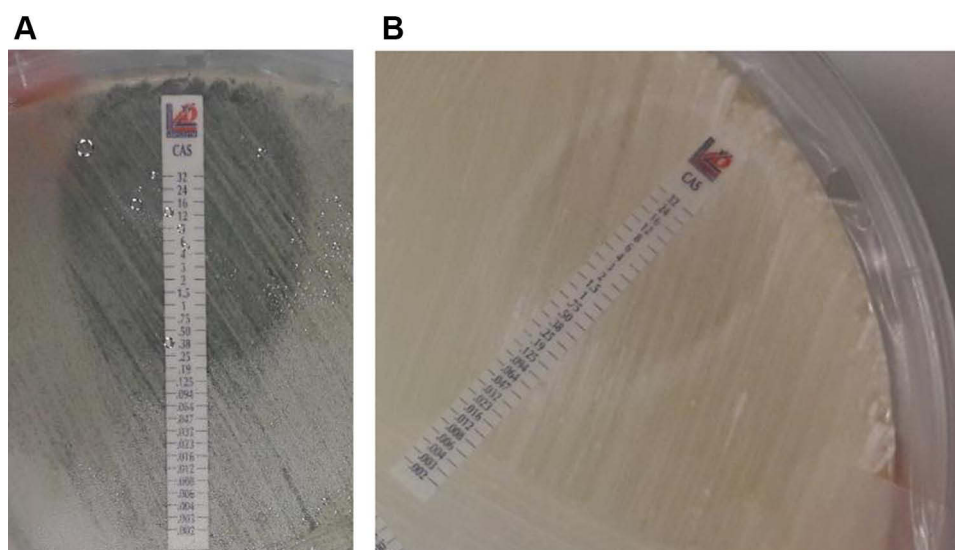


Figure 2 Caspofungin MIC test strip with *Aspergillus fumigatus* (A) and *Aspergillus terreus* (B).

Amphotericin B MIC₉₀ by the MIC Test Strip was >32 µg/mL for *A. flavus*, above the ECV value which should be ≤2. This is in line with the knowledge that *A. flavus* exhibits variable MIC to Amphotericin B with evidence of intrinsic resistance to polyenes.^{10,14} Caspofungin high MIC obtained by using SYO can be explained by 48 hours reading according to manufacturer's instructions, not specifying how to read the Minimal Effective Concentration (MEC). As reported by Siopi et al,⁹ concerning echinocandins the best agreement between CLSI and SYO was found with an incubation of 20 hours for *A. flavus* and after 30 hours for *A. fumigatus* and *A. terreus*. MEC shown in Table 1 were lower and in line with other recent reports.^{20,21}

MIC test strip showed Isavuconazole MIC lower than Voriconazole MIC, even if only slightly one 2-fold dilution. Conversely, MIC test strip showed high MIC for other drugs, especially amphotericin B in *A. flavus* and *A. niger* group. Few *A. fumigatus* evidenced Amphotericin B resistance, not seen in SYO BMD. MIC test strip overestimation was previously described by other authors.²² These phenotypes could be correlated to cryptic species as *A. lentulus*, morphologically indistinguishable from *A. fumigatus sensu stricto*, or other cryptic species in *flavi* and *nigri* sections, revealing the emerging importance of molecular species identification, as reported by Nargesi et al^{23,24}

Table 3 Essential Agreement (EA) and MIC₅₀/MIC₉₀ (µg/mL) by Sensititre (SYO) and MIC Test Strip for the Most Clinically Relevant *Aspergillus* spp

Antifungals	Species		<i>A. fumigatus</i>	<i>A. flavus</i>	<i>A. terreus</i>	<i>A. niger</i>	All
Isavuconazole	N° isolates		128	30	32	25	
	MIC Strip	MIC ₅₀	0.19	0.19	0.25	0.25	
		MIC ₉₀	0.25	0.25	0.5	0.75	
		MIC range	0.016–0.38	0.064–0.5	0.064–0.75	0.047–1.5	
	SYO	MIC ₅₀	0.25	0.5	0.12	1	
		MIC ₉₀	0.5	1	0.25	2	
		MIC range	0.06–2	0.25–2	0.06–0.5	0.25–4	
	EA	(%)	75.7	45.2	90.6	40	69.7
	ECV (CLSI)		≤1	≤1	≤1	≤4	
	ECV (EUCAST)		≤2	≤2	≤1	≤4	
Voriconazole	MIC Strip	MIC ₅₀	0.19	0.38	0.75	0.25	
		MIC ₉₀	0.38	0.5	1.5	1.5	
		MIC range	0.016–1	0.064–0.75	0.047–1.5	0.032–1.5	
	E-test ECV*		0.5	0.5	n.a.	1	
	SYO	MIC ₅₀	0.25	0.5	0.12	0.25	
		MIC ₉₀	0.5	0.5	0.25	0.5	
		MIC range	0.06–1	0.25–2	0.12–0.5	0.12–1	
	EA	(%)	78.9	77.4	50	50	70.2
	SYO ECV*	µg/mL	≤1	≤1	n.a.	≤1	
	ECV (CLSI)	µg/mL	≤1	≤1	≤1	≤2	
	ECV (EUCAST)	µg/mL	≤1	≤2	≤2	≤2	
Amphotericin B	MIC Strip	MIC ₅₀	2	>32	8	1.5	
		MIC ₉₀	6	>32	>32	3	
		MIC range	0.19–>32	0.5–>32	0.75–>32	0.125–6	
	E-test ECV*		2	8	16	2	
	SYO	MIC ₅₀	2	2	2	1	
		MIC ₉₀	2	4	4	2	
		MIC range	1–8	1–4	1–4	0.5–2	
	EA	(%)	67.2	12.9	34.4	55	52
	ECV (CLSI)		≤2	≤4	≤4	≤2	
	ECV (EUCAST)		≤1	≤4	≤8	(≤0.5)	

(Continued)

Table 3 (Continued).

Antifungals	Species		<i>A. fumigatus</i>	<i>A. flavus</i>	<i>A. terreus</i>	<i>A. niger</i>	All
Caspofungin	MIC Strip	MIC ₅₀	0.19	0.125	0.125	0.125	
		MIC ₉₀	0.38	0.75	0.25	0.25	
		MIC range	0.032–0.5	0.064–>32	0.047–>8	0.064–0.5	
	SYO	MIC ₅₀	>8	0.03	0.12	>8	
		MIC ₉₀	>8	>8	>8	>8	
		MIC range	0.03–16	0.015–16	0.03–16	0.015–16	
	EA MEC**	(%)	73.2	47.4	50	50	63
	EA MIC**	(%)	5.8	18.2	0	0	7
	ECV (CLSI)		n.a	n.a	n.a	n.a	
	ECV (EUCAST)		n.a.	n.a.	n.a.	n.a	

Notes: CLSI and EUCAST epidemiological cut-off (ECV) (µg/mL) values for each antifungal are shown. ECV in brackets are Tentative. * Method-specific ECV only if available. ** For Caspofungin, essential agreements (EA) were separately calculated for MIC and MEC interpretation. Isavuconazole MIC are in blue boxes.

Abbreviation: n.a, not available.

A limitation of our study is that the SYO and MIC Test Strip results were not compared to CLSI or EUCAST reference methods, although other authors showed good correlation of Isavuconazole MIC Test Strip and EUCAST method for *Aspergillus* and *Scedosporium* clinical isolates (18). In addition, no molecular study has been done to evaluate azole resistance and few clinical strains of *A. flavus* complex, *A. terreus* complex, and *A. niger* complex have been tested. Therefore, further studies are necessary to increase the susceptibility data of clinical isolates.

In conclusion, our results confirm that Isavuconazole has an excellent activity against *Aspergillus*, as previously reported by using other standard methods.^{11,25} We showed that commercial SYO and MIC Test Strip methods could be used to determine Isavuconazole and Voriconazole susceptibility in molds, even though a comparative evaluation with the standardized EUCAST or CLSI method is recommended, especially if high azole MIC are found. In contrast, more attention should be paid to test Amphotericin B and Caspofungin, in line with previous reports.^{8,9} Overestimation of Amphotericin B MIC with SYO and MIC test strip could determine a false resistant phenotype. The difficulty to establish a simple and standardized method to read Caspofungin MEC is a concern and allows laboratories to fall in false resistance pattern if commercial company do not upgrade their technical instructions on correct incubation time and reading modality.

Essential Agreement between the two methods is rather good for Isavuconazole and Voriconazole, with an EA of 69.7% and 70%, respectively, but not enough for Amphotericin B and Caspofungin.

To our knowledge, this is the first Italian study investigating Isavuconazole activity against clinical *Aspergillus* strains by using the two commercial assays most widely used, namely MIC Test Strip and Sensititre™ YeastOne™. This study showed the susceptibility patterns of the main frequent *Aspergillus* clinical isolates in Italy and may help to understand the operational relevance of commercially available methods in clinical setting where Isavuconazole susceptibility tests are urgently demanded.

Acknowledgments

Isavuconazole MIC Strip and Sensititre™ YeastOne™ plates were kindly provided at no cost by Basilea. We thank Dr. Tatiana Wictorowicz and Dr. Antonio Cassin for logistic and technical support. We thank Sofia Piffer, Ottolini Paola, and many other technicians for excellent technical assistance.

Disclosure

The authors report no conflicts of interest in this work.

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