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Evaluation of isavuconazole MIC strips for susceptibility testing of *Aspergillus* and *Scedosporium* species

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Abstract

Isavuconazole is a new triazole with an expanded-spectrum and potent activity against moulds and yeasts. It has been authorized for use in adults for the treatment of invasive aspergillosis and for mucormycosis. The only commercially available isavuconazole susceptibility test is the minimum inhibitory concentration (MIC) strip isavuconazole test. The objective of this study was to assess the *in vitro* activity of isavuconazole using gradient concentration MIC strips, compared with the EUCAST broth microdilution reference method. A total of 147 clinically relevant fungal isolates comprising 120 *Aspergillus* sp. and 27 *Scedosporium apiospermum* complex were tested for susceptibility to isavuconazole using the EUCAST broth microdilution method and by the MIC strip isavuconazole test. The percent essential agreement between the two methods was calculated within a 1-fold dilution. The geometric means for the MICs using the EUCAST reference methods and the strip test were respectively: 0.60 mg/l and 0.65 mg/l for *A. fumigatus*, 0.70 mg/l and 0.77 mg/l for *A. flavus*, 1.50 mg/l and 1.25 mg/l for *A. niger*, 0.41 mg/l and 0.38 mg/l for *A. terreus*, 1.22 mg/l and 1.08 mg/l for *S. apiospermum* complex. The isavuconazole MIC strips showed good agreement with the EUCAST reference method. Isavuconazole MIC strips could be useful for susceptibility testing of *Aspergillus* sp. and *S. apiospermum* complex.

Key words: antifungal susceptibility testing, isavuconazole, MIC strips test, EUCAST, *Aspergillus* and *Scedosporium*.

Introduction

Invasive fungal disease (IFD) is a major cause of mortality and morbidity in immunocompromised patients.[1,2] Aspergillosis is the most common infection type caused by moulds in patients undergoing hematopoietic stem cell transplant or solid organ transplant and is associated with high mortality in these patient groups.[1,2] Rare fungal species, such as *Scedosporium* spp., have limited treatment options and are associated with high mortality rates.[3,4] In recent years the emergence of azole-resistant strains of *Aspergillus* spp. has been increasingly reported in patients under long-term antifungal treatment.[5–9] However, even azole-resistent strains have been reported in naïve patients without any previous exposure to azole drugs, due to resistant strains acquired from the environment.[10–12] Triazole antifungals drugs (i.e., itraconazole, voriconazole, posaconazole, and isavuconazole) are the major compounds currently involved in the treatment and prophylaxis of aspergillosis. In particular, isavuconazole is a triazole antifungal agent active against a broad range of clinically relevant fungi including *Aspergillus* spp.[13–16] Based on the results from Phase 3 clinical trials, isavuconazonium sulfate has been approved by the US Food and Drug Administration for the treatment of adults with invasive aspergillosis (IA) and invasive mucormycosis.[17,18] In addition, the European Commission has approved isavuconazole for the treatment of adults with IA and adults with mucormycosis for whom amphotericin B is not appropriate.[19] A multicenter, randomized, double-blind, non-inferiority clinical trial compared the efficacy of isavuconazole versus voriconazole for the treatment of invasive fungal disease

caused by *Aspergillus* spp. or other filamentous fungi.[17] A total of 527 patients were randomized. The primary end point was all-cause mortality through day 42 in the intention-to-treat population. Isavuconazole was noninferior to voriconazole. Isavuconazole was also better tolerated by patients and showed a better safety profile. However, only three patients with *Scedosporium* infection were enrolled, thus precluding conclusions about efficacy for these infections. The management of these complex infections request the individualized care of patient with *Scedosporium*, including susceptibility testing of the isolate with clinical course. *In vitro* activity of isavuconazole has shown against some isolates of *S. apiospermum*,[16,20,21] but because of the different activity of antifungal agents against *S. apiospermum* and especially *Lomentospora prolificans*, it is usually useful to ask for the susceptibility testing of isolates in patients with infections caused by these pathogens. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has set clinical breakpoints for isavuconazole and *Aspergillus*.[22] In particular, for *A. fumigatus* the currently proposed clinical breakpoint is 1 mg/liter derived on epidemiological cutoff value (ECOFF) (2 mg/liter) and *in vitro* pharmacokinetic (PK)/pharmacodynamic (PD) studies. Also for *A. terreus* the currently clinical breakpoint is 1 mg/liter with an ECOFF of 1 mg/liter. Instead, interpretive clinical breakpoints for antifungal MICs and *Scedosporium* complex are not available.

The only commercially available isavuconazole susceptibility test is the MIC strip isavuconazole test (Liofilchem, Roseto degli Abruzzi, TE, Italy). In a study on evaluation of MIC strip isavuconazole test for susceptibility testing of *Aspergillus fumigatus* isolates was been highlighted a greater agreement with EUCAST using the full inhibition endpoint.[23]

The objective of this study was to assess the *in vitro* activity of isavuconazole comparing gradient minimum inhibitory concentration (MIC) strips with the EUCAST broth microdilution reference method.

Methods

In total, we evaluated the MICs of 147 clinically relevant fungi such as *Aspergillus fumigatus* ($n = 48$), *Aspergillus flavus* ($n = 40$), *Aspergillus niger* ($n = 14$), *Aspergillus terreus* ($n = 18$) and *Scedosporium apiospermum* complex ($n = 27$) isolates from patients admitted to Intensive Care Unit, and Hematology, Pneumology, Pediatric Pneumology, and Medicine were included. The isolates were obtained from various respiratory tract specimens and in particular bronchoalveolar lavage, bronchial aspirate and sputum. Identification was done using macro- and micromorphology, supplemented by thermo-tolerance (incubation at 37°C and 43°C) and β -tubulin sequencing[24] for cryptic *Aspergillus* spp. The use of the term "complex" is acknowledged for *Aspergillus* spp. other than *A. fumigatus*, in the absence of detailed molecular identification, although for simplicity, it is not used throughout this article. All isolates were tested for susceptibility to isavuconazole using the EUCAST broth microdilution method (E. Def 9.3) and by MIC strip isavuconazole test (Liofilchem, Roseto degli Abruzzi, TE, Italy). For EUCAST susceptibility testing, stock solution (5000 mg/l in dimethyl sulphoxide; Sigma-Aldrich, Brøndby, Denmark) of isavuconazole (Basilea Pharmaceutica Ltd., Basel, Switzerland) was prepared. Final drug concentration ranges for isavuconazole was 0.125–16 mg/l. MICs were determined visually applying a no growth endpoint after 48 hours of incubation (*Aspergillus*), as recommended. EUCAST control strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were routinely included and read after 24 hours.[22] For interpretation of susceptibility, the following EUCAST clinical breakpoints were used for *A. fumigatus* and *A. terreus*: isavuconazole MIC $\leq$ 1 mg/l (susceptible) and >1 mg/l (resistant). For the strip test cultures were prepared in a MacFarland 0.5 conidia suspension and grown on RPMI 1640 2% glucose agar gel (SSI Diagnostica, Hillerød, Denmark). MICs from the strips were read at 80% growth inhibition after 48 hours incubation at 35°C. For the analysis of essential agreement, values from the MIC strips were rounded up to the next corresponding dilution for the broth microdilution. The percent of essential agreement between the two methods was calculated within a $\pm$ onefold dilution. The categorical agreement between the methods was calculated as the percentage of isolates classified equally by both methods. Very major errors (VMEs) were defined as isolate categorization as resistant (R) by EUCAST but susceptible (S) by the MIC strip test, and major errors (MEs) were defined as isolate categorization as S by the EUCAST method but R by the MIC strip test. Data were analyzed using the MedCalc Statistical Software version 17.9.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2017).

Results

A total numbers and MIC distributions for *Aspergillus* spp. and *Scedosporium apiospermum* complex tested in the study is provided in Table 1. The MICs for *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were in the recommended ranges. Applying the clinical breakpoints for the *A. fumigatus* isolates that underwent EUCAST susceptibility testing and MIC test strip, the susceptibility profiles were respectively as follows: 8.3% and 6.2% (resistant; R), 91.7% and 93.7% (susceptible; S). For the *A. terreus* isolates the susceptibility profiles were: 5.5% resistant and 94.4% susceptibility for both methods. The geometric means for the MICs using the EUCAST reference methods and the MIC strip test were respectively: 0.60 mg/l and 0.65 mg/l for *A. fumigatus*, 0.70 mg/l and 0.77 mg/l for *A. flavus*, 1.50 mg/l and 1.25 mg/l for *A. niger*, 0.41 mg/l and 0.38 mg/l for *A. terreus*, 1.22 mg/l and 1.08 mg/l for *S. apiospermum* complex. The percentage of MICs above the ECOFFs for the two methods (EUCAST/ MIC strip test) were as follows: *A. fumigatus* (0/0)

Table 1. Isavuconazole susceptibility of *Aspergillus* spp. and *Scedosporium apiospermum* complex determined by EUCAST and MIC strip test.

	N (%)	EUCAST (E. Def. 9.3)					MIC strip test				
		MIC range	MIC_{50}	MIC_{90}	ECOFF	% >ECOFF	MIC range	MIC_{50}	MIC_{90}	ECOFF	% >ECOFF
A. fumigatus sensu stricto	48 (32.6%)	0.125 – 2	1	1	2	0	0.19 – 2	1	1	2	0
A. flavus species complex	40 (27.4%)	0.125 – 2	1	1.1	2	0	0.25 – 1	0.75	1	2	0
A. niger species complex	14 (9.5%)	0.5 – 8	1.5	4	4	7.1	0.125 – 8	1.25	5.4	4	14.3
A. terreus species complex	18 (12.2%)	0.125 – 2	0.5	1	1	5.5	0.19 – 2	0.5	1	1	5.5
S. apiospermum complex	27 (18.4%)	0.5 – 8	1	2.8	NA	NA	0.38 – 8	1	2.8	NA	NA
C. krusei ATCC 6258	…	0.015 – 0.06	0.03	…	…	…	0.015 – 0.06	0.03	…	…	…
C. parapsilosis ATCC 22019	…	0.008 – 0.03	0.015	…	…	…	0.008 – 0.03	0.015	…	…	…

ECOFF, epidemiological cutoff value; MIC_{50}, MIC value inhibiting the growth of >50% of isolates; MIC_{90}, MIC value inhibiting the growth of >90% of isolates; NA, not available.

Figure 1. Essential agreement between the EUCAST reference method and MIC strips.

Table 2. Essential agreement between the EUCAST reference method and MIC strips for the different species tested.

	N	% essential agreement (±one-fold dilution)
A. fumigatus sensu stricto	48	89.6%
A. flavus species complex	40	97.5%
A. niger species complex	14	85.7%
A. terreus species complex	18	94.4%
S. apiospermum complex	27	96.3%
Total	147	93.2%

and *A. flavus* (0/0); *A. niger* (7.1/14.3); *A. terreus* (5.5/5.5). The essential agreement between the isavuconazole MIC strips and the EUCAST reference method was 93.2%, regardless of the species tested (Fig. 1). The values of essential agreement between two methods for the different species tested are shown in Table 2.

Six isolates differed by > ±onefold dilution (all six isolates differed by ± twofold dilution). In particular, three isolated of the *A. fumigatus* they had MIC values + twofold dilution using the strip test compared to the EUCAST reference method (0.5 mg/l vs 0.125 mg/l); one isolate of the *A. flavus* (0.75 mg/l vs 0.125 mg/l) and one of the *A. terreus* (1 mg/l vs 0.25 mg/l) they had MIC + twofold dilution using the strip test compared to the EUCAST reference method. Finally one isolate of the *Scedosporium apiospermum* complex (0.38 mg/l vs 1 mg/l) had MIC - twofold dilution using the strip test compared to the EUCAST reference method. The categorical agreement for *A. fumigatus* was 97.9% when interpreting the MICs according to EUCAST breakpoints, with VMEs of 2%. For *A. terreus* the categorical agreement between the EUCAST and MIC strips was 100%.

Discussion

Isavuconazole is a new extended-spectrum triazole with activity against yeasts, moulds, and dimorphic fungi. The safety profile of this agent and its excellent pharmacokinetic characteristics make isavuconazole an attractive option for treatment of invasive fungal infections.[25] Similar to other azoles, isavuconazole inhibits cytochrome P450 (CYP)–dependent 14α-lanosterol demethylation, which is essential for fungal cell membrane ergosterol synthesis. This blockade produces methylated sterols in the fungal membrane, altering its function and allowing the accumulation of ergosterol toxic precursors in the cytoplasm, which leads to cell death.[26] The side arm of the active isavuconazole molecule allows for greater affinity of isavuconazole for the binding pocket in the fungal CYP51 protein increasing the antifungal spectrum and conferring activity against some isolates resistant to other triazoles.[25] The *in vitro* activity of isavuconazole has been demonstrated against most *Aspergillus* spp. including *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*.[17,27] In recent years, the emergence of azole resistant *Aspergillus* spp. has become a threat to patients with the majority of clinical cases to date reported from western Europe.[28] In these resistant isolates, mutations within the CYP51A gene (which encodes the enzyme targeted by azoles) or within the gene promoter, have been identified that reduce drug affinity or increase target quantity conferring azole resistance. In *Aspergillus* isolates, cross-resistance to triazoles is quite variable and related to the type of mutations. Therefore it would be useful the detection of *in vitro* susceptibility test to the triazoles and the detection of the corresponding mutations within the CYP51A gene or within the gene promoter.[29,30] Our study assessed the *in vitro* activity of isavuconazole using gradient concentration MIC strips, compared with the EUCAST broth microdilution reference method. According to EUCAST breakpoints 8.3% and 5.5% of all *A. fumigatus* and *A. terreus* isolates were isavuconazole resistant. The categorical agreement for *A. fumigatus* and *A. terreus* was >97% when interpreting the MICs according to EUCAST breakpoints, and notably, in one isolate of *A. fumigatus* a VME was found.

In this strain it could be useful to study the resistance mechanism considering that the isolates with a wild-type CYP51A target gene either may be harboring other resistance mechanisms or may be isolates that are truly susceptible but misclassified as R by the EUCAST reference method because of the conservative EUCAST susceptibility breakpoint.[12] In all of the *A. flavus* strains tested with both methods they were observed MIC values below the epidemiological cutoff (2 mg/l). In one isolate of *A. niger* a discrepancy between the MIC strip test and EUCAST method was found. In particular, in this isolate the MIC value obtained by MIC strip test were above the epidemiological cutoff than those obtained by EUCAST method. Finally, isavuconazole MICs obtained with MIC test strip and EUCAST method against *S. apiospermum* complex were comparable. Moreover, for *Scedosporium apiospermum* complex could be useful distinguish *Scedosporium* species (*S. apiospermum, S. boydii*, *S. minutisporum*, *S. aurantiacum*, *S. dehoogii*, etc.) by molecular biology considering that these species have variable susceptibility to antifungal.

In conclusion the isavuconazole MIC strips could be useful for susceptibility testing of *Aspergillus* spp. and *S. apiospermum* complex, also evaluating the strip full inhibition endpoint since the essential and categorical agreements appear best with EUCAST method.[23] Further studies are needed to increase the isavuconazolo *in vitro* data and to define clinical breakpoints for other *Aspergillus* species. Furthermore, the appropriate use of antifungal drugs could reduce the possible development of resistance mechanisms showed in some moulds isolated in critically ill patients.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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