Azole and amphotericin B minimum inhibitory concentrations against *Aspergillus fumigatus*: high agreement between spectrophotometric and visual readings using the EUCAST 9.3.2 procedure

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Abstract

The EUCAST 9.3.2 procedure recommends visual readings of azole and amphotericin B MICs against *Aspergillus* spp. Visual determination of MICs may be challenging. In this work, we aim to obtain and compare visual and spectrophotometric MICs readings of azoles and amphotericin B against *A. fumigatus sensu lato* isolates.

Eight hundred and forty-seven *A. fumigatus sensu lato* isolates (*A. fumigatus sensu stricto* [n=828] and cryptic species [n=19]) were tested against amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole using the EUCAST EDef 9.3.2 procedure. Isolates were classified as susceptible or resistant/non-wild-type according to the 2020 updated breakpoints. The area of technical uncertainty for the azoles was defined in the updated breakpoints. Visual and spectrophotometric (fungal growth reduction >95% compared to control; read at 540 nm) MICs were compared. Essential (±1 twofold dilutions) and categorical agreements were calculated.

Overall, high essential (97.1%) and categorical (99.6%) agreements were found. We obtained 100% categorical agreements for amphotericin B, itraconazole, and posaconazole and, consequently, no errors were found. Categorical agreements were 98.7% and 99.3% for voriconazole and isavuconazole, respectively. Most of misclassifications for voriconazole and isavuconazole were found to be associated with MIC results falling either in the area of technical uncertainty or in one two-fold dilutions above the breakpoint. Resistance rate was slightly lower when the MICs were obtained by spectrophotometric readings. However, all relevant *cyp51A* mutants were correctly classified as resistant.

58 Spectrophotometric determination of azole and amphotericin B MICs against *A.*
59 *fumigatus sensu lato* isolates may be a convenient alternative to visual endpoint
60 readings.

Introduction

Azoles are the backbone of treatment and prevention of *Aspergillus* spp. diseases and are to date the only available anti-*Aspergillus* oral drugs. The European Society of Clinical Microbiology and Infectious Diseases guidelines recommend itraconazole for the management of patients with chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillosis. Voriconazole and isavuconazole are indicated as the first-line treatment of pulmonary invasive aspergillosis. Voriconazole is also recommended for primary therapy in patients with central nervous system involvement and chronic pulmonary forms of the infection. Posaconazole is recommended for antifungal prophylaxis during prolonged neutropenia in high-risk patients or as salvage therapy in intolerant or non-responding individuals. Finally, liposomal amphotericin B is recommended in settings in which azoles are contraindicated - resistant isolates - and as salvage therapy (1). Some *Aspergillus* species are intrinsically resistant to polyenes (*A. terreus*, *A. nidulans*, and *A. flavus*) or azoles (*A. ustus*) (2). *A. fumigatus sensu lato*, the main etiological agents of aspergillosis, include *A. fumigatus sensu stricto* and cryptic species. Cryptic species commonly show intrinsic resistance to amphotericin B and azoles (3). In contrast, *A. fumigatus sensu stricto* isolates may acquire resistance following exposure to azoles, particularly with environmental azole fungicides (4). Azole resistance in *A. fumigatus sensu stricto* isolates has been increasingly reported worldwide (5-7).

Patients infected by azole-resistant *A. fumigatus sensu lato* isolates show higher mortality than those with azole-susceptible infections (8, 9). Thus, to improve patient care, detection of resistance is of paramount importance. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility

Testing (EUCAST) proposed standard methods for the study of azole and amphotericin B susceptibility of *Aspergillus* spp isolates. The EUCAST EDef 9.3.2 procedure includes clinical breakpoints to classify isolates either as susceptible or resistant and recommends visual determination of MICs (10). Visual inspection may be challenging and spectrophotometric readings may facilitate MIC determination and overcome subjectivity. However, there is a limited number of studies using the EUCAST methodology in which azole MICs against *A. fumigatus sensu lato* obtained by visual and spectrophotometric readings are compared; furthermore, the studies are thwarted by a low number of isolates and antifungal drugs tested (11-14).

We recently conducted a Spanish multicenter study of azole-resistance in which 847 *A. fumigatus sensu lato* clinical isolates were collected between February 15 and May 14, 2019 (15). Taking advantage of the large number of isolates, the objective in this work is to report and compare azole and amphotericin B MICs using visual and spectrophotometric readings following the EUCAST EDef 9.3.2 procedure.

Results

Tables 2 to 6 show MIC distributions of amphotericin B, itraconazole, posaconazole, voriconazole, and isavuconazole against the 847 isolates by regular/stringent visual and spectrophotometric readings. MICs against QC strains were within the acceptable limit.

Agreement between MICs by regular visual and spectrophotometric readings. Overall, both MIC endpoints showed high essential (97.1%) and categorical agreements (99.6%). Essential agreements for individual drugs were as follows: amphotericin B

98.8%, itraconazole 94.8%, posaconazole 97.3%, voriconazole 98.3%, and isavuconazole 96.1% (Table 7).

Categorical agreements for amphotericin B, itraconazole, and posaconazole were 100% and, consequently, resistance rates for both MIC endpoints were identical. Categorical agreement for voriconazole was 98.7% and the rate of resistance was slightly lower when spectrophotometric readings were used for MIC determination. Very major errors (n=6, 0.7%) and major errors (n=3, 0.4%) for voriconazole occurred in *A. fumigatus sensu stricto* isolates with MIC results falling in the ATU (MIC=2 mg/L). In cryptic species, very major errors occurred in two *N. udagawae* isolates (10.5%), one of them with MIC results falling in the ATU. Categorical agreement for isavuconazole was 99.3% and the rate of resistance was slightly lower when spectrophotometric readings were used for MIC determination. Very major errors in isavuconazole occurred in three *A. fumigatus sensu stricto* isolates and in three cryptic species isolates (two *N. udagawae* and one *A. fumigatiaffinis*). With the exception of the *A. fumigatiaffinis* isolate, very major errors for isavuconazole (n=5) were detected in isolates with MIC results in the ATU, which also revealed very major errors for voriconazole (Table 1S). None of the six isolates for which very major errors were detected in the azole categorical classification harboured relevant *cyp51A* mutations (Table 1S).

Agreement between MICs obtained by regular/stringent visual readings. Overall, both visual MIC endpoints showed high essential (97.7%) and categorical agreements (96.7%). Essential agreements for individual drugs were above 98% (itraconazole [98.9%], posaconazole [98.7%], and isavuconazole [98.6%]) with the exception of voriconazole (94.4%) (Table 7).

Categorical agreements for itraconazole and posaconazole were 99.4% (Table 7). Resistance rates obtained by both MIC endpoints were identical in *A. fumigatus sensu stricto,* but slightly higher with stringent visual readings in cryptic species. This led to major errors for both drugs in five isolates (three *A. lentulus*, one *A. novofumigatus* and one *A. fumigatiaffinis*). Although posaconazole MICs by both visual readings were identical (MIC=0.25 mg/L, ATU), the categorical classification differed due to the MICs of itraconazole in four out of the five isolates (Table 2S). Percentage of voriconazole resistance was overestimated with the visual stringent endpoint (6.6% vs 15.8%). Categorical agreement was 90.8%. Major errors were found exclusively in *A. fumigatus sensu stricto* isolates (n=78), in MIC results falling in the ATU. Likewise, the rate of isavuconazole resistance was overestimated when visual stringent endpoint was used, although to a lesser extent than in the case of voriconazole (4.1% vs 4.4%). Categorical agreement was 97.2%. Major errors were found in *A. fumigatus sensu stricto* isolates (n=22) and in two isolates of cryptic species (*N. tsurutae* and *A. fumigatiaffinis*; Table 2S). Similarly, most misclassifications (23/24 isolates) were associated to MIC results falling in the ATU and mostly affected isolates in which major errors for voriconazole were detected (21/24 isolates). Since stringent visual readings shifted azole MICs to higher values, no very major errors were found.

Discussion

In this study we show that MICs of azoles and amphotericin B against *A. fumigatus* obtained either by spectrophotometric or regular visual readings have very high essential and categorical agreement.

154 The increase of resistant *A. fumigatus* isolates worldwide has promoted antifungal
155 susceptibility testing (5). Azole resistance in *A. fumigatus* may occur during azole
156 therapy or exposure to azole fungicides in the environment (4). Furthermore, cryptic
157 species commonly show intrinsic resistance to amphotericin B and azoles (3). Although
158 the EUCAST EDef 9.3.2 procedure recommends visual inspection for azole and
159 amphotericin B MIC setting against *Aspergillus* species, spectrophotometric readings
160 may offer objectivity, quick automated readings, and overall better performance.
161 Previous studies comparing spectrophotometric and visual readings showed excellent
162 essential (92%-97%) and categorical (93-99%) agreements (11-14). Some of the studies
163 used the CLSI methodology and were undermined by the limited number of *A.*
164 *fumigatus sensu stricto* tested isolates (up to 133 isolates), the absence of both cryptic
165 species isolates and *cyp51A* mutants, and a low number of studied antifungal
166 drugs(amphotericin B and itraconazole) (12-14). One of the studies, in which the EUCAST
167 method was used, included the four anti-mold triazoles (itraconazole, posaconazole,
168 voriconazole, and isavuconazole) and a low number of *A. fumigatus sensu stricto*
169 isolates (n= 88). The work did not assess cryptic species, although 15 isolates with
170 *cyp*51A mutations including isolates with the dominant substitutions TR_{34}/L98H, G54,
171 M220, among others, were examined. Furthermore, since EUCAST has recently changed
172 azole breakpoints against *Aspergillus fumigatus sensu lato*, a validation of
173 spectrophotometric readings including a large number of isolates classified according to
174 the updated EUCAST breakpoints is needed.

175 We recently conducted a survey of azole resistance in *A. fumigatus sensu lato*
176 isolates collected in Spain in 2019 (15). Taking advantage of the large number of isolates
177 collected (n=847), we obtained and compared MICs using visual and spectrophotometric

readings. Nineteen strains were identified as cryptic species and 45 *A. fumigatus sensu stricto* proved to be azole resistant, being TR_{34}-L98H the dominant mechanism of resistance. Both MIC endpoints show high essential/categorical agreements for amphotericin B (98.8/100), itraconazole (94.8%/100%), posaconazole (97.3%/100%), voriconazole (98.3%/98.7%), and isavuconazole (96.1%/99.3%). No errors were found in amphotericin B, itraconazole, and posaconazole. Most misclassifications for voriconazole and isavuconazole are linked with MIC results falling either in ATU (10/12 isolates) or in just one two-fold dilutions above the breakpoint (2/12 isolates; MIC = 4 mg/L). Cross-resistance between voriconazole and isavuconazole is the norm in *A. fumigatus senso stricto* (16). Using voriconazole as a surrogate marker, spectrophotometric readings resulted in misdetection of voriconazole resistance in six *A. fumigatus senso stricto* isolates with either a wild-type *cyp51A* gene or genetic polymorphisms of dubious clinical implications (Table 1S).

The EUCAST has recently reviewed the antifungal breakpoints against *A. fumigatus sensu lato*. Breakpoints for amphotericin B, itraconazole, voriconazole, and posaconazole were lowered, while for isavuconazole it was increased (17). Based on the updated breakpoints, spectrophotometric MIC readings led to correctly classify all isolates with relevant *cyp51A* mutations as resistant. Interpretation uncertainties regarding MIC values may arise in the ATU, a newly introduced term, when the breakpoint of wild-type isolates and mutant isolates converge (17). Isolates with posaconazole and isavuconazole MICs of 0.25 mg/L and 2 mg/L, respectively, cannot be automatically reported as susceptible or resistant. MIC determinations using spectrophotometric readings frequently led to the underestimation of resistance for MIC values falling in the ATU. Here, we were able to easily clarify misclassifications by

visually inspecting the tray. False resistance was detected in four *A. fumigatus sensu stricto* isolates for which a voriconazole MIC of 2 mg/L was determined by spectrophotometric readings.

Higher mortality rate is observed in patients infected with azole-resistant *A. fumigatus sensu lato* isolates. Resistance is frequently caused by mutations in the *cyp51A* gene, some of which are associated with a pan-triazole-resistant phenotype (high-level resistance) (8). Some phenotypes only affect the activity of a single azole, or several triazoles with similar molecular structure, and the MIC is close to the clinical breakpoint, resulting in low-level resistance (18). Previous studies have shown that patients infected with low-level voriconazole-resistant *A. fumigatus* (MIC=2 mg) and low-level isavuconazole-resistant *A. fumigatus* (MIC=2 mg) may be treated with voriconazole or isavuconazole, respectively, provided that higher doses are administered (19, 20). In cryptic species, very major errors in voriconazole and isavuconazole were detected (Table 1S).

Visual MIC readings may be challenging and taking small colonies into account (stringent visual readings) may result in overestimation of resistance rates and increase the MIC of the isolates one or two two-fold dilutions, particularly for voriconazole. Thus, major errors in voriconazole and isavuconazole (MIC results falling in the ATU) against *A. fumigatus sensu stricto* may be detected. Correct classification of relevant *cyp51A* gene mutants was achieved by stringent visual readings.

We conclude that spectrophotometric determination is a useful alternative to visual inspection of azole and amphotericin MICs against *A. fumigatus sensu stricto*. Both endpoints show high essential and categorical agreements. Future studies

including more isolates from cryptic species and *A. fumigatus sensu stricto* with other kind of *cyp51A* mutations are warranted.

Materials and methods

Samples. Eight hundred and forty-seven *A. fumigatus sensu lato* clinical isolates, identified by MALDI-TOF, were collected in a 30-hospital survey conducted in Spain (15). Azole-resistant isolates (n=45 *A. fumigatus sensu stricto* and n=19 cryptic species) were molecularly identified. Isolate distribution as per species identification was as follows: *A. fumigatus sensu stricto* (n=828), *A. lentulus* (n=6), *A. fumigatiaffinis* (n=5), *Neosartorya tsurutae* (n=3), *N. udagawae* (n=2), *A. novofumigatus* (n=2), and *A. thermomutatus* (n=1).

The *cyp51A* gene sequence from 45 azole-resistant *A. fumigatus sensu stricto* isolates carried the following mutations: TR_{34}-L98H (n=24), G54R (n=5), TR_{46}/Y121F/T289A (n=1), F46Y/M172V/N248T/D255E/E427K (n=2), F46Y/M172V/N248T/D255E/E416Q/E427K (n=1), F165L (n=1), S496L (n=1), and wild-type *cyp51A* gene (n=10).

EUCAST Antifungal susceptibility testing. All isolates were subcultured on potato dextrose agar or Sabouraud dextrose agar and incubated at 35°C for 2 to 5 days. Isolates from cryptic species were incubated long enough to assure filtered conidia suspensions reaching a sufficient inoculum (equivalent to McFarland 0.5 using a spectrophotometer). Isolate antifungal susceptibilities to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole were determined following the EUCAST EDef 9.3.2 procedure (21). The inoculated trays were incubated for 48 hours at 35 ºC and MICs

obtained using a visual endpoint (defined as the concentration that completely inhibits fungal growth) and a spectrophotometric endpoint (≥ 95% inhibition of fungal growth compared to the drug-free control and read at 540 nm, as described elsewhere) (11). Although the EUCAST EDef 9.3.2 procedure recommends ignoring single colonies on the surface, sometimes it is difficult to discern real growth from small colonies. Thus, we interpreted visual MICs using two endpoints: regular endpoint (very tiny growth was disregarded) or stringent endpoint (a totally clear well), as exemplified in Figure 1. Quality control (QC) was ensured by testing the *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 strains (amphotericin B, itraconazole, voriconazole, and posaconazole), and *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 (isavuconazole).

Data analysis. Regular visual endpoint MICs were assumed as the gold standards and compared against MICs obtained by other endpoints; MICs (percentage) within ±1 two-fold dilutions were considered to be in essential agreement. Isolates were classified as resistant/non-wild-type according to the updated 2020 EUCAST breakpoints (Table 1); intermediate category for amphotericin B and azoles and the category of "susceptible increased exposure" are no longer available, and the term area of technical uncertainty (ATU) for the four azoles has been defined (10). ATU is a warning to laboratories on an uncertainty needing attention before reporting the results and represents an area of confluence of both wild-type and mutant isolates particularly for voriconazole, posaconazole, and isavuconazole. MIC results in the ATU were interpreted as follows: itraconazole and voriconazole (always resistant), posaconazole (resistant only if the isolate was also resistant to itraconazole), isavuconazole (resistant only if the isolate was also resistant to voriconazole). Categorical agreement between the three endpoints was assessed. The endpoints were in categorical agreement when the results were in the

same susceptibility category (regardless of the MIC). Errors were defined as very major errors (false susceptibility) when the gold standard endpoint classified an isolate as resistant and the other endpoints as susceptible, and as major errors (false resistance) when the gold standard endpoint classified an isolate as susceptible and the other endpoints as resistant (22).

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Tables and figures

Table 1. Azole and amphotericin B breakpoints chosen to classify *Aspergillus fumigatus sensu lato* isolates as susceptible, resistant, or non-wild-type (17)

Drug	ECOFF (mg/L)	Clinical breakpoints (mg/L)		
	WT ≤	S ≤	R ≥	ATU
Amphotericin B	1	1	2	ND
Itraconazole	1	1	2	2
Posaconazole	0.25	0.125	0.25	0.25
Voriconazole	1	1	2	2
Isavuconazole	2	1	4	2

ECOFF, epidemiological cut-off value; **WT,** wild-type; **S,** susceptible; **R,** resistant; **ATU,** area of technical uncertainty; **ND,** not defined.
Isolates with itraconazole and voriconazole MICs results that fall in the ATU were always considered resistant; isolates with isavuconazole MICs and posaconazole MICs results that fall in the ATU were considered as resistant when voriconazole-resistant or itraconazole-resistant, respectively.

426 **Table 2.** MIC distributions of amphotericin B against the 847 *A. fumigatus sensu lato* isolates. MIC distributions by regular visual readings and
427 their correspondent rates of resistance are reported elsewhere (15)

428

Amphotericin B	MIC distributions (number of isolates for each MIC, in mg/L)												No. of resistant isolates (%)*
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥ 16	
***A. fumigatus sensu lato* (n=847)**													
Regular visual readings	0	0	1	5	70	407	306	45	**7**	**5**	**1**	**0**	13 (1.5)
Spectrophotometric readings	0	0	0	7	96	452	248	31	**6**	**5**	**0**	**2**	13 (1.5)
***A. fumigatus sensu stricto* (n=828)**													
Regular visual readings	0	0	1	5	68	407	305	42	**0**	**0**	**0**	**0**	0 (0)
Spectrophotometric readings	0	0	0	7	94	452	246	29	**0**	**0**	**0**	**0**	0 (0)
Cryptic species (n=19)													
Regular visual readings	0	0	0	0	2	0	1	3	**7**	**5**	**1**	**0**	13 (68.4)
Spectrophotometric readings	0	0	0	0	2	0	2	2	**6**	**5**	**0**	**2**	13 (68.4)
Isolates with tandem repeats (n=25)													
Regular visual readings	0	0	0	0	2	10	13	0	**0**	**0**	**0**	**0**	0 (0)
Spectrophotometric readings	0	0	0	0	3	10	12	0	**0**	**0**	**0**	**0**	0 (0)

432

437 Underlined values indicate non-wild-type isolates according to tentative ECOFFs and values in bold indicate resistant isolates (EUCAST Breakpoint table v 10.0, 2020).
438 *Identical number of resistant isolates and non-wild-type isolates were obtained
439

Table 3. MIC distributions of itraconazole against the 847 *A. fumigatus sensu lato* isolates. MIC distributions by regular visual readings and their correspondent rates of resistance are reported elsewhere (15)

Itraconazole	MIC distributions (number of isolates for each MIC, in mg/L)												No. of resistant isolates (%) *
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥ 16	
***A. fumigatus sensu lato* (n=847)**													
Regular visual readings	0	0	0	0	26	427	328	21	2	**2**	**2**	**39**	45 (5.3)
Stringent visual readings	0	0	0	0	10	269	459	59	2	**2**	**2**	**44**	50 (5.9)
Spectrophotometric readings	0	0	15	22	49	412	287	17	3	**1**	**1**	**40**	45 (5.3)
***A. fumigatus sensu stricto* (n=828)**													
Regular visual readings	0	0	0	0	26	426	326	15	2	**1**	**1**	**31**	35 (4.2)
Stringent visual readings	0	0	0	0	10	269	457	57	2	**1**	**1**	**31**	35 (4.2)
Spectrophotometric readings	0	0	15	22	49	410	282	15	3	**0**	**1**	**31**	35 (4.2)
Cryptic species (n=19)													
Regular visual readings	0	0	0	0	0	1	2	6	0	**1**	**1**	**8**	10 (52.6)
Stringent visual readings	0	0	0	0	0	0	2	2	0	**1**	**1**	**13**	15 (78.9)
Spectrophotometric readings	0	0	0	0	0	2	5	2	0	**1**	**0**	**9**	10 (52.6)
Isolates with tandem repeats (n=25)													
Regular visual readings	0	0	0	0	0	0	1	0	0	**0**	**0**	**24**	24 (96)
Stringent visual readings	0	0	0	0	0	0	0	1	0	**0**	**0**	**24**	24 (96)
Spectrophotometric readings	0	0	0	0	0	0	1	0	0	**0**	**0**	**24**	24 (96)

Values shaded in grey indicate MICs in the area of technical uncertainty (ATU) and were classified as resistant isolates. Underlined values indicate non-wild-type isolates according to tentative ECOFFs and values in bold indicate resistant isolates (EUCAST Breakpoint table v 10.0, 2020).

*Identical number of resistant isolates and non-wild-type isolates were obtained.

459 **Table 4**. MIC distributions of posaconazole against the 847 *A. fumigatus sensu lato* isolates. MIC distributions by regular visual readings and
460 their correspondent rates of resistance or non-wild-type isolates are reported elsewhere (15)

Posaconazole	MIC distributions (number of isolates for each MIC, in mg/L)												No. of isolates (%)	
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥ 16	Resistant	Non-wild-type
***A. fumigatus sensu lato* (n=847)**														
Regular visual readings	0	2	46	441	279	42	**27**	**3**	**0**	**1**	**0**	**6**	46 (5.4)	37 (4.4)
Stringent visual readings	0	1	18	268	399	119	**23**	**12**	**0**	**1**	**0**	**6**	51 (6)	42 (5)
Spectrophotometric readings	1	1	70	476	225	36	**28**	**4**	**1**	**1**	**0**	**4**	47 (5.5)	38 (4.5)
***A. fumigatus sensu stricto* (n=828)**														
Regular visual readings	0	2	46	440	278	32	**20**	**3**	**0**	**1**	**0**	**6**	34 (4.1)	30 (3.6)
Stringent visual readings	0	1	18	268	398	111	**13**	**12**	**0**	**1**	**0**	**6**	34 (4.1)	32 (3.9)
Spectrophotometric readings	1	1	70	475	222	27	**22**	**4**	**1**	**1**	**0**	**4**	34 (4.1)	32 (3.9)
Cryptic species (n=19)														
Regular visual readings	0	0	0	1	1	10	**7**	**0**	**0**	**0**	**0**	**0**	12 (63.1)	7 (36.8)
Stringent visual readings	0	0	0	0	1	8	**10**	**0**	**0**	**0**	**0**	**0**	17 (89.5)	10 (52.6)
Spectrophotometric readings	0	0	0	1	3	9	**6**	**0**	**0**	**0**	**0**	**0**	12 (63.2)	6 (31.6)
Isolates with tandem repeats (n=25)														
Regular visual readings	0	0	0	0	0	3	**18**	**2**	**0**	**0**	**0**	**2**	24 (96)	22 (88)
Stringent visual readings	0	0	0	0	0	1	**11**	**11**	**0**	**0**	**0**	**2**	24 (96)	24 (96)
Spectrophotometric readings	0	0	0	0	0	1	**19**	**4**	**1**	**0**	**0**	**0**	24 (96)	24 (96)

461
462 Values shaded in grey indicate MICs in the area of technical uncertainty (ATU). MIC results against *A. fumigatus sensu lato* falling in the ATU were translated to resistant as
463 follows: regular visual readings (n=9/42), stringent visual readings (n=9/119), and spectrophotometric readings (n=9/36). Underlined values indicate non-wild-type isolates
464 according to tentative ECOFFs and values in bold indicate resistant isolates (EUCAST Breakpoint table v 10.0, 2020).
465

Table 5. MIC distributions of voriconazole against the 847 *A. fumigatus sensu lato* isolates. MIC distributions by regular visual readings and their correspondent rates of resistance are reported elsewhere (15)

Voriconazole	MIC distributions (number of isolates for each MIC, in mg/L)												No. of resistant isolates (%) *
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	
***A. fumigatus sensu lato* (n=847)**													
Visual reading	0	0	0	0	3	82	529	177	**19**	**27**	**7**	**3**	56 (6.6)
Stringent visual readings	0	0	0	0	1	17	302	394	**87**	**31**	**11**	**4**	133 (15.7)
Spectrophotometric readings	0	0	0	0	4	138	500	154	**18**	**24**	**6**	**3**	51 (6)
***A. fumigatus* sensu stricto (n=828)**													
Visual reading	0	0	0	0	3	82	529	176	**13**	**19**	**3**	**3**	38 (4.6)
Stringent visual readings	0	0	0	0	1	17	302	393	**86**	**18**	**7**	**4**	115 (13.9)
Spectrophotometric readings	0	0	0	0	4	138	498	153	**11**	**18**	**4**	**2**	35 (4.2)
Cryptic species (n=19)													
Visual reading	0	0	0	0	0	0	0	1	**6**	**8**	**4**	**0**	18 (94.7)
Stringent visual readings	0	0	0	0	0	0	0	1	**1**	**13**	**4**	**0**	18 (94.7)
Spectrophotometric readings	0	0	0	0	0	0	2	1	**7**	**6**	**2**	**1**	16 (84.2)
Isolates with tandem repeats (n=25)													
Visual reading	0	0	0	0	0	0	0	0	**5**	**15**	**2**	**3**	25 (100)
Stringent visual readings	0	0	0	0	0	0	0	0	**1**	**14**	**7**	**3**	25 (100)
Spectrophotometric readings	0	0	0	0	0	0	0	0	**6**	**14**	**3**	**2**	25 (100)

Values shaded in grey indicate MICs in the area of technical uncertainty (ATU) and were classified as resistant isolates. Underlined values indicate non-wild-type isolates according to tentative ECOFFs and values in bold indicate resistant isolates (EUCAST Breakpoints table v 10.0, 2020).

*The number of resistant isolates and non-wild-type isolates were identical.

Table 6. MIC distributions of isavuconazole against the 847 *A. fumigatus sensu lato* isolates. MIC distributions by regular visual readings and their correspondent rates of resistance/non wild-type isolates were reported elsewhere (15)

Isavuconazole	MIC distributions (number of isolates for each MIC, in mg/L)												No. of isolates (%)	
	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	≥16	Resistant	Non-wild-type
***A. fumigatus sensu lato* (n=847)**														
Regular visual readings	0	0	0	0	0	13	440	333	26	**14**	**17**	**4**	48 (5.6)	35 (4.1)
Stringent visual readings	0	0	0	0	0	2	146	572	90	**11**	**21**	**5**	72 (8.5)	37 (4.4)
Spectrophotometric readings	0	0	0	17	4	14	434	314	31	**12**	**18**	**3**	42 (5)	33 (3.9)
***A. fumigatus sensu stricto* (n=828)**														
Regular visual readings	0	0	0	0	0	13	440	327	18	**10**	**16**	**4**	35 (4.2)	30 (3.6)
Stringent visual readings	0	0	0	0	0	2	146	568	80	**7**	**20**	**5**	57 (6.9)	32 (3.9)
Spectrophotometric readings	0	0	0	17	4	14	433	306	24	**9**	**18**	**3**	32(3.9)	30 (3.6)
Cryptic species (n=19)														
Regular visual readings	0	0	0	0	0	0	0	6	8	**4**	**1**	**0**	13 (68.4)	5 (26.3)
Stringent visual readings	0	0	0	0	0	0	0	4	10	**4**	**1**	**0**	15 (78.9)	5 (26.3)
Spectrophotometric readings	0	0	0	0	0	0	1	8	7	**3**	**0**	**0**	10 (52.6)	3 (15.8)
Isolates with tandem repeats (n=25)														
Regular visual readings	0	0	0	0	0	0	0	0	1	**6**	**14**	**4**	25 (100)	24 (96)
Stringent visual readings	0	0	0	0	0	0	0	0	0	**2**	**18**	**5**	25 (100)	25 (100)
Spectrophotometric readings	0	0	0	0	0	0	0	0	0	**7**	**15**	**3**	25 (100)	25 (100)

Values shaded in grey indicate MICs in the area of technical uncertainty (ATU). MIC results against *A. fumigatus sensu lato* falling in the ATU were translated to resistant as follows: regular visual readings (n=13/26), stringent visual readings (n=35/90), and spectrophotometric readings (n=9/31). Underlined values indicate non-wild-type isolates according to tentative ECOFFs and values in bold indicate resistant isolates (EUCAST Breakpoint table v 10.0, 2020)

506 **Table 7.** Essential and categorical agreement between MICs by visual (regular and stringent) and spectrophotometric readings

MIC readings	*A. fumigatus sensu lato* (%)				*A. fumigatus sensu stricto* (%)				Cryptic species (%)			
	Essential agreement*	Categorical agreement*	VME	ME	Essential agreement	Categorical agreement	VME	ME	Essential agreement	Categorical agreement	VME	ME
Amphotericin B												
Regular visual vs spectrophot. readings	98.8	100	0	0	99	100	0	0	89.5	100	0	0
Itraconazole												
Regular visual vs spectrophot. readings	94.8	100	0	0	95.1	100	0	0	78.9	100	0	0
Regular visual vs stringent readings	98.9	99.4	0	0.6	99.6	100	0	0	68.4	73.7	0	26.3
Posaconazole												
Regular visual vs spectrophot. readings	97.3	100	0	0	97.1	100	0	0	100	100	0	0
Regular visual vs stringent readings	98.7	99.4	0	0.6	98.7	100	0	0	100	73.7	0	26.3
Voriconazole												
Regular visual vs spectrophot. readings	98.3	98.7	0.9	0.3	98.4	98.9	0.7	0.4	94.7	89.5	10.5	0
Regular visual vs stringent readings	94.4	90.8	0	9.2	94.3	90.6	0	9.4	100	100	0	0
Isavuconazole												
Regular visual vs spectrophot. readings	96.1	99.3	0.7	0	96.3	99.6	0.4	0	89.5	84.2	15.8	0
Regular visual vs stringent readings	98.6	97.2	0	2.8	98.5	97.3	0	2.7	100	89.5	0	10.5

507

508 *Regular visual endpoint MICs were assumed as the gold standards and compared against MICs obtained by other endpoints; MICs (percentage) within ±1 two-fold
509 dilutions were considered to be in essential agreement. Isolates were classified as resistant/non-wild-type according to the updated 2020 EUCAST breakpoints. The
510 endpoints were in categorical agreement when the results were in the same susceptibility category (regardless of the MIC). VME: very major error (false susceptibility); ME:
511 major error (false resistance).
512

513

514

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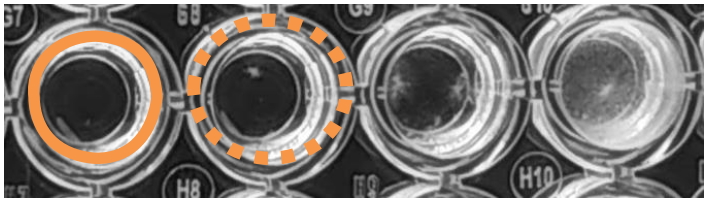
Figure 1. Example of fungal growth of an *A. fumigatus sensu stricto* isolate in the presence of itraconazole (1a), posaconazole (1b), voriconazole (1c), and isavuconazole (1d). Two MIC endpoints were used: the regular endpoint (gold standard) where tiny small colonies were disregarded (wells surrounded by rings of dashed lines) and the stringent endpoint where the tiny colonies were taken into account (wells surrounded by rings of solid lines)

1a

0.5 mg/L 0.25 mg/L 0.125 mg/L

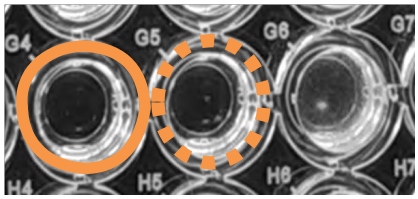
1b

0.125 mg/L 0.06 mg/L 0.03 mg/L 0.015 mg/L



1c

1 mg/L 0.5 mg/L 0.25 mg/L



1d

2 mg/L 1 mg/L 0.5 mg/L 0.25 mg/L 0.125 mg/L