



Evaluation of MIC Strip Isavuconazole Test for Susceptibility Testing of Wild-Type and Non-Wild-Type *Aspergillus fumigatus* Isolates

Maiken Cavling Arendrup,^{a,b,c} Paul Verweij,^d Henrik Vedel Nielsen^a

Unit of Mycology, Statens Serum Institut, Copenhagen, Denmark^a; Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark^b; Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark^c; Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands^d

ABSTRACT We evaluated the MIC Strip Isavuconazole test against EUCAST E.Def 9.3 by using 40 wild-type and 39 *CYP51A* mutant *Aspergillus fumigatus* strains. The strip full inhibition endpoint (FIE) and 80% growth inhibition endpoint were determined by two independent readers, reader 1 (R1) and R2. The essential (within ± 0 , ± 1 , and ± 2 twofold dilutions) and categorical agreements were best with the FIE (for R1/R2, 42%/41%, 75%/73%, and 90%/89% for essential agreement, and 91.1%/92.4% categorical agreement, with 6.3/8.9% very major errors and 0/1.3% major errors, respectively). The MIC Strip Isavuconazole test with the FIE appears to be useful.

KEYWORDS antifungal susceptibility testing, MIC, isavuconazole, gradient strip, EUCAST, *Aspergillus fumigatus*, *Cyp51A* mutants, wild type

Antifungal susceptibility testing of *Aspergillus fumigatus* has become increasingly important with the emergence of azole resistance (1–6). EUCAST has set clinical breakpoints for isavuconazole and *Aspergillus* (7). For *A. fumigatus*, the clinical breakpoint is 1 mg/liter, one step lower than the epidemiological cutoff value (ECOFF) (2 mg/liter) because the pharmacokinetic/pharmacodynamic breakpoint is 1 mg/liter and the MIC ranges for wild-type and resistant mutants overlap. Hence an MIC of 2 mg/liter may represent wild-type isolates as well as isolates with clinically relevant resistance mechanisms (1–3, 5, 8–15). In clinical practice, the adoption of a restrictive clinical breakpoint for interpretation of MICs generated by commercial tests may create a higher risk of misclassification unless the susceptibility test is very well standardized against the reference method and associated with low reader-to-reader and interlaboratory variations. An isavuconazole gradient strip (Etest; AB Biodisk, Solna, Sweden) was previously evaluated but is no longer available (16, 17). Thus, we evaluated the only commercially available isavuconazole susceptibility test, the MIC Strip Isavuconazole test (Liofilchem, Roseto degli Abruzzi, TE, Italy).

Forty wild-type and 39 *CYP51A* mutant *A. fumigatus* isolates with hot-spot alterations involving G54 ($n = 10$), M220 ($n = 10$), TR₃₄/L98H ($n = 9$), and TR₄₆/Y121F T289A ($n = 10$) were included. For the strip test (Liofilchem, Roseto degli Abruzzi, TE, Italy) a McFarland 0.5 conidial suspension and RPMI 1640 2% glucose agar (SSI Diagnostica, Hillerød, Denmark) were used. Strip MICs were read by two independent technicians (reader 1 [R1] and R2) blind to the *CYP51A* genotype at 24 and 48 h of incubation, with an 80% inhibition endpoint (80% IE) and a full inhibition endpoint (FIE). EUCAST testing was performed as previously recommended (7, 18). Four control strains were included (see Table S1 in the

Received 31 July 2016 Returned for modification 12 September 2016 Accepted 26 October 2016

Accepted manuscript posted online 31 October 2016

Citation Arendrup MC, Verweij P, Nielsen HV. 2017. Evaluation of MIC strip isavuconazole test for susceptibility testing of wild-type and non-wild-type *Aspergillus fumigatus* isolates. *Antimicrob Agents Chemother* 61:e01659-16. <https://doi.org/10.1128/AAC.01659-16>.

Copyright © 2016 Arendrup et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Maiken Cavling Arendrup, maca@ssi.dk.

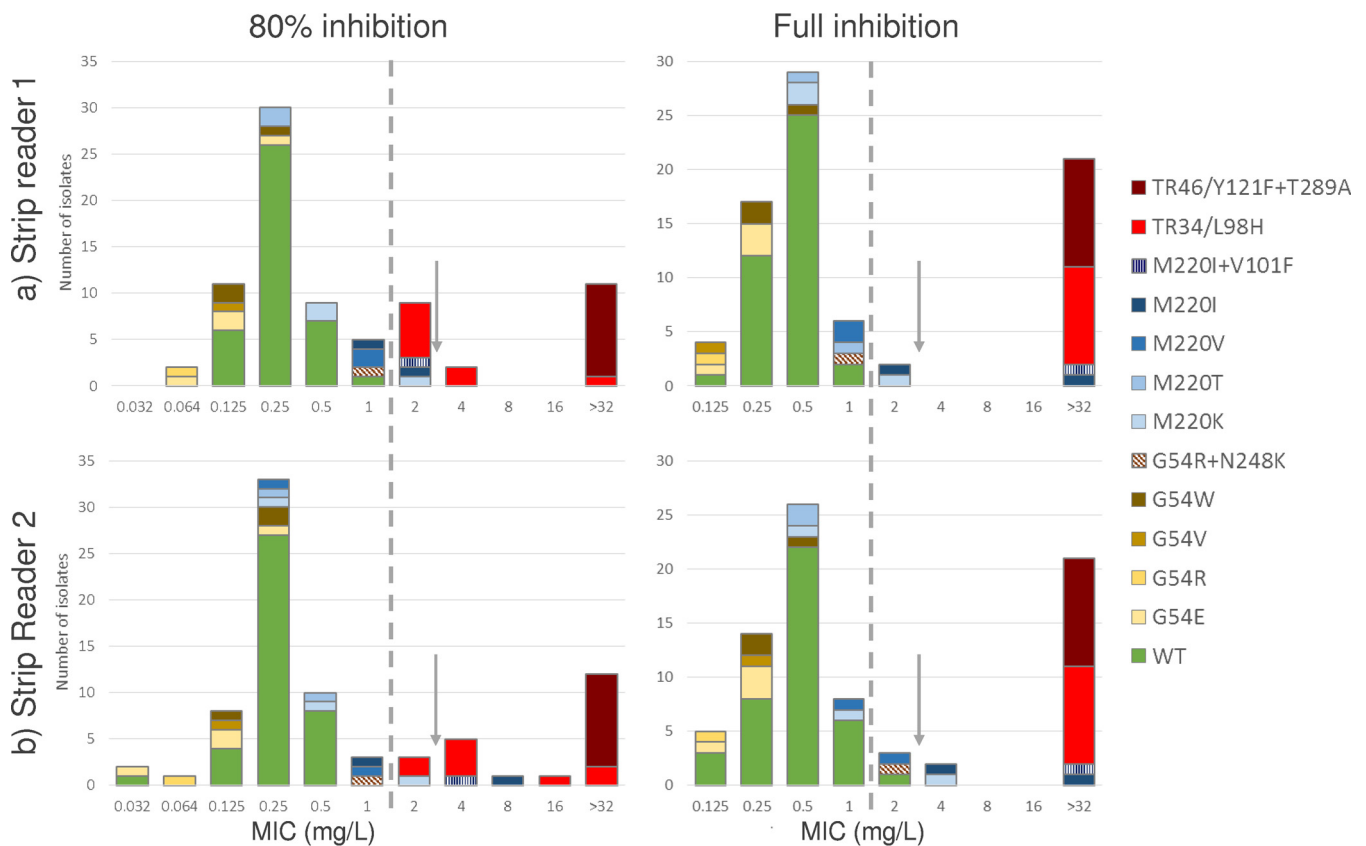


FIG 1 Isavuconazole strip MICs for wild-type and *CYP51A* mutant *A. fumigatus* isolates determined at 80% inhibition (left side) and full inhibition endpoints (right side) and by two independent readers, R1 (top) and R2 (bottom).

supplemental material) (7). The percent essential agreement between the tests was calculated. Isolates for which the MICs were above scale by both methods (EUCAST, >16 mg/liter; strip test, >32 mg/liter) were considered in agreement within ± 0 twofold 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 strip test, and major errors (MEs) were defined as isolate categorization as S by the EUCAST method but R by the strip test.

Most isavuconazole strip MICs were above the recommended ranges for the two control *Candida* strains (Table S1). In contrast, the strip MICs for *A. fumigatus* ATCC 204305 and *A. flavus* CM1813 were within ± 1 twofold dilution of the EUCAST MICs, suggesting better agreement for the *Aspergillus* strains and best when using the FIE for *Aspergillus*.

Nine isolates (11.4%) failed to grow sufficiently well to allow strip MIC reading on day 1, when, in general, zones were fuzzy and difficult to read. Day 2 MICs were lower with the 80% IE than with the FIE (Fig. 1). This was particularly evident for isolates harboring TR₃₄/L98H alterations, for which the modal 80% IE MICs were 2 and 4 mg/liter, respectively but >32 mg/liter for both readers with the FIE. The essential agreement between the strip MICs from the two readers was highest, 97% at ± 1 twofold dilution and 100% at ± 2 twofold dilutions, when using the FIE (Table 1).

Isavuconazole MICs for isolates with wild-type *CYP51A* or single alterations at the G54 codon were all below the EUCAST ECOFF for the strip test with the FIE, as well as for EUCAST (Table 2). Likewise, the MICs for isolates harboring M220I alterations or TR₃₄/L98H or TR₄₆/Y121F T289A were all above the clinical breakpoint for both methods when the FIE was used for the strip test. However, the MICs for TR₃₄/L98H isolates were higher when determined by the strip test (MIC range, >32 mg/liter)

TABLE 1 Essential agreement between R1 and R2 of MIC Strip Isavuconazole test and between strip and EUCAST MICs

CYP51A profile	n	Strip MIC agreement (%) ^a between R1 and R2						Agreement (%) ^a between Strip MIC and EUCAST MIC											
		R1 vs R2, FIE			R1 vs R2, 80% IE			R1 FIE vs EUCAST			R2 FIE vs EUCAST			R1 80% IE vs EUCAST			R2 80% IE vs EUCAST		
		±0 ^b	±1	±2	±0	±1	±2	±0	±1	±2	±0	±1	±2	±0	±1	±2	±0	±1	±2
Wild type	40	55	95	100	83	98	98	38	80	98	28	78	95	18	63	88	15	63	88
G54 alterations																			
G54E	4	100	100	100	75	100	100	75	100	100	75	100	100	0	100	100	0	75	100
G54R	1	100	100	100	100	100	100	0	0	100	0	0	100	0	0	0	0	0	0
G54V	1	0	100	100	100	100	100	0	100	100	100	100	100	0	100	100	0	100	100
G54W	3	100	100	100	67	100	100	33	100	100	33	100	100	67	100	100	100	100	100
G54R N248K	1	0	100	100	100	100	100	0	100	100	100	100	100	0	100	100	0	100	100
M220 alterations																			
M220K	3	33	100	100	67	100	100	0	100	100	67	100	100	0	100	100	0	67	100
M220T	2	50	100	100	50	100	100	100	100	100	50	100	100	0	50	100	50	50	100
M220V	2	50	100	100	50	50	100	0	50	100	50	50	100	0	50	100	0	50	50
M220I	2	50	100	100	50	50	100	50	50	50	0	50	50	0	50	100	50	100	100
M220I V101F	1	100	100	100	0	100	100	0	0	100	0	0	100	0	0	0	0	0	100
Mutants with TR ^c																			
TR ₃₄ /L98H	9	100	100	100	33	78	89	11	11	33	11	11	33	11	22	100	33	67	100
TR ₄₆ /Y121F T289A	10	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
All isolates	79	68	97	100	75	94	97	42	75	90	41	73	89	25	66	91	30	70	91

^aPercent essential agreement within ±0, ±1, and ±2 twofold dilutions, respectively. MICs were read after 2 days of incubation. Strip MICs were read by using the FIE or 80% IE endpoint.

^bNumber of twofold dilutions.

^cTR, tandem repeat in the *CYP51A* promoter region.

than when determined by EUCAST (MIC₅₀ of 8 mg/liter; range, 4 to >16 mg/liter) (Table 2). The overall essential agreement between strip MICs and EUCAST MICs within ±0, ±1, and ±2 twofold dilutions was best when using the FIE (R1/R2: 42/41, 75/73, and 90/89%) than when using the 80% IE (R1/R2: 25/30, 66/70, and 91/91%). At least 95% essential agreement between the strip test and EUCAST within ±2 twofold dilutions was seen for all *CYP51A* genotypes except those harboring the TR₃₄/L98H mechanism or the M220I alteration. Similarly, the categorical agreement was better for the FIE reading of the strip test (91.1 to 92.4% with 6.3 to 8.9% VMEs and 0 to 1.3% MEs) than for the 80% IE (89.9% with 10.1% VMEs and 0% MEs for both readers). VMEs included four isolates with the wild-type *CYP51A* genotype and one to four isolates harboring M220V, M220I, or G54R N248K alterations, respectively.

The MIC Strip Isavuconazole test manufacturer recommends an 80% IE reading, but in this study, higher interreader essential agreement, better separation between wild-type and resistant strains, and greater essential and categorical agreement compared to EUCAST results were achieved with the FIE. Thus, the FIE criterion was found to be superior although the MICs for the recommended *Candida* control strains were out of range (7). When using the FIE, the essential agreements with EUCAST within ±1 and ±2 twofold dilutions were 73 to 75% and 89 to 90% and thus better than previously found for the isavuconazole Etest versus the CLSI method, even though challenged here with a strain collection including a significant number of non-wild-type isolates (16). The categorical agreement was >91% when interpreting the MICs according to EUCAST breakpoints, and notably, among the 6 to 9% VMEs, half were isolates with a wild-type *CYP51A* target gene that 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 (7). Finally, the separation between wild-type and TR₃₄/L98H and TR₄₆/Y121F T289A mutant isolates

TABLE 2 Isavuconazole susceptibility of wild-type and *CYP51A* mutant *A. fumigatus* isolates determined by strip test^a and EUCAST E.Def 9.3

CYP51A profile ^a	No. of isolates	EUCAST E.Def 9.3			Gradient strip R1				Gradient strip R2				
		MIC ₅₀ ^b	MIC range	% >ECOFF ^c	% R	MIC ₅₀ ^b	MIC range	% >ECOFF	% R	MIC ₅₀ ^b	MIC range	% >ECOFF	% R
Wild type	40	0.5	0.25–2	0	10	0.5	0.125–1	0	0	0.5	0.125–2	0	3
G54 alterations													
G54E	4		0.125–0.25	0	0		0.125–0.25	0	0		0.125–0.25	0	0
G54R	1		0.5	0	0		0.125	0	0		0.125	0	0
G54V	1		0.25	0	0		0.125	0	0		0.25	0	0
G54W	3		0.125–0.25	0	0		0.25–0.5	0	0		0.25–0.5	0	0
G54R N248K	1		2	0	100		1	0	0		2	0	100
M220alterations													
M220K	3		1–4	33	33		0.5–2	0	33		0.5–1	33	33
M220T	2		0.5–1	0	0		0.5–1	0	0		0.5	0	0
M220V	2		2–4	50	100		1	0	0		1–2	0	50
M220I	2		2–8	50	100		2 to >32	50	100		4 to >32	100	100
M220I V101F	1		16	100	100		>32	100	100		>32	100	100
Mutants with TR ^d													
TR ₃₄ /L98H	9		4 to >16	100	100		>32	100	100		>32	100	100
TR ₄₆ /Y121F T289A	10		>16	>16	100		>32	>32	100		>32	>32	100
All isolates	79	1	0.125 to >16	29	38	0.5	0.125 to >32	27	29	0.5	0.125 to >32	29	33

^aPlates were read by two independent readers using the FIE endpoint after 2 days of incubation.

^bMIC₅₀ (mg/liter) are presented only for genotypes represented by ≥10 isolates.

^cPercentage of isolates with MICs above the EUCAST isavuconazole ECOFF (2 mg/liter) and clinical (1 mg/liter) breakpoints.

^dTR, tandem repeat in the *CYP51A* promoter region.

was greater for the MIC strip test, rendering it a potentially promising routine lab tool for detecting R environmental mutants, provided the FIE is used (2, 5, 19–21).

The *CYP51A* amino acid alterations have been associated with a codon-specific susceptibility pattern (4, 13). Here, both the strip and EUCAST isavuconazole MICs indeed straddled the clinical breakpoint for isolates harboring M220 alterations and for the G54R N24K double mutant, which will inevitably lead to the random classification of such isolates as S or R in routine testing. Hence, as long as clinical outcome data are unavailable for such mutants, other measures such as itraconazole MIC testing or *CYP51A* sequencing should be undertaken to detect these genotypes.

This study has limitations. We investigated strip test reader-to-reader agreement but no other factors associated with variation, such as variation across different lots and brands of RPMI agar plates, inoculum preparation, etc. Therefore, the promising performance reported here needs confirmation in a multicenter study.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01659-16>.

TEXT S1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

Isavuconazole MIC strips and the pure substance were kindly provided at no cost by Basilea. We thank Birgit Brandt and Désiré Mageme Nahimana for excellent technical assistance.

Maiken Cavling Arendrup has received research grants or speaker honoraria from Astellas, Basilea, Gilead, MSD, Novartis, Pfizer, and T2Biosystems. She is the current chairman of the EUCAST-AFST and has previously served on advisory boards for MSD (until 2014) and Pfizer (until 2012). Paul Verweij has received research grants from Astellas, Basilea, F2G, Gilead Sciences, Merck, and Pfizer; has been a consultant to Basilea, F2G, Gilead Sciences, Merck, and Pfizer; and has received speaker's fees from Basilea, Gilead Sciences, and Merck. Henrik Vedel Nielsen has no conflicts to declare.

REFERENCES

- Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW. 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. *J Antimicrob Chemother* 65:1–5. <https://doi.org/10.1093/jac/dkq279>.
- Astvad KM, Jensen RH, Hassan TM, Mathiasen EG, Thomsen GM, Pedersen UG, Christensen M, Hilberg O, Arendrup MC. 2014. First detection of TR46/Y121F T289A and TR34/L98H alterations in *Aspergillus fumigatus* isolates from azole-naïve patients in Denmark despite negative findings in the environment. *Antimicrob Agents Chemother* 58:5096–5101. <https://doi.org/10.1128/AAC.02855-14>.
- Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, Leatherbarrow H, Mellado E, Arendrup MC. 2011. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. *J Clin Microbiol* 49:2243–2251. <https://doi.org/10.1128/JCM.00213-11>.
- Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Alessandro C, Laverdiere M, Arendrup MC, Perlin DS, Denning DW. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg Infect Dis* 15:1068–1076. <https://doi.org/10.3201/eid1507.090043>.
- Verweij PE, Chowdhary A, Melchers WJ, Meis JF. 2016. Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? *Clin Infect Dis* 62:362–368. <https://doi.org/10.1093/cid/civ885>.
- van der Linden JWM, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, Chryssanthou E, Mellado E, Kidd SE, Tortorano AM, Dannaoui E, Gaustad P, Baddley JW, Uekötter A, Lass-Flörl C, Klimko N, Moore CB, Denning DW, Pasqualotto AC, Kibbler C, Arikian-Akdaglı S, Andes D, Meletiadiis J, Naumiuk L, Nucci M, Melchers WJ, Verweij PE. 2015. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg Infect Dis* 21:1041–1044. <https://doi.org/10.3201/eid2106.140717>.
- Arendrup MC, Meletiadiis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, Howard SJ, Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). 2016. EUCAST technical note on isavuconazole breakpoints for *Aspergillus*, itraconazole breakpoints for *Candida* and updates for the antifungal susceptibility testing method documents. *Clin Microbiol Infect* 22:571.e1-4. <https://doi.org/10.1016/j.cmi.2016.01.017>.
- Howard SJ, Lass-Flörl C, Cuenca-Estrella M, Gomez-Lopez A, Arendrup MC. 2013. Determination of isavuconazole susceptibility of *Aspergillus* and *Candida* species by the EUCAST method. *Antimicrob Agents Chemother* 57:5426–5431. <https://doi.org/10.1128/AAC.01111-13>.
- Kidd SE, Goeman E, Meis JF, Slavina MA, Verweij PE. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. *Mycoses* 58:350–355. <https://doi.org/10.1111/myc.12324>.
- Wu C-J, Wang H-C, Lee J-C, Lo H-J, Dai C-T, Chou P-H, Ko W-C, Chen Y-C. 2015. Azole-resistant *Aspergillus fumigatus* isolates carrying TR34/L98H mutations in Taiwan. *Mycoses* 58:544–549. <https://doi.org/10.1111/myc.12354>.
- Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DI, Sanders C, Fan H, Fothergill AW, Sutton DA. 2016. First detection of TR34 L98H and TR46 Y121F T289A Cyp51 mutations in *Aspergillus fumigatus* isolates in the United States. *J Clin Microbiol* 54:168–171. <https://doi.org/10.1128/JCM.02478-15>.
- Jensen RH, Hagen F, Astvad KM, Tyrone A, Meis JF, Arendrup MC. 2016. Azole resistant *Aspergillus fumigatus* in Denmark: a laboratory based study on resistance mechanisms and genotypes. *Clin Microbiol Infect* 22:570.e1-9. <https://doi.org/10.1016/j.cmi.2016.04.001>.
- Stensvold CR, Jørgensen LN, Arendrup MC. 2012. Azole-resistant invasive aspergillosis: relationship to agriculture. *Curr Fungal Infect Rep* 6:178–191. <https://doi.org/10.1007/s12281-012-0097-7>.
- Arendrup MC, Mavridou E, Mortensen KL, Snelders E, Frimodt-Møller N, Khan H, Melchers WJ, Verweij PE. 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS One* 5:e10080. <https://doi.org/10.1371/journal.pone.0010080>.
- Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ, Verweij PE. 2009. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol* 75:4053–4057. <https://doi.org/10.1128/AEM.00231-09>.
- Guinea J, Peláez T, Recio S, Torres-Narbona M, Bouza E. 2008. In vitro antifungal activities of isavuconazole (BAL4815), voriconazole, and fluconazole against 1,007 isolates of zygomycete, *Candida*, *Aspergillus*, *Fusarium*, and *Scedosporium* species. *Antimicrob Agents Chemother* 52:1396–1400. <https://doi.org/10.1128/AAC.01512-07>.
- Thompson GR, Fothergill AW, Wiederhold NP, Vallor AC, Wickes BL, Patterson TF. 2008. Evaluation of Etest method for determining isavuconazole MICs for *Cryptococcus gattii* and *Cryptococcus neoformans*. *Antimicrob Agents Chemother* 52:2959–2961. <https://doi.org/10.1128/AAC.00646-08>.
- Arendrup MC, Howard S, Lass-Flörl C, Mouton JW, Meletiadiis J, Cuenca-Estrella M. 2014. EUCAST testing of isavuconazole susceptibility in *Aspergillus*: comparison of results for inoculum standardization using conidium counting versus optical density. *Antimicrob Agents Chemother* 58:6432–6436. <https://doi.org/10.1128/AAC.03779-14>.
- van der Linden JW, Snelders E, Kampinga GA, Rijnders BJA, Mattsson E, Debets-Ossenkopp YJ, Kuijper EJ, van Tiel FH, Melchers WJ, Verweij PE. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007–2009. *Emerg Infect Dis* 17:1846–1854. <https://doi.org/10.3201/eid1710.110226>.
- Steinmann J, Hamprecht A, Vehreschild MJ, Cornely OA, Buchheidt D, Spiess B, Koldehoff M, Buer J, Meis JF, Rath PM. 2015. Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. *J Antimicrob Chemother* 70:1522–1526. <https://doi.org/10.1093/jac/dku566>.
- Verweij PE, Mellado E, Melchers WJ. 2007. Multiple-triazole-resistant aspergillosis. *N Engl J Med* 356:1481–1483. <https://doi.org/10.1056/NEJMc061720>.