Development and Validation of a High-Resolution Melting Assay To Detect Azole Resistance in Aspergillus fumigatus

1 Title

2 **Development and validation of a High Resolution Melting Assay to**

3 **detect azole resistance in *Aspergillus fumigatus***

4

5 Bernal-Martínez L‡[(1,2)], Gil H‡[(1, 3)], Rivero-Menéndez O[(1,2)], Gago S[(1)], Cuenca-Estrella

6 M[(1,2)], Mellado E[(1,2)] and Alastruey-Izquierdo A*[(1,2)]

7 [(1)] Mycology Reference Laboratory, National Centre for Microbiology. Instituto de Salud

8 Carlos III. Majadahonda, Madrid, Spain. [(2)] Spanish Network for the Research in

9 Infectious Diseases (REIPI RD12/0015), Instituto de Salud Carlos III, Madrid, Spain. [(3)]

10 European Program for Public health Microbiology Training (EUPHEM), European Centre

11 for Disease Prevention and Control, ECDC, Stockholm, Sweden

12 ‡ Both authors have contributed equally

13 *Corresponding author**:**

14 Ana Alastruey-Izquierdo

15 Mycology Reference Laboratory.

16 Centro Nacional de Microbiología. Instituto de Salud Carlos III

17 28220. Majadahonda. Madrid. Spain.

18 Phone: +34-91 822 3784

19 Fax: 91 509 7966

20 *E-mail*: anaalastruey@isciii.es

21 **Running title:** HRM assay for azole resistance detection in *A. fumigatus*

22 **Keywords:** Antifungal resistance, Azoles, HRM, *cyp*51A, Aspergillosis

ABSTRACT

The global emergence of azole resistant *Aspergillus fumigatus* strains is a growing public health concern. Different patterns of azole resistance are linked to mutations in *cyp*51A. Therefore, an accurate characterization of the mechanisms underlying azole resistance is critical to guide selection of the most appropriate antifungal agent in patients with aspergillosis. This study describes a new sequencing-free molecular screening tool for the early detection of the most frequent mutations known to be associated with azole resistance in *A. fumigatus*. PCRs targeting *cyp*51A mutations at positions G54, Y121, G448 and M220 and the promoter region targeting the different tandem repeats (TR) were designed. All PCRs were simultaneously performed using the same cycling conditions. Amplicons were then distinguished using a High Resolution Melting assay. For standardization, 30 well-characterized azole resistant *A. fumigatus* strains were used, obtaining melting curve clusters for different resistance mechanisms in each target and detecting the most frequent azole-resistance mutations: G54E, G54V, G54R, G54W, Y121F, M220V, M220I, M220T, M220K, G448S and the tandem repeats, TR_{34}, TR_{46} and TR_{53}. Validation of the method was performed using a blind panel of 80 *A. fumigatus* azole susceptible and resistant strains. All strains included in the blind panel were properly classified as susceptible or resistant by the developed method. The implementation of this screening method can reduce the time for the detection of azole resistant *A. fumigatus* isolates and therefore facilitate the selection of the best antifungal therapy in patients with aspergillosis.

47 **INTRODUCTION**

48 In the last years, azole resistance in *A. fumigatus* has been increasingly
49 reported in the clinical setting, representing a growing public health concern (4). In the
50 Netherlands azole resistance in *A. fumigatus* has been increasing since 1999 with
51 resistance rates ranging from 4.3% to 19.2% in 2013 and 3.8% to 13.3% in 2014 (5, 6).
52 Similarly, an increase from 5% in 2004 to 20% in 2009 was observed in the UK (7, 8).
53 Nowadays, the presence of azole resistant strains has been reported in many countries
54 from all continents, with strains isolated from both environmental and clinical samples
55 (9).

56 Azole targets 14 alpha sterol demethylase, a key enzyme of the ergosterol
57 biosynthesis pathway, which is encoded in *Aspergillus* by *cyp*51A. The alteration of this
58 gene is the major mechanism leading azole resistance (10). Several point mutations
59 have been described in *cyp*51A although only some of them have been confirmed as
60 responsible for the phenotypic resistance. Those comprise mutations at positions
61 Glycine 54 (G54), Methionine 220 (M220) and Glycine 448 (G448) (11-13). However,
62 the most frequent mechanism of resistance in *A. fumigatus* until now is a combination
63 of a point mutation at position Leucine 98 (L98) with a tandem repeat (TR) insertion of
64 34 base pairs (bp) in the promoter region of *cyp*51A (14). Recent studies indicate that
65 approximately 50% of the increase in azole resistant strains is due to the TR_{34}/L98H
66 alteration (15). Other emerging mechanisms of resistance have also been described,
67 such as two point mutations (Y121F and T289A) in combination with 46 bp TR in the
68 promoter (16) or point mutation Y121F alone (17) and another with 53 bp TR with no
69 associated point mutations (18, 19). Nevertheless, each of these mutations confers
70 different susceptibility profiles: G54 has been associated with resistance to

71 itraconazole and posaconazole (11, 20); M220 with resistance to itraconazole, high
72 Minimum Inhibitory Concentration (MIC) to voriconazole and variable MICs to
73 posaconazole (13). G448 with voriconazole resistance and some reduction in
74 itraconazole and posaconazole susceptibility (21); TR_{34}/L98H is described as pan-azole
75 resistant (14), TR_{46}/Y121F/T289A confers resistance to posaconazole and voriconazole,
76 with variable susceptibilities to itraconazole (22, 23) and TR_{53} confers resistance to
77 voriconazole and itraconazole and lower susceptibility to posaconazole (19).
78 The analysis of the High Resolution Melting curves (HRM) using fluorescent
79 DNA binding dyes with improved saturation properties allows a precise assessment of
80 the sequence and can be used to identify single point mutations (24), reducing the
81 time for characterization without need for sequencing. Fast and effective diagnosis
82 using HRM assays has been performed for microbiological applications using various
83 platforms (25-28). Since a fast identification of azole resistance is a critical point for the
84 selection of the proper antifungal drug in the IFD patients, we have developed a
85 screening tool based on this technology, targeting the most common *cyp*51A
86 mutations associated with azole resistance in *A. fumigatus*.
87
88 **MATERIALS AND METHODS**
89 **Strains analyzed in this study**
90 Thirty well characterized *A. fumigatus* clinical strains (26 azole resistant and 4
91 azole susceptible) from the Mold collection of the Spanish National Centre for
92 Microbiology were selected for setting up the method (Table 1). Azoles susceptibility
93 was assessed using the broth microdilution method of the European Committee on
94 Antimicrobial Susceptibility Testing (29). Strains were classified as susceptible or

resistant according to EUCAST breakpoints for *A. fumigatus* and azoles (resistance to itraconazole and voriconazole MIC >2 mg/L and resistant to posaconazole MIC > 0.25 mg/L) (30) (Table 1). The *cyp*51A gene including its promoter region was amplified and sequenced following the procedure previously described (14) for the detection of specific mutations associated to azole resistance (Table 1). In a second step a blinded panel of 80 strains of *A. fumigatus* including 46 susceptible and 34 azole resistant strains was used for the validation of the method.

DNA extraction from the *A. fumigatus* strains

Strains were subcultured in Glucose Yeast Extract Peptone medium (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich Química, Madrid, Spain) for 24 to 48 h at 30°C. After mechanical disruption of the fungus by vortexing the mycelium with glass beads, genomic DNA was extracted using a phenol-chloroform method, following the protocol previously described (31). The extracted DNA was quantified by a Nanodrop 8000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE; USA) and stored at -20ºC until processing.

Amplification of the targets

Five PCRs to amplify fragments containing the targeted mutations G54, Y121, M220 and G448 in *cyp*51A and its promoter region, in which the three tandem repeats (TR_{34}, TR_{46} and TR_{53}) are located, were developed. As M220 PCR failed to identify M220K, an extra PCR to specifically detect that mutation was also included in the method. Primers (Table 2) were designed using the Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Sigma-Aldrich (Madrid, Spain). Reactions were performed in a 20 µl final reaction volume containing 1 µl of Light Cycler 480 Resolight Dye (Roche Diagnostics, Mannheim, Germany), 10 µl of SensiMix

DNA (Quantace, Ecogen, Madrid, Spain), 1 µM of each primer, water (PCR Grade, Roche, Spain) and 25 ng of DNA. All reactions were performed with the same cycling conditions: an initial denaturation step for 5 min at 95^{o}C followed by 45 cycles of denaturation (10 s at 95^{o}C), annealing (10 s at 60^{o}C), and extension (10 s at 72^{o}C). All samples were performed in duplicate, including in each experiment positive controls, which contained the described mutations, and negative controls in which water was added as template.

High Resolution Melting analysis

After amplification, PCR products were maintained 1 min at 95^{o}C and 1 min at 40^{o}C, followed by a 10 min ramp from 65^{o}C to 95^{o}C at a rate of 0.002, 25 acquisitions per grade centigrade, and a final cooling step of 30 s at 40^{o}C. HRM analysis was performed using the LightCycler® 480 Gene Scanning Software (Roche, Madrid, Spain) in the LightCycler© 480 Instrument II (Roche, Madrid, Spain). The melting curve data was manually adjusted and the fluorescence was normalized, fixing the pre and post melt slider settings ranging from 77.4-87.9^{o}C and 83.7-91.1^{o}C, respectively, with threshold at 0 or 1 of the specific amplification and a sensitivity range between 0.20-0.30. In the case of the TR target, we performed a second analysis for discriminating between TR_{46} and TR_{53}, adjusting the pre and post melt slider to 84.3-85.3^{o}C and 89.3-90.3^{o}C, respectively. The HRM analysis optimized conditions for each assay are shown in Table 3.

RESULTS

Standardization: Azole-resistant mutation discrimination by HRM

142 The real time PCR (RT-PCR) amplified fragments ranged 70 - 144 bp, being the
143 cycle thresholds of the amplification below 30 for all the samples. After HRM analysis
144 and standardization, normalized melting curve clusters for different resistance
145 mechanisms were obtained for each target (Figure 1).

146 The average melting temperatures (Tm) obtained for each mutation are
147 summarized in Table 4. In position G54, the specific amino acid changes could not be
148 distinguished but any change from glycine was clearly differentiated from the wild type
149 (WT) strains (Figure 1, panel A). For the Y121 target, two melting curve clusters
150 allowed tyrosine differentiation from phenylalanine (Figure 1, panel B). This also
151 happened in the case of mutation G448S (Figure 1, panel E). The three TRs were
152 distinguished from the WT by the melting curve clusters (Figure 1 panel C and D). In
153 position M220, three of the four described mutations (M220/I/T/V) were specifically
154 differentiated, being able to distinguish all of them by the melting curves clusters
155 (Figure 1, Panel F). The mutation M220K presented a Tm of 82.9^{o}C (Table 4) and was
156 located in the same cluster as the WT strains, challenging its proper detection.
157 However, the PCR designed for M220K amplified only the strains which harbor this
158 mutation, distinguishing specifically from the others (Figure 1, Panel G).

159

160 **Validation of the method**

161 A total of 80 strains (34 azole resistant and 46 azole susceptible) were included
162 in the panel. All 46 azole susceptible strains tested were classified as WT (Table S1).
163 Nine strains harbored mutations in position G54: G54E (n=3), G54W (n=2), G54V (n=3)
164 and G54R (n=1). These mutations could not be distinguished among them, although
165 they were clearly differentiated from the WT and therefore characterized as resistant.

The two strains with Y121F were properly identified. For M220 target, this methodology was able to specifically differentiate the strains in the blind panel harboring M220V (n=2), M220I (n=1), M220T (n=1) and M220K (n=2). The only strain included in the panel with G448S mutation was also properly identified. Strains carrying the TR [TR_{34} (n= 14), TR_{46} (n= 2) and TR_{53} (n= 2)] were specifically identified in the panel.

DISCUSSION

Azoles are currently the preferred agents for treatment and prevention of invasive aspergillosis (IA). voriconazole is the first-line therapy of invasive aspergillosis and posaconazole is recommended for primary prophylaxis, with voriconazole and itraconazole as alternative agents (32, 33). Recently Isavuconazole has also being approved for the treatment of IA, showing similar MICs than the ones obtained for voriconazole {Maertens, 2016 #67} {Gregson, 2013 #66;Howard, 2013 #65}. The use of liposomal amphotericin B (L-AMB) is indicated as an alternative treatment, although it can be associated with high toxicity (34). Combined therapies of azoles with echinocandins, mainly voriconazole and anidulafungin , have been tested and seem to be beneficial for IA treatment (35-37). As alternative rescue therapies, posaconazole or itraconazole are prescribed (33).

Azole resistant *A. fumigatus* isolates have been increasingly reported in the last years (38-40). Although the number of patients affected is still limited (41), treatment options are clearly reduced and lead to poor outcomes (3, 8) as mutations or alterations in *cyp*51A gene lead to different azole susceptibility profiles (11, 19, 20, 23). Thus, early diagnosis and a proper antifungal prescription have a direct impact on

patient survival (42). In addition, susceptibility testing is not routinely performed in all clinical laboratories (especially for molds) and, when available, it takes 48-72 hours to be completed. This delays the detection of resistance and, therefore, the administration of an appropriate antifungal agent if different from the standard. Close surveillance of clinical azole resistant strains, as well as an exhaustive screening of environmental isolates are vital for the management of antifungal resistance in human pathogenic fungi (43).

To face all these challenges, we have developed a single HRM assay to detect each of the most frequent mutations related to azole resistance (changes at G54, Y121, M220, or G448 and promoter insertions of variable sizes, $TR_{34,}$ $TR_{46,}$ TR_{53}). As a result, we were able to clearly differentiate the azole resistant from the WT isolates by HRM, showing the reliability of this diagnostic tool for the rapid identification of known azole resistance mechanisms, in both clinical and environmental *A. fumigatus* strains. This assay could be used as a screening method, reducing the time for obtaining the information of the type of azole resistance profile to a single day. In addition, the method can also be useful to detect the molecular mechanism of phenotypic resistance when the susceptibility profile has already being determined, with the option of only testing the mutations that can explain the obtained resistance pattern.

Several molecular methods for the detection of mutations responsible for azole resistance have been developed previously: Tuohy et al. developed a HRM assay to characterize the G54 codon in *cyp*51A, but other mutations associated with resistance were not included (44). Nested PCR for detecting mutations in position M220 (without discriminating among different base changes), L98H and the TR_{34}, have also been described (45, 46). In contrast to nested PCR, our assay is a closed-tube technique that

reduces the possibility of contamination and the time to get results, since there is no need for sequencing. Other methods based on RT-PCR have been described but are able to detect fewer mutations, requires the use of expensive fluorescent probes or/and are performed in several steps (47-50)(51).

Another advantage of the method developed is the potential detection of additional mutations that have not been previously described in the amplified regions without the need of expanding the assay, as well as the possibility of including new amplification regions if new resistance mechanisms arise. Moreover, the technique is very flexible and can be adapted to particular situations were only some of the mechanisms need to be detected, optimizing assay costs.

Although our HRM assay is a useful method for the identification of azole resistant isolates reducing the time for characterization without need for sequencing, it also presents some limitations. Mutations which include changes between adenine and thymine are harder to detect with this methodology. Indeed, we could not differentiate properly the strains harboring the M220K mutation from the WT ones due to this change. Modification of $MgCl_2$ concentration or using some additives such as betaine or dimethyl sulfoxide, as previously suggested (52), did not improve the differentiation. To solve this limitation, we included an extra PCR that specifically detects this mutation and runs simultaneously with the rest of the assay.

Our method has not targeted L98 and T289 because these point mutations have never been reported alone. Moreover, laboratory generated strains containing these single point mutations are not azole resistant (14, 53). In our study, the use of the PCR targeting TR has been enough to identify all the included TR_{34}/L98H and TR_{46}/Y121F/T289A isolates.

238 In addition, for some mutations, such as G448, M220T, M220I or G54R or $TR_{53,}$
239 a limited number of strains were available for testing. Even though the results were
240 robust, more strains should be tested to confirm them. Another limitation is that this
241 technique still requires the isolation of the strains. The identification of resistance
242 mutations from direct clinical samples, such as blood, serum or bronchoalveolar lavage
243 among others, could clearly reduce the time response (47, 50). However, the low
244 amount of *Aspergillus* in clinical samples represents a challenge. A multicopy target
245 approach is recommended but not possible for azole resistance characterization since
246 it is based on a single copy target (54). Three studies detecting *cyp*51A mutations
247 directly from respiratory samples have been published recently (46-48). However,
248 these methods also have limitations: one of the studies detected only TR_{34}, L98H,
249 Y121F, and T289A mutations via melting curve analysis (47, 48) and the other used
250 nested PCR combined with sequencing (46).
251 In conclusion, the current study is the first to detect simultaneously the most
252 frequent azole resistant strains based on HRM technology. This method is simple to
253 perform, enabling the rapid and accurate detection of *A. fumigatus* resistant strains. It
254 is highly sensitive, specific and it is suitable for the screening routine clinical
255 diagnostics as the equipment needed is usually available at the hospitals. The fast
256 detection of resistant *A. fumigatus* strains with this technology will allow to select the
257 proper antifungal treatment, improving the management of the *A. fumigatus* infected
258 patients. Moreover, in the future, it could represent a good alternative to replace
259 phenotypic methods such as antifungal susceptibility testing in the clinical laboratory
260 allowing quick, precise and reliable detection of the most frequent mutations.
261

Funding

This study has been financed by a research project from the Fondo de Investigación Sanitaria (FIS ISCIII) [Exp. no. PI13/02145]. Supported by Plan Nacional de I+D+i and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015) - co-financed by European Development Regional Fund "A way to achieve Europe" ERDF. LBM has a research contract from the Spanish Network for Research in Infectious Diseases [REIPI RD12/0015]. ORM holds a predoctoral fellowship from the Fondo de Investigaciones Sanitarias (FI14CIII/00025). SG was supported by a research fellowship from the Fondo de Investigaciones Sanitarias of the Spanish Ministry of Science and Innovation (FI10/00464). EM is supported by a project from the Spanish Fondo de Investigación Sanitaria (FIS: PI15CIII/00019).

This work was presented in part in the 7th Advances Against Aspergillosis, Manchester, UK, 3-5th March 2016.

TRANSPARENCY DECLARATIONS

In the past 5 years, M Cuenca-Estrella has received grant support from Astellas Pharma, BioMérieux, Gilead Sciences, Merck Sharp & Dohme, Pfizer, Schering-Plough, Soria Melguizo SA, Ferrer International, the European Union, the ALBAN Program, the Spanish Agency for International Cooperation, the Spanish Ministry of Culture and Education, the Spanish Health Research Fund, the Instituto de Salud Carlos III, the Ramon Areces Foundation and the Mutua Madrileña Foundation. He has been an advisor/ consultant to the Panamerican Health Organization, Astellas Pharma, Gilead Sciences, Merck Sharp & Dohme. A. Alastruey-Izquierdo has received grant or travel

support from Gilead Sciences and Pfizer. The other authors declare no conflicts of interest.

Table 1: Phenotypic and genotypic characteristics of the strains used to standardize the method.

Modification	Number (n=30)	Triazole phenotype			Mutations in nucleotides
		ICZ	VCZ	PCZ	
WT	4	S	S	S	None
G54R	1	R	S	R	GGG to **A**GG
G54W	2	R	S	R	GGG to **T**GG
G54V	3	R	S	R	GGG to G**T**G
G54E	3	R	S	R	GGG to G**A**G
Y121F	2[a]	S[b]	R[b]	S[b]	TAT to T**T**T
M220K	2	R	S[c]	R	ATG to A**A**G
M220T	1	R	S[c]	S[c]	ATG to A**C**G
M220I	1	R	S[c]	R	ATG to AT**A**
M220V	2	R	S[c]	S[c]	ATG to **G**TG
G448S	1	S	R	R	GGT to **A**GT
TR_{34}	6	R	R	R	GAATCACGCGGTCCGATGTGTGCTGAGCCGAAT[d]
TR_{46}	2[a]	R[e]	R	R	GAATCACGCGGTCCGATGTGTGCTGAGCCGAATGAAAGTTGTCTA[d]
TR_{53}	2	R	R	S[c]	GAATCACGCGGTCCGATGTGTGCTGAGCCGAATGAAAGTTGTCTAATGTCTA[d]

ICZ: Itraconazole; VCZ: Voriconazole; PCZ: Posaconazole. R: Resistant; S; Susceptible

[a]: Same strains containing the mutations TR_{46} and Y121F .

[b:] Triazole phenotype is based on the results published for the only strain identified with this single point mutation (17)

[c]: Elevated Minimum Inhibitory Concentration

[d]: Sequence of the tandem repeated in the promoter region

[e]: Resistant phenotype variable

299 Table 2: Primers used in this study
300

Target	Primers	Amplicon length (bp)	Sequence (5´-3´)	Source
G54	G54F	70	TCATTGGGTCCCATTTCTG	(51)
	G54R		GCACGCAAAGAAGAACTTG	(51)
Y121	Y121F1	72	CATTGACGACCCCCGTTT	This study
	Y121R1		TTTTCTGCTCCATCAGCTTG	This study
M220	M220F	77	TCATGACCTGGACAAGGGC	(51)
	M220R		TCGCTTCTTGTTATGCGGC	This study
M220K	M220Kmod4F	144	TTTACTCCCATCAATTTTAA	This study
	M220KR		TGATTTCTGAGAGTCCTTGTCAC	This study
G448	G448F	131	TACTAAGGAGCAGGAGAA	This study
	G448R		TGACATAAGCGAATTTCT	This study
TR	TRF	117	GCACCACTTCAGAGTTG	This study
	TRR1		ACCGTAGTATGAGTTAGG	This study

301
302

303 Table 3: Parameters established for the analysis of HRM

HRM Target	**Pre-Melt T (^{o}C)**	**Post –Melt T (^{o}C)**	**Threshold/Sensitivity**
G54	77.4-78.4	83.7-84.7	0/0.30
Y121	78.2-79.2	84.2-85.2	0/0.20
M220	80.2-81.2	85.7-86.7	0/0.20
G448	86.9-87.9	90.1-91.1	0/0.20
TR_{34}	81.3-82.3	90.0-91.0	0/0.30
$TR_{46, 53}$	84.3-85.3	89.3-90.3	1/0.30

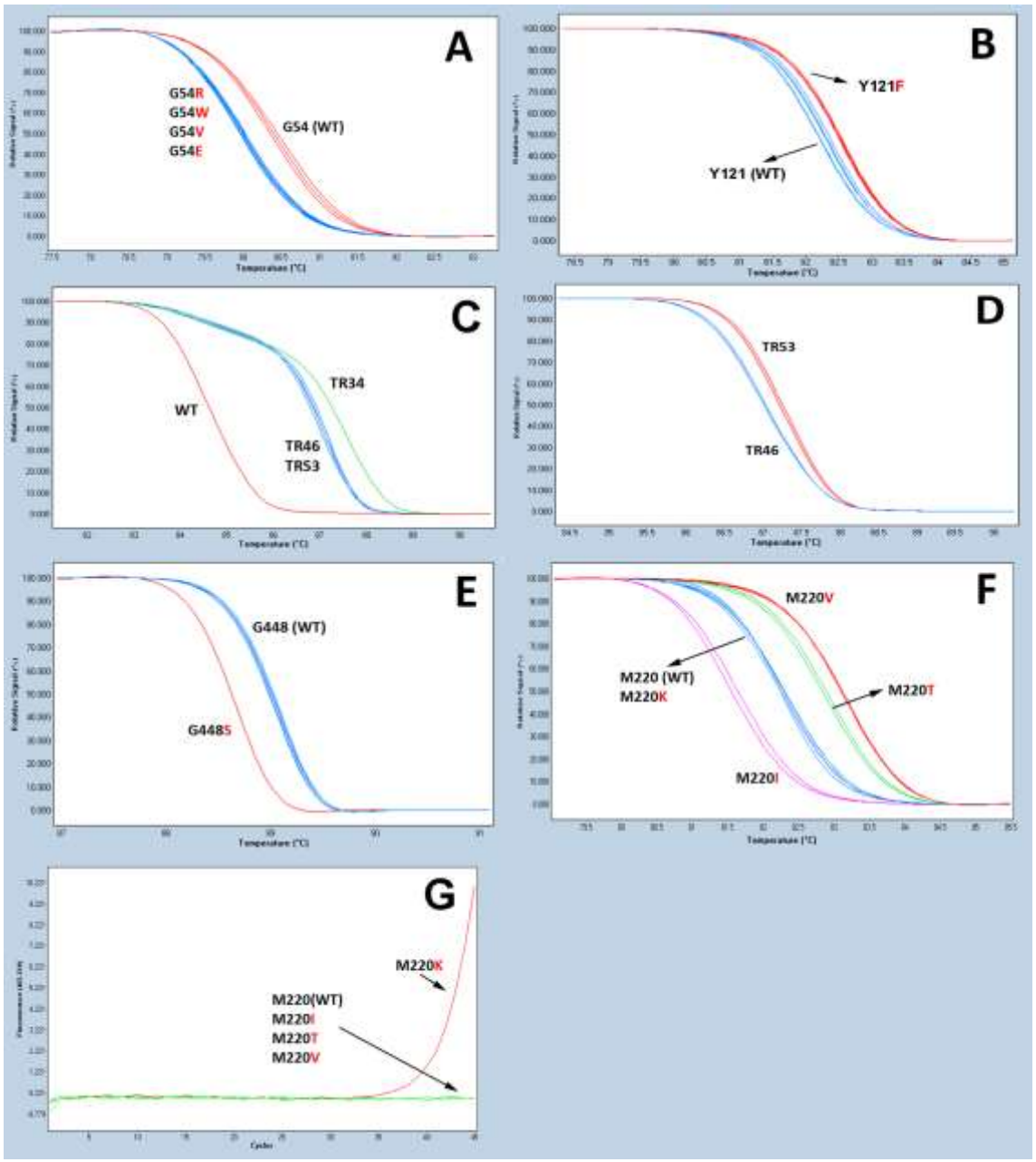
304
305
306

307 Table 4: The average Tm for the different target and mutation included in this study
308
309

HRM target	Mutations	Tm (ºC)
G54	Wild type	80.6
	G54R	80.1
	G54V	80.2
	G54E	80.3
	G54W	79.9
Y121	Wild type	82.2
	Y121F	82.5
M220	Wild type	82.9
	M220K	82.9
	M220I	82.1
	M220T	83.4
	M220V	82.6
G448	Wild type	89.2
	G448S	88.6
TR	Wild type	84.6
	TR_{34}	88.2
	TR_{46}	87.1
	TR_{53}	87.2

310 Tm: melting temperature. TR: tandem repeat

Figure 1: Representative HRM assays of the different targets used in the method with the normalized profiles obtained.



Panel A: G54 target; Panel B: Y121 target; Panel C: TR target; Panel D: TR target for differentiating TR46 and TR53; Panel E: G448 target; Panel F: M220 target and Panel G: Specific amplification of *A. fumigatus* strain harboring the M220K mutation.

References

1. **Akan H, Antia VP, Kouba M, Sinko J, Tanase AD, Vrhovac R, Herbrecht R.** 2013. Preventing invasive fungal disease in patients with haematological malignancies and the recipients of haematopoietic stem cell transplantation: practical aspects. JAntimicrobChemother **68 Suppl 3:**iii5-16.
2. **Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drgona L, Blijlevens N.** 2011. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. JAntimicrobChemother **66 Suppl 1:**i5-14.
3. **van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, 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. EmergInfectDis **17:**1846-1854.
4. **European Centre for Disease prevention and Control.** 2016. Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in Aspergillus species. Available online: http://ecdc.europa.eu/en/publicantions/Publications/risk-assessment-impact-environmental-usage-of triazoles-on- Aspergillus -spp-resistance-to-medical-triazoles.pdf (accessed on 1 May 2016).
5. **Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, Mellado E, Donders AR, Melchers WJ, Verweij PE.** 2008. Emergence of azole resistance in Aspergillus fumigatus and spread of a single resistance mechanism. PLoSMed **5:**e219.
6. **van der Linden JW, Arendrup MC, Melchers WJ, Verweij PE.** 2016. Azole Resistance of Aspergillus fumigatus in immunocompromised patients with invasive aspergillosis. EmergInfectDis **22:**158-159.
7. **Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, Denning DW.** 2010. Azole antifungal resistance in Aspergillus fumigatus : 2008 and 2009. JAntimicrobChemother **65:**2116-2118.
8. **Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW.** 2009. Frequency and evolution of azole resistance in Aspergillus fumigatus associated with treatment failure. EmergInfectDis **15:**1068-1076.
9. **Rivero-Menendez O, Alastruey-Izquierdo A, Mellado E, Cuenca-Estrella M.** 2016. Triazole resistance in Aspergillus spp.: a worldwide problem? Journal of Fungi **2:**1-21.
10. **Denning DW, Perlin DS.** 2011. Azole resistance in Aspergillus : a growing public health menace. FutureMicrobiol **6:**1229-1232.
11. **Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL.** 2003. A point mutation in the 14alpha-sterol demethylase gene cyp 51A contributes to itraconazole resistance in Aspergillus fumigatus AntimicrobAgents Chemother **47:**1120-1124.
12. **Manavathu EK, Alangaden GJ, Chandrasekar PH.** 2003. Differential activity of triazoles in two-drug combinations with the echinocandin caspofungin against Aspergillus fumigatus JAntimicrobChemother **51:**1423-1425.
13. **Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL.** 2004. Substitutions at methionine 220 in the 14alpha-sterol demethylase (cyp 51A) of Aspergillus fumigatus are responsible for

resistance in vitro to azole antifungal drugs. AntimicrobAgents Chemother **48:**2747-2750.

14. **Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, Rodriguez-Tudela JL.** 2007. A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp 51A alterations. AntimicrobAgents Chemother **51:**1897-1904.

15. **Jensen RH, Hagen F, Astvad KM, Tyron A, Meis JF, Arendrup MC.** 2016. Azole-resistant Aspergillus fumigatus in Denmark: a laboratory-based study on resistance mechanisms and genotypes. ClinMicrobiolInfect **22:**570-579.

16. **van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ, Haas PJ, Rijnders BJ, Kuijper EJ, Van Tiel FH, Varga J, Karawajczyk A, Zoll J, Melchers WJ, Verweij PE.** 2013. Aspergillosis due to voriconazole highly resistant Aspergillus fumigatus and recovery of genetically related resistant isolates from domiciles. ClinInfectDis **57:**513-520.

17. **Lescar J, Meyer I, Akshita K, Srinivasaraghavan K, Verma C, Palous M, Mazier D, Datry A, Fekkar A.** 2014. Aspergillus fumigatus harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. J Antimicrob Chemother **69:**3244-3247.

18. **Mellado, E.; Alcazar-Fuoli L.; Pajkrt, D.; verweij, P.E.; Melchers, WJ.; Cuenca-Estrella M.; Rodriguez-Tudela, J.L**. 2007. Alteration of the *cyp51*A gene promoter contribute to *Aspergillus fumigatus* multiple triazole resistance. In Proceedings of 47th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA..

19. **Hodiamont CJ, Dolman KM, Ten Berge IJ, Melchers WJ, Verweij PE, Pajkrt D.** 2009. Multiple-azole-resistant Aspergillus fumigatus osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. MedMycol **47:**217-220.

20. **Mann PA, Parmegiani RM, Wei SQ, Mendrick CA, Li X, Loebenberg D, DiDomenico B, Hare RS, Walker SS, McNicholas PM.** 2003. Mutations in Aspergillus fumigatus resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase. AntimicrobAgents Chemother **47:**577-581.

21. **Pelaez T, Gijon P, Bunsow E, Bouza E, Sanchez-Yebra W, Valerio M, Gama B, Cuenca-Estrella M, Mellado E.** 2012. Resistance to voriconazole due to a G448S substitution in Aspergillus fumigatus in a patient with cerebral aspergillosis. J Clin Microbiol **50:**2531-2534.

22. **Pelaez T, Monteiro MC, Garcia-Rubio R, Bouza E, Gomez-Lopez A, Mellado E.** 2015. First detection of Aspergillus fumigatus azole-resistant strain due to cyp 51A TR 46 /Y121F/T289A in an azole-naive patient in Spain. New MicrobesNew Infect **6:**33-34.

23. **van Ingen J, van der Lee HA, Rijs TA, Zoll J, Leenstra T, Melchers WJ, Verweij PE.** 2015. Azole, polyene and echinocandin MIC distributions for wild-type, TR 34 /L98H and TR 46 /Y121F/T289A Aspergillus fumigatus isolates in the Netherlands. JAntimicrobChemother **70:**178-181.

24. **Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ.** 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. ClinChem **49:**853-860.

25. **Erali M, Voelkerding KV, Wittwer CT.** 2008. High resolution melting applications for clinical laboratory medicine. ExpMolPathol **85:**50-58.
26. **Hoek KG, Gey van Pittius NC, Moolman-Smook H, Carelse-Tofa K, Jordaan A, van der Spuy GD, Streicher E, Victor TC, van Helden PD, Warren RM.** 2008. Fluorometric assay for testing rifampin susceptibility of Mycobacterium tuberculosis complex. JClinMicrobiol **46:**1369-1373.
27. **Lin JH, Tseng CP, Chen YJ, Lin CY, Chang SS, Wu HS, Cheng JC.** 2008. Rapid differentiation of influenza A virus subtypes and genetic screening for virus variants by high-resolution melting analysis. JClinMicrobiol **46:**1090-1097.
28. **Slinger R, Bellfoy D, Desjardins M, Chan F.** 2007. High-resolution melting assay for the detection of gyrA mutations causing quinolone resistance in Salmonella enterica serovars Typhi and Paratyphi. DiagnMicrobiolInfectDis **57:**455-458.
29. **Arendrup MC, Guinea J, Cuenca-Estrella M, Meletiadis J, Mouton JW, Lagrou K, Howard SJ, EUCAST A.** 2015. EUCAST DEFINITIVE DOCUMENT E.DEF 9.3: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_Mould_testing_definitive.pdf.
30. **EUCAST.** 2016. European committee on antimicrobial susceptibility testing. Antigungal breakpoint tables for interpretation of MICs v 8.0. Available online: http://www.eucst.org./fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/Antifungal_brealpoints_v8.0_November_2015.pdf (accessed on 25 April 2016).
31. **Tang CM, Cohen J, Holden DW.** 1992. An Aspergillus fumigatus alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. MolMicrobiol **6:**1663-1671.
32. **Leroux S, Ullmann AJ.** 2013. Management and diagnostic guidelines for fungal diseases in infectious diseases and clinical microbiology: critical appraisal. ClinMicrobiolInfect **19:**1115-1121.
33. **Patterson TF, Thompson GR, III, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, Segal BH, Steinbach WJ, Stevens DA, Walsh TJ, Wingard JR, Young JA, Bennett JE.** 2016. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the infectious diseases Society of America. ClinInfectDis **63:**e1-e60.
34. **Sabra R, Branch RA.** 1990. Amphotericin B nephrotoxicity. Drug Saf **5:**94-108.
35. **Ledoux MP, Toussaint E, Denis J, Herbrecht R.** 2017. New pharmacological opportunities for the treatment of invasive mould diseases. JAntimicrobChemother **72:**i48-i58.
36. **Seyedmousavi S, Bruggemann RJ, Melchers WJ, Rijs AJ, Verweij PE, Mouton JW.** 2013. Efficacy and pharmacodynamics of voriconazole combined with anidulafungin in azole-resistant invasive aspergillosis. JAntimicrobChemother **68:**385-393.
37. **Seyedmousavi S, Mouton JW, Melchers WJ, Bruggemann RJ, Verweij PE.** 2014. The role of azoles in the management of azole-resistant aspergillosis: from the bench to the bedside. Drug ResistUpdat **17:**37-50.

38. **Jeanvoine A, Rocchi S, Reboux G, Grenouillet F, Benassarou M, Chirouze C, Millon L.** 2016. Sinus aspergillosis due to an azole-resistant Aspergillus fumigatus strain carrying the TR 34 /L98H mutation in immunocompetent host. InfectDis(Lond) **48:**765-766.
39. **Mellado E, De La Camara R, Buendia B, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2013. Breakthrough pulmonary Aspergillus fumigatus infection with multiple triazole resistance in a Spanish patient with chronic myeloid leukemia. RevIberoamMicol **30:**64-68.
40. **Prigitano A, Esposto MC, Biffi A, De LG, Favuzzi V, Koncan R, Lo CG, Barao OM, Colombo C, Pizzamiglio G, Romano L, Tortorano AM.** 2016. Triazole resistance in Aspergillus fumigatus isolates from patients with cystic fibrosis in Italy. JCystFibros.
41. **Alanio A, Sitterle E, Liance M, Farrugia C, Foulet F, Botterel F, Hicheri Y, Cordonnier C, Costa JM, Bretagne S.** 2011. Low prevalence of resistance to azoles in Aspergillus fumigatus in a French cohort of patients treated for haematological malignancies. JAntimicrobChemother **66:**371-374.
42. **Bernal-Martinez L, Alastruey-Izquierdo A, Cuenca-Estrella M.** 2016. Diagnostics and susceptibility testing in Aspergillus FutureMicrobiol **11:**315-328.
43. **van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, Chryssanthou E, Mellado E, Kidd SE, Tortorano AM, Dannaoui E, Gaustad P, Baddley JW, Uekotter A, Lass-Florl C, Klimko N, Moore CB, Denning DW, Pasqualotto AC, Kibbler C, Arikan-Akdagli S, Andes D, Meletiadis J, Naumiuk L, Nucci M, Melchers WJ, Verweij PE.** 2015. Prospective multicenter international surveillance of azole resistance in Aspergillus fumigatus EmergInfectDis **21:**1041-1044.
44. **Tuohy MJ, Reja V, Park S, Perlin DS, Wnek M, Procop GW, Yen-Lieberman B.** 2010. Use of a high-resolution melt assay to characterize codon 54 of the cyp 51A gene of Aspergillus fumigatus on a Rotor-Gene 6000 instrument. AntimicrobAgents Chemother **54:**2248-2251.
45. **Spiess B, Seifarth W, Merker N, Howard SJ, Reinwald M, Dietz A, Hofmann WK, Buchheidt D.** 2012. Development of novel PCR assays to detect azole resistance-mediating mutations of the Aspergillus fumigatus cyp 51A gene in primary clinical samples from neutropenic patients. AntimicrobAgents Chemother **56:**3905-3910.
46. **Zhao Y, Garnaud C, Brenier-Pinchart MP, Thiebaut-Bertrand A, Saint-Raymond C, Camara B, Hamidfar R, Cognet O, Maubon D, Cornet M, Perlin DS.** 2016. Direct molecular diagnosis of aspergillosis and cyp 51A profiling from respiratory samples of French patients. Front Microbiol **7:**1164.
47. **Chong GL, van de Sande WW, Dingemans GJ, Gaajetaan GR, Vonk AG, Hayette MP, van Tegelen DW, Simons GF, Rijnders BJ.** 2015. Validation of a new Aspergillus real-time PCR assay for direct detection of Aspergillus and azole resistance of Aspergillus fumigatus on bronchoalveolar lavage fluid. JClinMicrobiol **53:**868-874.
48. **Chong GM, van der Beek MT, von dem Borne PA, Boelens J, Steel E, Kampinga GA, Span LF, Lagrou K, Maertens JA, Dingemans GJ, Gaajetaan GR, van Tegelen DW, Cornelissen JJ, Vonk AG, Rijnders BJ.** 2016. PCR-based detection of Aspergillus fumigatus cyp 51A mutations on bronchoalveolar lavage: a multicentre validation of the AsperGenius assay(R) in

201 patients with haematological disease suspected for invasive aspergillosis. JAntimicrobChemother.

49. **Klaassen CH, de Valk HA, Curfs-Breuker IM, Meis JF.** 2010. Novel mixed-format real-time PCR assay to detect mutations conferring resistance to triazoles in Aspergillus fumigatus and prevalence of multi-triazole resistance among clinical isolates in the Netherlands. JAntimicrobChemother **65:**901-905.

50. **White PL, Posso RB, Barnes RA.** 2015. Analytical and clinical evaluation of the PathoNostics AsperGenius Assay for detection of invasive aspergillosis and resistance to azole antifungal drugs during testing of serum samples. JClinMicrobiol **53:**2115-2121.

51. **Garcia-Effron G, Dilger A, Alcazar-Fuoli L, Park S, Mellado E, Perlin DS.** 2008. Rapid detection of triazole antifungal resistance in Aspergillus fumigatus JClinMicrobiol **46:**1200-1206.

52. **Song C, Castellanos-Rizaldos E, Bejar R, Ebert BL, Makrigiorgos GM.** 2015. DMSO increases mutation scanning detection sensitivity of high-resolution melting in clinical samples. ClinChem **61:**1354-1362.

53. **Snelders E, Camps SM, Karawajczyk A, Rijs AJ, Zoll J, Verweij PE, Melchers WJ.** 2015. Genotype-phenotype complexity of the TR 46 /Y121F/T289A cyp 51A azole resistance mechanism in Aspergillus fumigatus FungalGenetBiol **82:**129-135.

54. **Alanio A, Bretagne S.** 2017. Performance evaluation of multiplex PCR including Aspergillus -not so simple! MedMycol **55:**56-62.