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Culture-Based Methods and Molecular Tools for Azole-Resistant *Aspergillus fumigatus* Detection in a Belgian University Hospital

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ABSTRACT Azole-resistant *Aspergillus fumigatus* is an increasing worldwide problem with major clinical implications. Surveillance is warranted to guide clinicians to provide optimal treatment to patients. To investigate azole resistance in clinical *Aspergillus* isolates in our institution, a Belgian university hospital, we conducted a laboratory-based surveillance between June 2015 and October 2016. Two different approaches were used: a prospective culture-based surveillance using VIPcheck on unselected *A. fumigatus* (n = 109 patients, including 19 patients with proven or probable invasive aspergillosis [IA]), followed by molecular detection of mutations conferring azole resistance, and a retrospective detection of azole-resistant *A. fumigatus* in bronchoalveolar lavage fluid using the commercially available AsperGenius PCR (n = 100 patients, including 29 patients with proven or probable IA). By VIPcheck, 25 azole-resistant *A. fumigatus* specimens were isolated from 14 patients (12.8%). Of these 14 patients, only 2 had proven or probable IA (10.5%). Mutations at the *cyp51A* gene were observed in 23 of the 25 *A. fumigatus* isolates; TR_{34}/L98H was the most prevalent mutation (46.7%), followed by TR_{46}/Y121F/T289A (26.7%). Twenty-seven (27%) patients were positive for the presence of *Aspergillus* species by AsperGenius PCR. *A. fumigatus* was detected by AsperGenius in 20 patients, and 3 of these patients carried *cyp51A* mutations. Two patients had proven or probable IA and *cyp51A* mutation (11.7%). Our study has shown that the detection of azole-resistant *A. fumigatus* in clinical isolates was a frequent finding in our institution. Hence, a rapid method for resistance detection may be useful to improve patient management. Centers that care for immunocompromised patients should perform routine surveillance to determine their local epidemiology.

KEYWORDS VIPcheck, AsperGenius, *cyp51A*, *cyp51B*, *hapE*

Azole resistance in *Aspergillus fumigatus* is an emerging problem worldwide, with major epidemiological and clinical implications (1–6). Mold active triazoles are commonly used as first line treatment and prophylaxis of invasive aspergillosis (IA) (7). Mutations in the *cyp51A* gene, which encodes the target of azoles, the lanosterol 14α-demethylase, represent the most commonly reported mechanism conferring azole resistance and consequently treatment failure in *A. fumigatus* (8–10). The most prevalent mutation (TR_{34}/L98H) involves the insertion of a 34-bp tandem repeat (TR_{34}) in the promoter region of the *cyp51A* gene and a point mutation within the gene leading to an amino acid substitution (L98H). More recently, an additional *cyp51A*-mediated resistant genotype (TR_{46}/Y121F/T289A) has been reported: a 46-bp tandem repeat (TR_{46}) in the promoter of *cyp51A* gene and two point mutations within the gene

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(Y121F/T289A) (11–13). Other point mutations in the *cyp51A* gene are also responsible for azole resistance. For example, prolonged exposition to azole treatment is associated with selective pressure, resulting in the emergence of azole-resistance mediated by point mutations (10–14). Furthermore, *A. fumigatus* azole-resistant strains have also been isolated from azole-naive patients and from the environment due to mutant selection (mainly TR_{34}/L98H and TR_{46}/Y121F/T289A) caused by the use of fungicides in agriculture, as confirmed by molecular typing (12, 15).

Several studies performed in Belgium and in the Netherlands, a neighboring country, have reported the presence of azole-resistant *A. fumigatus* isolates both in patients and in the environment (16–18). A clinical case of IA due to *A. fumigatus* containing the TR_{46}/Y121F/T289A mutation in the *cyp51A* gene was detected in 2013 for the first time at Erasme Hospital in Brussels, Belgium (19). In order to investigate the occurrence of azole-resistant *A. fumigatus* at Erasme Hospital, resistance surveillance has been performed between June 2015 and October 2016 in the patient population attending to this Belgian University hospital. Two different approaches were used: a prospective culture-based surveillance of unselected azole-resistant *A. fumigatus* and a retrospective direct detection of azole-resistant *A. fumigatus* in bronchoalveolar lavage (BAL) specimens by using the commercially available AsperGenius PCR (Pathognostic, Maastrich, The Netherlands). Furthermore, we report the performance of AsperGenius PCR to diagnose proven and probable IA compared to an in-house-developed PCR and culture methods.

RESULTS

Mycological cultures and susceptibility testing by azole resistance screening. A total of 212 positive samples for *A. fumigatus* were isolated from 109 hospitalized patients' respiratory specimens, including 116 sputum samples, 51 bronchopulmonary aspirate samples, 41 BAL fluid samples, 2 pleural fluid samples, and 2 pulmonary biopsy specimens. Most prevalent underlying diseases among these 109 patients were as follows: 23% ($n = 25$) were cystic fibrosis patients, 21% ($n = 23$) were lung transplant patients, and 13% ($n = 14$) had chronic obstructive pulmonary disease (COPD). Four of the 23 lung transplant recipients had undergone a lung transplant due to the cystic fibrosis. Seventeen percent of the patients were diagnosed with proven ($n = 5$) or probable ($n = 14$) IA, 4% ($n = 5$) with allergic bronchopulmonary aspergillosis, and the remaining 78% of patients ($n = 85$) were considered to be colonized by *A. fumigatus*.

The 212 *A. fumigatus* isolates recovered from respiratory specimens were screened by VIPcheck: 25 specimens from 14 patients had azole-resistant *A. fumigatus* isolates, translating into a prevalence of azole resistance of 12.8% (14 of 109) among patients and of 10.5% (2 of 19) among patients with proven or probable IA. Resistance genotyping was then performed. Mutations at the *cyp51A* gene were observed in 23 *A. fumigatus* isolates from 12 patients, while no missense mutations were observed in two cases. MICs, resistance genotyping results, and clinical and demographic data from patients harboring azole-resistant *A. fumigatus* are summarized in Table 1. Genotyping allowed us to determine the presence of one or two isogenic strains per patient: 15 different strains were obtained from 14 patients. Among these 15 strains, the TR_{34}/L98H was the most prevalent mutation (46.7%), followed by TR_{46}/Y121F/T289A (26.7%). An isolate with a TR_{34}/L98H mutation from one patient showed also a deletion of eight nucleotides in the *cyp51B* promoter. No isolates showed mutations in *hapE*.

Regarding the patients with proven or probable IA, a 58-year-old heart transplant patient with a probable IA during a neutropenic episode harbored first an *A. fumigatus* wild type. After 5 weeks of treatment with voriconazole, *A. fumigatus* TR_{46}/Y121F/T289 was detected. The patient was treated with voriconazole for 6 weeks in all and cured despite the mutations at *cyp51A*. The other patient harboring *A. fumigatus* TR_{34}/L98H, was a 67-year-old lung transplant recipient diagnosed with probable IA and treated and cured with voriconazole associated with caspofungin.

AsperGenius PCR. Twenty-seven patients (27%) were determined to be positive by AsperGenius for *Aspergillus* species detection using a standard threshold cycle (C_T)

TABLE 1 *cyp51A* mutations, MIC results, and demographic data from patients ($n = 14$) harboring azole-resistant *A. fumigatus* isolated at Erasme Hospital as detected by VIPcheck from June 2015 to October 2016

Patient	Age (yr)	Underlying disease	Source[a]	Colonization/IA	Prior azole exposition	MIC (mg/liter)[b]			*cyp51A* mutation(s) (no. of isolates)[c]
						ITC	VRC	POS	
1	39	Cystic fibrosis	BA/S	Colonization	VRZ, ITZ	1	2	0.5	TR_{34}/L98H (5)
						0.5	1	0.25	G448S (2)
2	81	Solid malignancy	BA/S	Colonization		1	2	0.5	
3	53	Hematological malignancy	BA/S	Colonization	POS	1	2	0.5	TR_{34}/L98H
4	47	Cystic fibrosis	BA/S	Colonization		1	1	0.5	
5	18	Cystic fibrosis	BA/S	Colonization		1	2	0.5	TR_{34}/L98H
6	26	Intestinal malabsorption	BA/S	Colonization		1	2	0.5	TR_{34}/L98H
7	67	Lung transplant	BAL	Probable IA		1	1	0.5	TR_{34}/L98H
8	58	Heart transplant	BAL	Probable IA	VRZ	0.5	>8	0.5	TR_{46}/Y121F/T289A
9	26	Cystic fibrosis	BA/S	Colonization		0.5	>8	0.5	TR_{46}/Y121F/T289A
10	86	Solid malignancy	BA/S	Colonization		16	1	1	TR_{34}/L98H[d]
11	66	COPD	BAL	Colonization		1	2	0.5	TR_{34}/L98H (2)
12	48	Lung transplant	BA/S	Colonization	VRZ	0.5	>8	0.5	N248K
13	55	Lung transplant	BA/S	Colonization	VRZ	0.5	>8	0.5	TR_{46}/Y121F/T289A (5)
14	65	Lung transplant	BA/S	Colonization		0.5	>8	0.25	TR_{46}/Y121F/T289A

[a]BA/S, bronchial aspirate or sputum; BAL, bronchoalveolar lavage fluid.
[b]ITC itraconazole; VRC, voriconazole; POS, posaconazole.
[c]The number of isolates when more than one strain was recovered with the same characteristics is indicated within parentheses.
[d]A deletion of eight nucleotides in the *cyp51B* promoter was also observed for this isolate.

value of <36. More specifically, *A. fumigatus* was detected in 20 patients, and 3 of them (15%) harbored *cyp51A* mutations. Two of these patients were already detected for azole resistance by culture-based screening (see above). The remaining case was a patient with a galactomannan (GM)-negative BAL fluid sample, probably colonized by *A. fumigatus* TR_{34}/L98H. However, mycological cultures for this patient were negative. Among these 27 patients, a total of 19 patients were diagnosed with proven or probable IA, 47% of whom were lung transplant recipients. The resistance frequency of *A. fumigatus* detected by AsperGenius among patients with proven or probable IA was 11.7% (2/17).

The receiver operating characteristic (ROC) curve for AsperGenius showed an optimal cutoff C_T value of ≤36 (Fig. 1). Considering the EORTC/MSG (European Organization for Research and Treatment of Cancer/Invasive Fungal Infection Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group) defini-

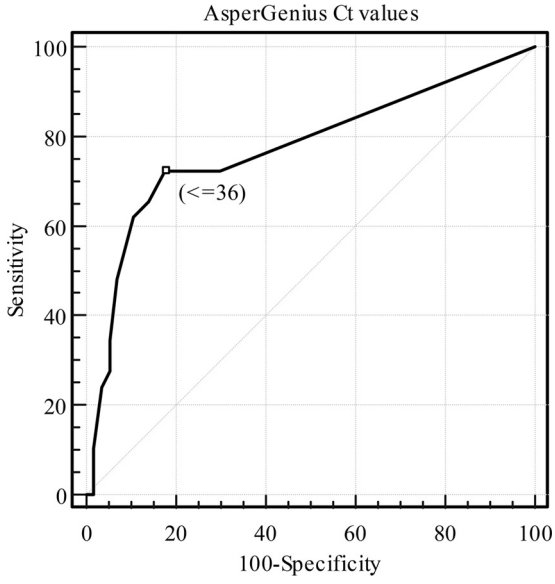

FIG 1 ROC curve for AsperGenius C_T results.

TABLE 2 Clinical characteristics of patients included in the AsperGenius evaluation study and results of AsperGenius, in-house PCR, galactomannan antigen detection, and cultures

EORTC/MSG classification and underlying disease (*n*)[a]	AsperGenius PCR (*n*)[b]	In-house PCR (*n*)[c]	GM (*n*)[d]	Culture and *cyp51A* genotype (*n*)[e]
Proven IA (5)				
Lung transplantation (3)	*A. fumigatus* WT[7] (5)	5	5	*A. fumigatus* WT (5)
Sarcoidosis (1)				
Acute alcoholic hepatitis (1)				
Probable IA (24)				
Lung transplantation (10)	*A. fumigatus* WT (10)			*A. fumigatus* WT (8)
Hematological malignancy (5)	*A. fumigatus* TR_{46}/Y121F/T289A (1)	14	24	*A. fumigatus* TR_{46}/Y121F/T289A (1)
Solid malignancy (3)	*A. fumigatus* TR_{34}/L98H (1)			*A. fumigatus* TR_{34}/L98H (1)
Acute alcoholic hepatitis (2)	*Aspergillus* spp. (2)			*A. flavus* (1)
Others (4)				
Possible IA (14)				
Hematological malignancy (4)				
Lung transplantation (2)	0	0	0	0
Solid malignancy (2)				
Acute alcoholic hepatitis (2)				
Others (4)				
Unclassifiable (57)				
Autoimmune disease (9)				
COPD (9)				
Lung transplantation (7)				
Solid malignancy (4)	*A. fumigatus* WT (2)			
Liver cirrhosis (4)	*A. fumigatus* TR_{34}/L98H (1)	8	16	*A. fumigatus* WT (1)
Ischemic heart disease (3)	*Aspergillus* spp. (5)			*A. niger* (2)
Liver transplantation (1)				
Others (13)				
No underlying diseases (7)				

[a]EORTC/MSG, European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group; *n*, number of patients in each category. A total of four lung transplant patients were diagnosed with cystic fibrosis.
[b]That is, the number of patients determined to be positive with the AsperGenius PCR for *Aspergillus* (cutoff C_T value of <36) and resistance detection. WT, wild type without *cyp51A* mutations.
[c]*n*, the number of patients determined to be positive with the in house real-time PCR (cutoff C_T value of <45).
[d]*n*, the number of patients determined to be positive for galactomannan (GM) antigen detection (OD index ≥1).
[e]*n*, the number of mycological cultures positive for *Aspergillus* spp. and *cyp51A* genotypes. WT, wild type without *cyp51A* mutations.

tions for clinical classification as the gold standard, the sensitivity and specificity for AsperGenius (C_T values of <36 and ≤36), in-house PCR, and mycological culture for diagnosis of IA are shown in Table 2. The sensitivity and specificity of AsperGenius using the standard C_T value were 65.5% (95% confidence interval [95% CI] = 48.2 to 82.8%) and 86% (95% CI = 76.9 to 95%), respectively (Table 3).

Excellent agreement and correlation between AsperGenius and the in-house PCR results for *Aspergillus* detection were observed independently of the cutoff C_T value used (Fig. 2). By using a standard C_T value (<36) for AsperGenius, the correlation with the in-house PCR generated a kappa statistic of 0.85 (95% CI = 0.73 to 0.96) and a Spearman coefficient of $r = 0.81$ (95% CI = 0.61 to 0.91; $P < 0.001$). By using the

TABLE 3 Clinical performance of AsperGenius, in-house PCR, and culture, considering EORTC/MSG definitions for clinical classification of invasive aspergillosis as the gold standard

	% Sensitivity or specificity (95% CI)	
Technique (C_T)	Sensitivity	Specificity
AsperGenius PCR		
<36	65.5 (48.2–82.8)	86 (76.9–95)
≤36	72.4 (52.8–87.2)	82.5 (70.1–91.2)
In-house PCR (<45)	65.5 (48.2–82.8)	86 (76.9–95)
Culture	58.6 (40.7–76.5)	94.7 (88.9–100)

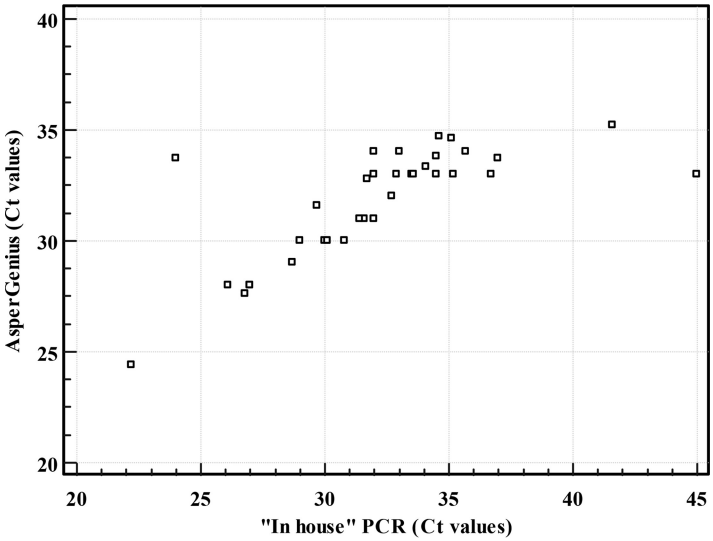

FIG 2 Scatter diagram showing correlation between cycle threshold values (C_T) obtained by AsperGenius ($C_T < 36$) and in-house PCR ($C_T < 45$).

optimal C_T value (≤36) for AsperGenius, the correlation with the in-house PCR generated a kappa statistic of 0.80 (standard error = 0.066; 95% CI = 0.67 to 0.93) and a Spearman coefficient of $r = 0.79$ (95% CI = 0.57 to 0.9; $P < 0.001$).

DISCUSSION

Invasive aspergillosis due to azole-resistant isolates is a growing concern in the clinical setting (20). Two approaches have been used in this study to assess this problem in our institution. The first approach was a prospective culture-based surveillance of unselected azole-resistant *A. fumigatus*, and the second approach was a retrospective direct detection of azole-resistant *A. fumigatus* in BAL fluid specimens by using AsperGenius PCR, a commercial real-time PCR that detects *Aspergillus* spp. and simultaneously identifies the most prevalent *cyp51A* mutations in *A. fumigatus*. The two approaches both showed an alarmingly high frequency of azole resistance occurrences among patients with proven or probable IA in our institution.

The prospective culture-based surveillance of unselected azole-resistant *A. fumigatus* and screening with VIPcheck identified a high prevalence of azole-resistance among all patients (approximately 13%) and among patients with probable or proven IA (approximately 11%). Similar surveillance studies in unselected isolates have shown a lower prevalence of azole-resistant *A. fumigatus* among patients, such as 1.8% in France and 2.1% in Denmark (21, 22). Furthermore, van der Linden et al. (4) reported an overall prevalence of 3.2% of azole-resistant *A. fumigatus* among patients in a prospective international surveillance study conducted in 19 countries, the majority of them being European. Similarly, the prevalence of azole-resistant *A. fumigatus* in a Belgian multicenter prospective cohort of patients with *Aspergillus* disease was 5.5% (17). Higher prevalences (8 to 30%) have been reported in other surveillance studies and particularly in those involving hematological and intensive care unit patients (10, 23–25). The prevalence of resistance may vary between units, hospitals, and geographical regions. To explain these variations, the bias related to laboratory procedures or the surveillance systems applied has to be considered (26, 27). The higher prevalence observed in the present study can be due to the different patient populations investigated. Here, hematological patients represented only 8% of the cohort, whereas cystic fibrosis and lung transplant patients accounted for nearly 50% of the population investigated. The prevalences of azole resistance among cystic fibrosis and lung transplant patients were 16 and 17%, respectively. Indeed, other studies in cystic fibrosis patients have shown prevalences of azole resistance in *A. fumigatus* varying between 0 and 20%, depending

on previous azole exposure. Nevertheless, a prevalence of approximately 5% is the most common in this population (28–32). In the present study, TR_{34}/L98H was the most frequent mutation observed, as has been observed in other studies (4, 23–25, 31).

AsperGenius PCR was tested retrospectively in the same period of the study in order to detect *Aspergillus* spp in BALs. The performance of AsperGenius for identifying patients with probable or proven IA was identical with the in-house PCR when a standard C_T value ($<$36) was applied and slightly better than the culture. A better sensitivity was found when a cutoff C_T value of $\leq$36 was used following ROC curve results. A similar clinical performance for the AsperGenius assay was determined in other studies (33–36). Chong et al. (34) favored a cutoff value of $\leq$38 to increase sensitivity to 84% without harming specificity, while in another study a cutoff value of 39 was chosen for testing AsperGenius in serum samples (35). Diagnostic performances of *Aspergillus* PCRs in the literature are often contradictory and difficult to compare, mainly because of differences in the protocols used. Some meta-analysis and reviews have shown sensitivities and specificities also around 76 to 79% and 93 to 94%, respectively, when BAL specimens were tested (37–39).

AsperGenius identified similar azole resistance occurrences among patients with proven or probable IA compared to VIPcheck. The advantage of AsperGenius is the combination of a sensitive method with the detection of resistance in the same test. Culture-based methods are theoretically less sensitive and slower than molecular methods, essentially due to the well-reported poor sensitivity of culture for IA diagnosis (40). However, culture-based methods are cheaper than molecular methods, and they allow the identification of new resistance mutations and trends. Moreover, unselected culture of different respiratory samples from the same patient could improve the detection of azole-resistant *A. fumigatus* isolates in patients at high risk for IA.

In the past, microbial surveillance systems have improved infection control by helping acquire knowledge concerning risk factors, trends in resistance, and how to improve therapeutics (41). A recently published international expert opinion on the management of infection due to azole-resistant *A. fumigatus* recommends reconsidering azole monotherapy in regions where azole resistance rates exceed 10% (42). However, some authors consider that before making decisions on therapeutics, a harmonization of surveillance approaches in terms of mycological procedures and/or the use of an appropriate denominator is required (27, 28).

This study has some limitations since the AsperGenius method was performed retrospectively, and not all GM-negative samples in the study period were included. Therefore, we do not know the exact azole resistance frequency over this period of time. However, this does not change the high azole frequency observed in patients with proven or probable IA.

In conclusion, a high level of azole resistance ($>$10%) was found at Erasme Hospital in unselected clinical respiratory specimens positive for *A. fumigatus* and among patients with proven and probable IA, regardless of the method used: unselected *A. fumigatus* culture-based screening by VIPcheck and direct detection of azole-resistant *A. fumigatus* by AsperGenius. These methods could be combined for resistance surveillance and diagnosis in hospitals with a high risk of IA. For clinical decisions concerning management of patients with azole-resistant IA, harmonization of azole resistance surveillance systems is required. Further surveillance of azole resistance in *A. fumigatus* at Erasme Hospital is warranted.

MATERIALS AND METHODS

Culture-based study on resistance mechanisms. (i) Clinical samples. This study took place at Erasme Hospital, an 858-bed university hospital in Brussels, Belgium. All culture-positive respiratory samples for *A. fumigatus* were prospectively screened for azole resistance from June 2015 to October 2016.

(ii) Mycological cultures. Respiratory specimens were processed and inoculated on Sabouraud dextrose sheep agar plates supplemented with chloramphenicol (bioTRADING, Mijdrecht, The Netherlands). The inoculated plates were incubated at 35°C for at least 15 days. *Aspergillus* isolates were identified first by microscopic and then by macroscopic morphological methods and confirmed by β-tubulin sequencing (43).

(iii) Susceptibility testing by azole resistance screening. All *A. fumigatus* isolates were screened for azole resistance on VIPcheck (Balis Laboratorium, Boven-Leeuwen, The Netherlands). These plates contain four wells with itraconazole (4 mg/liter), voriconazole (1 mg/liter), posaconazole (0.5 mg/liter), or no antifungal agent (growth control well). In each well, 25 μl of a suspension with a turbidity standard of 0.5 McFarland (prepared with four to five colonies of *A. fumigatus*) was inoculated. All isolates able to grow on at least one of the azole-containing wells were further investigated for their MICs by Sensititre YeastOne (Trek Diagnostic Systems, Cleveland, OH) according to the manufacturer's instructions. Epidemiological cutoffs based on Clinical and Laboratory Standards Institute guidelines were used for the interpretation of the MIC values (0.5 μg/ml for posaconazole and 1 μg/ml for voriconazole and itraconazole) (44).

(iv) Resistance genotyping. The promoter and/or full coding sequences of *cyp51A*, *cyp51B*, and *hapE* were amplified using primers and conditions previously described (8, 9, 14, 45). Amplicons were obtained by using the PCR master mix Promega (Promega Benelux, Leiden, The Netherlands) and later purified with an illustra ExoStar 1-Step kit (GE Healthcare Europe, Diegem, Belgium). Custom primers were designed to sequence each entire gene. Sequencing was performed by using a Dye Terminator DNA sequencing kit (v1.1; Applied Biosystems), followed by purification using a Performa DTR Ultra 96-well plate kit (Edge Bio, Gaithersburg, MD). Sequences were assembled and analyzed using BioNumerics 7.6 software (Applied Maths NV, Sint-Martens-Latem, Belgium). The sequences obtained were compared to *cyp51A* (AF338659), *cyp51B* (AF338660), and *hapE* (CM000174) sequences of reference strains from the NCBI database (http://www.ncbi.nlm.nih.gov/GenBank/index.html).

Evaluation of AsperGenius PCR. (i) Clinical samples. BALs from 489 hospitalized patients were tested for galactomannan (GM) antigen detection (Platelia Aspergillus; Bio-Rad, Marnes-la-Coquette, France) between June 2015 and October 2016. GM was considered positive at an optical density (OD) index cutoff value of $\geq$1. A retrospective selection of the BALs analyzed during that period was performed in order to include 100 hospitalized patients: all patients with GM positive BALs (n = 45), as well as the first 55 patients with GM-negative BAL specimens (12%). When the BAL specimens were assessed, three aliquots of 50 ml were sampled and diluted in saline. The second and third aliquots were sent to the microbiology laboratory for GM analysis and culture.

(ii) DNA extraction of BAL specimens. DNA was extracted from 800-μl BAL samples (elution volume, 60 μl) with a QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen, Westburg, The Netherlands) according to the manufacturer's instructions.

(iii) AsperGenius PCR. The commercially available AsperGenius assay is a PCR that targets *Aspergillus* species DNA (including specific detection of *A. fumigatus* and *Aspergillus terreus*) in combination with the detection of the most prevalent mutations (TR_{34}/L98H and TR_{46}/Y121F/T289A) conferring azole resistance. This real-time PCR was performed according to the manufacturer's instructions using a LC480 sequence detector system. A C_T cutoff value of $<$36 for the *Aspergillus* probe is recommended by the manufacturer.

The performance of this commercial PCR assay to diagnose IA was compared to mycological culture results and an "in-house"-developed PCR on the QuantStudio Dx (Qiagen) using primers and probe as described in the literature (46, 47). The PCR mix contained 7.5 μl pf TaqMan Fast Virus Mix (Life Technologies), primers (0.25 μmol/liter), probe (20 μmol/liter), internal control primers and probe for detection of inhibition (0.1 μmol/liter, template Phocine herpesvirus 1), and 10 μl of DNA template in a total volume of 30 μl. PCR conditions were one cycle of 95°C for 20 s, followed by up to 45 cycles of 95°C for 3 s and finally 60°C for 30 s, while acquiring the fluorescence data each cycle.

Patients and clinical aspects. All patient characteristics were acquired retrospectively by reviewing medical charts including age, gender, underlying disease, immunosuppressive therapy, antifungal treatment, laboratory results, radiological presentation, and final clinical diagnosis. Patients were classified as having proven, probable or possible IA according to the revised EORTC/MSG definitions. Briefly, proven IA is defined as the demonstration of fungal elements in diseased tissue. Probable IA requires a host factor, clinical features, and mycological evidence of infection. Finally, possible IA are patients with appropriate host factors, with sufficient clinical evidence consistent with IA, but for whom mycological evidence is lacking. Host factors are the characteristics by which individuals predisposed to acquire IA can be recognized. Patients with host factors include those with cancer, recipients of hematopoietic stem cell transplants, recipients of solid-organ transplants, patients with hereditary immunodeficiencies, patients with connective tissue disorders, and those who are treated with immunosuppressive agents (corticosteroids or T cell immunosuppressants) (48). Patients who could not be included in these three categories were defined as unclassifiable. Classification was performed by an infectious disease physician with experience in infectious lung disease in immunocompromised hosts and who was blind to PCR results.

Statistical analysis. An ROC curve was constructed and used to define AsperGenius optimal cutoff C_T values for IA diagnosis. Correlation between the two real-time PCR results was analyzed by Spearman coefficient rank correlation test. The sensitivity and specificity for diagnosing IA were calculated for each real-time PCR performed in this study and for the culture method. EORTC/MSG clinical classification was considered as the gold standard; proven and probable IA were considered true positives. Patients who did not meet the EORTC/MSG clinical criteria for proven and probable IA and who were not treated with antifungals were considered true negatives. A P value of $<$0.05 was considered statistically significant. Statistical analysis was performed by using the MedCalc software (Mariakerke, Belgium).

Accession number(s). The *cyp51A*, *cyp51B*, and *hapE* sequences generated in this study were deposited in GenBank (nucleotide accession numbers MF070870 to MF070914).

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