Chapter 5.1

Direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. Validation of a new *Aspergillus* real-time PCR.

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ABSTRACT

Introduction

Azole resistance in *Aspergillus fumigatus* is increasingly reported. We describe the validation of AsperGenius®, a new multiplex real-time polymerase chain reaction (PCR) assay consisting of two multiplex real-time PCRs: one which identifies the clinically relevant *Aspergillus* species, and one which detects the TR_{34}, L98H, T289A, Y121F mutations in *Cyp51A* and differentiates susceptible from resistant *A. fumigatus* strains.

Methods

The diagnostic performance was tested on 37 bronchoalveolar lavage (BAL) samples from hematology patients and on 40 BAL samples from intensive care unit (ICU) patients using BAL galactomannan ≥1.0 or positive culture as the gold standard for the presence of *Aspergillus*.

Results

In the hematology and ICU groups combined, there were 22 BAL samples with IA (2 proven, 9 probable and 11 non-classifiable). Nineteen of the 22 BAL samples were positive according to the gold standard. The optimal cycle threshold value for the presence of *Aspergillus* was <36. Sixteen of the 19 BAL samples had a positive PCR (2 *Aspergillus* species and 14 *A. fumigatus*). This resulted in a sensitivity, specificity, positive and negative predictive value of 88.9%, 89.3%, 72.7% and 96.2% for the hematology group and 80.0%, 93.3%, 80.0% and 93.3% in the ICU group, respectively. The *Cyp51A* real-time PCR confirmed 12 wildtype and 2 resistant strains (1 TR_{34}/L98H and 1 TR_{46}/Y121F/T289A mutant).

Conclusion

The AsperGenius® multiplex real-time PCR allows for a sensitive and fast detection of *Aspergillus* species directly in BAL samples. More importantly, this assay detects and differentiates wildtype from resistant strains even if BAL cultures remained negative.

INTRODUCTION

Aspergillus fumigatus is the most frequent cause of invasive mould infections in immunocompromised patients. Its mortality varies substantially and depends on patient characteristics and the extent of disease. Mortality in intensive care unit (ICU) patients with invasive aspergillosis (IA) can be as high as 90% [1]. In hematology patients, a relatively low mortality is observed when the diagnosis is made early and treatment with voriconazole, the current standard of care [2], is initiated promptly [3]. In 2002, the landmark study by Herbrecht *et al*. showed that treatment of IA with voriconazole resulted in an improved survival [4]. However, a series of recent publications described the appearance of azole resistance in *A. fumigatus* [5-10]. This resistance is caused by a mutation in the *Cyp51A* gene of *A. fumigatus* at position 98 (L98H) together with a 34-basepair tandem repeat (TR) in the promoter region (TR_{34}). *Cyp51A* encodes for cytochrome p450 sterol 14α-demethylase, the target of azoles. The majority of these mutated strains were cultured from patients never exposed to azoles. It is assumed that resistance development is caused by environmental azole exposure [11]. More recently, van der Linden *et al*. described a second mutation, a 46-basepair TR combined with the point mutations Y121F and T289A [12]. In this study, 47 of 921 patients (5.1%) were diagnosed with TR_{34}/L98H and 13 (1.4%) with the TR_{46}/Y121F/T289A mutation. Occasionally, other mutations have also been described [13-16]. Infections with azole resistant strains are associated with a very high mortality [17].

Currently, the absence of a non-culture based, fast and readily available azole susceptibility testing method compromises the identification of azole resistance. This is a major limitation as the mortality of IA increases substantially when the initiation of adequate therapy is delayed [18]. Furthermore, most *Aspergillus* infections are diagnosed indirectly using galactomannan (or beta 1-3 d-glucan) testing because cultures remain negative in most patients. Therefore, even if culture based azole resistance testing would become broadly available, this would only be helpful in a subset of patients.

This study describes the laboratory and first clinical validation of AsperGenius®, a new *Aspergillus* real-time polymerase chain reaction (PCR) that detects *Aspergillus* species directly from bronchoalveolar lavage (BAL) and simultaneously identifies the most prevalent *Cyp51A* mutations in *A. fumigatus*.

METHODS

This retrospective study was performed at the Erasmus University Medical Center in the Netherlands. The following information was obtained: age, sex, mortality, underlying disease, reason for ICU admission, hospital admission duration, presence and treatment

of IA. For the *Aspergillus* PCR, we used stored BAL samples of historical patients. BAL samples of hematological and ICU patients were selected because these patients are at high risk for IA.

Processing of BAL samples

BAL samples from ICU patients (1-2 ml) were incubated with 0.1 M dithiothreitol (DTT) to reduce viscosity. This was not needed for BAL samples from neutropenic hematology patients. Subsequently, all BAL samples were centrifuged at high speed (10 min at 13400 g). After centrifugation, the supernatant and the pellet were processed in different ways. The supernatant was added to 2 ml NucliSENS® lysis buffer (BioMérieux, Boxtel, the Netherlands) and incubated for 10 min at room temperature. An Internal Control (IC) was added to the BAL to monitor PCR inhibition, DNA-extraction efficiency and manual handling errors. The pellet was transferred to green bead tubes (Roche Diagnostics, Indianapolis, USA) and 500 µl NucliSENS® lysis buffer was added together with 5 µl of IC. The pellets were subsequently bead-beaten in a MagNA Lyser instrument (Roche Diagnostics) for 45 sec at 6500 rpm. Proteinase K (Roche Diagnostics) was added and incubated for 10 min at 65 °C, and subsequently inactivated for 10 min at 95 °C. After centrifugation, the supernatant of the lysed pellet suspension was transferred to a new tube. DNA from both supernatant and pellet was extracted with the NucliSENS® miniMAG magnetic extraction (BioMérieux) according to the manufacturer's instructions. The DNA from both the pellet and supernatant were tested separately.

AsperGenius® multiplex real-time PCR assay

The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) was used for the detection of *Aspergillus* species and the identification of prevalent mutations conferring resistance against triazoles. The AsperGenius® species multiplex assay allows for specific detection of *A. fumigatus* complex, *A. terreus* and *Aspergillus* species by targeting the 28S rRNA multicopy gene. The *A. fumigatus* probe detects the most relevant species of the *Fumigati* complex: *A. fumigatus, A. lentulus, A. udagawae* and *A. viridinutans.* The *Aspergillus* species probe specifically detects *A. fumigatus, A. terreus, A. flavus* and *A. niger.* In addition the *A. nidulans* could be detected based on sequence information. Detection of the IC is included. The AsperGenius® resistance multiplex assay targets the single copy *Cyp51A* gene of *A. fumigatus* and detects TR_{34}, L98H, Y121F and T289A to differentiate wildtype from mutant *A. fumigatus* strains via melting curve analysis. The real-time PCR was performed according to the manufacturer's instructions. Detection of four different fluorescent labels (emission spectra: 495 nm, 530 nm, 598 nm, 645 nm) was enabled by using the Rotor-Gene Q (Qiagen, Heidelberg, Germany) for all experiments. The real-time PCR assay was first validated on DNA of 131 *A. fumigatus* cultures including resistant strains (Erasmus University Medical Center) before testing BAL samples.

These strains were identified to the species level on morphology and by sequencing of the internal transcribed spacer region. Furthermore, the assay was tested for cross reactivity with species selected on their prevalence in the respiratory tract and/or their genomic similarity. The specificity was tested for the following species: *P. marneffei, P. chrysogenum, Fusarium* species, *Scedosporium* species, *R. oryzae*, *S. cerevisae, C. neoformans, C. albicans ,C. lustaniae, C. krusei*, *C. dubliniensis, C. guilliermondii, C. tropicalis, C. glabrata, C. parapsilosis, P. jirovecii*, *B. pertussis, E. coli, H. influenzae, M. catarrhalis, P. aeruginosa, S. aureus*, *S. pneumoniae, K. pneumoniae, C. pneumoniae, L. pneumoniae*, *M. pneumoniae.*

Each extracted BAL sample was tested in duplicate and a no template control (blank) was included in each run to exclude contamination. A sample was considered positive when one of the duplicates showed increased fluorescence above the threshold. Synthetic single-stranded DNA targets (IDT, Heverlee, Belgium) were included in the assay as a standard (positive control) for the melting peaks. These positive control sequences were tested simultaneously with the BAL samples to determine if the melting peak represents wildtype or resistant *A. fumigatus* strains.

Gold standard

BAL samples with a positive galactomannan (≥ 1.0) or positive BAL or sputum cultures for *Aspergillus* were selected as the gold standard for positivity. True negatives were BAL samples with a negative BAL galactomannan in combination with a negative culture from BAL or sputum. BAL samples with only a positive serum galactomannan (≥ 0.5) were considered to be negative as there was no microbiological evidence of the presence of *Aspergillus*.

Classification of IA

The European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria for IA were used to classify patients into *possible*, *probable* or *proven* IA. A patient is considered to have ***possible IA*** if a new and otherwise unexplained well-defined intrapulmonary nodule (with or without halo sign), an air-crescent sign, or a cavity within an area of consolidations is radiologically documented in an immunocompromised host. ***Probable IA*** is diagnosed when on top of these radiological findings microbiological proof of *A. fumigatus* infection is documented by galactomannan antigen detection (Platelia™ Bio-Rad inc.) or positive cultures of *A. fumigatus.* Galactomannan was considered positive in BAL fluid if ≥ 1.0 and in serum when ≥ 0.5. ***Proven IA*** is defined as histopathologic evidence of invasive mould infection and microbiological proof of *A. fumigatus* infection. Immunocompromised patients who had a positive galactomannan test but who could not be categorized into probable IA, because the radiology of the lungs was non-specific, were referred as ***non-classifiable IA***, a category not included in the EORTC/MSG definitions. In clinical practice, these patients

are treated similarly to patients with probable IA because the outcome of these patients is comparable to patients with probable IA [19]. Note that most ICU patients with a clinical diagnosis of IA will fall into this non-classifiable category because in contrast to the neutropenic patients, the findings on high-resolution computed tomography or chest X-ray in ICU patients with IA is mostly non-specific [1].

PCR cut-off analysis

To determine the most appropriate *Aspergillus* PCR cut-off for clinically significant positivity, we first analysed the cycle threshold (Ct) values of the *Aspergillus* PCR on 37 BAL samples of 34 hematological patients. Three patients in the hematology group underwent a BAL twice at different times and therefore contributed two BAL samples each. The lowest Ct value of the pellet or supernatant was used. The optimal cut-off was assessed with receiver operator characteristic (ROC) curves. Areas under the curve (AUC) were estimated to determine discriminatory power (IBM® SPSS® statistics, version 21). In a second set of 40 BAL samples from 39 ICU patients we confirmed the usefulness of the cut-off that was obtained in the hematology population. The sensitivity, specificity, positive and negative predictive values (PPV, NPV) were calculated for the two groups separately and combined.

RESULTS

The AsperGenius® multiplex real-time PCR assay was first tested on 131 *A. fumigatus* strains. Based on the PCR assay, 17 resistant strains were identified which all carried the TR_{34}/L98H mutation. These data were confirmed by sequencing the *Cyp51A* regions. Furthermore, the specificity of the assay was tested with species selected on their prevalence in the respiratory tract and/or their genomic similarity. No cross-reactivity was observed for *the A. fumigatus*, *A. terreus* and the resistant probes. Limited cross-reactivity was observed with the *Aspergillus* species probe for *R. oryzae and P. chrysogenum* that resulted in false positive signals when using a 1000 times higher DNA load (50 picogram) than the A. fumigatus DNA load needed to get a PCR positive results with a CT of 36 (50 fentogram or 2 DNA copies/ml).

In the hematology and ICU groups combined, there were 22 BAL samples with proven, probable or non-classifiable IA (table 1). More detailed information on the complete set of hematology and ICU BAL samples can be found in the supplement. There were three patients with non-classifiable IA, one in the hematology group and two in the ICU group who had negative BAL galactomannan and culture, but had a positive serum galactomannan. Because BAL galactomannan tests and cultures were negative, there was no microbiological evidence that *Aspergillus* was present in these BAL samples. Therefore, these three BAL samples were counted as negatives in the statistical analysis.

Table 1. Clinical characteristics, radiological findings and results of the galactomannan and AsperGenius® multiplex real-time polymerase chain reaction (PCR) for 22 bronchoalveolar lavage (BAL) samples of patients with proven, probable and non-classifiable invasive aspergillosis (IA).

Patient no.	Clinical setting[a]	IA diagnosis[b]	Underlying disease/ host factor(s)[c]	Radiological findings	BAL or sputum culture[d]	Pathology[e]	AsperGenius® multiplex real-time PCR[f]						
							Galactomannan level		Ct value of *Aspergillus* species PCR		Ct value of *A. fumigatus* PCR		*Cyp51A* PCR
							Serum	BAL	Supernatant	Pellet	Supernatant	Pellet	
1	HEM	Probable	MM, allogeneic HSCT	Specific	Pos	NA	0.2	1.5	Pos (29)	Pos (28)	Pos (29)	Pos (29)	WT
2	HEM	Probable	AML	Specific	Neg	NA	NA	1.3	Pos (32)	Pos (34)	Pos (33)	Pos (35)	WT
3	HEM	Probable	AML	Specific	Pos	NA	0.3	0.2	Pos (33)	Pos (27)	Pos (33)	Pos (28)	WT
4	HEM	Probable	AML	Specific	Neg	NA	1.0	7.1	Pos (29)	Pos (31)	Pos (30)	Pos (32)	WT
5	HEM	Non-classifiable	APL	Not specific	Neg	NA	0.1	1.1	Neg	Neg (39)	Neg	Neg	
6	HEM	Probable	AML	Specific	Neg	Neg	0.4	2.0	Pos (31)	Pos (32)	Pos (33)	Pos (33)	TR_{34}/L98H
7	HEM	Non-classifiable	CLL, allogeneic HSCT	Not specific	Pos	NA	1.3	5.1	Pos (27)	Pos (27)	Pos (29)	Pos (29)	WT
8	HEM	Probable	MM, allogeneic HSCT	Specific	Neg	NA	0.9	5.7	Pos (33)	Pos (35)	Neg (39)	Neg (42)	
9	HEM	Non-classifiable	AML	Not specific	Neg	NA	0.6	0.1	Neg	Pos (34)	Neg	Neg	
10	HEM	Non-classifiable	CLL	Not specific	Pos	NA	0.1	1.3	Pos (35)	Pos (32)	Neg (38)	Pos (34)	WT
11	ICU	Proven	Lung transplantation	Specific	Pos	Pos	NA	0.4	Neg (42)	Pos (35)	Neg	Neg (38)	
12	ICU	Non-classifiable	AML	Not specific	Neg	NA	0.1	1.6	Neg	Neg	Neg	Neg	
13	ICU	Probable	HL, allogeneic SCT	Specific[g]	Pos	NA	7.2	7.5	Pos (31)	Pos (23)	Pos (31)	Pos (24)	TR_{46}/Y121F/ T289A
14	ICU	Non-classifiable	Liver cirrhosis	Not specific	Neg	NA	0.6	0.3	Neg	Neg	Neg	Neg	
15	ICU	Non-classifiable	Lung transplantation	Not specific	Neg	Neg	0.1	1.2	Pos (31)	Pos (30)	Pos (32)	Pos (32)	WT
16	ICU	Proven	Dermatomyositis	Not specific	Neg	Pos	0.8	5.0	Pos (29)	Pos (27)	Pos (30)	Pos (29)	WT
17	ICU	Non-classifiable	HIV	Not specific	Pos	NA	NA	0.2	Pos (33)	Pos (33)	Neg	Pos (35)	WT
18	ICU	Probable	Dermatomyositis	Specific	Neg	NA	NA	5.9	Neg (36)	Neg (36)	Neg	Neg (40)	
19	ICU	Probable	Dermatomyositis	Specific	Neg	NA	5.2	5.9	Pos (33)	Pos (34)	Pos (35)	Neg (38)	WT

Table 1. Clinical characteristics, radiological findings and results of the galactomannan and AsperGenius® multiplex real-time polymerase chain reaction (PCR) for 22 bronchoalveolar lavage (BAL) samples of patients with proven, probable and non-classifiable invasive aspergillosis (IA). (*continued*)

Patient no.	Clinical setting[a]	IA diagnosis[b]	Underlying disease/ host factor(s)[c]	Radiological findings	BAL or sputum culture[d]	Pathology[e]	AsperGenius® multiplex real-time PCR[f]						
							Galactomannan level		Ct value of *Aspergillus* species PCR		Ct value of *A. fumigatus* PCR		*Cyp51A* PCR
							Serum	BAL	Supernatant	Pellet	Supernatant	Pellet	
20	ICU	Non-classifiable	AML	Not specific	Neg	NA	8.1	0.2	Neg	Neg (37)	Neg	Neg	
21	ICU	Non-classifiable	Vasculitis	Not specific	Pos	NA	NA	6.5	Pos (26)	Pos (24)	Pos (26)	Pos (25)	WT
22	ICU	Non-classifiable	MM	Not specific	Pos	NA	0.3	22.7	Pos (32)	Pos (26)	Pos (34)	Pos (27)	WT

[a] HEM, hematology. ICU, intensive care unit.

[b] IA, invasive aspergillosis.

[c] MM, multiple myeloma. HSCT, hematopoietic stem cell transplantation. AML, acute myeloid leukaemia. APL, acute promyelocytic leukaemia. CLL, chronic lymphatic leukaemia. HL, Hodgkin lymphoma.

[d] Pos, positive. Neg, negative.

[e] NA, not available.

[f] WT, wildtype. Ct, cycle threshold. Ct value < 36 was considered positive and Ct value ≥ 36 negative.

[g] No specific radiological findings in the lung, but specific cerebral findings on magnetic resonance imaging.

The ROC curves for the different groups are shown in figure 1. The diagnostic accuracy as given by the AUC for the hematology group was 0.92 (95% CI 0.79 – 1.00; p-value < 0.001). The AUC was 0.91 (95% CI 0.76 – 1.00; p-value < 0.001) for the ICU group and 0.91 (95% CI 0.81 – 1.00; p-value < 0.001) for the two groups combined. The most optimal Ct value cut-off for the *Aspergillus* species PCR was < 36 for the 37 BAL samples of the hematology patients. Table 2 shows the IA classification related to the Ct < 36 cut-off and table 3 shows the BAL samples according to the gold standard related to the Ct < 36 cut-off. In the hematology group, the Ct < 36 cut-off resulted in a sensitivity, specificity, PPV and NPV of 88.9%, 89.3%, 72.7% and 96.2%, respectively. In the ICU group (n=40), the Ct < 36 cut-off value resulted in a sensitivity, specificity, PPV and NPV of 80.0%, 93.3%, 80.0% and 83.3%, respectively. Therefore, the overall sensitivity, specificity, PPV and NPV was 84.2%, 91.4%, 76.2% and 94.6%, respectively.

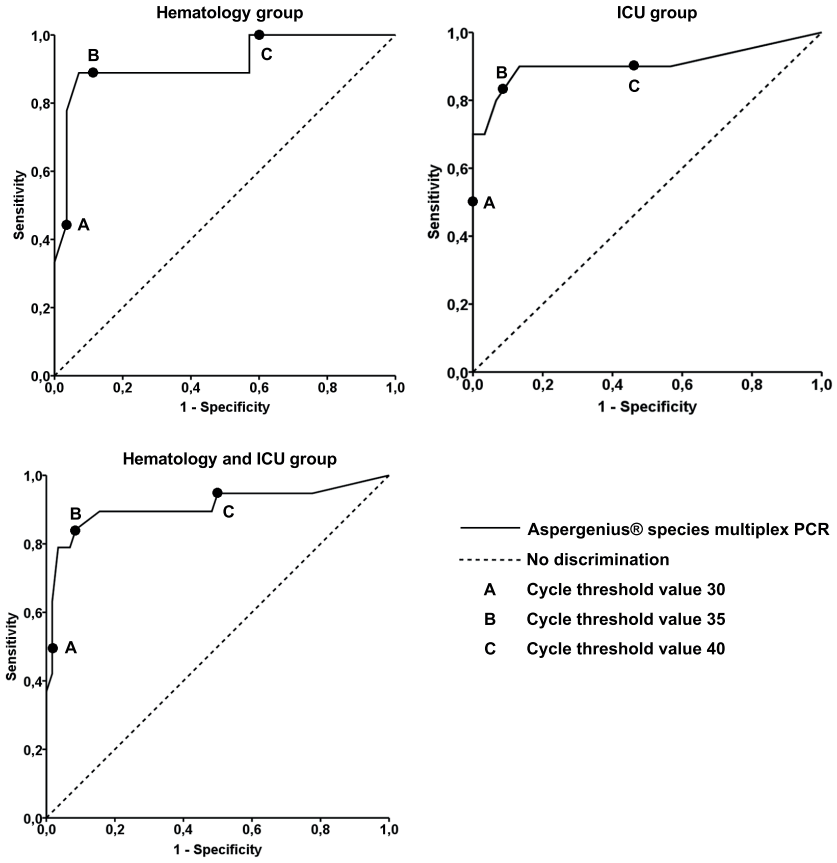

Figure 1. Receiver operator characteristic curves of AsperGenius® species multiplex real-time polymerase chain reaction (PCR) in bronchoalveolar lavage in the hematology, intensive care (ICU) group and combined.

Table 2. Epidemiological classification of invasive aspergillosis (IA) related to cycle threshold (Ct) cut-off of 36.

Classification of IA	Hematology group BAL sample n = 37			ICU group BAL samples n = 40		
	Ct < 36 cut-off	Ct ≥ 36 cut-off	Total	Ct < 36 cut-off	Ct ≥ 36 cut-off	Total
Proven IA	0	0	0	2	0	2
Probable IA	6	0	6	2	1	3
Non-classifiable IA	3	1	4	4	3	7
Possible	2	1	3	0	5	5
No IA	0	24	24	2	21	23

Note: BAL, bronchoalveolar lavage. ICU, intensive care unit.

Table 3. Bronchoalveolar lavage (BAL) samples with positive galactomannan or cultures according cycle threshold (Ct) cut-off of 36.

BAL samples	Hematology group BAL sample n = 37			ICU group BAL samples n = 40		
	Ct cut-off < 36	Ct cut-off ≥ 36	Total	Ct cut-off < 36	Ct cut-off ≥ 36	Total
BAL samples with positive galactomannan ≥ 1.0 and/or with positive culture	8	1	9	8	2	10
BAL samples with negative galactomannan < 1.0 and negative culture	3	25	28	2	28	30

Note: ICU, intensive care unit

In the hematology and ICU patients combined, 19 BAL samples of patients with proven, probable or non-classifiable IA were identified based on the gold standard. From these 19 BAL samples, 16 had a positive *Aspergillus* PCR (15 positive for both the pellet and supernatant, 1 positive for only the pellet). Fourteen of the 16 BAL samples had a positive *A. fumigatus* PCR and the remaining two were *Aspergillus* species. In all 14 positive *A. fumigatus* PCR BAL samples, the *Cyp51A* resistance PCR was successful. Twelve strains were determined as wildtype and two as mutant strains. One sample had a TR_{46}/Y121F/T289A mutation and the other had a TR_{34}/L98H mutation. More information on the melting curves of the mutant strains can be found in the supplement. Both patients of these BAL samples showed clinical failure of voriconazole therapy. The first patient was treated with allogeneic stem cell transplantation for acute myeloid leukaemia. The patient died of culture positive pulmonary and cerebral IA developed during voriconazole prophylaxis (3 days before he died, the single serum voriconazole level was high at 8 mg/L). The second patient developed IA during remission induction chemotherapy for acute myeloid leukaemia. The patient showed an increased serum galactomannan level,

progressive pulmonary infiltrates and pleural effusion (galactomannan levels in pleural fluid were > 2.0) despite 14 days of therapeutic voriconazole serum levels (>4 mg/L, supplement figure S4). Cultures were repeatedly negative. The patient survived after surgical drainage and 8 weeks of liposomal amphotericin-B combined with voriconazole.

DISCUSSION

In this study, the AsperGenius® multiplex real-time PCR showed good performance characteristics for the diagnosis of IA directly on clinical samples in 2 distinct patient populations at risk for this infection. In addition to the fast and correct identification of *A. fumigatus*, this PCR simultaneously differentiated azole susceptible from resistant strains. In contrast, current conventional microbiological tools for susceptibility testing of *A. fumigatus* are rarely helpful because they are time-consuming, not widely available and patients with IA are mostly culture negative. This multiplex real-time PCR assay tackles these problems by enabling diagnosis within hours after a BAL sample is submitted to the laboratory.

In the last decade, the use of galactomannan testing and high-resolution computed tomography of the lungs allowed for an early diagnosis of IA. Together with the availability of voriconazole as the preferred therapy, this resulted in a major decrease in IA-related mortality [3,20]. However, now that azole resistance in *A. fumigatus* is increasing [5-10], the availability of azole resistance testing with a short turn-around time is critical to secure this improved survival in patients with IA. The PCR that was validated in this study can serve this purpose. In accordance with the PCR results, patients can be switched to other non-azole antifungal therapy in an early phase and not when patients clinically deteriorate.

The ROC curves showed that the most optimal Ct value cut-off was 36. This cut-off was comparable to the cut-off described for the 2 *Aspergillus* PCR assays tested by Torelli *et al.* [21]. Moreover, these PCR assays were validated on BAL samples of hematology and ICU patients, the same subset of patients as in the present study. Given these observations, the 36 Ct value cut-off is probably accurate.

In the hematology group, there were one false negative and two false positive *Aspergillus* PCR results when compared with the gold standard. The false negative result was in a BAL sample of a patient with non-classifiable IA (patient no. 5 in table 1). The lung abnormalities could also be a side effect of the chemotherapy that the patient received for her acute promyelocytic leukaemia. It is possible that this patient did not have IA and that the galactomannan of 1.1 in BAL was false positive. The two false positive *Aspergillus* PCR BAL samples were from patients with possible IA. The BAL galactomannan of the first patient was 0.7 and of the second was 0.4. The first patient was treated with

antifungal therapy because there was no alternative diagnosis. In the second patient, a lung biopsy was performed that showed an organizing pneumonia without signs for a fungal infection. As with every diagnostic test, there is no galactomannan cut-off with a 100% diagnostic accuracy. A BAL galactomannan cut-off of 0.5 has an increased sensitivity but a somewhat lower specificity [22]. For PCR validation purpose of this study, we considered a higher specificity more important and therefore we selected the 1.0 galactomannan cut-off as the gold standard.

The PCR assay was performed on DNA extracted from both the pellet and the supernatant of the BAL. The DNA extraction from the pellet is more labour intensive than the extraction of free-circulating DNA from the supernatant. Therefore, it is reassuring that 15 of 16 supernatants were PCR positive. In the remaining patient only the PCR of the DNA extracted from the pellet was positive (Ct value < 36). In this particular patient, the PCR positivity of the pellet was corroborated by a positive sputum culture. Therefore, it may be reasonable and more time efficient to first test the supernatant and only test the pellet if the supernatant is negative in patients with a positive *Aspergillus* culture, a positive galactomannan, or a very high clinical suspicion.

The *Aspergillus* species probe showed cross-reactivity for the *P. chrysogenum* and *R. oryzae*. *P. chrysogenum* is rarely pathogenic in humans. *R. oryzae* can cause comparable symptoms as invasive aspergillosis, but the clinical prevalence is low. Furthermore, for both these species, a 1000 times higher load of DNA was needed to get a Ct-value result of 35 with the Aspergillus species probe. Thus, we believe that these species will not compromise the performance of the PCR.

This study has also some limitations. The validation was performed on readily available leftover BAL fluids from historical patients. At the clinical microbiology laboratory of the Erasmus University Medical Center, all superfluous BAL fluids are stored at -20 ^{o}C for future research purposes. Therefore, no selection bias occurred during storage of the samples. Nevertheless, the results of this study should be confirmed on a larger sample set from different hospitals and ideally prospectively collected across different countries. Another limitation is the fact that only the *Cyp51A* mutations that are included in the PCR, will be detected. As such, this PCR will not replace culture-based sensitivity testing and when this PCR is used, the results should be interpreted in the epidemiological context. Finally, the diagnostic characteristics of every test and in particular the PPV will depend on the background incidence of the population tested. Therefore, the PPV and NPV we describe may be different in other patient populations.

When confirmed in a larger study, this PCR may be incorporated in the EORTC/MSG criteria. The *Aspergillus* PCR could be used in combination with galactomannan testing as it provides information on the *Aspergillus* species involved and azole resistance.

In conclusion, this new multiplex real-time PCR allows for a sensitive and fast detection of *Aspergillus*. Furthermore, it can differentiate wildtype from resistant strains

even on culture negative BAL samples. This enables on-time and targeted therapy in IA-patients.

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SUPPLEMENTARY MATERIAL

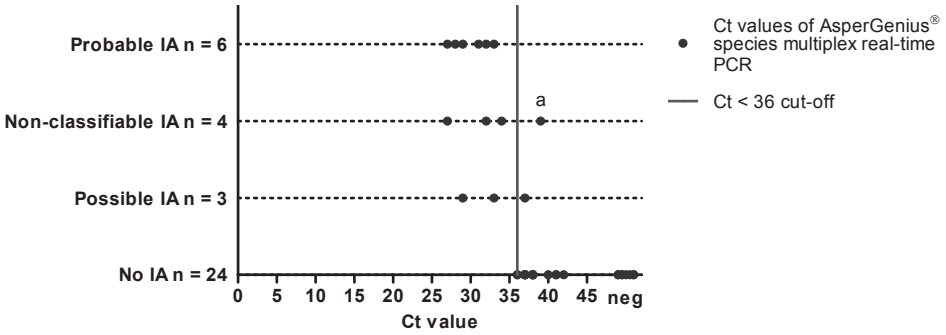

Figure S1. Ct-values of AsperGenius® species multiplex real-time polymerase chain reaction (PCR) in the hematology group.
37 bronchoalveolar lavage samples of hematology patients with probable IA (n=6), with non- classifiable IA (n=4) and no IA (n=24). "a" = patient (no. 5 in table 1) with galactomannan in BAL of 1.1 and in serum of 0.1. The vertical line represents the Ct < 36 cut-off. Ct ≥ 36 are negative (neg) samples.

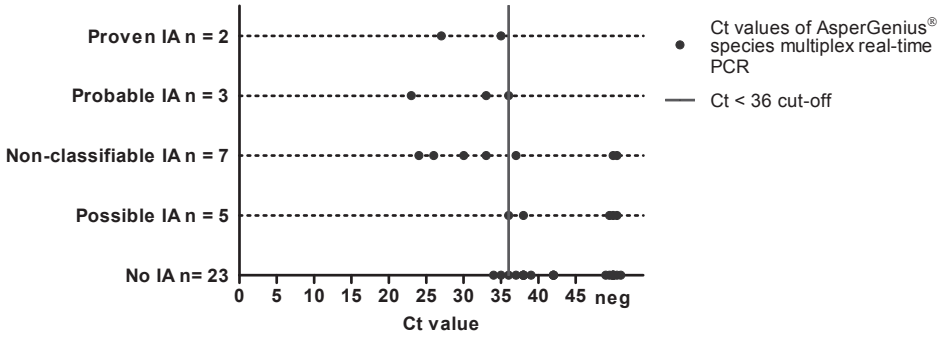

Figure S2. Ct-values of AsperGenius® species multiplex real-time polymerase chain reaction (PCR) in the intensive care (ICU) group.
40 BAL samples of ICU patients with proven (n = 2), probable IA (n=3), non-classifiable IA (n=7), possible IA (n=5) and no IA (n=23). The vertical line represents the Ct < 36 cut-off. Ct ≥ 36 are negative (neg) samples.

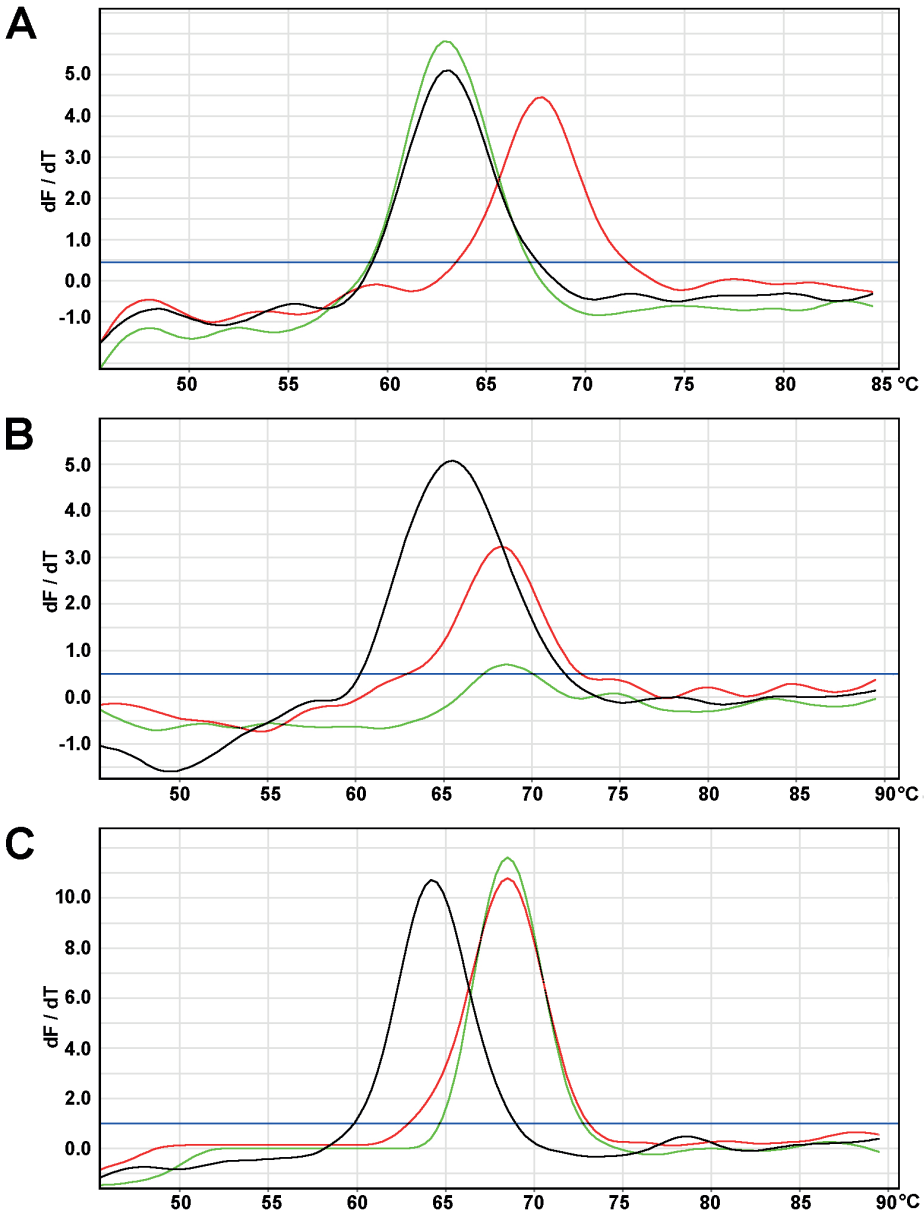

Figure S3. Melting curves of the *A. fumigatus Cyp51A* region.
(A) Patient with an azole susceptible *A. fumigatus* (green) compared to the mutant (red) and wildtype L98 control (black).
(B) Patient infected with an azole resistant *A. fumigatus* TR_{34}/L98H (green) compared to mutant (red) and wildtype TR_{34} control (black).
(C) Patient infected with an azole resistant *A. fumigatus* TR_{46}/Y121/T289 (green) compared to mutant (red) and wildtype T289 control (black).

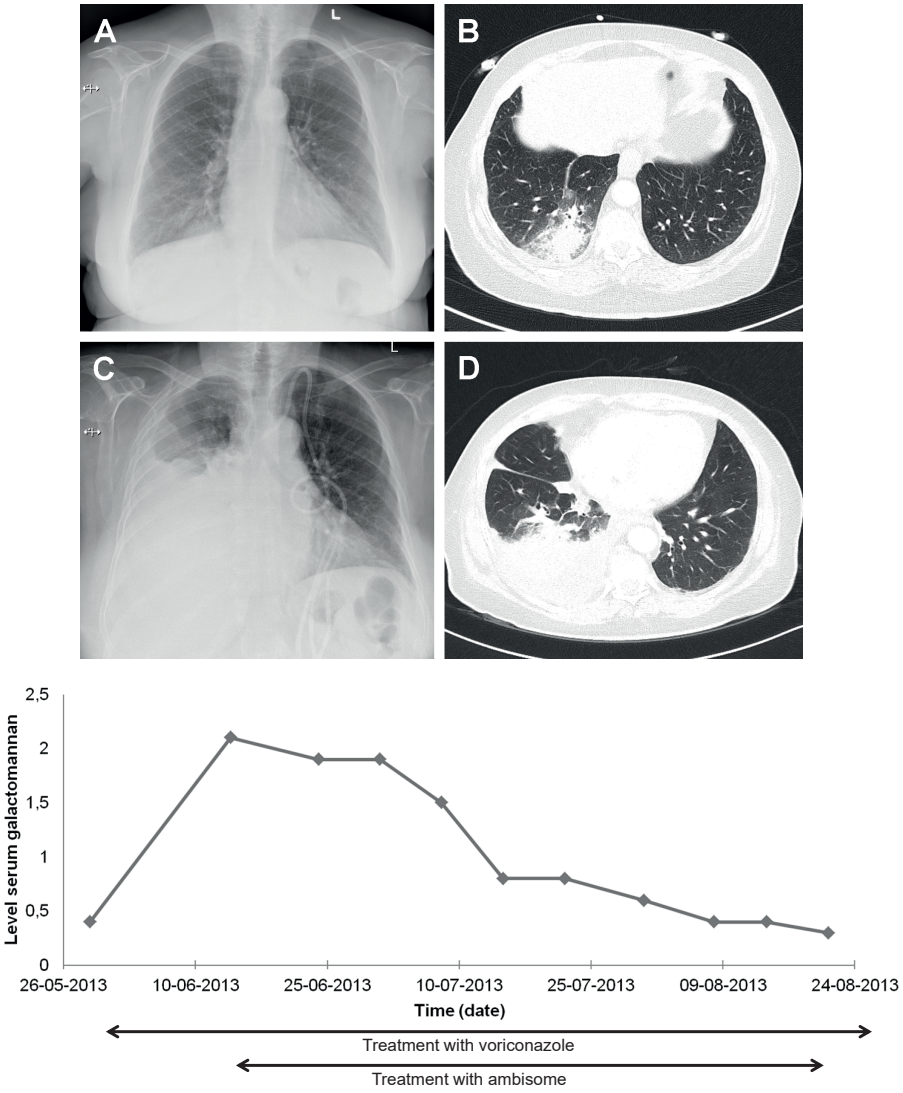

Figure S4. Patient with azole resistant invasive aspergillosis, bronchoalveolar lavage sample showed a TR34/L98H mutation.

Patient was initially treated with voriconazole. After 15 days of treatment, X-ray and computed tomography of the chest showed progressive pulmonary infiltrates and pleural effusion. Liposomal amphotericin-B was added to the treatment. Serum galactomannan eventually became negative after 2 months of voriconazole-liposomal amphotericin-B double therapy.

(A) Chest X-ray before start treatment with voriconazole.

(B) Computed tomography of the chest before start treatment with voriconazole.

(C) Chest X-ray 15 days after start treatment with voriconazole.

(D) Computed tomography of the chest 15 days after start treatment with voriconazole.

(E) Serum galactomannan levels during treatment.

Table S1. Characteristics of the patients of the bronchoalveolar (BAL) samples of the hematology and ICU group.

	Hematology group BAL samples n = 37	ICU group BAL samples n = 40
Mean age in years	58.9 (19.8-83.2)	52.8 (18.3-73.2)
Underlying hematological disease (%)		
Acute myeloid leukaemia	24 (63.2%)	-
Acute lymphatic leukaemia	2 (5.3%)	-
Chronic lymphatic leukaemia	2 (5.3%)	-
Non-Hodgkin Lymphoma	2 (5.3%)	-
Multiple myeloma	4 (10.5%)	-
Myeloproliferative neoplasms	3 (7.9%)	-
Myelodysplastic syndrome	1 (2.6%)	-
Reason ICU admission (%)		
Pneumonia	-	22 (55.0%)
Interstitial lung abnormalities	-	3 (7.5%)
Pulmonary edema	-	3 (7.5%)
Livercirrosis / failure / transplantation	-	2 (5.0%)
Neurological cause	-	3 (7.5%)
Sepsis	-	6 (15.0%)
Multiorgan failure	-	1 (2.5%)
Invasive aspergillosis (%)		
Proven	0	2 (5.0%)
Probable	6 (16.2%)	3 (7.5%)
Possible	3 (8.1%)	5 (12.5%)
Non classifiable	4 (10.8%)	7 (17.5%)
No IA	24 (64.9%)	23 (57.5%)
Treated with following antifungal therapy[a]		
Abelcet	1 (2.7%)	0
Ambisome	6 (16.2%)	6 (15.0%)
Anidulafungin	0	3 (7.5%)
Caspofungin	2 (5.4%)	5 (12.5%)
Fungizone	2 (5.4%)	1 (2.5%)
Micafungin	0	5 (12.5%)
Posaconazol	1 (2.7%)	1 (2.5%)
Voriconazol	22 (59.5%)	18 (45.0%)
No antifungal therapy	14 (37.8%)	18 (45.0%)
Mortality (%)		
At 6 weeks	7 (18.4%)	20 (50.0%)
At 12 weeks	10 (26.3%)	22 (55.0%)
Hospital admission duration (days)		
Hospital stay in total	47.9 (0-388.0)	49.0 (3.0-216.0)
IC stay	-	25.7 (2.0-133.0)

[a] Some patients were treated with more than one antifungal therapy.