Chapter 5.2

PCR-based detection of *A. fumigatus Cyp51A* mutations on bronchoalveolar lavage. A multi-center validation of the AsperGenius assay® in 201 patients with hematological disease suspected for invasive aspergillosis.

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ABSTRACT

Objectives

In patients with invasive aspergillosis (IA), fungal cultures are mostly negative. Consequently, azole resistance often remains undetected. The AsperGenius® multiplex real-time PCR assay identifies clinically relevant *Aspergillus* species and 4 resistance associated mutations (RAM: TR_{34}/L98H/T289A/Y121F) in the *Cyp51A* gene. This multicenter study evaluated the diagnostic performance of this assay on bronchoalveolar lavage (BAL) fluid and correlated the presence of RAMs with azole treatment failure and mortality.

Methods

Stored BAL samples from patients with hematological diseases suspected for IA were used. BAL samples that were galactomannan/culture positive were considered positive controls for the presence of *Aspergillus*. Azole treatment failure and 6-week mortality was compared in patients with and without RAMs that had received ≥5 days of voriconazole monotherapy.

Results

201 patients contributed each 1 BAL sample, of which 88 were positive controls and 113 negative controls. The optimal cycle threshold cut-off value for the *Aspergillus* species PCR was <38. With this cut-off, the PCR was positive in 74/88 positive controls. The sensitivity, specificity, positive and negative predictive values were 84%, 80%, 76% and 87%, respectively. 32/74 BAL samples were culture negative. Azole treatment failure was observed in 6/8 patients with a RAM compared to 12/45 patients without RAMs (p-value=0.01). Six-week mortality was 2.7 times higher in in patients with RAMs (50.0% versus 18.6%, p-value=0.07).

Conclusion

The AsperGenius® assay had a good diagnostic performance on BAL and differentiated wildtype from *A. fumigatus* with RAMs, also in culture negative BAL samples. Most importantly, detection of RAMs was associated with azole treatment failure.

INTRODUCTION

Invasive aspergillosis (IA) is the most frequent pulmonary mold infection among immunocompromised patients with hematological diseases, and is usually caused by *Aspergillus fumigatus* [1,2]. The triazole voriconazole is currently recommended for first line therapy [3]. However, (pan)azole resistance in *A. fumigatus* has been reported increasingly over the past decade with a prevalence ranging from 1.0% to as high as 20.0% [4-11]. This is worrisome because a study showed that the mortality in culture positive IA caused by an azole resistant strain was 88% [11]. Azole resistance is often caused by mutations in the *Cyp51A* gene that encodes for the lanosterol 14α-demethylase, the target enzyme for azoles. Two mutation patterns in this gene account for a large part of azole resistance mechanisms: TR_{34}/L98H and TR_{46}/T289A/Y121F [6,9-12].

Aspergillus cultures of respiratory specimens are positive in at most 26% of the IA cases [13,14]. Given the low sensitivity of the cultures, most cases are diagnosed indirectly by detection of galactomannan (GM) [13]. However, in the absence of a positive culture, azole resistance remains undetected. Thus, the lack of a fast and readily available azole susceptibility test compromises the initiation of adequate treatment in case of azole resistance. The commercially available AsperGenius® multiplex real-time polymerase chain reaction (PCR) assay consists of 2 PCRs: the species PCR identifies the clinically relevant *Aspergillus* species, and the resistance PCR detects the TR_{34}, L98H, T289A and Y121F resistance associated mutations (RAM) that represent the prevalent mutation combinations TR_{34}/L98H and TR_{46}/T289A/Y121F in the *Cyp51A* gene. In a recent single-center study, the diagnostic performance of the species PCR on bronchoalveolar lavage (BAL) samples of patients with hematological diseases showed a sensitivity, specificity, positive and negative predictive values (PPV, NPV) of 89%, 89%, 73% and 96%, respectively, when a cyclic threshold (Ct) value of <36 was used [15]. Moreover, the resistance PCR was able to detect RAMs in a culture negative patient with IA [15].

The purpose of this retrospective multicenter study was to confirm the diagnostic performance of the AsperGenius® assay in a large population of patients with hematological diseases and to evaluate if the molecular detection of the above-mentioned RAMs correlate with azole treatment failure and mortality.

METHODS

Study design

This retrospective study was performed at 3 Dutch and 2 Belgian hospitals (Erasmus University Medical Center, Leiden University Medical Center, University Medical Center Groningen, Ghent University Hospital and University Hospitals Leuven). The AsperGenius® assay was

performed on 1 milliliter stored leftover BAL samples on which GM (Platelia™ Bio-Rad inc.) and culture had already been performed because of a clinical suspicion of IA. Only BAL samples from patients with hematological diseases were included. Collecting BAL samples ended on 31st of May, 2015. All available leftover BAL samples with a minimum volume of 1 milliliter before this date were obtained. BAL samples were stored at -20 degrees in 4 hospitals and at -80 degrees in 1 hospital. The following information was retrieved from medical files: age, sex, underlying disease, duration of hospitalization, documentation of IA and the antifungal treatment given. In addition, 6 and 12-week mortality was documented. Because this was a retrospective study, no data on the BAL procedure itself was registered.

The study consisted of 2 parts. First, the optimal Ct value and diagnostic performance of the species probe of the PCR were determined. Secondly, treatment failure and 6-week mortality were determined in all patients who had received azole monotherapy for at least 5 days and in whom the resistance PCR successfully discriminated wildtype from *Cyp51A* mutated *A. fumigatus*. Patients with *A. fumigatus* without RAM (=wildtype) were compared to those with *A. fumigatus* containing a RAM. Patients were excluded from the azole treatment failure analysis if (i) a non-*A. fumigatus* or mixed infection was present (e.g. *A. fumigatus* and *A. terreus*), (ii) patients were treated with non-azole therapy or combination therapy, (iii) the antifungal therapy or duration was unknown, or (iv) patients received no therapy. Azole treatment failure was defined as a switch from an azole to any other antifungal drug class. Data of the patient population with hematological diseases of the previous study (n=10) were pooled with the data of the current study for the specific analysis of azole treatment failure and 6-week mortality [15]. The pooling of data was deemed necessary and appropriate because of (i) the rarity of patients infected with RAMs and (ii) the identical methodology and same study site in both studies.

One BAL sample per patient was included in the study. If for a given patient multiple BAL samples were available, the BAL sample of the period with the highest IA classification was selected. In case of multiple BAL samples for a given patient with the same IA classification, 1 BAL sample was randomly selected.

PathoNostics tested the BAL samples blindly and was not involved in the analysis of the results. GMC and BJAR analyzed the data.

Ethics

The medical ethics committees approved the study under the reference numbers MEC-2014-628, P14.337, UC UZG 2014/1217 and S57319. For one Dutch center, local approval was not necessary as approval given by another Dutch medical ethics committee also implied approval for that center. In centers with an opt-out system, all included patients were cross-checked with the list of patients that had objected to the opt-out system. In one center, opt-out forms were sent to the surviving patients to give them the opportunity to refuse the use of their clinical data.

Categorization of BAL samples

BAL samples with a positive GM (≥1.0) and/or a positive *Aspergillus* culture of the BAL, sputum or lung biopsy (<6 days after the date of the BAL) were considered positive controls for the presence of *Aspergillus* in BAL samples. Negative controls were BAL samples with a negative BAL GM in combination with a negative culture from BAL, sputum or biopsy. BAL samples from patients with only a positive serum GM (≥0.5) but a negative BAL GM were considered as negative controls as there was no microbiological evidence of the presence of *Aspergillus* in the BAL sample itself on which the PCR was performed.

Definitions of invasive fungal disease

Patients were categorized as having proven, probable or possible invasive fungal disease according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) consensus criteria [16]. In addition, patients with appropriate host criteria and positive microbiological findings but with non-specific radiological features were classified as *non-classifiable* disease. Although this category is not included in the EORTC/MSG definitions, in clinical practice, these patients are treated similarly to those with probable IA given their similar outcome [17].

Processing of BAL samples

One milliliter of the BAL sample was used for DNA-extraction. Samples were processed as described previously [15], with the exception that DNA was extracted from the BAL supernatant and pellet by using the NucliSENS® easyMag® (bioMérieux). The onboard lysis protocol and 50 microliter elution was selected for this purpose before the DNA eluate was used in the AsperGenius® assay. The AsperGenius® assay was performed on the BAL supernatant and pellet separately. A LightCycler 480 II (Roche) was used to perform the AsperGenius® assay. For the determination of the Ct values, the 2nd derivative function of the LightCycler 480 software (v1.5.62) was applied.

AsperGenius® multiplex real-time PCR assay

The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) was used to detect *Aspergillus* species and *Cyp51A* gene mutations. The species PCR allows for 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,* and *A. felis*. The *Aspergillus* species probe specifically detects *A. fumigatus, A. terreus, A. flavus* and *A. niger.* An internal control is included to monitor for inhibition or manual handling errors. The resistance PCR targets the single copy *Cyp51A* gene of *A. fumigatus* and detects TR_{34}/L98H/Y121F/T289A mutations to differentiate wildtype from mutant *A. fumigatus* via melting curve analysis.

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. The positive control from the assay was used as a standard for the melting peaks and was tested simultaneously with the BAL samples to determine if the melting peak represents wildtype or *A. fumigatus* with RAM. The resistance PCR was deemed successfully when the supernatant or pellet showed melting peaks for (i) at least 1 of the TR_{34} or L98H, together with (ii) at least 1 of T289A or Y121F resistance markers.

Statistical analysis

The optimal Ct cut-off and diagnostic performance was determined for the species probe of the PCR using the earlier Ct value of the supernatant or pellet. Using the controls as described above, the receiver operator characteristic (ROC) curve and its area under the curve (AUC) were determined (IBM® SPSS® statistics, version 21). The closest to (0,1) point and Youden index were used to further assess the optimal Ct cut-off [18]. The sensitivity, specificity, PPV and NPV were calculated for all BAL samples in total and per hospital. The positive and negative likelihoods were calculated for the most optimal Ct value.

As an additional sensitivity analysis, we determined the ROC curves, AUC and diagnostic performance when using the patients with EORTC/MSG proven, probable IA versus patients without IA. This was thought to be appropriate because the EORTC/MSG criteria are often used for antifungal therapy studies. Because clinicians tend to treat patients with non-classifiable IA in the same way as proven or probable IA [17], we did a second sensitivity analysis in which patients with proven, probable or non-classifiable IA were compared to patients without IA.

For the azole treatment failure and 6-week mortality analysis, the 2-sided Fisher's exact test was used, with a p-value<0.05 considered statistically significant.

RESULTS

In total, 228 BAL samples from 201 patients were available. Samples were obtained between December 2007 and May 2015. No patients refused to have their clinical data used for the purpose of this study. As only 1 BAL per patient was used, 201 BAL samples were available for the analysis. Seven patients with proven, probable or non-classifiable IA were counted as negative controls since there was no evidence of *Aspergillus* in the BAL itself from culture or GM (5 positive serum GM, 1 positive sinus culture and 1 positive lung biopsy culture obtained 16 days after the BAL). The clinical characteristics of the 201 patients are summarized in table 1.

Table 1. Clinical characteristics of the 201 hematology patients who contributed bronchoalveolar lavage (BAL) fluid samples.

	Patients (n = 201)
Age, mean years (range)	56.6 (17.5 – 82.6)
Male gender (n,%)	132 (65.7)
Diagnosis (n,%)	
Acute myeloid leukemia	78 (38.8)
Acute lymphocytic leukemia	16 (8.0)
Chronic lymphocytic leukemia	13 (6.5)
Myelodysplastic syndrome	17 (8.5)
Hodgkin's lymphoma	10 (5.0)
Non-Hodgkin's lymphoma	38 (18.9)
Myeloproliferative disorders	8 (4.0)
Plasma cell disorders	11 (5.5)
Aplastic anemia	3 (1.5)
Other[a]	7 (3.5)
Allogeneic stem cell transplantation (n,%)	82 (40.8)
Invasive aspergillosis (n,%)	
Proven	9 (4.5)
Probable	43 (21.4)
Non-classifiable	43 (21.4)
Possible	32 (15.9)
No IA	74 (36.8)
BAL galactomannan and/or culture positive or negative (n,%)	
Galactomannan and culture positive	28 (13.9)
Galactomannan positive and culture negative	56 (27.9)
Galactomannan negative and culture positive	4 (2.0)
Galactomannan and culture negative	113 (56.2)
Treated with following antifungal therapy (n,%)[b]	
Amphotericin B lipid complex	6 (3.0)
Liposomal amphotericin B	40 (19.9)
Conventional amphotericin B deoxycholate	4 (2.0)
Caspofungin	26 (12.9)
Itraconazole	3 (1.5)
Posaconazole	18 (9.0)
Voriconazole	112 (55.7)
Study anidulafungin versus placebo	3 (1.5)
Study voriconazole versus isavuconazole	3 (1.5)
Study voriconazole versus posaconazole	6 (3.0)
No antifungal therapy	56 (27.9)
Mortality (%) after IA diagnosis	
At 6 weeks	50 (24.9)
At 12 weeks	65 (32.3)
Hospital admission duration (days, range)	
Hospital stay in total	40.7 (2 – 236)

[a] Monoclonal B-cell lymphocytosis, auto-immune hemolytic anemia, sickle cell disease, hemophagocytic lymphohistiocytosis**,** T-cell prolymphocytic leukemia.

[b] There were patients who were treated with more than one antifungal therapy. Some patients were treated within a clinical trial in which the prescribed antifungal therapy was unknown for physicians.

Eighty-eight BAL samples were positive controls, of which 74 (84.1%) were positive for the species PCR (58 positive in supernatant and pellet, 10 only positive in supernatant and 6 only positive in pellet). The species PCR detected 66 *A. fumigatus*, 2 *A. fumigatus* combined with *A. terreus,* 2 *A. terreus* and 4 *A.* species. Thirty-two of these 74 (43.2%) BAL samples were culture negative and only GM positive.

Twenty-three BAL samples from negative controls were species PCR-positive. Five of these 23 BAL samples were from patients with proven, probable or non-classifiable IA (3 diagnosed on positive serum GM, 1 on positive sinus culture, and 1 on positive lung biopsy pathology plus culture). Eleven BAL samples were from patients without IA and 7 from patients with possible IA.

The ROC curve of the species PCR is shown in figure 1. The diagnostics accuracy as given by AUC was 0.890 (CI 95% 0.842–0.939; p-value<0.001). The closest to (0,1) point designated Ct<38 as optimal cut-off, while the Youden index designated Ct<36 as optimal cut-off (table S1). The Ct cut-off of <38 resulted in a sensitivity, specificity, PPV and NPV of 84.09%, 79.65%, 76.29% and 86.54%, respectively. A Ct cut-off of <36 gave values of 70.45%, 95.56%, 92.58% and 80.60%, respectively (table 2). Because a higher sensitivity was preferred over a higher specificity, the Ct value of <38 was chosen for the purpose of subsequent analyses that we performed. The positive likelihood ratio was 4.13 and the negative likelihood ratio 0.20. Table 3 shows the distribution of the BAL samples according to their IA classification. Table S2 shows the diagnostic performance per hospital. As a sensitivity analysis, the diagnostic characteristics were calculated when

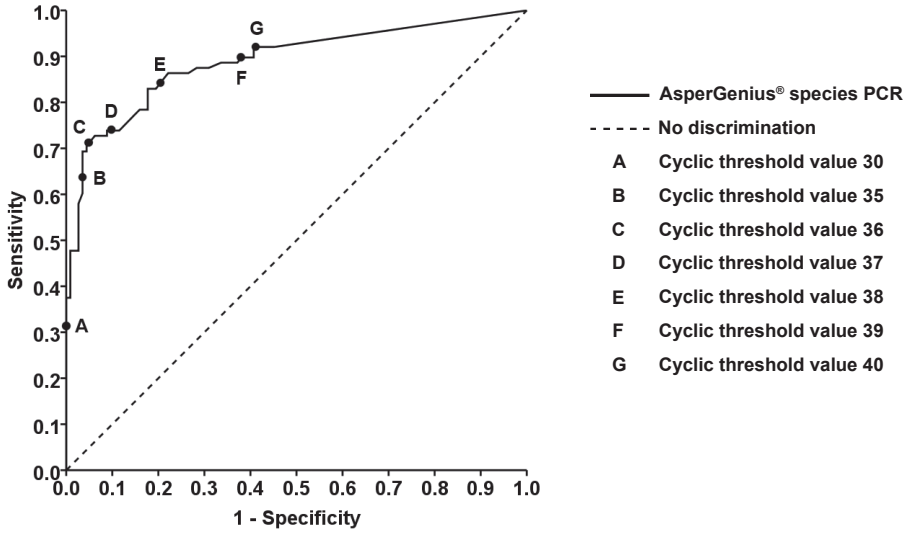

Figure 1. Receiver operator characteristic curve of species probe of the AsperGenius® PCR in the bronchoalveolar lavage fluid samples of the 201 patients with hematological diseases.

patients with proven or probable IA were considered as positive controls and compared to patients without IA. The sensitivity increased to 88% when the <38 Ct cut-off was used. See table 2 and figure S1 for the ROC curves when patients with proven, probable or non-classifiable IA were considered as positive controls.

Table 2. Diagnostic performance of the species probe of the AsperGenius® PCR according to different cycle threshold (Ct) cut-offs and positive/negative controls.

Ct value cut-off of the AsperGenius® species PCR	Diagnostic performance[a]	Positive control versus negative control BAL samples as defined in this study[b] n = 201	Proven, probable or non-classifiable IA versus no IA[c] n = 169	Proven or probable IA versus no IA n = 126
<36	Sensitivity (%)	70.45	68.42	76.92
	Specificity (%)	95.58	98.65	98.65
	PPV (%)	92.54	98.48	97.56
	NPV (%)	80.60	70.87	85.88
<37	Sensitivity (%)	73.86	71.58	78.85
	Specificity (%)	90.27	94.59	94.59
	PPV (%)	85.53	94.44	91.11
	NPV (%)	81.60	72.16	86.42
<38	Sensitivity (%)	84.09	83.16	88.46
	Specificity (%)	79.65	85.14	85.14
	PPV (%)	76.29	87.78	80.70
	NPV (%)	86.54	79.75	91.30
<39	Sensitivity (%)	88.64	87.37	90.38
	Specificity (%)	62.83	72.97	72.97
	PPV (%)	65.00	80.58	70.15
	NPV (%)	87.65	81.82	91.53

[a] PPV, positive predictive value. NPV, negative predictive value.

[b] Bronchoalveolar (BAL) samples with a positive GM (≥1.0) and/or positive culture for *Aspergillus* of BAL, sputum or lung biopsy at most 5 days from date of the BAL were considered positive controls. BAL samples with a negative BAL GM in combination with a negative culture from BAL, sputum or lung biopsy were considered negative controls.

[c] Proven and probable IA was defined according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria. Non-classifiable is defined as a patient with EORTC/MSG host and microbiological criteria fulfilled and a pulmonary infiltrate without a halo or air-crescent or well-defined nodule. IA, invasive aspergillosis. No IA was defined as no proven IA, no probable IA, no non-classifiable IA or no possible invasive fungal disease.

Table 3. Distribution of the bronchoalveolar lavage (BAL) samples according to their invasive aspergillosis (IA) classification and species probe of the AsperGenius® PCR using cycle threshold (Ct) value of <38 as cut-off.

Classification of IA	BAL samples n = 201		
	Ct <38	Ct ≥38	Total
Proven	9	0	9
Galactomannan and culture positive	5	0	
Only galactomannan positive	3	0	
Only culture positive	1	0	
Probable	37	6	43
Galactomannan and culture positive	11	0	
Only galactomannan positive	22	6	
Only culture positive	4	0	
Non-classifiable	33	10	43
Galactomannan and culture positive	12	0	
Only galactomannan positive	20	10	
Only culture positive	1	0	
Possible	7	25	32
No IA	11	63	74
Total	97	104	201

Note: galactomannan in BAL and serum. Culture in BAL and elsewhere in the body.

In the 201 BAL samples, the species PCR was positive in 97 BAL samples (figure 2). The resistance PCR was successfully performed in 68/97 BAL samples. Fifty-seven patients had a wildtype *A. fumigatus* and 11 patients had an *A. fumigatus* with RAM (TR_{34}/L98H mutation (n=7), TR_{46}/T289A/Y121F mutation (n=1) and TR_{34}/L98H mutation combined with a wildtype *A. fumigatus* (n=3)). One or two resistance markers were not detected in 9/73 BAL samples (L98H in 4, TR_{34} in 5 and T289A in 2). But based on the detection of the other corresponding resistance marker (e.g. successful amplification of L98H in 4 patients in combination with unsuccessful TR_{34} amplification), a conclusion could be drawn on the presence of *A. fumigatus* wildtype or resistant due to TR_{34}/L98H or TR_{46}/T289A/Y121F.

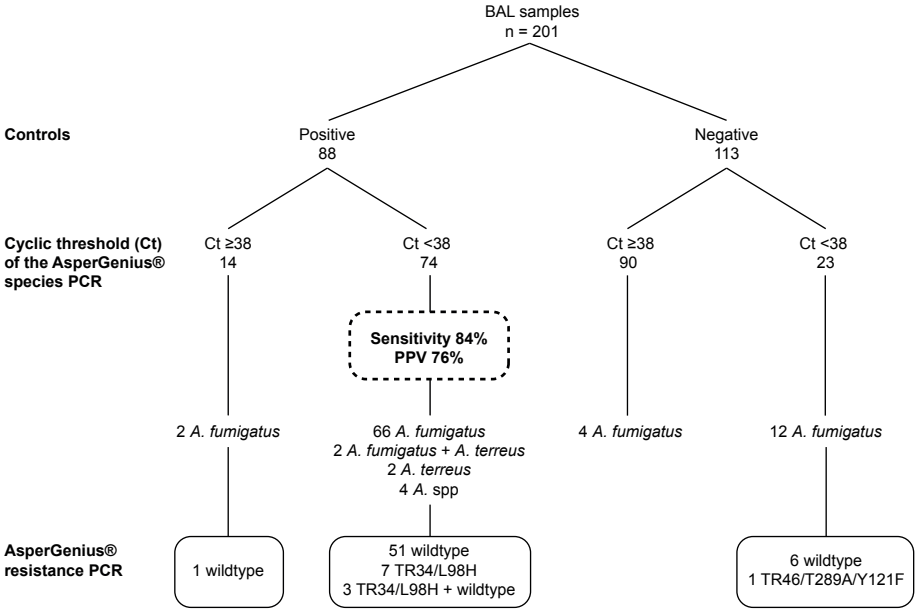

Figure 2. Bronchoalveolar lavage (BAL) samples divided according to positive/negative controls and species probe of the AsperGenius® PCR and resistance PCR.
Two patients had a co-infection with an *A. fumigatus* and *A. terreus*.

For the azole treatment failure and 6-week mortality analysis, resistance data from the 68 patients were included together with 10 patients with hematological diseases from the previous study (8 wildtype, 1 TR_{34}/L98H and 1 TR_{46}/T289A/Y121F).[15] As such, the total group consisted of 78 patients of whom 65 were infected with wildtype *A. fumigatus* and 13 with *A. fumigatus* carrying a RAM. Twenty-five of these 78 patients were excluded from the analysis as described in the methods and figure 3. After exclusion, 45 patients with wildtype *A. fumigatus* remained and 8 with an *A. fumigatus* that contained RAMs. The patients infected with wildtype had 5 proven, 20 probable, 17 non-classifiable IA and 3 possible IA. In patients infected with *A. fumigatus* containing RAM, 4 had probable, 3 had non-classifiable and 1 had possible IA. *A. fumigatus* was cultured in 23/53 patients (3 with and 20 without RAMs). Data on antifungal susceptibility testing were available for 7/23 *A. fumigatus* cultures (5 azole susceptible and 2 azole-resistant) and correlated with the results of the resistance PCR. Azole treatment failure was observed in 12/45 patients with wildtype *A. fumigatus* compared to 6/8 patients with *A. fumigatus* with RAM (p-value=0.01). Six-week mortality was 2.7 times higher in patients with detected RAM (18.6% without versus 50.0%, p-value=0.07).

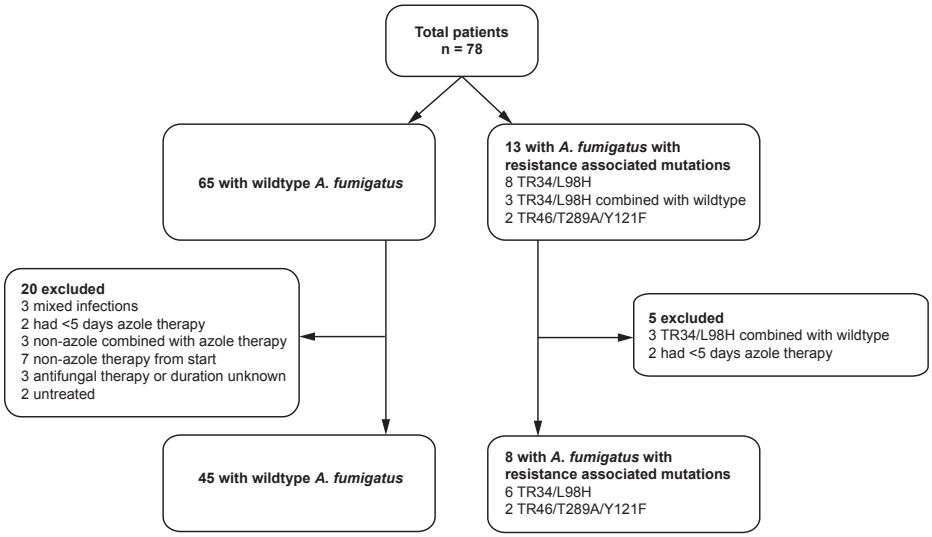

Figure 3. Inclusion for the azole treatment failure and 6-week mortality analysis.

DISCUSSION

This retrospective multicenter study showed that the AsperGenius® species PCR has a good diagnostic performance on BAL samples of patients with hematological diseases. The sensitivity, specificity, PPV and NPV were 84%, 80%, 76% and 87%, respectively, when a Ct cut-off of <38 was used.

The 2 most frequently used statistical methods to analyze a ROC curve are the closest to (0,1) and Youden index. In this study, they led to contradictory results on the most appropriate Ct cut-off values (36 and 38, respectively). Given the important clinical consequences of a missed IA diagnosis, a later Ct cut-off of 38 that results in a better sensitivity may be preferred by the clinician if the resulting loss in specificity is acceptable. Therefore, we favor the use of the Ct cut-off of <38 which is later than the Ct cut-off of <36 that we reported in the previous smaller single center study.[15] The current sensitivity was somewhat lower than in the previous single center study (89%),[15] and may be explained by the fact that a standard volume of 1 milliliter BAL was used in this study versus 1-2 milliliter in the previous study. The lower volume may have decreased the sensitivity. The difference in sensitivity observed *between* the study centers may be explained by differences in the way the BAL is performed in each center. For example, centers may differ in the volume used to perform the BAL, or a bronchoscopist may lavage 2 different parts of the lung but send it in 1 container to the laboratory which may result in a diluted DNA content if *Aspergillus* is present in only 1 part of the lung. Unfortunately, the way the BAL procedure was done in each patient was not registered.

The difference in sensitivity could not be explained by the differences in BAL storage methods. One hospital stored its BAL samples at -80 degrees and had a sensitivity of 75%, while the other 4 hospitals stored at -20 degrees and had a sensitivity of 71% to 100%. The difference could neither be explained by the duration of BAL storage *before* the PCR was performed because 2 hospitals contributed BAL samples from the period 2014 to 2015 and had a different sensitivity of 88% and 100%.

As with all diagnostic tests, a test should be interpreted within the context of the prevalence of the disease. When azole resistance is low in a certain area, it is expected that the PPV of the PCR will probably drop. Based on the positive and negative likelihood ratios of 4.13 and 0.20, respectively, one can determine the post-test probability in a Fagan nomogram to take the prevalence in the patient population into account.

On top of the detection of *Aspergillus*, the AsperGenius® resistance PCR was able to differentiate *A. fumigatus* without a RAM (=wildtype) from RAM-positive *A. fumigatus*, even in culture negative BAL samples. Most importantly, patients infected with RAM-positive *A. fumigatus* failed significantly more often on azole treatment than those infected with a wildtype *A. fumigatus* (75% versus 27%, p-value=0.01). Therefore, this study is the first to show that PCR-detected resistance is clinically relevant. The incidence of azole treatment failure and the 6-week mortality was determined in the pooled data of the current and previous study [15]. The sole reason to pool the data of both studies is the fact that azole resistant IA is still a rare disease with only 13 cases detected in the 83 PCR-positive patients of the 251 patients included both studies. Given the small numbers, a multivariate analysis was not possible to investigate other predictors of azole treatment failure.

To date, the AsperGenius® assay has been studied on serum samples of patients with hematological diseases in which the species PCR had a sensitivity and specificity of 79% and 100%, respectively, when a Ct cut-off of 39 was used [19]. Next to the AsperGenius® assay, other *Aspergillus* PCRs have been tested on BAL samples and sensitivities varied between 38% and 94% [20-22]. Therefore, the sensitivity of the species PCR found in this study is comparable but with the added advantage that RAMs are detected simultaneously. There are other PCRs alike the AsperGenius® assay that detect *Cyp51A* mutations directly on BAL samples [23-25]. Spiess et al. described the detection of *Cyp51A* mutations on 189 clinical samples in their 1st and 2nd studies combined and found the TR_{34}/L98H mutation on 2 BAL samples and 1 cerebral biopsy [23,24]. Zhao et al. found in their study the *Cyp51A* mutations M220 and PL216 which are also associated with azole resistance, on 2/94 BAL samples. These studies along with the current study show that detection of *Cyp51A* mutation on BAL samples is possible, also on culture negative BAL samples. The current study is the first to show that the detection of RAMs is clinically associated with azole treatment failure.

The study has limitations. First, only *Cyp51A* mutations included in the assay can be detected. To date, more than 15 *Cyp51A* gene-mediated resistance mechanisms have been described [26]. The included mutations TR_{34}/L98H and TR_{46}/T298A/Y121F originate from the environment in contrast to *Cyp51A* mutations that are patient acquired after prolonged azole treatment [7,27,28]. The prevalence for the TR_{34}/L98H mutation can account up to 90.2% of the azole resistant *A. fumigatus*, while for the TR_{46}/T289A/Y121F up to 26.9% [6,9-12]. However, the prevalence varies per region. For example, a study from the United Kingdom found only 2 TR_{34}/L98H mutations among the 45 azole-resistant *A. fumigatus* isolates [7]. The assay should therefore be interpreted within the context of the local prevalence of the *Cyp51A* mutations. Besides the *Cyp51A* mutations, non-*Cyp51A* mechanisms that confer azole resistance have been reported [4,6,8-11]. Therefore, PCR testing does not replace culture-based sensitivity testing that should be performed as well. Second, we studied the BAL samples from December 2007 to May 2015. Azole resistance has increased over the past decade [4-11]. Eleven of the 201 (5.5%) patients were infected with an *A. fumigatus* containing a RAM, which may be an underestimation of RAMs in the current population. Lastly, the retrospective nature of the study is another limitation. Antifungal susceptibility testing was not performed routinely in the past. Therefore, the PCR results could only be correlated with the phenotypical resistance in a small portion of the patients. However, in a prospective study, it would be unacceptable to test BAL samples real-time without reporting the detected *Cyp51A* mutations back to the clinician which obviously will lead to a switch from an azole to a non-azole therapy. Therefore, this retrospective study had the advantage that it became possible to report on 8 patients treated with azoles despite the fact that, in retrospect, they had been infected with RAM-positive and therefore azole resistant *A. fumigatus*.

In conclusion, the AsperGenius® assay showed a good diagnostic performance in detecting IA in patients with hematological diseases and the detection of RAM-positive *A. fumigatus* was associated with azole treatment failure, also when patients were culture negative. Therefore, early detection of RAMs by PCR can lead to a prompt adaptation of the antifungal regimen, hopefully contributing to a more favorable outcome of azole resistant *A. fumigatus* in future patients.

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SUPPLEMENTARY MATERIAL

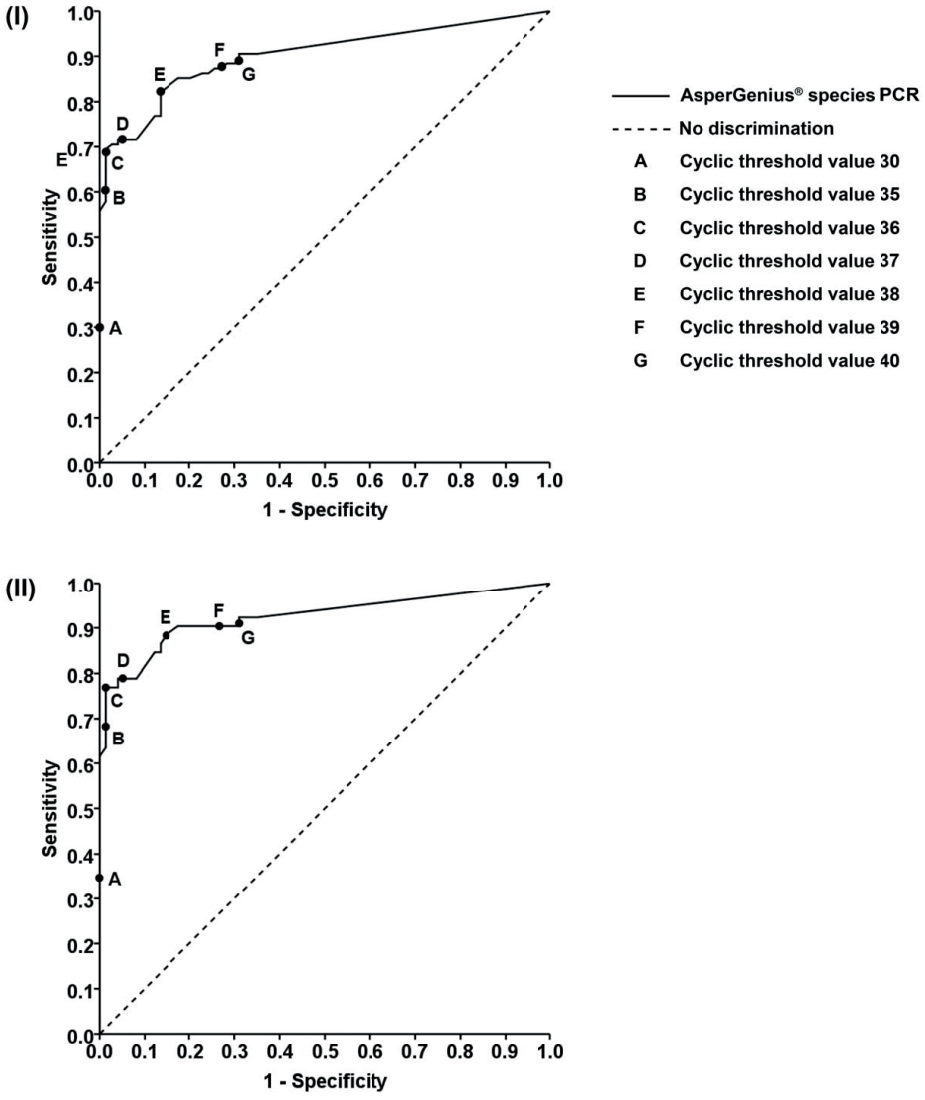

Figure S1. Additional receiver operator characteristic (ROC) curves of species probe of the AsperGenius® PCR in the bronchoalveolar lavage fluid samples.

ROC curve I uses proven (n=9), probable (n=43) and non-classifiable IA (n=43) as positive controls versus no IA (n=74) as negative controls. The diagnostics accuracy as given by area under the curve (AUC) was 0.902 (CI 95% 0.855 – 0.949; p-value<0.001). ROC curve II uses proven and probable IA as positive controls versus no IA as negative controls. The AUC was 0.925 (CI 95% 0.871 – 0.979; p- value<0.001).

Online supplement table S1. Selection of the best cycle threshold (Ct) cut-off using the closest to (0,1) point and Youden index criteria.

Criteria to test optimal cut-off[a]	Positive versus negative BAL samples as defined in this study[b] n = 201	Proven, probable or non-classifiable IA versus no IA[c] n = 169	Proven or probable IA versus no IA n = 126
Closest to (0,1) point			
Ct <36	0.2988	0.3161	0.2312
Ct <37	0.2789	0.2893	0.2183
Ct <38	0.2583	0.2246	0.1881
Ct <39	0.3887	0.2984	0.2869
Youden index			
Ct <36	0.6603	0.6707	0.7557
Ct <37	0.6413	0.6617	0.7344
Ct <38	0.6374	0.6830	0.7360
Ct <39	0.3887	0.6034	0.6335

[a] The closest to (0,1) point selects the lowest distance between a point on a receiver operator curve (ROC) and the point (0,1) as best cut-off. The Youden index selects the maximum vertical distance between the ROC curve and the diagonal or chance line as best cut-off.

[b] Bronchoalveolar (BAL) samples with a positive galactomannan (GM) of ≥1.0 and/or positive culture for *Aspergillus* of BAL, sputum or lung biopsy at most 5 days from date of the BAL were considered gold standard positive. BAL samples with a negative BAL GM in combination with a negative culture from BAL, sputum or lung biopsy were considered gold standard negative.

[c] Proven and probable IA was defined according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria. Non-classifiable is defined as a patient with EORTC/MSG host and microbiological criteria fulfilled and a pulmonary infiltrate without a halo or air-crescent or well-defined nodule. IA, invasive aspergillosis. No IA was defined as no proven IA, no probable IA, no non-classifiable IA or no possible invasive fungal disease.

Online supplement table S2. Diagnostic performance of the AsperGenius® species PCR per hospital using cycle threshold value cut-off of <38.

Diagnostic performance[a]	Overall n = 201	Hospital 1 n = 34	Hospital 2 n = 34	Hospital 3 n = 37	Hospital 4 n = 33	Hospital 5 n = 63
Sensitivity (%)	84.09	87.50	90.00	100.00	75.00	70.59
Specificity (%)	79.65	61.11	75.00	82.14	76.47	86.96
PPV (%)	76.29	66.67	96.43	64.29	75.00	66.67
NPV (%)	86.54	84.62	50.00	100.00	76.47	88.89

[a] PPV, positive predictive value. NPV, negative predictive value.