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Clinical Infectious Diseases

MAJOR ARTICLE

Clinical Impact of Polymerase Chain Reaction–Based *Aspergillus* and Azole Resistance Detection in Invasive Aspergillosis: A Prospective Multicenter Study

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Background. Invasive aspergillosis (IA) by a triazole-resistant *Aspergillus fumigatus* is associated with high mortality. Real-time resistance detection will result in earlier initiation of appropriate therapy.

Methods. In a prospective study, we evaluated the clinical value of the AsperGenius polymerase chain reaction (PCR) assay in hematology patients from 12 centers. This PCR assay detects the most frequent *cyp51A* mutations in *A. fumigatus* conferring azole resistance. Patients were included when a computed tomography scan showed a pulmonary infiltrate and bronchoalveolar fluid (BALf) sampling was performed. The primary end point was antifungal treatment failure in patients with azole-resistant IA.

Results. Of 323 patients enrolled, complete mycological and radiological information was available for 276 (94%), and probable IA was diagnosed in 99/276 (36%). Sufficient BALf for PCR testing was available for 293/323 (91%). *Aspergillus* DNA was detected in 116/293 (40%) and *A. fumigatus* DNA in 89/293 (30%). The resistance PCR was conclusive in 58/89 (65%) and resistance detected in 8/58 (14%). Two had a mixed azole-susceptible/azole-resistant infection. In the 6 remaining patients, treatment failure was observed in 1. Galactomannan positivity was associated with mortality ($P = .004$) while an isolated positive *Aspergillus* PCR was not ($P = .83$).

Conclusions. Real-time PCR-based resistance testing may help to limit the clinical impact of triazole resistance. In contrast, the clinical impact of an isolated positive *Aspergillus* PCR on BALf seems limited. The interpretation of the EORTC/MSGERC PCR criterion for BALf may need further specification (eg, minimum cycle threshold value and/or PCR positive on >1 BALf sample).

Keywords. invasive aspergillosis; azole resistance; *Aspergillus* PCR; clinical impact.

Invasive aspergillosis (IA) is the most common mold infection in immunocompromised patients and is associated with significant morbidity and mortality. Over 15 years, azoles have been used as first-line therapy [1, 2]. Azole resistance in *Aspergillus fumigatus* is increasingly reported [3]. It is mostly caused by resistance-associated mutations (RAMs) in the *cyp51A* gene encoding for the target enzyme of azoles. We previously noted that 6 weeks after diagnosis, the mortality of culture-positive voriconazole-resistant IA was 21% higher compared with voriconazole-susceptible IA [4]. A delay in appropriate antifungal therapy was associated with 23% higher mortality compared with patients who received appropriate

Received 14 November 2022; editorial decision 06 March 2023; published online 11 March 2023

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Clinical Infectious Diseases® 2023;77(1):38–45

https://doi.org/10.1093/cid/ciad141

antifungal therapy immediately and median time to switch to appropriate antifungal therapy was 10 days [4]. This delay is mainly caused by slow growth of fungal cultures, transportation time to a mycology reference laboratory, and susceptibility testing on that isolate. The resistance polymerase chain reaction (PCR) assay could result in a faster and more sensitive diagnosis of azole resistance, enabling early appropriate antifungal therapy and reducing the need for upfront combination therapy [5]. A PCR assay also enables azole-resistance detection in culture-negative samples. AsperGenius is a multiplex, real-time, PCR assay that allows for simultaneous detection of *A. fumigatus* and other *Aspergillus* species as well as mutations in the *A. fumigatus cyp51A* gene, which confers resistance to azoles [6]. This PCR test can be implemented in any molecular diagnostics laboratory without the need for specific expertise in mycology. In the Azole Resistance Management study, we evaluated the clinical value of this PCR assay in patients suspected of having IA.

METHODS

Study Design and Population

A prospective multicenter study was performed in 12 centers in the Netherlands and Belgium. The study was approved by the institutional review boards at all sites, and patients provided written informed consent.

We included adult patients with a hematological malignancy and a new pulmonary infiltrate on a computed tomography (CT) scan for which bronchoalveolar fluid (BALf) sampling was planned or performed within 48 hours. Invasive fungal disease (IFD) was classified according to the updated Consensus Definitions of Invasive Fungal Disease From the European Organization for Research and Treatment of Cancer and the Mycoses Study group Education and Research Consortium (2020 EORTC/MSGERC) (Supplementary Methods 1) [7]. A diagnostic and therapeutic protocol was agreed on by those at the study sites. This incorporated the AsperGenius PCR on BALf. Operational information on this PCR assay is available in the manufacturer's instructions and Supplementary Methods 2. This consensus protocol also provided a guideline on antifungal treatment (Supplementary Methods 3, Supplementary Figure 1) [8].

Baseline characteristics, serum and BALf galactomannan (GM), culture results, antifungal treatment, and mortality up to week 12 were registered. GM testing on serum and BALf with the Bio-Rad Platelia *Aspergillus* Ag assay and fungal culture was performed at the study site. Phenotypic resistance testing with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard was performed at the Dutch and Belgian Mycology Reference Center. Some of the centers also used the VIPcheck (Mediaproducts BV, the Netherlands) to screen for possible azole resistance, with confirmation at the reference center.

Primary and Secondary End Points

The primary end point was the proportion of patients with probable IA by an azole-resistant *A. fumigatus* in whom antifungal treatment failure, defined as death or switch to antifungal agent from another class after at least 5 days of first-line therapy, was observed in the 6 weeks following diagnosis.

Secondary end points were overall prevalence of azole resistance and outcome of patients with an isolated, positive *Aspergillus* PCR on BALf. For this, the 6-week overall mortality in patients with a negative test result for all 3 mycological tests (culture/GM/PCR) was compared with mortality in patients with an isolated positive PCR (single as well as duplicate positive PCR). Furthermore, in a post hoc analysis, we evaluated the influence of low versus high fungal loads based on cycle threshold (Ct) values of the PCR.

Statistical Analyses

For the primary end point, the incidence of treatment failure was compared with a fixed hypothetical 75% incidence that can be expected when patients with an azole-resistant *A. fumigatus* are treated with azoles and switch to a non-azole therapy when treatment failure is clinically diagnosed [9]. Patients with IA in whom a mixed azole-susceptible/azole-resistant *Aspergillus* infection was demonstrated were excluded because it could not be excluded that only the azole-susceptible strain was causing IA while the resistant strain was a colonizer. Because the use of real-time detection of azole resistance in patients allows for a proactive change from the first-line therapy with voriconazole to other agents as soon as resistance is detected, a lower incidence of treatment failure can be expected. The goal of the study therefore was to demonstrate that this PCR-based approach reduces the incidence of treatment failure compared with the presumed 75%. We anticipated that failure would be reduced to 35% when the antifungal therapy was changed to liposomal amphotericin B (L-AmB) as soon as resistance was documented. Using these percentages, at least 15 cases of azole resistance would have to be enrolled to have 90% power to show that treatment failure is significantly less than 75%. To compare the observed treatment failure with this 75%, the exact Clopper–Pearson 95% confidence interval (CI) for the observed proportion of treatment failure was calculated, and the *P* value of the observed proportion vs 75% was calculated using a general *z* test.

Secondary end points were analyzed using Mann–Whitney *U* and Pearson χ^2 tests as appropriate. All tests were 2-tailed with a significance level of 0.05. A receiver operating characteristic (ROC) curve was generated to evaluate the discriminative power of the fungal load (reported as Ct value) to

Table 1. Baseline Patient Characteristics

Characteristic	Total Patients, N = 323
Age, median (interquartile range), y	63 (53–69)
Male sex (%)	219/320 (68)
Allogeneic stem cell transplant (%)	102/322 (32)
Autologous stem cell recipient (%)	13/322 (4)
Underlying hematological disease (%)	
Acute myeloid leukemia	163/321 (51)
Myelodysplastic syndrome	40/321 (13)
Acute lymphoblastic leukemia	20/321 (6)
Other	98/321 (30)
Acute GvHD, grade II–IV, n (%)	23/321 (7)
Chronic GvHD, n (%)	19/321 (6)
Mild	6/321 (2)
Moderate	5/321 (2)
Severe	8/321 (3)
Use of prednisolone[a] (%)	
<0.3 mg/kg/d	41/310 (13)
>0.3 mg/kg/d	51/310 (17)
Chemotherapy in last 90 d[b] (%)	195/273 (71)
Neutropenia,[c] Yes (%)	170/293 (58)

Abbreviation: GvHD, graft-versus-host disease.

[a]Median dose of prednisolone in the 21 days preceding bronchoalveolar fluid (BALf) sampling.

[b]Chemotherapy received in the last 3 months prior to BALf sampling.

[c]Neutropenia (<500/μL) on day of BALf sampling.

predict 6-week mortality or successful resistance testing (Supplementary Data S9). Analysis was performed with SPSS version 28 (IBM, Armonk, NY)

RESULTS

From April 2017 to March 2021, 323 patients who underwent bronchoscopy with BALf sampling and fulfilled the host factor criterion of the EORTC/MSGERC definitions were enrolled. Two-thirds were male (68%), and the median age was 63 years. Seventy percent had an acute leukemia or myelodysplastic syndrome, and 32% had received an allogeneic stem cell transplantation (Table 1).

Aspergillus PCR

Sufficient BALf remained for *Aspergillus* PCR testing for 293, and this was used as the denominator for the performance of the PCR. Overall, *Aspergillus* DNA was detected in BALf of 116 (40%) patients and *A. fumigatus* DNA was detected in 89 (30%). Patients were categorized in three groups according to the GM on BALf (<0.5, 0.5–0.99 and ≥1.0) and *Aspergillus* DNA was more frequently detected with increasing GM (Table 2). In patients with a BALf GM <1.0, *Aspergillus* DNA could still be detected in 66 of 224 (29%).

EORTC/MSGERC Classification

Data on radiological findings were available for 302 patients and lesions suspect for IFD (which in the 2020 criteria also include a wedge-shaped, lobar, or segmental infiltrate) were present in 291, while 11 patients had atypical findings (eg, ground glass opacities). Results of the chest CT scan in combination with complete mycological data (ie, PCR, GM, and culture result) were available for 276. Of these patients, 99 (36%) were classified as probable IA based on the radiological findings in combination with a positive GM, culture, or duplicate PCR test. Possible IFD was diagnosed in 169 of 276 (61%). Eight of 276 patients showed atypical radiological findings, of whom 2 had a positive mycological criterion. Supplementary Data 4 and Supplementary Data 6 (Supplementary Tables 1 and 2, respectively) provide more information on the classification of these patients and Supplementary Data S8 provides a visual overview of all positive diagnostics (Supplementary Figure 2). When we used the previous (2008) version of the EORTC/MSGERC criteria in which a positive *Aspergillus* PCR was not included, only 72 (26%) had a probable IA.

Azole Resistance

The *A. fumigatus* resistance PCR was performed on the BALf of the 89 patients in whom *A. fumigatus* DNA was detected. A conclusive resistance PCR for both *cyp51A* mutation patterns (ie, showing wild type or a resistance marker) was obtained in 58 of 89 (65%) patients. The resistance PCR was more often conclusive when BALf GM was higher (44% with GM <1.0% vs 92% when ≥1.0). In 8 of 58 patients (14%), resistance markers were detected (Table 2); all of these patients were categorized as probable IA.

Aspergillus was cultured from BALf in 7.5% (24 of 323) of patients. *Aspergillus fumigatus* was cultured in 21, while *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus niger* were each cultured in 1 patient. Even in patients with GM ≥1.0 on BALf, cultures were positive in only 23% (17 of 74). Supplementary Data 6 (Supplementary Table 3) provides additional information on the success rate of the resistance PCR and phenotypic resistance testing.

As shown in Table 3, a RAM was detected in 8 patients by PCR. Six had a positive culture for *A. fumigatus*. Unfortunately, phenotypic resistance testing was also performed in only 4 of them. Resistance was confirmed in 3 of 4. In 1 patient, the BALf culture showed no phenotypic resistance; however, a sputum sample gathered 14 days after inclusion showed phenotypic resistance. In our cohort, there were no patients in whom phenotypic resistance was shown that could not be confirmed by the *cyp51A* resistance PCR. For details on treatment of these patients, refer to Supplementary Data 5.

Primary End Point

After exclusion of patients with the mixed azole-susceptible/azole-resistant infection as predefined in the protocol, 6 patients with probable azole-resistant IA remained. In 1 of these 6, treatment failure was observed, and an echinocandin was

Table 2. Microbiology Results Including Bronchoalveolar Fluid Galactomannan, AsperGenius Polymerase Chain Reaction, and Culture

	BALf GM		
	<0.5	0.5–0.99	≥1
Number of patients (n)[a]	215	32	74
Aspergenius performed	193	31	68
PCR *Aspergillus* species–positive	50 (26%)	16 (52%)	50 (74%)
PCR *Aspergillus* species–negative	143 (74%)	15 (48%)	18 (26%)
PCR *Aspergillus fumigatus*–positive	38 (20%)	12 (39%)	39 (57%)
PCR *Aspergillus fumigatus*–negative	156 (80%)	19 (61%)	29 (43%)
PCR *Aspergillus terreus*–positive	1 (0.5%)	0 (0%)	2 (3%)
TR_{34}/L98H PCR successful[b]	19 (50%)	8 (67%)	36 (92%)
TR_{46}/T289A/Y121F PCR successful[b]	21 (55%)	5 (42%)	36 (92%)
TR_{34}/L98H and TR_{46}/T289A/Y121F both WT	16	4	32
TR_{34}/L98H and TR_{46}/T289A/Y121F both not successful	15	3	3
TR_{34}/L98H WT and TR_{46}/T289A/Y121F not successful	1	4	0
TR_{34}/L98H not successful and TR_{46}/T289A/Y121F WT	3	1	0
TR_{34}/L98H resistant and TR_{46}/T289A/Y121F WT	1	0	5 (2[c])
TR_{34}/L98H WT and TR_{46}/T298A/y121F resistant	1	0	1
Culture positive for *Aspergillus* species	6	1	17
Culture positive for *Aspergillus fumigatus*	5	0	16
Culture positive for *Aspergillus niger*	1	0	0
Culture positive for *Aspergillus terreus*	0	0	1
Culture positive for *Aspergillus flavus*	0	1	0

Abbreviations: BALf, bronchoalveolar fluid; GM, galactomannan; PCR, polymerase chain reaction; WT, wild type.

[a]Bronchoalveolar fluid (BALf) volume is occasionally too small to perform all tests in all patients. BALf PCR was performed in 293 patients. In 1 patient, galactomannan (GM) was not available; therefore, total number of patients in this table is 292. In this patient, *Aspergillus* species PCR was positive and *A. fumigatus* PCR was negative.

[b]The number of patients in the GM subgroup for whom *A. fumigatus* PCR was positive was used as the denominator (39, 12, and 37 for GM <0.5, GM 0.5–0.99, and GM ≥ 1.0, respectively).

[c]In 2 patients, DNA of WT *A. fumigatus* and the TR_{34}/98H mutation were detected simultaneously.

added to L-AmB 42 days after the initiation of antifungal therapy. This patient eventually died on day 64. Compared with the predefined historical treatment failure rate of 75%, the treatment failure of 16.7% (95% CI, .5%–64%) we observed was significantly lower ($P = .005$) [9].

Secondary End Points

Overall Prevalence of Azole Resistance

RAMs associated with azole resistance were found in 8 of 58 (14%) with successful resistance PCR results available and in 8 of 293 (2.7%) in whom the PCR was performed. Phenotypic resistance testing (VIPcheck and/or EUCAST method) was available for 18 of 21 (86%) of the culture-positive cases; in 3, resistance to 1 or more azoles was documented (2 of 3 voriconazole, 1 of 3 posaconazole, 3 of 3 isavuconazole) in BALf. Unfortunately, phenotypic resistance testing was not performed on culture for 2 patients in whom a RAM was detected by PCR.

Outcome of Patients With an Isolated Positive Aspergillus *Species PCR*

Characteristics of Patients With an Isolated Positive* Aspergillus *Species PCR. We analyzed the clinical impact of a positive PCR result in patients with a negative BAL GM and a negative BALf culture (Table 4, Supplementary Data 7). In our cohort, 240 patients had a negative BALf GM in combination with a negative culture. Because the PCR could be performed in 216 of 240, 216 was used as the denominator. Sixty-two (29%) of them had an isolated positive *Aspergillus* species and/or *fumigatus* PCR. In 52 of 62 (84%), *Aspergillus* therapy was initiated in the 14 days following BALf sampling. Significantly fewer of those with a negative PCR received antifungal therapy (105 of 154, 68%, $P = .019$). The median duration of therapy in patients with an isolated positive PCR was 34 days (interquartile range [IQR], 10–123), and this was 18 days (IQR, 7–63) in patients with a negative PCR ($P = .045$).

Mortality According to Subgroup. Of 321 patients with available data on mortality, 89 (28%) died within 12 weeks. The 6-week as well as the 12-week overall mortality were significantly higher in patients with BALf GM ≥1.0 compared with patients with a lower BALf GM (6 weeks: 32% vs 16%, $P = .004$; 12 weeks: 40% vs 24%, $P = .010$). In contrast, the mortality was not significantly higher in patients with a positive PCR in duplicate vs a negative PCR (6 weeks: 24% vs 19%, $P = .324$; 12 weeks: 31% vs 27%, $P = .457$). Furthermore, the 6-week mortality in patients with an isolated positive PCR result was comparable to that for patients who lacked any

Table 3. Overview of Baseline Characteristics, Treatment, and Outcome on the 8 Azole-Resistant Cases

Age, y	Sex	Disease	Stem Cell Transplant	Bronchoalveolar Fluid Galactomannan	Culture	Resistance Testing on Culture	AsperGenius Resistance Testing	Initial Therapy	Subsequent Therapy	Time of Antifungal Switch, d	Therapy Failure	6-Week Mortality	12-Week Mortality
66	M	AML	-	1.6	+	Azole resistant	TR_{46}	Azole	L-AmB	1	No	No	No
53	F	Non-Hodgkin lymphoma	+	0.3	+	Azole resistant	TR_{34}	Azole + L-AmB	L-AmB	4	No	No	No
54	M	Hodgkin lymphoma	+	4.8	+	Azole resistant	TR_{34}	Azole + L-AmB	L-AmB[a]	5	Yes on day 42	No	No
48	F	AML	-	5.6	-	-	TR_{34}	Azole + echinocandin	L-AmB	2	No	No	Yes
64	F	AML	+	0.07	-	-	TR_{46}	Azole		NA	No	No	No
57[b]	M	Mantle cell lymphoma	-	3.08	+	Not tested	Mixed pattern: WT and TR_{34}	Azole		NA	No	No	No
23	M	T-cell acute lymphocytic leukemia	-	8	+	Azole susceptible[c]	TR_{34}	Azole	Azole + L-AmB	15[d]	No	No	No
79	M	MW	-	5.6	+	Not tested	Mixed pattern: WT and TR_{34}	Azole		NA	No	Yes	Yes

Abbreviations: AML, acute myeloid leukemia; azole, voriconazole or posaconazole; culture, culture of bronchoalveolar fluid; L-AmB, liposomal amphotericin B; WT, wild type; MW, morbus waldenström.

[a]Treatment failure: anidulafungin was associated with L-AmB on day 42 due to lack of clinical improvement.

[b]Wild-type and TR34/L98H-resistant strains both present in the bronchoalveolar fluid sample.

[c]Azole resistance was proven on sputum sample 14 days after start of azole treatment.

[d]In this patient, the result of the resistance test became available 14 days after start of initial antifungal therapy with voriconazole. The switch was therefore not due to clinical treatment failure but based on the resistance test which became available at that time. This patient was not counted for treatment failure in the analysis.

Table 4. Outcome of Patients According to the Mycological Test That Was Positive

	GM-Positive[a] (N = 77)	Culture-Positive[a] (N = 24)	*Aspergillus* PCR-Positive[a,b] (N = 119)	*Aspergillus* PCR-Positive in Duplicate[a] (N = 67)	GM- and Culture-Negative but *Aspergillus* PCR[b]-Positive (N = 62)	GM- and Culture-Negative but *Aspergillus* PCR-Positive in Duplicate (N = 28)	GM-, Culture-, and *Aspergillus* Species PCR-Negative (N = 154)
Antifungal therapy started around bronchoalveolar lavage (−5, + 14 d) (n/N)	72/77 (94%)	23/24 (96%)	105/119 (88%)	62/67 (93%)	52/62 (84%)	24/28 (86%)	105/154 (68%)
Median duration of antifungal treatment (d)—median (interquartile range)	27 (11–73)	38 (17–88)	32 (10–89)	33 (12–89)	34 (10–123)	71 (15–135)	18 (7–63)
6-week mortality (n/N)	23/76 (30%)	8/24 (33%)	26/119 (22%)	16/67 (24%)	9/62 (15%)	4/28 (14%)	24/153 (16%)

Abbreviations: GM, galactomannan; PCR, polymerase chain reaction.

[a]Irrespective of the other mycological tests. GM in serum and/or bronchoalveolar fluid.

[b]*Aspergillus* species– and/or *Aspergillus* fumigatus PCR–positive.

mycological evidence (15% when positive vs 16% when negative, $P = .829$; Table 4). The results were comparable when we restricted the analysis to patients with an isolated duplicate positive PCR (mortality 14% with duplicate positive PCR and 16% when negative, $P = .851$). Supplementary Data 7 (Supplementary Tables 4 and 5) provides additional information on outcome for subgroups defined by combinations of positive diagnostic tests.

Relevance of the* Aspergillus *PCR Ct Value. In patients with an isolated positive PCR, the median Ct value was higher (36.4, IQR, 35.1–37.5) compared with patients with a positive GM or culture (33.8, IQR, 31.8–36.1 and 33.4, IQR, 32.6–36.4, respectively). In the entire patient cohort, a cutoff of 33.11 had the best Area Under the Curve (AUC) (65.9%; 95% CI, 53.4–78.3) to predict 6-week mortality but with a low sensitivity (52%) and moderate specificity (80%). In patients with an isolated positive PCR, there was no statistical difference in 6-week mortality based on the cutoff of 33.11, but Ct values <33.11 were present in only 6 patients (see Supplemental data S9 for the complete Ct value analysis). Finally, a Ct value of 34.6 predicted that the resistance PCR would be the most successful (AUC, 79.3%; CI, 79.8–94.3; sensitivity, 79.3%; specificity, 90%).

DISCUSSION

Patients with an azole-resistant IA are characterized by an excess mortality of greater than 20% compared with an azole-susceptible IA. Early switch to appropriate antifungal therapy may reduce the resistance-attributable mortality [4, 10, 11]. We therefore determined if the use of an azole-resistance PCR improves the outcome of patients with IA.

Compared with historical data on the incidence of treatment failure in patients with azole-resistant IA, use of PCR was associated with a better treatment response. Indeed, treatment failure was observed in only 1 of 6 patients [9]. This observation should be interpreted cautiously. First, a randomized trial rather than a comparison with a historical cohort would have been preferred. However, the required sample size (>1000 patients) was deemed unrealistic. Also, current Dutch guidelines recommend combination therapy or L-AmB if real-time resistance testing is not performed. Consequently, despite its clear limitations, a prospective observational study with a historical control group was considered the only realistic design.

Despite the inclusion of 323 patients, only 8 patients with probable IA by an azole-resistant *A. fumigatus* were identified. Since 2 of the patients had a mixed azole-susceptible/azole-resistant infection, 6 were left for the primary analysis. Therefore, the patient group was smaller than anticipated. Also, in contrast to what was suggested in the flow diagram of the protocol (Supplementary Methods 3, Supplementary Figure 1), 3 of 8 patients with azole-resistant IA received L-AmB or an echinocandin rather than an azole as their initial antifungal therapy before resistance had been detected. So, despite the agreed-on flow diagram, some clinicians took the risk for azole resistance into account before it was documented. The positive outcome of these patients can therefore potentially be explained in part by this initial treatment choice.

Of all 72 patients with a probable IA in which *A. fumigatus* was demonstrated, the culture was positive in 20 (28%). However, focusing on the 8 patients with azole-resistant probable IA, cultures were positive in 6 of 8 (75%). This means that in 2 of 8 (25%) azole resistance would have been missed by culture alone. Moreover, as phenotypic resistance testing takes time

and is frequently done at a reference laboratory, PCR-based testing can decrease the time it takes to demonstrate azole resistance. Historical data on azole resistance are mostly culture based. Since cultures may be more frequently positive if the IA is caused by azole-resistant *A. fumigatus*, this may lead to an overestimation of the actual azole resistance frequency in patients with IA. However, the PCR-based resistance prevalence of 14% that we observed is comparable to the prevalence based on culture results described previously in the Netherlands [12].

Our study provides insight into the potential value of the systematic use of an *Aspergillus* PCR on BALf as well as the association with the quantitative GM result and Ct value of the PCR [13]. In 116 patients (40%), *Aspergillus* species DNA was detected, and this percentage increased when GM was higher (Table 2). While the *A. fumigatus* PCR was positive in 89 (30%) patients, resistance testing was successful in only 58 (65%). Also, this resistance PCR was more successful with higher GM levels. The lower sensitivity of the resistance PCR is in line with a previous study and can be explained by the single-copy nature of the *cyp51A* gene target in contrast to the *fumigatus* DNA probe that targets a multicopy gene [9, 14].

A positive PCR test result on BALf in a patient with a negative GM and culture was observed in 1 of every 5 patients (in 1 of every 10 when the duplicate positivity criterion was used). However, it was not associated with any increase in overall mortality. We cannot conclude whether this lack of clinical impact on mortality of a positive PCR was the result of the antifungal therapy that 85% of these patients received or if it reflects colonization instead of IPA in most of these patients. The value of antifungal therapy for these patients remains uncertain. During the conduct of our study, the EORTC/MSGERC definitions were updated, and the 2020 version now includes a positive *Aspergillus* PCR as a mycological criterion. For blood samples, the guideline clearly mentions that 2 consecutive samples should be PCR-positive; for BALf, the criterion is "2 or more duplicate PCR tests positive." Because BALf sampling is invasive, it is almost never done twice. We therefore interpreted this criterion as "2 positive PCRs on a single BALf sample." This EORTC/MSGERC criterion will need further specification as we found no association with mortality in patients with a negative GM and culture and therefore only fulfilled the PCR BALf criterion. These specifications may consist of a certain minimum Ct value threshold because the median Ct value of patients with an isolated positive PCR was much higher (36.4) than in patients with additional mycological evidence (33.1). Also, the guideline could perhaps state that the PCR should be positive on BALf from 2 different bronchoscopies or 2 different lobes. In all scenarios, the PCR criteria that are included should be associated with a clinical impact and ideally mortality because the criteria in the guidelines are used for registration trials.

Despite the reasonably large number of patients enrolled across 12 sites over 4 years, only 1 patient with azole resistance died of this infection within 6 weeks. This suggests that azoles can continue to be the initial therapy for the large majority of patients in the Netherlands and Belgium, as long as real-time resistance testing is possible and a change to appropriate antifungal therapy is initiated promptly when resistance is detected. Our observations therefore are in support of the Dutch antifungal treatment guideline in which this approach is considered reasonable. This approach also has the advantage of reducing overexposure to antifungal drugs that can have side effects while only few patients will benefit.

In conclusion, real-time PCR-based resistance testing may help to limit the clinical impact of triazole resistance. In contrast, the clinical impact of an isolated positive *Aspergillus* PCR on BALf seems limited. The EORTC/MSGERC PCR criterion for BALf may need further specification (eg, minimum Ct value and/or PCR positive on >1 BALf sample).

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. B. R., A. S., and A. D. contributed to study design. A. S., A. D., and S. H. did the data extraction and analysis. B. R. supervised the statistical analysis. B. R., A. S., A. D., and S. H. wrote the original draft. A. D., S. H., J. B. B., C. K., P. V., K. D., N. J., J. J., W. V., B. B., A. B., A. H. W. B., P. H., A. D., G. O., P. B., M. B., S. K., P. G., L. S., D. P., G. K., J. M., K. L., T. M., I. M., J. B., D. S., M. R., W. Z., J. D., J. C., A. S., and B. R. reviewed and edited the manuscript. All authors read and approved the final version. B. R., A. D., and S. H. had full access to the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgments. The authors acknowledge all patients and participating centers for their efforts during the trial. They thank the Department of Medical Microbiology and Infectious Diseases of Erasmus Medical Center for technical support and performing the majority of the AsperGenius polymerase chain reaction assays.

Financial support. This work was supported by Gilead Sciences, Inc, under grant number [IN-NL-131-4187].

Potential conflicts of interest J. M. reports grants from Gilead Sciences, Inc; consulting fees, payment for lectures/presentations, and support for meetings/travel expenses from Gilead Sciences, Inc, MSD, Pfizer, Takeda, and F2G; and participation on a data safety monitoring board or advisory board for Gilead Sciences, Inc, MSD, Pfizer, Takeda, F2G, and Cidara. P. G. reports partial support for travel to Trends In Medical Mycology conference. K. L. reports grants from Thermo Fisher Scientific and TECOmedical paid to their institution; consulting fees from Gilead, MSD, and MRM Health, all paid to their institution; and personal fees for lectures/presentations for Pfizer, Gilead, and FUJIFILM Wako. M. R. reports support for travel to Trends In Medical Mycology conference. J. J. reports grants from Novartis and BMS, both paid to their institution; payment for lectures from AbbVie, Novartis, Pfizer, and Incyte; and serving as president of the Apps for Care and Science, a nonprofit organization, supported by AbbVie, Alexion, Amgen, Astellas, BMS, Daiichi-Sankyo, Janssen-Cilag, Olympus, Incyte, Sanofi Genzyme, Servier, Jazz, and Takeda. J. B. reports research grants from Gilead Sciences, Inc, and F2G. P. V. reports research grants from F2G and Gilead, paid to their institution; honoraria for lectures from F2G, Gilead, and Pfizer, all paid to their institution; and participation on a data safety monitoring board for F2G,

paid to their institution. S. H. reports support from Gilead for travel to the International Society for Human and Animal Mycology 2022 conference. B. R. reports research grants from Gilead Sciences, Inc; support for meetings/travel expenses from Gilead Sciences, Inc, F2G, and Pfizer; consulting fees from F2G; payment/honoraria for lectures/presentations from Gilead Sciences, Inc; and participation on a data safety monitoring board/advisory board for Exevir. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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