Azole resistance in *Aspergillus fumigatus* is increasingly reported. Here, we describe the validation of the AsperGenius, a new multiplex real-time PCR assay consisting of two multiplex real-time PCRs, one that identifies the clinically relevant *Aspergillus* species, and one that detects the TR34, L98H, T289A, and Y121F mutations in CYP51A and differentiates susceptible from resistant *A. fumigatus* strains. The diagnostic performance of the AsperGenius assay was tested on 37 bronchoalveolar lavage (BAL) fluid samples from hematology patients and 40 BAL fluid samples from intensive care unit (ICU) patients using a BAL fluid galactomannan level of ≥1.0 or positive culture as the gold standard for detecting the presence of *Aspergillus*. In the hematology and ICU groups combined, there were 22 BAL fluid samples from patients with invasive aspergillosis (IA) (2 proven, 9 probable, and 11 nonclassifiable). Nineteen of the 22 BAL fluid 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 fluid samples had a positive PCR (2 *Aspergillus* species and 14 *A. fumigatus* samples). This resulted in a sensitivity, specificity, and positive and negative predictive values of 88.9%, 89.3%, 72.7%, and 96.2%, respectively, for the hematology group and 80.0%, 93.3%, 80.0%, and 93.3%, respectively, in the ICU group. The CYP51A real-time PCR confirmed 12 wild-type and 2 resistant strains (1 TR34-L98H and 1 TR46-Y121F-T289A mutant). Voriconazole therapy failed for both patients. The AsperGenius multiplex real-time PCR assay allows for sensitive and fast detection of *Aspergillus* species directly from BAL fluid samples. More importantly, this assay detects and differentiates wild-type from resistant strains, even if BAL fluid cultures remain negative.

*Aspergillus fumigatus* is the most frequent cause of invasive mold infections in immunocompromised patients. The mortality rate from these infections 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. (4) showed that the treatment of IA with voriconazole resulted in improved survival. 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-bp tandem repeat (TR) in the promoter region (TR34). CYP51A encodes cytochrome P450 sterol 14α-demethylase, the target of azoles. The majority of these mutated strains were cultured from patients who were never exposed to azoles. It is assumed that resistance development is caused by environmental azole exposure (11). More recently, van der Linden et al. (12) described a second mutation, a 46-bp TR combined with the point mutations Y121F and T289A (12). In this study, 47 of 921 patients (5.1%) were diagnosed with TR34-L98H and 13 (1.4%) with the TR46-Y121F-T289A mutations. Other mutations have also been described (13–16). Infections with azole-resistant strains are associated with a very high mortality rate (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 β-1,3-D-glucan) testing, because cultures remain negative in most patients. Therefore, even if culture-based azole resistance testing became broadly available, this would be helpful in only a subset of patients.

This study describes the laboratory and first clinical validation of the AsperGenius, a new *Aspergillus* real-time PCR assay that detects *Aspergillus* species directly from bronchoalveolar lavage fluid samples.
(BAL) fluid and simultaneously identifies the most prevalent CYP51A mutations in *A. fumigatus*.

(Join this work was presented at the 24th European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Barcelona, 10 to 13th May 2014, abstr. 0216 [19].)

**MATERIALS AND METHODS**

This retrospective study was performed at the Erasmus Medical Center in the Netherlands. The following patient 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 fluid samples from historical patients. The BAL fluid samples from hematological and ICU patients were selected because these patients are at high risk for IA.

**Processing of BAL fluid samples.** The BAL fluid samples from ICU patients (1 to 2 ml) were incubated with 0.1 M dithiothreitol (DTT) to reduce viscosity. This was not needed for BAL fluid samples from neutropenic hematologic patients. Subsequently, all BAL fluid samples were centrifuged at high speed (10 min at 13,400 g). After centrifugation, the supernatant and the pellet were processed in different ways. The supernatant was added to 2 ml of NucliSENS lysis buffer (bioMérieux, Boxtel, the Netherlands) and incubated for 10 min at room temperature. An internal control (IC) was added to the BAL fluid to monitor PCR inhibition, DNA extraction efficiency, and manual handling errors. The pellet was transferred to green bead tubes (Roche Diagnostics, Indianapolis, IN, USA), and 500 μl of 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 s at 6,500 rpm. Proteinase K (Roche Diagnostics) was added, 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 the supernatant and pellet was extracted with the NucliSENS miniMAG magnetic extraction (bioMérieux), according to the manufacturer’s instructions.

**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 the specific detection of *A. fumigatus* complex, *Aspergillus terreus*, and other *Aspergillus* species by targeting the 28S rRNA multicopy gene. The *A. fumigatus* probe detects the most relevant species of the *Fumigati* complex: *A. fumigatus*, *Aspergillus lentulus*, *Aspergillus udagawae*, and *Aspergillus viridinutans*. The *Aspergillus* species probe specifically detects *A. fumigatus* complex, *A. terreus*, *Aspergillus flavus*, and *Aspergillus niger*. In addition, *Aspergillus nidulans* can be detected based on sequence information. The detection of the IC is included. The AsperGenius resistance multiplex assay targets the single-copy CYP51A gene of *A. fumigatus* and detects the TR34, L98H, Y121F, and T289A mutations to differentiate wild-type from mutant *A. fumigatus* strains via melting curve analysis. The real-time PCR was performed according to the manufacturer’s instructions. The detection of four different fluorescent labels (emission spectra, 495 nm, 530 nm, 598 nm, and 645 nm) was enabled by using the Rotor-Gene Q (Qiagen, Heidelberg, Germany) for all experiments. The real-time PCR assay was first validated using the DNA of 131 *A. fumigatus* cultures, including resistant strains (Erasmus Medical Center) before testing BAL fluid samples. These strains were identified to the species level based on morphology and by sequencing of the internal transcribed spacer region. Furthermore, the assay was tested for cross-reactivity with species selected based on their prevalences in the respiratory tract and/or their genomic similarity. The specificity was tested for the following species: *Penicillium marneffei*, *Penicillium chrysogenum*, *Fusarium* species, *Scedosporium* species, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Candida albicans*, *Candida lusitaniae*, *Candida krusei*, *Candida dubliniensis*, *Candida guilliermondii*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Pneumocystis jirovecii*, *Bordetella pertussis*, *Escherichia coli*, *Haeomophilus influenzae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Chlamyphilia pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*.

Each extracted BAL fluid sample was tested in duplicate, and a nontemplate 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, Herlev, 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 fluid samples to determine if the melting peak represents wild-type or resistant *A. fumigatus* strains.

**Gold standard.** BAL fluid samples with a positive galactomannan level (≥1.0) or positive BAL fluid or sputum cultures for *Aspergillus* were selected as the gold standard for positivity. True negatives were BAL fluid samples with a negative BAL fluid galactomannan level in combination with a negative culture from BAL fluid or sputum. BAL fluid samples with a positive serum galactomannan level only (≥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 as 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 consolidation 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 (Platelet; Bio-Rad, Inc.) or positive cultures of *A. fumigatus*. Galactomannan was considered positive if at a level of ≥1.0 in BAL fluid and ≥0.5 in serum. Proven IA is defined as histologic or pathologic evidence of invasive mold 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 nonspecific, were referred to as nonclassifiable 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 that of patients with probable IA [20]. Note that most ICU patients with a clinical diagnosis of IA will fall into this nonclassifiable category because, in contrast to the neutropenic patients, the findings on high-resolution computed tomography or chest X ray in ICU patients with IA are mostly nonspecific (1).

**PCR cutoff analysis.** To determine the most appropriate *Aspergillus* PCR cutoff for clinically significant positivity, we first analyzed the cycle threshold (Ct) values of the *Aspergillus* PCR on 37 BAL fluid samples from 34 hematological patients. Three patients in the hematology group underwent a BAL twice at different times and therefore contributed two BAL fluid samples each. The lowest Ct value of the pellet or supernatant was used. The optimal cutoff was assessed with receiver operator characteristic (ROC) curves. The areas under the concentration-time curve (AUCs) were estimated to determine discriminatory power (IBM SPSS Statistics, version 21). In a second set of 40 BAL fluid samples from 39 ICU patients, we confirmed the usefulness of the cutoff that was obtained in the hematology population. The sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) were calculated for the two groups both 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, all of which carried the L98H–TR34 mutations. These data were confirmed by sequencing of the CYP51A
regions. Furthermore, the specificity of the assay was tested with species selected based on their prevalences in the respiratory tract and/or their genomic similarity. No cross-reactivity was observed for *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 DNA load that was 1.000 × higher (50 pg) than the *A. fumigatus* DNA load needed to get a PCR-positive result with a *C_T* of 36 (50 fg or 2 DNA copies/ml).

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

The ROC curves for the different groups are shown in Fig. 1. The diagnostic accuracy as given by the AUC for the hematology group was 0.92 (95% confidence interval [CI], 0.79 to 1.00; *P* < 0.001). The AUC was 0.91 (95% CI, 0.76 to 1.00; *P* < 0.001) for the ICU group and 0.91 (95% CI, 0.81 to 1.00; *P* < 0.001) for the two groups combined. The most optimal *C_T* value cutoff for the *Aspergillus* species PCR was <36 for the 37 BAL fluid samples of the hematology patients. Table 2 shows the IA classification related to the *C_T* cutoff of <36, and Table 3 shows the BAL fluid samples according to the gold standard related to the *C_T* cutoff of <36. In the hematology group, the *C_T* cutoff of <36 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 *C_T* cutoff of <36 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 were 84.2%, 91.4%, 76.2%, and 94.6%, respectively.

In the hematology and ICU patients combined, 19 BAL fluid samples of patients with proven, probable, or nonclassifiable IA were identified based on the gold standard. From these 19 BAL fluid samples, 16 had a positive *Aspergillus* PCR result (15 positive for both the pellet and supernatant, and 1 positive for the pellet only). Fourteen of the 16 BAL fluid samples had a positive *A. fumigatus* PCR result, and the remaining two were *Aspergillus* species. In all 14 positive *A. fumigatus* PCR BAL fluid samples, the CYP51A resistance PCR was successful. Twelve strains were determined to be wild type and two to be mutant strains. One sample had the TR46-Y121F-T289A mutations, and the other had the TR34-L98H mutations. More information on the melting curves of the mutant strains can be found in the supplemental material. Both patients from whom these BAL fluid samples were obtained showed clinical failure with voriconazole therapy. The first patient was treated with allogeneic stem cell transplantation for acute myeloid leukemia. The patient died of culture-positive pulmonary and cerebral IA that developed during voriconazole prophylaxis (3 days before he died, the single serum voriconazole level was high, at 8 mg/liter). The second patient developed IA during remission induction chemotherapy for acute myeloid leukemia. The patient showed an increased serum galactomannan level, progressive pulmonary infiltrates, and plural effusion (galactomannan levels in pleural fluid, >2.0) despite 14 days of therapeutic voriconazole serum levels (>4 mg/liter; see Fig. S4 in the supplemental material). The 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 assay showed good performance characteristics for diagnosing 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 and not widely available, and because patients with IA are mostly culture negative. This multiplex real-time PCR assay tackles these problems by enabling diagnosis within hours after a BAL fluid 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, 21). However, now that azole resistance in *A. fumigatus* is increasing (5–10), the availability of azole resistance testing with a short turnaround 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 nonazole antifungal therapies in an early phase and not when patients clinically deteriorate.

The ROC curves showed that the most optimal *C_T* value cutoff was 36. This cutoff was comparable to the cutoff described for the 2 *Aspergillus* PCR assays tested by Torelli et al. (22). Moreover, these PCR assays were validated on BAL fluid samples from hematology and ICU patients, the same subset of patients as in the present study. Given these observations, the *C_T* cutoff of 36 is probably accurate.

In the hematology group, there was one false-negative and two false-positive *Aspergillus* PCR results compared with the gold standard. The false-negative result was in a BAL fluid sample from a patient with nonclassifiable IA (Table 1, patient 5). The lung abnormalities might also be a side effect of the chemotherapy that the patient received for her acute promyelocytic leukemia. It is possible that this patient did not have IA and that the galactomannan level of 1.1 in BAL fluid was false positive. The two false-positive *Aspergillus* PCR BAL fluid samples were from patients with possible IA. The BAL fluid galactomannan level of the first patient was 0.7, and that 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 of a fungal infection. As with every diagnostic test, there is no galactomannan level cutoff with 100% diagnostic accuracy. A BAL fluid galactomannan level cutoff of 0.5 has increased sensitivity but somewhat lower specificity (23). For the purpose of validating the PCR in this study, we considered a higher specificity to be more important, and therefore, we selected the galactomannan level cutoff of 1.0 as the gold standard.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical setting</th>
<th>IA diagnosis</th>
<th>Underlying disease/host factor(s)</th>
<th>Radiological findings</th>
<th>BAL fluid or sputum culture</th>
<th>Pathology</th>
<th>Galactomannan level in:</th>
<th>AsperGenius multiplex real-time PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HEM</td>
<td>Probable</td>
<td>MM, allogeneic HSCT</td>
<td>Specific</td>
<td>Pos NA 0.2</td>
<td>1.5</td>
<td>Pos (29)</td>
<td>Pos (29)</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>ICU</td>
<td>Probable</td>
<td>AML</td>
<td>Neg NA 0.1</td>
<td>1.6</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>ICU</td>
<td>Probable</td>
<td>HL, allogeneic SCT</td>
<td>Specific</td>
<td>Pos NA 7.2</td>
<td>7.5</td>
<td>Pos (31)</td>
<td>Pos (23)</td>
</tr>
<tr>
<td></td>
<td>ICU</td>
<td>Nonclassifiable</td>
<td>Liver cirrhosis</td>
<td>Not specific</td>
<td>Neg</td>
<td>NA 0.6</td>
<td>0.3</td>
<td>Neg</td>
</tr>
<tr>
<td></td>
<td>ICU</td>
<td>Nonclassifiable</td>
<td>MM</td>
<td>Not specific</td>
<td>Pos NA 0.3</td>
<td>22.7</td>
<td>Pos (32)</td>
<td>Pos (26)</td>
</tr>
</tbody>
</table>

**Notes:**
- **HEM:** hematology; **ICU:** intensive care unit.
- **IA:** invasive aspergillosis.
- **MM:** multiple myeloma; **HSCT:** hematopoietic stem cell transplantation; **AML:** acute myeloid leukemia; **APL:** acute promyelocytic leukemia; **CLL:** chronic lymphatic leukemia; **HL:** Hodgkin lymphoma.

- **Pos:** positive; **Neg:** negative.
- **WT:** wild type; **CT:** cycle threshold. A CT of $>H_11021 36$ was considered positive, and $<H_11350 36$ was considered negative.
- **NA:** not available.
- **No specific radiological findings were observed in the lung, but specific cerebral findings were observed on magnetic resonance imaging.**

TABLE 1: Clinical characteristics, radiological findings, and results of the galactomannan and AsperGenius multiplex real-time PCR assay for 22 bronchoalveolar lavage fluid samples of patients with proven, probable, and nonclassifiable invasive aspergillosis.
The PCR assay was performed on DNA extracted from both the pellet and the supernatant of the BAL fluid. DNA extraction from the pellet is more labor-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 (C<sub>T</sub> < 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 test the pellet only if the supernatant is negative in patients with a positive Aspergillus culture, a positive galactomannan level, or very high clinical suspicion of infection.

The Aspergillus species probe showed cross-reactivity for <i>P. chrysogenum</i> and <i>R. oryzae</i>. <i>P. chrysogenum</i> is rarely pathogenic in humans. <i>R. oryzae</i> can cause symptoms comparable to those of invasive aspergillosis, but the clinical prevalence is low.

### TABLE 2 Epidemiological classification of invasive aspergillosis related to a C<sub>T</sub> cutoff of 36

<table>
<thead>
<tr>
<th>Classification of IA</th>
<th>Hematology group (n = 37)</th>
<th>ICU group (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;36</td>
<td>≥36</td>
</tr>
<tr>
<td>Proven</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Probable</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Nonclassifiable</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Possible</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
<td>24</td>
</tr>
</tbody>
</table>

*BAL, bronchoalveolar lavage; ICU, intensive care unit.

### TABLE 3 Bronchoalveolar lavage fluid samples with positive galactomannan level or cultures according to a C<sub>T</sub> cutoff of 36

<table>
<thead>
<tr>
<th>BAL fluid sample results by C&lt;sub&gt;T&lt;/sub&gt; cutoff in*</th>
<th>Hematology group (n = 37)</th>
<th>ICU group (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive galactomannan level (≥1.0) and/or positive culture</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Negative galactomannan level (&lt;1.0) and negative culture</td>
<td>3</td>
<td>25</td>
</tr>
</tbody>
</table>

*BAL, bronchoalveolar lavage; ICU, intensive care unit.
more, for both of these species, a 1,000/H11003 fumigatus strains.

in IA patients.

differentiate wild-type from resistant strains even on culture-negative 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 that since only the CYP51A mutations are included in the PCR, those are the only mutations that 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 into the EORTC-MSG criteria. The Aspergillus PCR could be used in combination with galactomannan testing, as it provides information on the 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 wild-type from resistant strains even on culture-negative BAL fluid samples. This enables on-time and targeted therapy in IA patients.

ACKNOWLEDGMENTS

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