DIAGNOSTIC APPROACHES FOR ASPERGILLUS INFECTIONS

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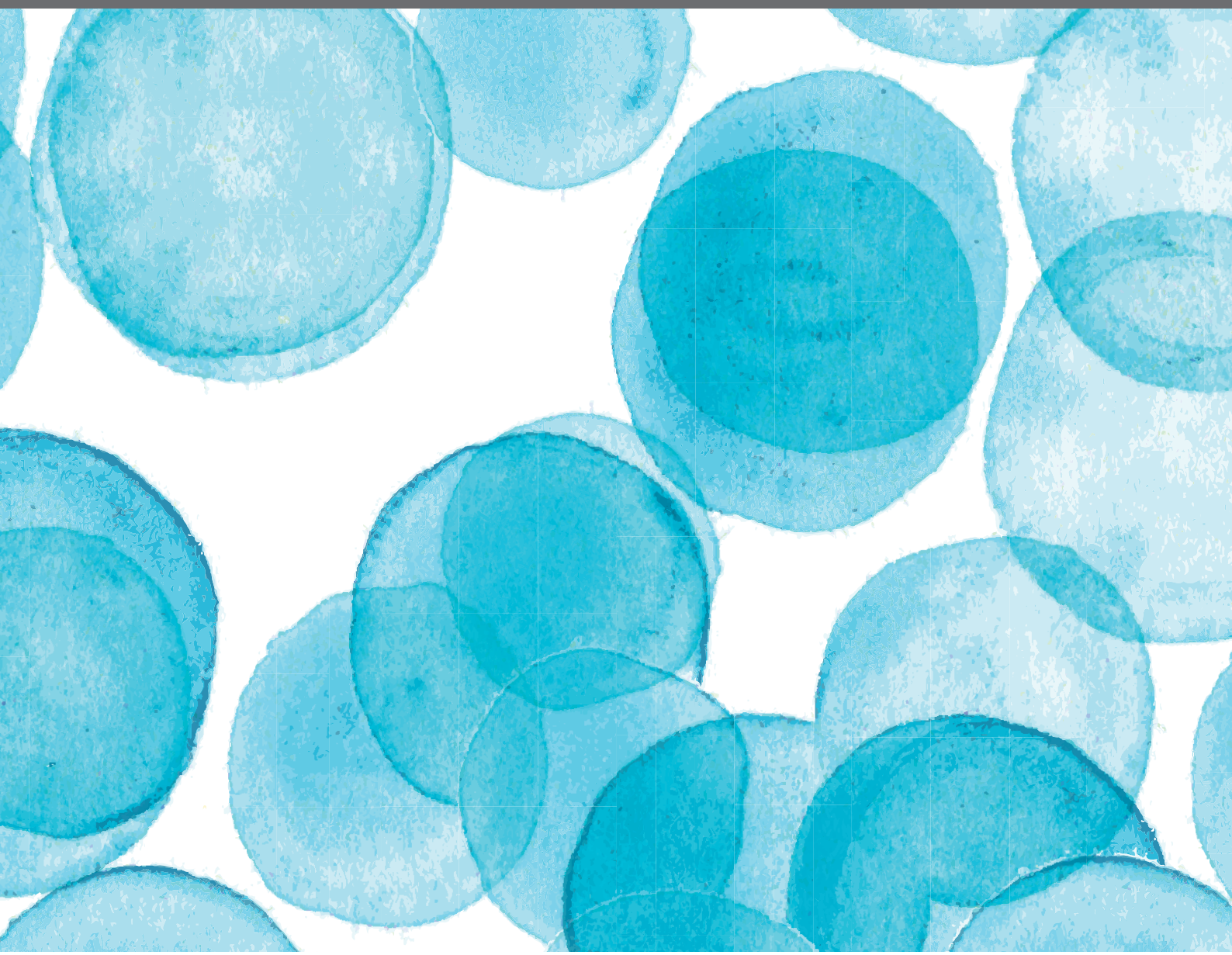

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DIAGNOSTIC APPROACHES FOR ASPERGILLUS INFECTIONS

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Infections caused by Aspergillus spp. are associated with devastating mortality rates. Early and reliable diagnosis and subsequent rapid initiation of appropriate antifungal therapy has shown to improve survival significantly, at least for invasive Aspergillosis. Early diagnosis of Aspergillus associated infections, therefore, represents a cornerstone in successful management of these diseases.

Current state of the art diagnostic approaches, new insights in epidemiology and established biomarkers as well as an outlook for future diagnostic options in the armamentarium of diagnostics tools are highlighted in this eBook.

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EDITORIAL
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Editorial: Diagnostic Approaches for Aspergillus Infections

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Keywords: galactomannanan, azole resistance detection, PCR, chronic pulmonary aspergillosis, immunoPET

Editorial on the Research Topic

Diagnostic Approaches for Aspergillus Infections

Infections caused by *Aspergillus* spp. affect immunocompromised patients, patients with certain genetic defects including CARD-9 deficiency (Vaezi et al.,) and patients with pre-existing lung conditions or liver cirrhosis, and are associated with devastating mortality rates (Cornely et al., 2017; Prattes et al., 2017; Hoenigl et al., 2018b). Early and reliable diagnosis and subsequent rapid initiation of appropriate antifungal therapy has shown to improve survival significantly, at least for invasive Aspergillosis (Heldt et al., 2018). However, invasive *Aspergillus* infections progress rapidly and are difficult to diagnose especially at early stages (Hoenigl et al., 2019). Culture based approaches are important for detection of fungal species and resistance testing, however they are limited by low sensitivities—in particular during early phases of infection—and long turnaround time (Eigl et al., 2017). Important advances to the field were brought by the introduction of non-cultural diagnostic tests for aspergillosis in blood and bronchoalveolar lavage fluid, including, galactomannan antigen testing, PCR, and 1,3-ß-D-glucan testing in patients at risk (Eigl et al., 2017; Heldt et al., 2018; Prattes et al., 2018; Jenks et al., 2019b). Complicating is the fact that performance of these tests may vary not only by fungal disease, but also by risk group (e.g., neutropenic patients vs. non-neutropenic patients, neonates vs. adults, antimould prophylaxis vs. no antimould prophylaxis) (Eigl et al., 2015).

The current Research Topic includes in total 19 high quality manuscripts, ranging from reviews of current state of the art of treatment of aspergillosis in solid organ transplant recipients (Herrera and Husain), the pediatric population (Lehrnbecher et al.), and the veterinary setting (Elad and Segal), to a variety of original articles focusing on new diagnostics of invasive and chronic forms of aspergillosis, including detection of azole resistance.

Importantly, several new diagnostic approaches for diagnosis of invasive aspergillosis have been studied within the last years (e.g., novel *Aspergillus* PCR (Rath and Steinmann) assays for which results were shown to be quantitatively correlated to galactomannan levels in one study of this Research Topic (Alanio et al.), the *Aspergillus* specific lateral flow assay (Hoenigl et al., 2018a; Jenks et al., 2019a,b; Salzer et al.), Triacetylfusarinine C (Skriba et al.; Hoenigl et al., 2019), Bis(methylthio)gliotoxin (Vidal-Garcia et al.), PET imaging studies (Thornton), interleukins (Goncalves et al.; Heldt et al., 2017, 2018). These new diagnostic approaches may overcome the limitations observed with the currently available diagnostic tools, like e.g., decreased sensitivities under antifungal prophylaxes/treatment, low specificity or long turn-around times (Hoenigl et al., 2018a, 2019; Jenks et al., 2019b). Our Research Topic includes also a review on serum galactomannan testing in a promising indication other than diagnosis, namely for outcome prediction and treatment stratification (Mercier et al.).

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Another focus of this Research Topic is the molecular detection of azole resistance, which is infected by *Aspergillus fumigatus (A. fumigatus)* (Tsitsopoulou et al.), but—even more threatening—as reported in this Research Topic also those infected by *A. terreus* (Zoran et al.), which is non-susceptible to Amphotericin B. Knowledge about epidemiology on *Aspergillus* susceptibility patterns represents a corner stone for appropriate antifungal prophylaxis and treatment as in areas with high rates of environmental azole resistance primarily antifungal treatment with azoles may be reconsidered. Data on environmental azole resistance rates, however, are lacking for many geographic regions. Tsitsopoulou et al. therefore investigated the rate of azole resistant *A. fumigatus* strains in South Wales, UK (Tsitsopoulou et al.). Screening 715 environmental soil and air samples from various regions of South Wales, they found a prevalence of azole resistant *A. fumigatus* strains of 6%. In some areas azole resistance rates were even >10%, including botanic gardens in public parks and a public garden within the grounds of a hospital. Those findings are of concern as susceptible patients may be colonized or infected with resistant *A. fumigatus* strains. Rapid detection of azole resistance in *A. fumigatus* strains in such cases is important for initiation of appropriate antifungal treatment. As *Aspergillus* culture requires up to 7 days followed by antifungal susceptibility testing, molecular assays have been developed for rapid detection of mutations associated with azole resistance among *A. fumigatus*. The performance of two commercially available *Aspergillus* PCR assays including detection of resistance mechanism, the AsperGenius® (PathoNostics, Maastricht, Netherlands) and the MycoGENIE® (Ademtech, Pessac, France) was reviewed by Buil et al. and by Rath and Steinmann. The AsperGenius® represents a multiplex qPCR assay targeting *Aspergillus* DNA, differentiates *A. fumigatus* and *A. terreus* DNA, and detects the most common mutations in the Cyp51A gene associated with azole resistance in *A. fumigatus* (TR_{34}/L98H and TR_{46}/Y121F/T289A). In addition, the assay is able to detect wildtype (WT) and mutations in Cyp51A DNA simultaneously, enabling to detect a co-infection with a WT and an azole resistant strain. In a prospective multicenter study of bronchoalveolar lavage samples from patients with hematological malignancies the AsperGenius® assay showed a promising diagnostic performance with a sensitivity of 84% and specificity of 80% (Chong et al., 2016). Importantly, detection of azole resistance molecular patterns was associated with treatment failure and higher mortality rates compared to infections with WT. The amount of *Aspergillus* DNA in blood samples is relatively lower compared to respiratory samples, complicating, and reducing the sensitivity of the resistance PCR compared to the *Aspergillus* PCR. Even though, sensitivity was 79% and specificity 100% in serum samples in another retrospective study, no resistance patterns could be observed, probably due to the lower fungal burden (White et al., 2015). The MycoGENIE® assay was superior to the AsperGenius® study in BALF samples in another study (Guegan et al., 2018). In a mixed cohort of hematological and non-hematological patients, sensitivity in of the MycoGENIE® assay was 53.7% in hematological patients, and 75% in non-hematological patients while the sensitivity of the AsperGenius® was 41.5 and 60%, respectively. Compared to the AsperGenius® assay, the MycoGENIE® assay however misses WT probes for the resistance marker. In addition, the TR_{46}/Y121F/T289A mutation in the Cyp51A gene is not detected by this assay compared to the AsperGenius® assay. Besides the most common Cyp51A mutations are detected by the AsperGenius® assay, other mutations in this gene may lead to azole resistance. Thus, a group of Germany developed six different in-house PCR assays to detect not only TR34, L98H, Y121F, T289A (all also covered by the AsperGenius® assay) mutations, but also M220 and TR46 mutations (not detected by the AsperGenius® assay) (Postina et al.). The in-house PCR assays resulted positive in 61% of biopsies, 29% in cerebrospinal fluid samples (CSF), 67% in BALF samples, and 100% in isolates. AsperGenius® resulted positive in 47% of biopsies, 42% in CSF samples, 58% in BALF samples, and 100% in isolates. Interestingly, the in-house assays detected more Cyp51A mutations compared to the AsperGenius® assay (17 vs. 10). Nevertheless, in-house assays had a significantly longer hands on time compared to the more time saving AsperGenius® assay, currently limiting the use in daily clinical routine.

Aspergillus infections and colonization is also causing disease progression and limitation of live quality in patients with cystic fibrosis as *Aspergillus* may lead to severe asthma or allergic bronchopulmonary aspergillosis in up to 15% of these patients. One article of this Research Topic evaluated the performance of two in-house PCR assays as well as the performance of the AsperGenius® and the MycoGENIE® assay for *Aspergillus* detection and detection of azole resistance in sputum samples from cystic fibrosis patients (Guegan et al., 2018). They found comparable performance for all four assays. Of note, a large number of culture negative samples turned out positive with the PCR assays indicating a higher sensitivity within these assays. Azole resistance was present in five cultured isolates recovered from patients with long term azole treatment, of which three displayed mutations in the Cyp51A gene.

In contrast to the invasive form chronic pulmonary aspergillosis (CPA) is usually seen in immunocompetent or mildly immunocompromised patients with underlying respiratory diseases (Salzer et al., 2017). Disease severity and progression is highly variable with a 5-year fatality rate between 40 and 60% (Lowes et al., 2017). Estimated three million people are suffering from CPA globally, but precise epidemiological data are lacking (Godet et al., 2018). Reasons include lack of evidence, but also lack of awareness on the disease itself and the challenge to establish the diagnosis. There is no single test or biomarker that allows CPA diagnosis so far. It needs a combination of clinical, radiological, and mycological characteristics (Denning et al., 2016). It is crucial to be aware of recent advances to apply diagnostic methods and interpret test results correctly.

Takazono and Izumikawa reviewed recent advances in diagnostic methods and proposed an algorithm for the diagnosis of CPA (Takazono and Izumikawa). First, it is important to identify patients at risk including immunocompetent or mildly immunocompromised patients with any underlying respiratory disorder, unspecific symptoms (e.g., fever, cough), inflammatory markers, and radiological deterioration. Second, *Mycobacterium*

infections should be excluded. Third, an *Aspergillus* IgG antibody assay (EIA) should be performed in blood. If positive, an optional bronchoscopy should be considered offering further diagnostic methods including histopathology/cytology, fungal culture for drug sensitivity testing, galactomannan (GM), 1,3-ß-D-glucan, PCR, and PCR of azole resistant related gene (e.g., AsperGenius©). If *Aspergillus* IgG antibody test result is negative, a bronchoscopy should be performed including further diagnostic methods. If all diagnostic tests from bronchoscopy and the *Aspergillus* IgG antibody are negative, differential diagnosis should be reconsidered, and patients should be followed up. Fourth, patients at risk with CPA typical radiological findings and symptoms with a positive *Aspergillus* IgG test result and/or a positive test result from bronchoscopy with proven mycological evidence should be treated primarily with azoles for at least 6 months.

Radiological presentation is a key diagnostic criterion and most often the first indication of CPA. If available a computed tomography (CT) scan of the chest should be performed. However, different technologies such as [^{18}F] fluorodeoxyglucose positron emission tomography ([18F]FDG-PET) CT scan or molecular imaging using antibody-guided PET/magnetic resonance imaging (immunoPET/MRI) are promising new techniques (Thornton). So far, most data are related to IA, but the principal could be used for CPA too.

Most tests used to prove mycological evidence in CPA patients were originally developed for the diagnosis of IA. GM testing from BAL is frequently used in CPA patients with a reported sensitivity and specificity between 78–92 and 76–90%, respectively using an cut-off of 0.5 optical density index (ODI). The most recent study by Salzer et al. showed lower rates of sensitivity of GM testing from BALF with 41 and 30% with a cut off level of 0.5 ODI and 1.0 ODI, respectively (Salzer et al.). However, the specificity was high with 100%. Comparable results were shown for the first time in CPA patients for the newly formatted CE marked *Aspergillus* lateral flow device (LFD). Previous studies included a higher proportion of subacute invasive aspergillosis (SAIA) patients (formally chronic necrotizing or semi-invasive pulmonary aspergillosis), which is a plausible explanation for higher sensitivity rates since SAIA is in fact an invasive form of the disease and very similar to IA. This study has direct implications for clinical routine since GM testing and LFD show an insufficient performance for diagnosing CPA, but it they can contribute to the diagnostic work-up by excluding invasive disease.

Any diagnostic test to proof mycological evidence needs to be interpreted in the clinical and radiological context since direct or indirect *Aspergillus* detection can reflect colonization, allergic- or infectious disease. Barac et al. conducted a prospective cohort study including 75 patients with ABPA with the aim to clarify if allergic bronchopulmonary aspergillosis (ABPA) and allergic fungal rhinosinusitis (AFRS) could be considered as a common disease entity (Barac et al.). AFRS was confirmed in 80% of patients with ABPA. Therefore, specialists have to consider both presentations in their clinical management.

Collectively, the studies described in original research and review articles in this topic describe recent advances and provide optimism for the future of diagnosis of Aspergillus infections. We hope these articles will stimulate further research with the ultimate goal of improving outcomes for patients with Aspergillus disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Current State of the Diagnosis of Invasive Pulmonary Aspergillosis in Lung Transplantation

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As the number of lung transplants performed worldwide each year continues to grow, the success of this procedure is threatened by the incidence of non-CMV infections such as invasive aspergillosis. Despite tremendous efforts and the availability of numerous diagnostic tests (especially in hematological malignancies) the diagnosis of invasive aspergillosis continues to be a challenge. Lung transplantation remains a unique clinical scenario, where additional host defenses are immunocompromized, making many of the available tests unsuitable. In this review we will navigate through the myriad of diagnostic tests currently available and how they apply to this unique patient population, as well as have a look into what the future holds.

Keywords: lung transplant, invasive aspergillosis, PCR, *Aspergillus*, immunocompromized, galactomannan, BD-glucan, cytokines

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INTRODUCTION

Lung transplantation is an established modality for end-stage pulmonary disease. With over 4600 lung transplants performed in 2016 worldwide (Lund et al., 2017) the number of these procedures carried out annually continues to grow. One of the greatest challenges for the survival of these patients during the first year after transplant are non-CMV infections, especially invasive pulmonary aspergillosis (IPA), which portends a higher mortality than bacterial or viral infections (Mattner et al., 2007).

Several strategies are being used to prevent the development of IPA including universal and pre-emptive prophylaxis (Husain et al., 2016). However, the diagnosis of IPA in lung transplantation remains challenging. *Aspergillus* is a ubiquitous organism, and is often found in sputum or bronchoalveolar lavage (BAL) samples. The presence of *Aspergillus* in these respiratory samples does not necessarily represent a true infection, but in most cases is merely indicative of colonization. Nevertheless, colonization itself is not a risk factor to be overlooked. In the context of immunosuppression and decreased mucociliary motility, colonization and other risk

Abbreviations: BAL, bronchoalveolar lavage; BG, beta-glucan; CF, cystic fibrosis; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; EORTC, European Organisation for Research and Treatment of Cancer; GM, galactomannan; HSCT, hematopoietic stem cell transplantation; IL, interleukin; IPA, invasive pulmonary aspergillosis; ISHLT, international society for heart and lung transplantation; LAL, *Limulus* amebocyte lysate; LTRs, lung transplant recipients; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MRI, magnetic resonance imaging; PCR, polymerase chain reaction; PET, positron emission tomography; PTLD, post-transplantation lymphoproliferative disease; PTX3, pentraxin-3; SOT, solid organ transplant; STAT3, signal transducer and activator of transcription 3; TAFC, triacetylfusarinine C; Th17, T-helper cell 17; TNFα, tumor necrosis factor alpha; ZNF77, zinc finger protein-77.

factors can often lead to a true invasive infection; highlighting the importance of having precise diagnostic tools. Moreover, diagnostic tests used in other patient populations such as hematological malignancies cannot be readily applied in Lung transplant recipients (LTRs).

In this paper we will review the current state of the diagnosis of invasive aspergillosis in lung transplantation, and will comment on the currently available tests as well as diagnostic tests in development, that will available in the near future.

DIAGNOSIS OF INVASIVE PULMONARY ASPERGILLOSIS IN LUNG TRANSPLANT RECIPIENTS

Invasive pulmonary aspergillosis in LTRs has several particularities that are not accounted for in The European Organisation for Research and Treatment of Cancer (EORTC) guidelines, such as colonization, tracheobronchitis and bronchial anastomotic infections. It is therefore preferred to use the International Society for Heart and Lung Transplantation (ISHLT) guidelines (Husain et al., 2011) for the diagnosis of these patients, as it includes diagnostic criteria for all these entities. For the diagnosis of 'proven IPA' there must be a biopsy showing histologic evidence of parenchymal invasion by fungal hyphae, or pseudohyphae, or positive culture from sterile tissue *ALONE*; *OR* with sign/symptoms AND radiological criteria AND laboratory criteria [single positive culture for mold BAL/blood *OR* single positive PCR for mold BAL/blood *OR* positive galactomannan in the BAL; *OR* at least TWO positive sputum cultures/PCRs of fungal organisms (excluding *Candida* species)]. For 'probable IPA' their criteria are the same as for proven, except there is negative or absent histology.

MICROBIOLOGY AND MICROSCOPY

There have been immense advances in the past decade in the development of molecular and immunological tests for the diagnosis of IPA. However, these are not widely available or standardized, and culture and microscopy still play an important role in the diagnosis of IPA. Microscopic methods such as wet mounts, Gram stains, and conventional histopathology, are useful suggesting the presence of *Aspergillus* spp. in parenchyma biopsies or BAL samples for the diagnosis of IPA (McClenny, 2005). *Aspergillus* spp. grows as septate hyphae 2.5–4.5 μm in diameter and can be characterized as branching dichotomously (∼ 45°C angle) (Lass-Flörl and Mayr, 2009). Demonstration of *Aspergillus* spp. in tissue is the only way to prove IPA, however, a negative result cannot rule it out. For a definite identification of the species, a culture or the use of molecular methods is required, in addition to histology. *Aspergillus* spp. can grow from fungal media and also from sheep blood agar commonly used for bacterial culture. Culture has the advantage of speciation and ability to perform susceptibility testing. Their sensitivity nevertheless is far from ideal with a positive culture in sputum in 8 to 34% of patients with IPA and 45 to 62% in BAL (Horvath and Dummer, 1996; Hoenigl et al., 2014). Sensitivity is significantly higher in samples from lung biopsies (Lass-Flörl et al., 2007). The main caveat for these methods are the processing times, especially in immunocompromized patients where diagnosis is truly time-sensitive. Additionally in LTRs culture is unable to differentiate colonization from invasive disease, unless the culture is done directly from a tissue sample. Since *Aspergillus* spp. is ubiquitous, contamination of samples can also occur. Trained staff in the lab are essential for better performance of the aforementioned tests.

SEROLOGICAL AND MOLECULAR TESTS

Serological tests are among the most widely used tests for the diagnosis of IPA. This is in part the result of the poor yield of respiratory sample cultures.

Aspergillus Galactomannan

The major antigenic component secreted by *A. fumigatus* into the growth medium is galactomannan (GM) – a soluble polysaccharide that is present in the cell wall of most *Aspergillus* and *Penicillium* species (Latge et al., 1994). We can detect GM in biological fluids by a commercial sandwich enzyme-linked immunosorbent assay (ELISA) such as the Platelia™ *Aspergillus* (Bio-Rad, Marnes-la-Coquette, France) (Stynen et al., 1995). Despite being used extensively, the ELISA lacks some of the advantages that cultures provide; notably speciation and sensitivities. Therefore, it is generally used in combination with cultures or histological samples. In LTRs GM has been studied both in serum and in BAL, having a better performance in the latter (Clancy et al., 2007). It's sensitivity and specificity range from 60 to 100% and 85 to 98%, respectively, according to different studies including several meta-analysis (Pfeiffer et al., 2006; Clancy et al., 2007; Husain et al., 2007, 2008; Zou et al., 2012; see **Table 1**). It is to note that some of the studies were not done purely on LTR and some included other SOT and some hematological malignancies (Pfeiffer et al., 2006; Zou et al., 2012).

Although an optical density cut-off of 0.5 is generally used in both serum and BAL assays, several of these studies showed that increasing the optical density cut-off to 1.0 in BAL increased the specificity of the assay (Clancy et al., 2007; Husain et al., 2007, 2008). Husain et al. (2007) studied 116 LTRs, 6 with probable or proven IPA, finding sensitivity of 60% and specificity of 95%. By increasing the index cutoff value for BAL to >0.66 or $= 1.0$ a specificity of 98% was achieved, while maintaining a sensitivity of 60%. Similar findings were reported by Clancy et al. (2007) who noted that increasing the cut-off to ≥ 1 improved the specificity to 90.8%, and by Husain et al. (2008), who demonstrated a higher specificity of 96.6% with cut-off of 1.0 in a larger cohort of LTRs.

As the *Aspergillus* GM assay is a widely used tool, it is often used to monitor therapeutic response in LTRs. In practice, serum GM is more feasible for monitoring than BAL GM, as it does not require the repeated bronchoscopies necessary to obtain multiple BAL samples. However, GM has several limitations that need to be taken into account. Firstly, a positive BAL GM does not

TABLE 1 | Summary of human studies assessing galactomannan, BD-glucan and *Aspergillus* PRC in lung transplantation.

Study	Number patients recruited	Sample	Sensitivity	Specificity	Outcome
GM					
Bhimji et al., 2018	197 LTR	EBC			GM detectable in EBC but no correlation with IA
Zou et al., 2012	3344 Patients or controls (614 with IPA)	BAL	87%	89%	Meta-analysis including 30 studies until 2012, mainly hematological but also SOT
Pasqualotto et al., 2010	60 LTRs, 8 with probable or proven IPA	BAL	100%	40%	Increasing the cutoff to 1.5 improved the specificity (90.4%) maintaining sensitivity
Husain et al., 2008	11 Patients and 185 controls (119 LTRs)	BAL	81.8%	95.8%	Higher specificity with cut-off of 1.0 (96.6%)
Clancy et al., 2007	5 Patients with IPA and 76 controls (16 LTRs)	BAL	100%	90.8%	The sensitivity of BAL GM testing was better than serum GM or BAL cytology and culture. Increasing the cutoff to $\geq$1 improved the specificity (90.8%)
Husain et al., 2007	116 LTR, 6 with probable or proven IPA	BAL	60%	95%	Increasing the index cutoff value to 1.0 or more yielded a sensitivity of 60%, a specificity of 98%
Pfeiffer et al., 2006		Serum	71%	85%	Meta-analysis including 27 studies from 1996 to 2005, mainly hematological but also SOT
BD glucan					
Bhaskaran et al., 2017	195 Samples LTR, and 10 episodes of IPA	BAL	80%	53%	Using 41 pg/ml as cut-off, sensitivity and specificity improved to 75 and 91%, respectively, at a 524 pg/ml cut-off
Mutschlechner et al., 2015	135 SOT patients including LTR	BAL and serum	79.2% 79.2%	38.5% 81.8%	233 BAL and 109 serum specimens. Multicenter studies. 135 SOT patients, 114 LTRs. Based on a 100-pg/ml positive cutoff
Alexander et al., 2010	14 LTRs with proven or probable IFI and 59 LTRs controls	Serum	71%	59%	756 Specimens from 59 subjects without IFI and 41 specimens from 14 patients with proven or probable IFI. Based on a 60-pg/ml positive cutoff
PCR					
Lass-Flörl et al., 2011	9 SOT and 33 patients with HM	Lung and skin	82%	79%	Sensitivity of the MycAssay *Aspergillus* test was 82% and specificity 79% relative to microscopy and 90 and 64%, respectively, compared with *Aspergillus* culture
Luong et al., 2011	150 LTRs (16 proven/probable IPA, 26 colonized, 11 non-*Aspergillus* mold colonization, and 97 negative controls)	BAL	100%	88%	The sensitivity and specificity of *A. fumigatus*-specific PCR were 85 and 96%, respectively

BAL, bronchoalveolar lavage; EBC, exhaled breath condensate; GM, galactomannan; HM, hematological malignancy; IFI, invasive fungal infection; IPA, invasive pulmonary aspergillosis; LTR, lung transplant recipient; PCR, polymerase chain reaction; SOT, solid organ transplant.

necessarily mean there is an invasive disease as a positive BAL GM result can also be found in colonized patients (Herrera and Husain, 2018). Secondly, both false positive and false negative results have been reported with varying frequency. Cross reactivity has been reported in patients with *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Cryptococcus* (Xavier et al., 2009). Use of Piperacillin-tazobactam has also been associated with false positive GM in the past (Sulahian et al., 2003), as its production is the result of the fermentation product of molds of the genus *Penicillium* that also contain GM in their wall (Aubry et al., 2006). This issue seems to be less of a problem recently with new formulations of Piperacillin-tazobactam (Vergidis et al., 2014). In children, it has been hypothesized that GM present in some food (such as milk, rice, or protein nutriments) can possibly pass through the intestinal mucosa and in turn actuate a false positive result (Siemann et al., 1998; Gangneux et al., 2002). Finally, Plasma-Lyte an electrolyte replacement solution containing sodium gluconate, has also been identified as a cause of false positive GM in BAL samples (Muñoz et al., 2003).

In LTRs, false positivity of the GM assay was reported in 20% of the patients in a 2004 study by Husain et al. (2004) Importantly, most false-positive tests occurred in the early post-transplant period, with 79% occurring in the first 14 days following transplantation. This is a limitation of the assay as it is the time period where patients at are at higher immunosuppressive risk, and a false positive result would prompt the physicians into initiating unnecessary treatment. This phenomenon was more likely to happen in patients with the underlying diseases of cystic fibrosis (CF) or chronic obstructive pulmonary disease (COPD),

as these patient populations are known to have higher rates of *Aspergillus* colonization. Pasqualotto et al. (2010) also found a high rate of false positivity, in a 2010 study 55.5% of the cases were associated with *Aspergillus* colonization when using a cut-off of 1.0. False positivity was also noted to be higher in single lung transplant recipients.

On the other hand, patients receiving anti-mold prophylaxis or treatment often have false negative GM results (Duarte et al., 2014). Other causes of false negative results include patients who have been in contact with *Aspergillus* before, as they may develop antibodies, which reduces the GM available for the assay to detect (Mennink-Kersten et al., 2004). Both situations are common scenarios after lung transplantation and may reduce the sensitivity of the assay. A previous study in 2013 also suggested that the GM optical index cut-off might vary according to the species of *Aspergillus* spp. (Xavier et al., 2013).

(1-3)-β-d-Glucan

(1-3)-β-d-glucan is another cell wall polysaccharide that is found in most fungi, with the exception of *Cryptococcus, Zygomycetes,* and *Blastomyces dermatitidis*. Several (1-3)-β-d-glucan assays have been developed by different companies and use different cut-offs: Fungitell 60–80 pg/mL (Associates of Cape Cod, East Falmouth, MA, United States), Endosafe-PTS 10–1000 pg/mL (Charles River Laboratories, Charleston, SC), Fungitec-G 20 pg/mL (Seikagaku Biobusiness, Tokyo, Japan), beta-Glucan Test 11 pg/mL (Waco Pure Chemical Industries, Osaka, Japan), and BGSTAR β-Glucan Test 11 pg/mL (Maruha, Tokyo, Japan). It is a chromogenic kinetic assay based on the *Limulus* test. To summarize, β-Glucan (BG) activates factor G, a protease of *Limulus* amebocyte lysate (LAL), which is extracted from the amebocytes of horseshoe crab species. This triggers the activation of a coagulation cascade, and the activity of this reaction is measured (Hope et al., 2005).

The *Limulus* test has been used for the diagnosis of numerous invasive fungal infections, including IPA. Its performance has been shown to be best in hematological malignancies (Senn et al., 2008). In solid organ transplantation and lung transplant in particular, it has several limitations that have lead GM to be a better choice for the diagnosis of IPA. The studies performed, in both serum and BAL in LTRs have shown poor results, with sensitivities and specificities ranging from 71 to 80% and 38 to 81%, respectively (Alexander et al., 2010; Mutschlechner et al., 2015; Bhaskaran et al., 2017; **Table 1**). One study achieved an increased sensitivity and specificity in LTR's by increasing the cut-off of the Fungitell assay to 524 pg/ml (Bhaskaran et al., 2017), a nearly sevenfold increase from the cut-off of 40–80 pg/ml used as standard. All studies have consistent results showing a very low specificity, which limits the use of this assay for IPA diagnosis. Conversely, it has in fact been used in other invasive fungal infections, such as Candida infections (Ahmed et al., 2017) and *Pneumocystis jirovecii* pneumonia, where it has an excellent performance according to a recent meta-analysis (Karageorgopoulos et al., 2013).

Other issues that limit the use of this assay include false positive results due to cross reactions with certain hemodialysis filters, beta-lactam antimicrobials, and immunoglobulins (Theel and Doern, 2013), all of them frequently used in the lung transplant population. Additionally, it is unable to differentiate colonization from invasive disease in BAL (Herrera and Husain, 2018). Its role in the management of lung transplant recipients is not yet well defined.

Aspergillus PCR

Many polymerase chain reaction (PCR) tests for *Aspergillus* spp. have been developed with great expectations as a promising tool to solve the enigma of IPA diagnosis. However, to date they have failed to live up to those expectations. Several PCRs have been developed according to primer selection (panfungal, genus specific, or species specific), and PCR formats (qualitative, quantitative, and real-time). DNA in respiratory samples of LTRs, has been studied by two standardized *Aspergillus* assays, Viracor (Viracor-IBT Laboratories) and MycAssay (Myconostica). One of the main caveats that this test faces is the lack of standardization in the procurement of samples. Its costs exceed those of GM or β-d-glucan, and it is not available in most centers. Furthermore, as with the aforementioned tests it is unable to differentiate colonization from invasive disease (Herrera and Husain, 2018) and unable to identify subspecies; requiring additional specific subspecies PCR and unable to provide antifungal susceptibilities. Despite these limitations and the lack of studies that have been performed in the LTR population, the few available studies have shown very good outcomes with sensitives and specificities of 100 and 88%, respectively, for diagnosis of IPA; and sensitivity and specificity for *A. fumigatus*-specific PCR of 85 and 96%, respectively (Luong et al., 2011; **Table 1**). Additional advantages include the ability to identify mutations associated with treatment resistance (Bernal-Martínez et al., 2017; Dannaoui et al., 2017) that are usually time consuming using conventional methods alone. Although AsperGenius (PathoNostics, Maastricht, Netherlands), has not been yet tested in LTRs for the identification of azole resistance in *A. fumigatus*, this could have a broad potential in this population, especially in colonized cystic fibrosis patients undergoing lung transplantation (Chong et al., 2016). Multiple PCR tests are being developed, but unfortunately none of these have been tested in LTRs (Salehi et al., 2016). All of these factors indicate that this test will be a very promising tool in the near future.

Table 1 summarizes the studies available in LTRs for the different serological and molecular markers available.

NEWER TESTS

Monoclonal Antibodies

Use of monoclonal antibodies has changed many fields in medicine over the past decades. Some centers have attempted to develop monoclonal antibodies against *Aspergillus* spp. for the diagnosis of IPA.

The first of these developed was the lateral flow device, an immunochromatographic assay that uses JF5, a monoclonal antibody that binds to an extracellular glycoprotein secreted during active growth of *Aspergillus* spp. This assay has been tested in several populations, including SOT (Willinger et al.,

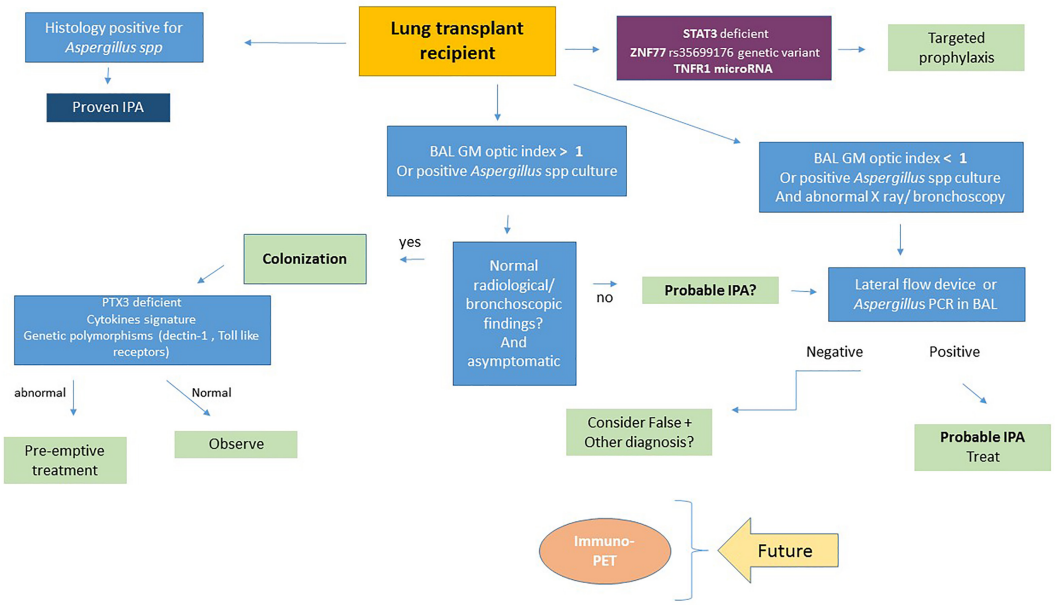

FIGURE 1 | Hypothetic algorithm of future diagnosis and management of invasive aspergillosis.

2014). It seems to display a better performance in BAL, where pooled sensitivity and specificity for the proven/probable IPA was assessed in a recent meta-analysis showing 86 and 93%, respectively (Pan et al., 2015). Its main strength is high specificity, and a new version of the assays shows improved specificity compared to the older assay according to a recent study (Hoenigl et al., 2018). However, the noted sensitivity of this assay is not as good, and is further decreased in patients receiving anti-mold prophylaxis (Castillo et al., 2017). Additionally, cross-reactivity has been reported with *Penicillium* spp. (Heldt and Hoenigl, 2017). So far the lateral flow device has only been studied in cohorts including a mix of LTR's, patients with hematological malignancies, or other solid organ transplants (Castillo et al., 2017). Thus, extrapolating the results for these mixed cohorts may not give an accurate indication of the sensitivity and specificity for LTR's alone.

Most recently, a group from Baltimore United States used an anti-*A. fumigatus* antibody (mAb476) that rapidly detects a fungal antigen in urine (Marr et al., 2018), avoiding the need for more invasive tests. The use of this antibody test in IPA had previously been proven possible only in mice and guinea pig models (Dufresne et al., 2012). In their human study, they found that the best performance was in patients who had hematological malignancies, whereas the sensitivity from the small subgroup with other underlying diseases was lower at 63.6%. Several false negative results were found in LTRs who had non-invasive forms of the disease. Excluding these patients gave a higher sensitivity of 88.5% for the test. Specificities where high overall, at around 92%. However, false positive results were also found in patients with Histoplasmosis. The advantages offered by this test are, simplicity and the possibility of earlier diagnosis. Nevertheless, further studies are needed in LTRs as with the scarce available data, it seems more advantageous in hematological malignancies.

Volatile Organic Compounds

A very original test has been able to identify a profile of volatile organic compounds exhaled in the breath of patients with pathogenic *Aspergillus*. These compounds are present during the growth phase of the fungus, in patients undergoing evaluation for IPA. Detection of α-trans-bergamotene, β-trans-bergamotene, a β-vatirenene-like sesquiterpene, or trans-geranylacetone identified IPA patients with 94% sensitivity and 93% specificity (Koo et al., 2014). The study was done in a heterogeneous cohort of immunocompromized patients. In LTRs volatile organic compounds have been studied for diagnosis of chronic lung allograft dysfunction (Kuppers et al., 2018), but to the date there are no studies assessing this test for IPA diagnosis.

PTX3 and Cytokines

Other tests that have been explored by researchers to refine the diagnosis of IPA include the detection of biomarkers, such as Pentraxin-3 (PTX3) and cytokines. These tests are used in conjunction with standardized serological or other fungal diagnostic tests. One of these tests is monitoring the levels of PTX3. PTX3 is a soluble pattern recognition receptor, expressed after induction of inflammatory cytokines in response to inflammatory stimuli from endothelial cells and mononuclear phagocytes. Animal models showed PTX3 enhanced survival rate and reduced the lung fungal burden of infected rats (Lo Giudice et al., 2010). In a 2014 study in the stem cell transplant population, genetic deficiency of PTX3 was associated with increased risk of IPA in hematopoietic stem cell transplantation (HSCT) (Cunha et al., 2014). While not specific for *Aspergillus*

spp., PTX3 has been used as diagnostic tool and for therapeutic monitoring in stem cell transplant studies (Biagi et al., 2008), where PTX3 levels were high in patients with IPA and decreased after treatment. However, only one group has explored this biomarker in the LTR population (Kabbani et al., 2017), where it was noted that PTX3 levels were significantly higher in BAL samples of LTRs with IPA. Patients with high levels of PTX3 in the BAL with positive GM or positive *Aspergillus* culture were 4.5 and 5.5 times more likely to have invasive pulmonary aspergillosis, respectively.

Identification of a specific signature of cytokines for IPA may be of tremendous help in the diagnosis of IPA. Cytokines have been studied more in depth in hematological malignancies; however, in these studies, a noted association has been made that higher levels of interleukin 10 (IL-10) were found in BAL of patients who also had IPA (Cunha et al., 2017) or worst outcomes in patients with persistently elevated IL-6 and IL-8 (Chai et al., 2010). To date, there haven't been any studies assessing the role of cytokines in IPA in LTRs exclusively. One study that included LTRs among other solid organ transplant (SOT) recipients and hematological malignancies concluded that IL-1β, IL-6, IL-8, IL-17A, IL-23, and tumor necrosis factor alpha (TNFα) levels in BAL were significantly increased among patients with IPA, with IL-8 being the best marker (Gonçalves et al., 2017).

MALDI-TOF

The incorporation of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to microbiology laboratories has been a revolution in the bacterial diagnosis of infections. Efforts are being made to apply this technique to other fields such as mycology. If implemented, this form of mass spectrometry will be a great advance in reducing the time of diagnosis. Yet a positive test will not be able to differentiate between colonization and disease in LTRs (Sanguinetti and Posteraro, 2014).

TAFC Triacetylfusarinine C

Triacetylfusarinine C (TAFC) is a siderophore secreted by *A. fumigatus* after conidiospore germination in media where iron is scarce. Its synthesis is required for fungal germination, and it is crucial for the virulence factor of *A. fumigatus* in a mice models of IPA (Schrettl et al., 2004). Studies performed in patients with systemic erythematous lupus have proved that TAFC can be measured in the serum of these patients (Carroll et al., 2016). In immunocompromized patients it has been tested in a small cohort of patients with hematological malignancies, where it was found to increase the sensitivity of BAL GM (Orasch et al., 2017). Limitations of this test include a lack of larger studies, and the fact that it is mainly produced by *A. fumigatus* and *A. nidulans* (Charlang et al., 1981), meaning that some other species of *Aspergillus* that are also known to be etiological for IPA are excluded. To date there have been no studies in LTRs.

Radiology

Radiology represents one of the pillars of IPA diagnosis. Most guidelines agree that while not specific to IPA alone, the typical radiological presentations in IPA include nodules, air crescent sign, cavity and halo sign. To complicate matters, however, the radiological presentation of IPA may be different in LTRs as compared to hematological malignancies or other SOT recipients. A 2014 study showed that bilateral bronchial wall thickening and centrilobular opacities with a tree-in-bud pattern were the most common radiological findings in a cohort of LTRs (Gazzoni et al., 2014). Another study that included LTRs among other SOT patients, found that halo sign was only observed in 8% of SOT recipients, whereas peri-bronchial consolidations were observed in 31% and ground-glass opacities in 38% (Park et al., 2010).

Positron emission tomography (PET) scans have also been studied for the diagnosis of IPA (Hot et al., 2011), but have a limited utility due to false positive results in patients with post-transplant lymphoproliferative disease (PTLD) or lung cancer (Baxter et al., 2011), and therefore must be used with caution. To avoid false positive results, immunological tests combined with diagnostic imaging such as PET scans or magnetic resonance imaging (MRI) are being developed. Using the monoclonal antibody JF5 combined with PET scan, researchers have found that the antibody binds to the antigenic determinant β1,5-galactofuranose present in a diagnostic antigen that is released by the pathogen during invasive growth in the lung (Davies et al., 2017). This strategy overcomes the problems of specificity faced by a computed tomography (CT) scans of the chest or MRI alone, or with the false positive results seen with the PET scan alone. However, the availability of this developing tool will be a limiting factor for its widespread use.

Proposed Algorithm

Figure 1 shows a proposed algorithm for the future management and diagnosis of IPA. This algorithm includes some of the newer and developing diagnostic tools. One of the emerging factors that will likely play a large role in the future, is the detection of biomarkers related to a patient's susceptibility to the disease, and the host's potential to control angioinvasion. This will also be a determining factor in changing the landscape of how we manage prophylaxis of these patients, as it can help tailor a more individualized approach to treatment.

CONCLUSION

What the Future Holds

Despite the advances in transplantation and treatment of IPA, the development of an accurate diagnostic or prognostic tool for IPA continues to be a struggle.

New areas of research include identification of genetic susceptibility markers and prognostic indicators of IPA. One of those markers is Signal transducer and activator of transcription 3 (STAT3), a member of the STAT protein family and an important transcription activator has been found to have an important role in invasive fungal infections (Puel et al., 2011) as it is associated to the T-helper cell 17 (Th17) pathway (Yang et al., 2007). Inhibition of STAT3 had been shown to impair hyphal killing (Taylor et al., 2016) in animal models. Autosomal-dominant *STAT3*-deficient hyper-IgE syndrome is associated to susceptibility of late-onset

mold infections, in particular *Aspergillus* (Vinh et al., 2010). STAT3 may hold the key to the timing when *Aspergillus* hyphae stops being a bystander, and starts to become an invasive disease.

Another set of biomarkers that can stratify the risk and prognosis of IPA is microRNA. MicroRNAs are non-coding RNAs capable of influencing gene expression through a variety of mechanisms (Lu and Rothenberg, 2013). Recent studies have examined MicroRNAs and identified those responsible for the regulation genes involved in the pulmonary immune responses following sub chronic inhalation exposure to *A. fumigatus* (Croston et al., 2016). Identification of microRNA's regulation of, and impact on target genes might be able to assist in the diagnosis and treatment of IPA. Recently, zinc finger protein-77 (ZNF77) has been identified as a key player in *Aspergillus* colonization, by causing a loss of integrity of the bronchial epithelium and an increase in the levels of extracellular matrix proteins (Gago et al., 2018). These changes promote *A. fumigatus* conidial adhesion, germination and growth. However, these data have so far only been examined in limited sample sizes and need to be scrutinized in a larger cohort.

Lastly, there is an omnipresent need to perform larger studies in the LTR population. As transplant numbers continue to rise, larger studies would allow an improved comprehension of many of the tests described above which so far, have only been studied in small cohorts.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and revision of this manuscript.

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REVIEW
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Diagnostic Approaches for Invasive Aspergillosis—Specific Considerations in the Pediatric Population

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Invasive aspergillosis (IA) is a major cause of morbidity and mortality in children with hematological malignancies and those undergoing hematopoietic stem cell transplantation. Similar to immunocompromised adults, clinical signs, and symptoms of IA are unspecific in the pediatric patient population. As early diagnosis and prompt treatment of IA is associated with better outcome, imaging and non-invasive antigen-based such as galactomannan or ß-D-glucan and molecular biomarkers in peripheral blood may facilitate institution and choice of antifungal compounds and guide duration of therapy. In patients in whom imaging studies suggest IA or another mold infection, invasive diagnostics such as bronchoalveolar lavage and/or bioptic procedures should be considered. Here we review the current data of diagnostic approaches for IA in the pediatric setting and highlight the major differences of performance and clinical utility of the tests between children and adults.

Keywords: ***Aspergillus*, child, cancer, diagnostics, galactomannan, ß-D-glucan, polymerase chain reaction, imaging**

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INTRODUCTION

Invasive fungal disease (IFD), in particular invasive aspergillosis (IA), is a major cause of morbidity and mortality in immunocompromised children and adults (Pagano et al., 2007; Sung et al., 2007; Upton et al., 2007). The definite diagnosis of IA ("proven aspergillosis") is extremely difficult and requires the visualization of the organism in tissue or its microbiological detection in an otherwise sterile compartment. In daily clinical practice, the diagnosis of IA is mostly made by appropriate imaging studies and diagnostic biomarkers in blood and other readily available specimens ("probable or possible aspergillosis"), and these methods are important guiding decisions about the institution and the choice of antifungal agents as well as regarding the duration of therapy. The rationale for this clinical practice is the fact that early institution of appropriate antifungal agents is the factor that is most strongly associated with favorable outcome (Caillot et al., 1997).

Different strategies have been developed in order to decrease the incidence of IA and to improve outcome. Many experts in the field administer prophylactic mold-active antifungals to patients at high-risk for IA. In the pediatric setting, this population includes patients with acute myeloid leukemia (AML), relapsed acute leukemia [both AML and acute lymphoblastic leukemia (ALL)]

as well as children undergoing allogeneic hematopoietic stem cell transplantation (HSCT), all of which have a risk >10% for IA (Sung et al., 2007; Hol et al., 2014; Fisher et al., 2017). The situation is more complex in children undergoing therapy for ALL, the largest patient population in pediatric hematology. Depending on the protocol used and some additional factors such as age, the presence of prolonged bone marrow failure, and concomitant use of corticosteroids, some of these patients are at high risk for IA, whereas others are at low risk; unfortunately, however, these subgroups are poorly defined to date (Groll et al., 2014). In contrast, children undergoing autologous HSCT and children diagnosed with non-Hodgkin and Hodgkin lymphoma as well as children with solid tumor have a low risk for IA, although IA is sometimes seen in these patients (Groll et al., 2014). Some institutions may not use antifungal prophylaxis but screen afebrile and asymptomatic neutropenic patients at high-risk for IA using non-culture based methods, and institute mold-active compounds only if the patient develops fever and screening tests turn positive ("pre-emptive therapy"). However, pediatric data of pre-emptive therapy are scarce, and therefore, in children, the approach to use empirical antifungal therapy is more common. With this concept, antifungals are started in all high-risk neutropenic patients who develop fever which does not respond to broad spectrum antibiotics.

The result of a diagnostic tool is not only important in the decision to start antifungal therapy, but may also help to modify and guide the use of antifungals. Although, as compared to studies with adult patients, considerably less data are available in the pediatric population, it has been recognized that the performance and the usefulness of diagnostic tools to detect IA may differ between children and adults. This has led to several pediatric specific guidelines for the diagnosis and treatment of IFD (Groll et al., 2014; Science et al., 2014; Lehrnbecher et al., 2017b), and at present, the European Organisation for Research and Treatment of Cancer (EORTC) and the Mycosis Study Group (/MSG) (EORTC/MSG) consensus definitions for the diagnosis of IFD undergo a second revision in which pediatric specific diagnostic considerations will be included. The present review will focus on the value of diagnostic tests to detect IA in the pediatric setting and highlight major differences between children and adults.

CLINICAL SIGNS AND SYMPTOMS OF INVASIVE ASPERGILLOSIS

Similar to adults, clinical signs, and symptoms of IA in children are often non-specific and cannot be readily distinguished from other infections caused by bacteria, viruses, and other fungi or from certain non-infectious complications caused by the underlying disease or sequelae of its treatment. Importantly, fever during neutropenia which is unresponsive to broad-spectrum antibiotics may be the only sign of IA. Other clinical symptoms depend of the site of infection: as IA most often involves the lung, patients may complain about pleural or back pain or dyspnea, whereas headache, altered mental state, seizure, or focal neurologic signs may indicate central nervous system (CNS) involvement (Hassler et al., 2015; Lehrnbecher et al., 2017a).

MICROSCOPY AND FUNGAL CULTURE

Overall, standard diagnostic techniques such as microscopy and culture to detect fungal organisms do not differ between children and adults. A microbiologist experienced in the diagnosis of fungal infections should microscopically examine all samples from invasive diagnostic procedures such as bronchoalveolar lavage (BAL) or biopsies (Ruhnke et al., 2012). Special "fungal stains" such as periodic acid—Schiff, Grocott's methenamine silver, or optical brighteners (e.g., calcofluor white) are recommended to visualize hyphae. Despite the fact that microscopy can already provide important information (e.g., presence of septa, hyphal diameter, ramification pattern), a reliable differentiation between *Aspergillus* spp. and other filamentous fungi is impossible; in individual situations, although not standardized, and validated, immunohistochemical examination may be helpful (Hayden et al., 2003).

Although *Aspergillus* species are rarely recovered from the blood, all clinical samples from children who are at high risk for invasive fungal infections have to be cultured for fungi (Kontoyiannis et al., 2000). Blood samples and cerebrospinal fluid (CSF) should be processed immediately, and issue specimens have to be kept moist and must not be placed in fixatives. Importantly, negative culture results do not reliably exclude IA. If *Aspergillus* can be cultured, the isolate should be identified at the species level and antifungal susceptibility testing should be performed as this might impact therapy (Ruhnke et al., 2012).

FUNGAL BIOMARKERS

Due to the difficulties in isolating and culturing *Aspergillus*, there has been much effort to develop and evaluate various non-culture based assays. These assays include the serological detection of fungal antigens to support the diagnosis of IA in immunocompromised patients.

GALACTOMANNAN

Galactomannan (GM) is a carbohydrate constituent of the cell-wall of *Aspergillus* spp. and is released by the fungus during cell growth. An FDA-approved enzyme immunoassay which employs the ß-1–5 galactofuranosyl specific EB-A2 rat monoclonal antibody has been evaluated in the clinical setting to detect the molecule (Platelia™ Aspergillus Enzyme Immunoassay, Bio-Rad). A number of studies evaluated the usefulness of GM as screening test in asymptomatic neutropenic adults (Maertens et al., 1999, 2001, 2004, 2005; Sulahian et al., 2001). Specificity and sensitivity of a positive test result were between 90 and 100% and 80 and 100%, respectively, and the negative predictive value (NPV) was greater than 90%. More importantly, positive test results were seen before patients developed clinical symptoms

of IA or before abnormalities could be detected in a chest CT scan (Hayden et al., 2003). The EORTC/MSG consensus group included positivity of GM assessed in serum, BAL fluid and CSF in their revised definitions of IFD (De Pauw et al., 2008). In serum, a cut-off of 0.5 is recommended by the manufacturer, whereas a cut-off of ≥1.0 might be more appropriate for BAL fluid (Maschmeyer et al., 2015). In the adult setting, a number of guidelines recommend the use of GM for the diagnosis and management of IA (Maertens et al., 2010; Ruhnke et al., 2012; Maschmeyer et al., 2015).

A recent systematic review has scrutinized the available data of serum GM in pediatric cancer patients and children undergoing HSCT (Lehrnbecher et al., 2016). Eligible for the analysis were studies which included only patients up to 25 years of age, included at least 50% of patients suffering from cancer or undergoing HSCT, used EORTC/MSG criteria to define IA, and included patients without IA as a control group. The authors analyzed the test characteristics of 10 studies in which GM was used for screening and of eight studies which evaluated GM as a diagnostic tool, e.g., in patients with prolonged febrile neutropenia or in patients with pulmonary signs and symptoms. In the screening setting, a total of pediatric 688 patients were included, with the number of patients ranging between 17 and 198 per study; and in the diagnostic setting, 733 pediatric patients were included with a range of 38–145 patients per study. The prevalence of proven and probable IA ranged from 0.5 to 30.4 in the screening setting and from 0.0 to 30.8 in the diagnostic setting, respectively. Specificities and sensitivities ranged from 50 to 100% and from 0 to 100% in the screening setting and from 35 to 100% and from 14% to 100% in the diagnostic setting, respectively. Pooled specificity and sensitivity in the screening setting were 91% [95% confidence interval (CI), 86–94%] and 68% (95% CI, 51–81%), and 85% (95% CI, 51–97%), and 89% (95% CI, 79–95%) in the diagnostic setting, respectively. In adults, a meta-analysis has reported comparable data with a specificity of 81% (95% CI 72–90%) and a sensitivity of 82% (95% CI 73–90%) (Maertens et al., 2005). Positive predictive values (PPVs) in children were rather low and ranged between 0 and 100% in each setting, while the negative predictive values (NPVs) were considerably higher and ranging from 85 to 100% and from 70 to 100% in the screening and diagnostic setting, respectively. It is well known that there are a number of reasons for false-positive results, such as the concomitant use of some batches of beta-lactam antibiotics or the cross-reactivity of the assay with other fungi (e.g., *Penicillium* spp., *Fusarium* spp., *Histoplasma capsulatum*, *Cryptococcus neoformans*) (Viscoli et al., 2004). On the other hand, the use of mold-active prophylaxis may result in a high rate of false-negative results (Lass-Florl, 2017), although this could not be proven in the meta-analysis of pediatric studies as the antifungal prophylaxis was not standardized in most of the included studies. It is important to note that predictive values were similar when pediatric patients with possible IFD were classified as not having IFD or when patients with possible IFD were excluded from analysis. Similarly, predictive values were comparable in both screening and diagnostic testing setting, and test characteristics did not systematically improve as disease prevalence increased. Based on the data of this meta-analysis, recent recommendations of an international expert panel recommended to consider not using GM testing in children with prolonged febrile neutropenia (weak recommendation, moderate quality of evidence), because (1) due to the poor PPV, the clinical consequence based on test results is often incorrect (e.g., to institute mold-active antifungals) and (2) despite the high NPV, a negative GM test does not rule out non-*Aspergillus* molds, which clearly limits the value of the assay. However, as the diagnosis of IA is extremely difficult and does not depend on a single parameter alone, and as the local epidemiology plays an important role, GM testing may have an impact in daily clinical decision making with consideration of its limitations.

It is important to mention that the design of the available studies is in part highly problematic, as many used GM as the reference parameter to define IA. Similarly, in studies which required two positive samples to define a positive test result of GM, it often remains unclear during which time period the two samples had to be drawn. Another problem causing heterogeneity of the results of different studies is the variable aggressiveness to use CT scans or the invasive diagnostic procedures to prove or to exclude IA, all of which may bias the results.

Limited pediatric data are available on the assessment of GM in BAL. One study retrospectively analyzed the GM results in BAL fluid, which was obtained in 85 children (59 among them were immunocompromised) (Desai et al., 2009). Proven IA was diagnosed in three patients, probable IA in 6, whereas 39 children had possible IA and 39 no evidence of fungal disease. Receiver-operating characteristics demonstrated that in immunocompromised children, a BAL GM cut-off value of 0.87 resulted in a sensitivity, specificity, PPV and NPV for probable/proven IA of 78, 100, 58, and 96%, respectively. Although similar results were reported in another retrospective analysis of 72 bronchoscopies performed in immunocompromised children, the study demonstrated that the result of the GM assay in BAL had only little impact on the clinical decision, as in a considerable number of patients antifungal agents were continued or even started despite a negative GM result (de Mol et al., 2013); however, the duration of antifungal therapy was not considered in this analysis. Although colonization might be a bias for positive results of GM in BAL fluid, the promising results in children are supported by adult data on the use of GM testing in BAL fluid in patients in whom pulmonary aspergillosis was suspected (Maertens et al., 2009).

To date, relatively little data are published on the utility of GM testing in the CSF in order to diagnose CNS aspergillosis. A 18-months-old boy was reported with pulmonary and cerebral aspergillosis, in whom GM in the CNS was elevated (Roilides et al., 2003). In addition, one small case series reported on five patients with probable CNS aspergillosis (Viscoli et al., 2002). In these patients, GM levels in the CNS were significantly higher than GM levels of 16 control patients, suggesting the potential value of GM in CSF. Similarly, the testing of GM in urine specimens allows an easy and non-invasive sample collection, which would be interesting in particular in neonates and young children. In this respect, a recent study reported on promising

results of measuring the urine GM/creatinine ratio in 71 adult patients with underlying hematological malignancies, but these results have to be validated in a pediatric patient population (Reischies et al., 2016).

Finally, there is growing interest in using serum GM as a surrogate marker for the effectiveness of antifungal therapy (Nouér et al., 2011; Chai et al., 2012; Kovanda et al., 2017). For example, one clinical trial in 158 adult patients demonstrated that an increase of the GM index of more than 0.25 from baseline by day 7 was associated with a significant increase of death compared to patients in whom the GM index increased less than 0.25 (Kovanda et al., 2017). Although not shown in the pediatric population, GM could individualize antifungal therapy as increases by day 7 could trigger treatment changes.

1-3-β-D-GLUCAN

As compared to GM, the use of 1-3-ß-D-glucan (BG) as a biomarker allows the detection of a wider array of pathogenic fungi, including *Aspergillus* spp, *Candida* spp, *Fusarium* spp, *Trichosporon,* or *Pneumocystis jirovecii* since BG is present in the cell wall of many fungi. However, BG remains low or even negative in patients with cryptococcosis or mucormycosis. Positive values of BG can also be assessed during various bacterial infections, for example in infections due to *Streptococcus pneumoniae* or *Pseudomonas aeruginosa*, and additionally, BG may be detected in healthy individuals (Oz and Kiraz, 2011). Similar to GM, the EORTC/MSG consensus group included BG as mycological criterion in the revised definitions of IFD (De Pauw et al., 2008). A meta-analysis in adult patients demonstrated that different BG test assays have a similar performance [e.g., Fungitell™ (Associates of Cap Cod, Inc. Falmouth, MA), Fungitec-G (Seikagaku)], and that BG might be a useful tool to exclude IFD in the clinical setting, as for two consecutive tests, sensitivity and specificity were 50 and 99%, respectively, and estimated PPV and NPV for an IFD prevalence of 10% were 84% and 95%, respectively (Lamoth et al., 2012). However, it has to be noted that the assay has major limitations due to multiple factors which may cause false-positivity. For example, in addition to contamination, bacteremia, severe mucositis, the infusion of platelets with leukocyte-removing filters, the administration of albumin and immunoglobulins, or the administration of antibiotics such as amoxicillin-clavulanate or piperacillin-tazobactam have all been described as potential cause for false-positive results (Koltze et al., 2015). In addition, positive BG levels have to be interpreted with caution in patients who have had prior transient candidemia or have mucosal colonization with *Candida* species (Naselli et al., 2015).

There are limited data on the use of BG in children. It has been shown that mean values of BG are higher in normal healthy children than in healthy adults (Smith et al., 2007). It was suggested that the optimal cut-off level to identify neonates with invasive candidiasis was 125 pg/ml as compared to the 80 pg/ml recommended for adults, which resulted in a sensitivity and specificity of 84 and 75%, respectively (Goudjil et al., 2013). A total of three studies evaluated BG in children receiving therapy for a malignancy or undergoing HSCT, among them 38 patients with proven/probable IFD (Zhao et al., 2009; Badiee et al., 2012; Koltze et al., 2015). Overall, wide ranges were found for specificity (29–82%), sensitivity (50–83%), PPV (17–49%), and NPV (84–96%), respectively. Due to the limited pediatric data and the poor performance of the test in this patient population, BG testing is currently not recommended in the pediatric population as a routine diagnostic test, although it may be helpful under certain circumstances under awareness of its limitations (Groll et al., 2014; Lehrnbecher et al., 2017b).

MOLECULAR DIAGNOSTIC TOOLS

Polymerase chain reaction (PCR) has become a promising diagnostic tool for the early detection of IA. However, the method is not included to date as mycological criterion in the current EORTC/MSG definitions for IFD. This is due to a number of methodological issues, such as the optimal clinical sampling (e.g., whole blood, serum, plasma), the best DNA extraction method, and the optimal primer design (De Pauw et al., 2008; White et al., 2010, 2015b). In addition, multiple PCR approaches have been described, such as the use of nested PCR which detects genus-specific genomic sequences or single-copy genes, the use of "panfungal" PCR which detects multiple-copy genes identifiable in many fungal species and is followed by hybridization with species-specific probes, and real-time PCR technologies such as LightCycler™ or TaqMan™ which even may allow the quantification of fungal burden.

A total of 11 studies of PCR using blood samples of pediatric cancer patients were analyzed in a recent systematic review (Lehrnbecher et al., 2016). Three of the studies enrolling a total of pediatric 147 patients were performed in a screening setting, whereas 8 studies enrolling a total of pediatric 539 patients evaluated PCR samples in children with high suspicion of IA, such as children with prolonged febrile neutropenia or children with lung infiltrates. In the screening setting, specificity, sensitivity, PPV, and NPV ranged from 43 to 85%, 11 to 80%, 22 to 50% and 60 to 96%, respectively. In the diagnostic setting, specificity, sensitivity, PPV and NPV ranged from 36 to 83%, 0 to 100%, 0 to 71% and 88 to 100%, respectively. Due to these results, a recent international expert group gave a strong recommendation with a moderate quality of evidence for not using fungal PCR testing in blood (Lehrnbecher et al., 2017b). Whether the combined use of GM and PCR improves the test results as it was shown in a study in adult high-risk hematological patients needs to be evaluated in the pediatric population (Morrissey et al., 2013).

As compared to blood, the performance of PCR in BAL samples seems to be superior. For example, it has been demonstrated in 226 adult patients that BAL PCR testing had a sensitivity of 69% and a specificity of 87% in patients who did not receive antifungals prior to BAL (Reinwald et al., 2012). Unfortunately, the performance of the test significantly decreased

in patients who had received at least two antifungals, which may be the situation in a considerable proportion of pediatric patients prior to receiving a BAL.

It is important to mention that PCR evaluation of biopsy samples may have an important impact in the correct diagnosis and in the treatment of pulmonary infections with a fungal pathogen, as it was demonstrated in adult patients (Lass-Flörl et al., 2007). In addition, PCR based methods have also been investigated and are of major usefulness for the identification of mutations conferring resistance to specific antifungals (e.g., azole resistance in *A. fumigatus*) (White et al., 2015a).

IMAGING STUDIES

As autopsy data demonstrate that the lung is affected in almost 90% of the patients with IA (Groll et al., 1996; Lehrnbecher et al., 2010), imaging of the lung is an important tool in the early diagnosis of the infection. In adults, chest computerized tomography (CT) scans reveal pneumonia due to a fungal pathogen earlier as conventional chest radiographs (Heussel et al., 1999), which is important in order to institute early antifungal therapy which impacts upon outcome. In adults, CT findings such as the "halo sign," the "air-crescent sign", or "cavitation" have been included in the current EORTC/MSG definitions of IFD (Caillot et al., 1997; De Pauw et al., 2008). Nevertheless, these findings are not specific for pulmonary aspergillosis and may also be seen in other pulmonary infections or due to the progression and relapse of the underlying malignancy. In addition, the appearance of these signs depends on the time of imaging (Caillot et al., 1997).

Data on CT findings in children with IA are conflicting. One retrospective study in 139 children with IA demonstrated that the halo sign was seen in only 6.4% of the patients, the air-crescent sign in 1.6% of children and a cavitation in 14.4% of patients, respectively (Burgos et al., 2008). In contrast, nodules were found in 21% of the pediatric patients, corroborating the results of another report (Taccone et al., 1993). In contrast, a more recent pediatric study found that the halo sign in the chest CT was significantly associated with pulmonary IA (78.4 vs. 40.7%, $P < 0.001$) (Han et al., 2015). It is unclear whether the different results among the studies simply reflect different time points of CT imaging or are due to other, yet unknown reasons. Nevertheless, the appearance of any new infiltrate in the chest CT in children with prolonged febrile neutropenia not responding to broad-spectrum antibiotics should be considered as possible pulmonary fungal disease and trigger diagnostic procedures. Notably, in adults, high resolution CT angiography may improve radiological diagnosis by detecting vessel occlusions caused by invasive aspergillosis, but to date, no data are available in the pediatric population (Stanzani et al., 2012, 2015).

Regarding the radiologic evaluation of other sites in children with prolonged febrile neutropenia and a high suspicion of IFD, a recent pediatric guideline included a weak recommendation for routine abdominal imaging, as in four studies, imaging findings in a significant proportion of patients without localizing signs or symptoms were consistent with IFD (Bartley et al., 1982; Archibald et al., 2001; Ahmad Sarji et al., 2006; Cohn et al., 2016; Lehrnbecher et al., 2017b). In contrast, the guideline panel gave a weak recommendation against routine sinus imaging in persistently febrile neutropenic patients in the absence of localizing signs or symptoms, which is based on the observation that sinus imaging is frequently abnormal in these patients and that the findings do not distinguish between children with and without invasive fungal infection of the sinus (Kavanagh et al., 1991; Archibald et al., 2001; Park et al., 2005; Ahmad Sarji et al., 2006; Cohn et al., 2016; Lehrnbecher et al., 2017b).

Whereas the lung is the primary portal of entry of *Aspergillus*, an autopsy study demonstrated that the infection is confined to the lung in only 41%, but disseminated in 53% of the patients, and that the CNS is one of the most common sites of disseminated involvement (Groll et al., 1996). The clinical presentation of cerebral aspergillosis, which is associated with an extremely high mortality, depends on the site and extent of involvement and can be classified in a number of defined syndromes, such as fungal abscesses, granulomas, or cavitated lesions (Armenian et al., 2009; Castagnola et al., 2010). For these patients, magnetic resonance imaging (MRI) of the CNS is the most important diagnostic tool in order to identify and monitor cerebral fungal infections. There is an ongoing debate whether MRI of the CNS should routinely be performed in asymptomatic patients with pulmonary aspergillosis.

INVASIVE DIAGNOSTIC PROCEDURES

Invasive diagnostic procedures such as bronchoscopy combined with BAL or lung biopsy should be considered in the work-up of children with pulmonary infiltrates. Whereas, the isolation of *Aspergillus* spp. from the sputum of neutropenic patients with clinical symptoms consistent with IFD is only an indicator of fungal pneumonia, the histopathological identification and/or the culture of *Aspergillus* spp. from lung tissue remains the diagnostic "gold standard" for proof of pulmonary aspergillosis (Ruhnke et al., 2012).

A recent meta-analysis on the diagnostic yield and the complication rate of BAL and lung biopsies performed in both pediatric and adult patients with cancer or HSCT recipients included 72 studies on BAL and 31 studies on lung biopsies (Chellapandian et al., 2015). When comparing the two diagnostic tools, the proportion of procedures leading to any diagnosis including non-infectious diagnoses was similar. However, BAL led significantly more often to an infectious diagnosis than did lung biopsy (49 vs. 34%; $P < 0.001$). A significant difference was seen for the identification of a bacterial infection, but not for the detection of viral and fungal infection, respectively. In contrast, lung biopsies resulted significantly more often in a non-infectious diagnosis than BAL (43 vs. 7%; $P < 0.001$), but, at the same time, was associated with a significantly higher complication rate (15 vs. 8%; $P = 0.006$). Whereas the diagnostic yield of BAL and lung biopsy seems to be similar between the pediatric and adult population, children had a higher complication rate during biopsy procedures than adults ($P = 0.003$). Studies

which included the assessment of GM in BAL reported on a significantly higher detection rate of proven/probable IA than studies in which GM was not used (31%; 95% CI, 23–43% vs. 18%; 95% CI, 15–22%; $P = 0.005$), whereas the impact of PCR as a diagnostic tool in BAL samples is less clear. Notably, as mentioned above, the diagnostic performance of the PCR in BAL samples decreased in adult patients who had received at least two antifungal agents (Reinwald et al., 2012). In contrast, the diagnostic yield of invasive diagnostic procedures increased when different diagnostic tools (e.g., histopathology, antigen- and molecular testing) were combined (Lass-Flörl et al., 2007). On the other hand, according to a prospective study in 55 adult patients, a positive mycologic result in respiratory samples depended on the underlying malignancy and the leukocyte count, with significantly higher yields in non-acute leukemia and in patients with leukocyte counts of more than 100 per μl (Bergeron et al., 2012). A meta-analysis showed that results of lung biopsies led significantly more often to a change in the clinical management (48% vs. 31%; $P = 0.002$), but it remains unclear whether this was associated with an improved overall outcome in this highly selected patient population.

SUMMARY AND FUTURE PERSPECTIVES

The early and reliable diagnosis of IA is difficult in immunocompromised patients, in particular in the pediatric population. In high risk patients with prolonged febrile neutropenia or signs and symptoms for IA, available diagnostic tools include imaging procedures and non-culture based techniques such as antigen-based and molecular biomarkers. The performance and clinical utility of these tests may be different between children and adults, and they may also be different for invasive diagnostic procedures such as BAL or biopsy. However, it is important to note that considerably less data are available for children as compared to adults. Although the combination of diagnostic methods seems to improve sensitivity and specificity, new diagnostic tools are needed that will improve the early and reliable diagnosis of IA. In this respect, the lateral-flow device assay which is based on the JF5 antibody and detects an extracellular glycoprotein antigen secreted during active growth of *Aspergillus* spp, is a promising platform that is to be tested in the pediatric population, as it may allow for a rapid and simple bed-side testing of IA (Heldt and Hoenigl, 2017; Hoenigl et al., 2018). In addition, specific host response biomarkers may be an interesting approach to diagnose IA, as it has been demonstrated for the monitoring of *Aspergillus*-specific $CD4^+$ T-cells (Bacher et al., 2015). Another interesting approach is the use of *A. fumigatus*-specific antibodies labeled with a radionuclide, which can be visualized by Positron Emission Tomography (PET)/MRI. Although not tested yet in humans, this approach could clearly distinguish invasive pulmonary aspergillosis from bacterial lung infection and other non-specific lung inflammation in the mouse model (Rolle et al., 2016). However, in order to perform meaningful studies to evaluate these diagnostic tools in the pediatric population, a comprehensive and necessarily international effort is necessary.

AUTHOR CONTRIBUTIONS

TL, AH, AG, and KB designed the manuscript, wrote parts of the manuscript, reviewed, and approved the last version of the manuscript.

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REVIEW
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Diagnostic Aspects of Veterinary and Human Aspergillosis

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The genus *Aspergillus* is composed of more than 300 species, a fraction of which are involved in animal or human infections mostly following environmental exposure. Various risk factors (i.e., immunosuppression, tuberculosis) have been recognized for human whereas for veterinary infections, unhygienic management, trauma, anatomical conformation of the skull, or suspected immunological deficiencies have been suggested. In animals, aspergillosis is mostly sporadic but in some circumstance such as infections on poultry farms may involve the whole flock. Since the high prevalence of immunosuppression in human patients has not been mirrored in veterinary medicine, and although to the best of our knowledge, no comprehensive data on the prevalence of aspergillosis in animals has been published, their epidemiology has not changed during the last decades. The impact of these infections may be economic or if they are incurable, sentimental. The objective of the first part is to describe the diagnosis of the main clinical entities caused by *Aspergillus* spp. in animals. It includes disseminated canine aspergillosis, canine and feline sino-nasal and sino-orbital aspergillosis, guttural pouch mycosis in horses, mycotic abortion in cattle, mycotic keratitis in horses, and avian aspergillosis. When pathogenesis and clinical aspects are relevant for diagnosis—they will be addressed as well. The second part deals with human aspergillosis, which is a multifaceted disease, manifested in a spectrum of clinical entities affecting one or more organs. Diagnosis is based on the clinical manifestation, supported and confirmed by laboratory means, involving the classical approach of demonstrating the etiological agent in the clinical specimens and in culture. Noncultural methods, such as antigen detection and/or molecular assays to detect fungal nucleic acids or protein profiles, are used as well. The isolation and identification of the fungus allows the determination of its susceptibility to antifungal drugs. Thus, antifungal susceptibility testing maybe considered as part of the diagnostic process, which is of relevance for management of the infection. In this review article, the part dealing with diagnostic aspects of aspergillosis in humans concentrates on susceptibility testing of *Aspergillus* spp. to antifungal drugs and drug combinations. The technologies and methods of susceptibility testing are described and evaluated.

Keywords: aspergillosis, animal, human, diagnosis, antimycotic drugs

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VETERINARY INFECTIONS CAUSED BY *ASPERGILLUS* SPP.

Introduction

Veterinary mycology differs from human mycology mainly in two important aspects. Since the 1980s the phenomenon of immunosuppression, pathological or iatrogenic, has affected an ever-increasing number of people. This has led to the emergence of fungal infections many of which were caused by opportunistic fungi, previously not or rarely involved in human mycoses (Enoch et al., 2017). A byproduct of this change in fungal infection epidemiology was the discovery of a large variety of antimycotic drugs, many of which are prohibitively expensive for veterinary use. This phenomenon has bypassed animals since the gamut of fungi involved in veterinary mycoses has remained largely unchanged, with differences stemming primarily from factors such as improved diagnostic methods and raised awareness (Elad, 2011). Susceptibility testing of animal *Aspergillus* isolates is exceptional due to their limited use and their low predictive value stemming from the animals' different anatomical and physiological characteristics.

The second aspect that differentiates veterinary from human mycology is the economic one. The price has a significantly higher impact on decisions to diagnose, identify the etiology, and treat disseminated fungal infections in animals, especially livestock. Thus, the economic or sentimental value of the animal/s dictates the actions (such as environmental decontamination, antimycotic therapy, or euthanasia) to be taken in case of animal aspergillosis.

An accurate diagnosis of animal fungal infections in general and aspergillosis in particular is of significant importance to determine the prognosis, remedial steps to be taken, and the choice of therapy, especially considering the differences in susceptibility of the various species of the fungus. Moreover, the importance of the etiology's identification stems from the fact that some *Aspergillus* infections cannot be differentiated clinically from infections caused by other pathogens that require a different therapeutic approach and/or pose a zoonotic threat (see mycotic abortion and brucellosis below). The primary identification of the fungus may be based on morphology (this may necessitate the use of specific media such as Czapek-Dox agar). Due to differences in the prognosis and susceptibility of morphologically undistinguishable fungi, the exact identification of the etiology is of importance. It may require the sequencing of the Internal Transcribed Spacer gene and often additional genes such as those of tubulin and/or calmodulin (Barrs et al., 2014). Serological methods were tested in animals in a limited number of cases and do not allow obtaining unequivocal conclusions as to the mycoses' nature (Schultz et al., 2008).

Most cases of animal aspergillosis are sporadic. Immunosuppression, either pathologic or iatrogenic, that has been the main predisposing factor to human mycoses in general and aspergillosis in particular, is much rarer in animals, and thus is not considered as a significant risk factor. Infection source may vary but is mostly environmental. Heavy fungal loads may be the source of massive poultry infections whereas trauma is usually assumed to cause ophthalmic infections. Disseminated or rhino-nasal/rhino-orbital aspergillosis seem to be associated with breed predisposition related to immune deficiencies the former and skull conformation the latter. The detailed basis of this predisposition is still to be elucidated.

Human activities that may promote aspergillosis include the use of contaminated intramammary tubes (Elad et al., 1995) or unhygienic management, especially of poultry farms, but in most cases such activities have no bearing on other infections.

Several well-defined clinical entities have been described and are reviewed.

Disseminated Canine Aspergillosis

Disseminated canine aspergillosis (DCA) affects primarily German Shepherd breed dogs, with females being over-represented (Taylor et al., 2015). It is caused most frequently by *Aspergillus terreus* or, more rarely by two other species belonging to the *Terrei* group, namely *A. carneus* and *A. alabamensis*. Other *Aspergillus* spp. that may cause disseminated canine mycoses include *A. deflectus*, *A. fumigatus*, *A. niger*, *A. flavus*, *A. flavipes*, *A. versicolor*, or unspecified *Aspergillus* spp. Clinical signs are non-specific and include lethargy, weight loss, central nervous system signs, and ataxia due to musculoskeletal lesions (Schultz et al., 2008). Abnormal clinical pathological test results may vary and result from the inflammatory process and/or dysfunctions of the affected organs. Since these signs are non-specific, diagnosis of DCA is often made after prolonged periods of therapeutic attempts aimed at other pathogens, with advanced dissemination by the time the correct etiology is identified (Zhang et al., 2012).

In vivo diagnosis may be based on the isolation of the fungus from biopsies and/or urine (Bruchim et al., 2006) reflecting the dissemination of the fungus to the kidneys. In urine sampled by cystocentesis to avoid contamination, the clusters of hyphae seen microscopically are a clear indication of a disseminated fungal infection, albeit not necessarily caused by *Aspergillus* spp. Thus if positive, this test that can easily perform animal-side provides a quick diagnosis of the infection's nature. For some *Aspergillus* spp., especially *A. terreus*, the presence of accessory conidia (aleurioconidia) in tissue and/or culture is an important indication as to the identity of the infection's etiology (Elad et al., 2008). In addition, a commercial kit (Platelia Aspergillus, BioRad, United States), aimed at the detection of *Aspergillus* galactomannan in serum or bronchoalveolar lavage, by an immunoenzymatic sandwich microplate assay has been assessed by Garcia et al. (2012) in dogs. They found that dogs with DCA had significantly higher values than suspected or control animals. Sensitivity was 92 and 88% for serum and urine samples, respectively, whereas specificity for the same samples was 86 and 92%. Increasing the cutoff value for human samples from 0.5 to 1.5 raised the specificity of the serum and urine samples to 93% without impacting the sensitivity. Corrigan et al. (2016) reported that when serial samples from two dogs under antimycotic therapy were examined with the kit, the values decrease for one and remained high for the other. The latter showed some clinical improvement but relapsed whereas the former survived. Conclusive diagnosis of CDA should, however, not be based exclusively on serodiagnosis (Bentley et al., 2018).

Taylor et al. (2015) examined seven dogs with CNS aspergillosis by magnetic resonance imaging (MRI). No aberrant findings were present in the MRI examination of the central nervous system of three dogs and those found in three others were variable (the 7th dog had discospondylitis). Moreover, they note that, unlike human CNS aspergillosis, in which typical changes can be observed by MRI, similar infections in dogs cannot be diagnosed solely by MRI and the latter should be complemented by other test in cases of suspected aspergillosis.

Post mortem diagnosis is based on the presence of hyphae in the organ lesions, their isolation, and identification. It should be stressed that other molds cannot be differentiated from *Aspergillus* spp. at the histopathological examination unless immunohistochemistry is applied (Pérez et al., 1996). Moreover, to accurately identify the mold species involved, such as those belonging to the section *Terrei*, sequencing the ITS gene alone may be insufficient and additional genes such as that encoding for tubulin should be sequenced (Burrough et al., 2012).

Due to the normally late presentation of the dogs and the poor record of antimycotic therapy success, animals are only rarely treated (Zhang et al., 2012). Recently, treatments with novel drugs, including voriconazole, posaconazole, and echinocandin have been attempted (Schultz et al., 2008). These drugs are currently too expensive for veterinary use, especially considering the fact that treatment has to be prolonged, possibly for the animal's whole life. Even if seemingly successful, animals have relapsed after the treatment's cessation, sometimes after years. Only three dogs have been reported to survive three or more years after therapy cessation (Kelly et al., 1995; Watt et al., 1995; Corrigan et al., 2016). Nevertheless, further therapeutic protocols including antimycotic drug combinations, dosages, and treatment periods should be tested to find a suitable treatment for DCA. Currently this is possible, due to economic considerations, only in an experimental setup and thus in a limited number of cases.

Sino-Nasal and Sino-Orbital Aspergillosis

Sino-nasal aspergillosis (SNA) afflicts more frequently dolichocephalic and mesocephalic dogs (Peeters and Clercx, 2007). They are caused primarily by *A. fumigatus*, although other molds are occasionally isolated (Talbot et al., 2014). Animals are regularly exposed to the fungal spores that are common in the environment. The mucociliary apparatus and the innate immune reaction of the lungs are usually sufficient to prevent the development of the infection and if they fail, SNA may develop (Sharman and Mansfield, 2012). In addition to host risk factors, fungal virulence factors such as the small size of conidia allowing them easy propagations in the host and metabolites such *A. fumigatus* gliotoxin that inhibits the mucociliary clearing capabilities may play a significant role in allowing the fungus to colonize the mucosae and lead to the establishment of a fungal mat (Peeters and Clercx, 2007). Deeper tissues are usually not invaded but occasionally the cribriform plate may be affected, most probably by fungal metabolites and the inflammatory reaction, resulting in the opening of a passageway to the central nervous system (Sharman and Mansfield, 2012). This process may have therapeutic implications (see below).

Initial clinical signs, sometimes present for prolonged periods (months, years) are non-specific and include those characterizing upper respiratory tract infections (URTIs): mucopurulent nasal discharges that may become hemorrhagic with eventual depigmentation of the nasal plane (Sharp et al., 1991). A differential diagnosis has to be made with other clinical entities with similar symptoms such as malignancies or foreign bodies (Cohn, 2014).

For the diagnosis of SNA various techniques may be employed, usually requiring the combination of more than one method to be precise.

Diagnostic methods involving imaging procedures include radioscopy, computerized tomography (CT), and MRI. The latter two, although more expensive and less available, are superior to the former, each having advantages and disadvantages in revealing the variety of possible lesions associated with SNA (Cohn, 2014). Karnik et al. (2009), however, report that CT was not always able to discriminate between SNA and neoplasia. These techniques do not discriminate between cases of SNA caused by *Aspergillus* spp. and other fungi. *Aspergillus* spp., however, cause most of these infections (Talbot et al., 2014) and thus may be empirically considered as the etiology until proven otherwise.

Among non-cultural methods, cytology of nasal discharge or blind swabs has low sensitivity and specificity as finding fungal elements may not be linked unequivocally to infection since their presence may be the result of other factors such as environmental contamination. Examining brush smears or squash biopsies significantly increased the sensitivity of cytology (De Lorenzi et al., 2006) especially if incubated at 37°C (Sharman and Mansfield, 2012). Comparably to the imaging techniques mentioned above, fungal hyphae found in cytological samples cannot unequivocally be identified as *Aspergillus* spp. since they are indiscernible from several other mold species (albeit with much lower prevalence), possibly having different prognostic and therapeutic significance. The sensitivity and specificity of serological tests are not uniform and quantitative DNA assays do not discriminate between infected and uninfected dogs as well as dogs suffering from some other nasal afflictions (Sharman and Mansfield, 2012).

Various approaches for SNA therapy, local and systemic, mostly based on azoles, have been reported (Peeters and Clercx, 2007). When administered alone, the therapeutic value of systemic drugs is limited, possibly due to the non-invasive nature of the mycosis. Topical treatment may be administered through catheters or by trephination of the frontal sinuses. They allow direct contact between the drugs and the fungal mat and thus are considered to be more efficacious. Additional treatment techniques have been described. Topical therapy is contraindicated in cases of cribriform plate involvement since the drug may leak into the cranium (Peeters and Clercx, 2007). One feline case, caused by *A. fumigatus*, was treated successfully, after debridement, with posaconazole, following a relapse after itraconazole therapy (Tamborini et al., 2016).

Sino-orbital aspergillosis (SOA) afflicts mostly cats and may be a complication of SNA. Brachycephalic skull conformation may increase the risk (Barrs et al., 2012). While SNA is mostly caused by *A. fumigatus*, the development of SOA is associated with a recently described species, *Aspergillus felis* (*Neosartorya*-morph). *A. felis* is a heterothallic mold, able to grow at 45°C but not 50°C, differentiating it from the morphologically similar *A. udagawae* and *A. fumigatus*, respectively (Barrs et al., 2013). *A. felis* is more invasive than *A. fumigatus* and it may cause lysis of the orbital lamina and pass into the orbital cavity, resulting in the development of a granuloma and exophthalmos (Hamilton et al., 2000). Subsequently other adjacent organs may be invaded (Barrs et al., 2013). Moreover, *A. felis* is more resistant to azoles than *A. fumigatus*, and thus the prognosis is poorer (Barrs et al., 2007). This emphasizes the clinical importance of the exact taxonomic identification of the infection's etiology, preferably by molecular methods. In dogs, although the lysis of the orbital lamina may be present in SNA caused by *A. fumigatus*, the development of SOA aspergillosis is rare (Barrs et al., 2014).

Whitney et al. (2013) assessed the possibility of sero-diagnosis of SOA by a commercial ELISA kit (Platelia Aspergillus, BioRad, United States) that detects galactomannan. They examined cats with proven URTIs caused by (URTIs) aspergillosis (confirmed by cultural and molecular methods), cats with URTIs not caused by *Aspergillus* spp., cats without URTIs but treated with β-lactam antibiotics, and healthy cats. Only 3 cats out of 13 of the *Aspergillus* spp. URTI had positive results as did almost one-third of the non-*Aspergillus* spp. infected groups. Consequently, this method was found by the authors as having low sensitivity and limited specificity.

Barrs et al. (2015) evaluated the sensitivity, specificity and positive and negative predictive value of indirect ELISA and agar gel immunodiffusion (AGID) to diagnose SNA or SOA. They compare sera of cats affected by SNA or SOA caused by *A. fumigatus* or several cryptic species including *A. felis*, to those of cats with non-fungal upper respiratory infections and healthy cats. The sensitivities were 43 and 95.2% and the specificities were 100% and 92–92.9% for the AGID and ELISA test, respectively. No differences between the tested species were found. Assessing IgA titers did not improve the results obtained by IgG alone (Taylor et al., 2016).

The difficulty and complexity of SOA treatment are epitomized in a series of SNA or SOA cases in cats (Barrs et al., 2012). Various treatment protocols including amphotericin B, itraconazole, posaconazole, and terbinafine alone or in combination have been reported. Other methods include out of seven SOA cases, one cat responded to amphotericin B and itraconazole, relapsed after 8 months, then was treated apparently successfully with posaconazole and terbinafine, relapsed after 19 months and did not improve with liposomal amphotericin B and posaconazole. Finally, treatment with caspofungin followed by posaconazole resulted in remission for the 12 months, when the article was written. It has to be noted, however, that some of these drugs such as liposomal amphotericin B and caspofungin are currently too expensive to be used in veterinary medicine except under experimental conditions.

Guttural Pouch Mycosis

The guttural pouch (GP) is a diverticulum of the Eustachian tubes in one toed ungulates (*Peryssodactila*), hyraxes, some bats, and the American forest mouse. Among these, horses are the only domestic animals that have this organ (Nation, 1978). The GPs are in contact with some major arteries and nerves which may be affected when the walls of the GP are eroded by the fungi (Davis and Legendre, 1994). This may result in potentially fatal epistaxis (while the animal is at rest) and/or paralysis of various cranial nerves leading, among others, to dysphagia (Freeman, 2015). Microorganisms, including fungi, have been found in healthy GPs (Manglai et al., 1999) and thus predisposing factors are necessary to allow the evolution of an infection. Although several hypotheses were suggested as to the nature of such factors, they thus far have remained unidentified (McLaughlin and O'brien, 1986). Fungi involved are primarily *A. fumigatus* and *A.* (*Emericella*) *nidulans* but other *Aspergillus* spp. have been reported (Freeman, 2015).

Presentation and initial diagnosis are based on the clinical signs (epistaxis, paralysis). Thus, in most cases the infection is already in an advanced stage (Lepage et al., 2004). In the subsequent endoscopy areas of necrotic tissue with fungal mats may be seen (Dobesova et al., 2012). The definitive diagnosis is based on the histopathological demonstration of hyphal invasion of tissue and the fungus' isolation. It is noteworthy that, similar to what has been described in canine SNA, some samples do not yield fungal growth in culture (Seyedmousavi et al., 2015).

Guillot et al. (1997) compared three serological tests to diagnose equine aspergillosis. They examined 12 horses with endoscopically verified fungal plaques in the GP and 12 healthy controls by counter immunoblot and ELISA. *Aspergillus* spp. was isolated from only two horses (the remaining were either culture negative or not cultured). Antibodies were found by the ELISA test in both infected and healthy horse, possibly indicating previous exposure to the fungus. In the immunoblot tests, two antigens (22 and 26 kd) were detected in all the horse with GP mycosis. While the test for the 22 kd antigen resulted positive in two control samples, the one for 26 kd was negative in all the samples of this group.

Therapy is mostly surgical and aimed at preventing or treating epistaxis (Freeman, 2015). This may be accompanied by antimycotic treatments by various drugs (Greet, 1987). Surgical removal of fungal plaques followed by antimycotic treatment prevented their reformation (Church et al., 1986). In cases of dysphagia, antmycotic therapy is the only alternative but the chances of recovery are poor (Freeman, 2015) since this approach can solve the fungal infection but it has no impact on the paralysis poor (Church et al., 1986). One case was, however, reported in which the fungal infection was successfully treated with a combination of itraconazole and topical enilconazole (Davis and Legendre, 1994) and subsequently the dysphagia regressed within a few weeks.

Mycotic Abortion

Mycotic abortion with *Aspergillus* spp. as etiological agent was reported in various animals such as horses (Monga and

Mohapatra, 1980; Hong et al., 1993) and pigs (Todd et al., 1985). Mycotic abortions in cattle, however, have been the focus of most publications. *A. fumigatus* is the most prevalent etiology, although other species such as *A. terreus* have been reported as well (Elad and Bernstein, 1987).

The pathogenesis of mycotic abortions in ruminants has significant implication for the interpretation of the diagnostic results. The contact between the fetal and the maternal part of the ruminant placenta is special mainly in two characteristics: (a) it is not contiguous but occurs in contact organs called placentomes, composed of the fetal part—the cotyledon and the maternal part—the caruncle and (b) there is no direct contact between the fetal and the maternal blood vessels. Thus, infectious agents that spread hematogenously do not pass from the maternal to the fetal blood but infect the placentome and, if the pathological process is acute enough to lead to an early abortion, the agent will not be found in the fetal organs. This happens in about 70% of cases. If, however, the abortion is delayed, the microorganism will spread through the amniotic fluid first to the fetus' stomach (abomasum), then to the lungs, and finally to the other organs. Since the placenta is often not found or is in a state that does not permit its microbiological examination, examining the fetus alone does not reveal the abortive agent (Sheridan et al., 1985).

Bovine mycotic abortions (BMAs) occur mostly in the third trimester of pregnancy and are mostly sporadic although they are an important cause of mycotic abortions in some countries (Hugh-Jones and Austwick, 1967). Symptoms of BMA include modifications to the placenta that becomes thick and "leathery." Cotyledons are thickened and may have necrotic centers. This may also occur in abortions caused by *Brucella* spp. and, considering the significant zoonotic potential of this microorganism, appropriate precautions should be taken until the abortion's etiology is ascertained. In addition, raised, hyperkeratotic plaques may be present (Glover et al., 2011) on the fetus' skin although the frequency of this symptom is uncertain.

Laboratory diagnosis is based on culture of the fungus from the placenta and/or the fetus and its identification. Since the culture of *Aspergillus* spp. may be the result of contamination, it is imperative to demonstrate tissue invasion by histopathology (Jensen et al., 1991). The mycological identification of the fungus causing the abortion may sometimes not be possible due to contaminant overgrowth. In a comparison between three diagnostic methods, Jensen et al. (1991) found a relatively low (kappa = 0.28) relationship between the attempted histopathological and mycological identification of the fungi. A better agreement was found between immunofluorescence and histopathology or mycology (kappa = 0.4 and 0.48, respectively). Jensen et al. (1993) assessed by inhibition ELISA the presence of galactomannan in the sera and urine of calves infected experimentally intravenously with *A. fumigatus*, cows with mycotic placentitis and abortion (confirmed by histopathology and culture), cows that aborted for other reasons, cows that did not abort but had other infections and healthy cows at the slaughterhouse. The results showed that the test was neither sensitive nor specific enough to be considered a reliable means to diagnose mycotic abortions in cattle.

In addition to the clinical and microbiological diagnosis, the possibility of serological diagnosis of mycotic abortions in cattle and sheep was assessed (Corbel et al., 1973; Wiseman et al., 1984; Jensen et al., 1991, 1993), but to the best of our knowledge, these experiments did not result in diagnostic tests.

Mycotic Keratitis

Mycotic keratitis (MK) may afflict a variety of animal species with trauma providing the portal of entry for the infecting agent (Aho et al., 1991). Horses seem to be the animals most frequently affected, possibly due to the lateral location and protrusion of their eyes (Sherman et al., 2017). In fact, this syndrome comprises about one-third of cases of equine keratitis. *Aspergillus* spp., especially *A. fumigatus*, often the etiological agent of these infections (Aho et al., 1991) has been isolated also from eyes of healthy horses (Sherman et al., 2017). Proteases produced by these fungi provide an important pathogenic mechanism (Gopinathan et al., 2001).

Gaarder et al. (1998) divided MK cases into five types. The most severe form is characterized by the development of a furrow in the cornea. Most of these cases necessitated enucleation or even exenteration. The authors suggested that blocking of deep vascularization by the furrow impeded the formation of the granulation tissue required for healing.

Medical treatment with drugs such as voriconazole, miconazole, natamycin, itraconazole, or oral fluconazole suffices to heal some cases while surgical intervention may be necessary in other cases (Sherman et al., 2017).

Avian Aspergillosis

Avian aspergillosis afflicts domestic and wild birds, free and captive. Some species such as penguins seem to be over-represented (Samanta and Bandyopadhyay, 2017) but their increased susceptibility has not been unequivocally confirmed (Fischer and Lierz, 2015). Young birds seem to be more susceptible to acute aspergillosis (Fischer and Lierz, 2015). It may be chronic, resulting from the action of predisposing factors such as stressors, husbandry, or other pathogens or acute due to exposure to high spore concentrations. A possible connection between spore rich environments and human colonization has been suggested (Cafarchia et al., 2014). Although not a mycotic infection and thus beyond the scope of this review, it is noteworthy that *Aspergillus* spp. may contaminate hatcheries causing significant economic damages (Hamet et al., 1991). Fungi infecting birds must be able to grow at the relatively high body temperature of these animals that may surpass 40°C (Prinzinger et al., 1991). *Aspergillus* spp. in general and *A. fumigatus* in particular are the main etiological agents involved (Neumann, 2016).

A special feature of bird anatomy, the air sacs or coelomic cavities, facilitates the systemic dissemination of fungal spores. Air sacs are hollow organs, distributed throughout the body and inside bones. In pelicans they are present also in the subcutis. They communicate with the lungs and thus spores that reach the lower respiratory tract can disperse to other body compartments (König et al., 2016).

Clinically, the first signs include breathing difficulties and wheezing due to the obstruction of airways by fungal granulomata (Girma et al., 2016). Subsequently general symptoms such as lethargy, inappetence, diarrhea, and feather ruffling may appear (Pardeike et al., 2016). In other cases, wasting may be the only symptom (Neumann, 2016). Pathological changes include granulomata in the lungs and fungal plaques in the air-sacs are (Girma et al., 2016).

Since clinical symptoms are not specific for aspergillosis, additional tests are necessary to confirm the diagnosis (Neumann, 2016). In cases of flocks, husbandry deficiencies can provide the initial indication to fungal infections (Fischer and Lierz, 2015).

For individual birds, tests may include radiology, CT, or MRI scans (Schwarz et al., 2016) and endoscopy to reveal occlusions in birds with straight tracheae (if this organ is convoluted, such as in some crane species, this approach is not possible). In addition, endoscopy allows sampling of the lesions and culturing of the etiological agent. The detection of a fungal toxin, fumigaclavin, has been suggested to indicate avian aspergillosis but the conditions in which it may be detected have to be further clarified (Seyedmousavi et al., 2015). Aberrant clinical pathological test results are mostly the function of the organ involved. Other methods include protein electrophoresis on cellulose acetate or agarose gel film (for a detailed description of the method see Werner and Reavill, 1999). A decrease in albumin or serum proteins was found to be negative prognostic indicators in penguins (Werner and Reavill, 1999). These findings were confirmed by Naylor et al. (2017) who assessed the prognostic value of the plasma protein value in 183 Gentoo penguins (*Pygoscelis papua papua*), as determined by agarose gel electrophoresis. They found that the negative predictive value of an increase in the albumin/globulin ratio to be high whereas the positive predictive value was limited. It is noteworthy, however, that since standard plasma protein profiles for many bird species have not been defined, the interpretation of these tests may be problematic (Fischer and Lierz, 2015).

Biopsies may reveal fungal hyphae whereas immunohistochemistry may indicate whether the infecting fungus belongs to the genus. Challa et al. (2015) assessed the specificity of these tests by using polyclonal anti-*Aspergillus* rabbit antibodies on 50 paraffin-embedded samples. Forty-seven of these consisted of tissue samples in which hyphae were seen and three of cultures. *Aspergillus* spp. were cultured from 25 of the tissue samples. The remaining tissue samples and the cultures were of a variety of fungi. They found that 88% of the proven aspergillosis cases were positive and no cross reaction with the other fungi. Consequently they concluded that immunohistochemistry has the potential to be used as a supplementary test to diagnose avian aspergillosis. Moreover, they reviewed previously published studies and stressed the impact of the method employed on the results.

The therapeutic approach to avian aspergillosis differs in flocks or individual birds. In the former, husbandry improvements such as decrease in stressors, better ventilation, and uncontaminated feed and environment are required. For individual birds, various treatments have been described (Krautwald-Junghanns et al., 2015). In some cases, such as wild birds, their periodic capture, necessary for repeated administration of parenteral antimycotic drugs during long periods, may be impractical due to the resulting stress. Moreover, the fungus' sequestration from the blood stream may result in it not being exposed to the drug (Girma et al., 2016). Consequently, oral antimycotics are preferred and various approaches such as nebulization or the use of nanosuspensions have been attempted (Wlaź et al., 2015; Pardeike et al., 2016). Since therapeutic protocols have not been validated specifically for birds in general and for the various species in particular, necessary care should be taken, especially considering the prolonged time required to complete the treatment (Tartor and Hassan, 2017). In cases in which anesthesia is necessary such as surgery for air sac exposure to alleviate respiratory distress or imaging tests, the eventual impact of air way obstructions or other lung lesions upon the anesthetic process must be considered.

Future Prospects

Improved diagnostic methods and economically sustainable therapeutic options could significantly improve the prognosis of animal aspergillosis. Reports of adapting modifications of human kits such as that aimed at galactomannan detection to animals, especially pets, are few and should be the object of more detailed assessment resulting in clear use recommendations. In addition, results of imaging techniques have also been found to be often unreliable and should be further investigated.

While great advances in antifungal therapy have been made in the last years, the price of the newer drugs is prohibitive for animal use especially considering the necessity of extended, possibly life-long, treatments. This has limited their use to a very low number of cases, mostly for scientific studies. It is expected that the price of these drugs will decrease in the future, but the development of specific antifungal drugs for veterinary use should be considered as well.

A special case of future research should try to define the immunologic basis of canine disseminated aspergillosis. This would permit the exclusion of dogs carrying the faulty gene to be excluded from the pedigree lists (as has been done for hip dysplasia in GS dogs) and reduce the number of cases.

HUMAN ASPERGILLOSIS: *IN VITRO* ASSESSMENT OF SUSCEPTIBILITY TO DRUGS AND DRUG COMBINATIONS AGAINST *ASPERGILLUS*

Introduction

Human aspergillosis is a multifaceted disease, including ear, sinus, eye, skin, lung, or disseminated infection (Patterson, 2010; Denning, 2015). The pulmonary tract is the major target system for *Aspergillus* spp. Pulmonary involvement is represented in several clinical entities: the allergic broncho-pulmonary aspergillosis (ABPA), aspergilloma, chronic pulmonary aspergillosis (CPA), and invasive pulmonary aspergillosis (IPA), leading possibly to disseminated invasive aspergillosis (IA). Each of these entities, differing in the symptoms and

epidemiological parameters, differ as well in regard to diagnosis and therapy.

Diagnosis of human fungal infections in general, including that of *Aspergillus* spp. infections, is based on demonstration of the etiological agent directly in the clinical specimen by various microbiological or histopathological techniques, followed by culturing the agent from the clinical sample and identifying it (Balaye and Brandt, 2011).

Current laboratory diagnosis of aspergillosis employs also non-cultural methods, such as immunological assays, including detection of anti-*Aspergillus* antibodies and the *Aspergillus* antigen-galactomannan (the Platelia test). Additional non-cultural tests include detection of *Aspergillus* nucleic acids in patients' blood or other clinical samples, by using PCR technology (Balaye and Brandt, 2011; Denning, 2015).

At the situation of success of the classical diagnostic venue leading to isolation and identification of the etiological agent—the rational approach is attempting to manage the infection with specific therapy. Specific antifungal therapy relies on determination of the sensitivity of the isolated fungal agent to antifungal drugs. Hence, assessment of susceptibility of the identified agent to the available antifungal agents is an essential step of the diagnosis in the process of management of the specific infection, as was pointed out in a recent publication in regard to diagnosis of aspergillosis (Powers-Fletcher and Hanson, 2016).

Therapy of pulmonary aspergillosis is based on the use of voriconazole as a drug of choice. Posaconazole, isavuconazole, liposomal amphotericin B, or amphotericin are used as well. For allergic forms of aspergillosis such as ABPA or allergic *Aspergillus* sinusitis, the recommended treatment is itraconazole. Corticosteroids may also be helpful.

Invasive pulmonary aspergillosis and IA are infections associated with high mortality rates, in many cases even under treatment (Denning, 2015). Thus, combination therapy, with more than a single antifungal drug, particularly with drugs of different modes of activity, seems as a sensible approach that could improve the management of such infections (Spitzer et al., 2017). As a consequence, susceptibility testing of antifungal drug-combinations against fungi evolved as part of the management of aspergillosis.

The following text will concentrate on description of susceptibility assessment of antifungal drugs and antifungal drug-combinations against *Aspergillus* spp.

Susceptibility Testing of Antifungals—General Overview

Susceptibility of antimicrobial drugs has historically started with the era of antibiotics in use against bacterial infections, both in terms of methodology, evaluation, and breakpoint determination.

Susceptibility of antifungal drugs was introduced into research and clinical use significantly later. The major reason for this delay was the paucity of availability of antifungal drugs in addition to technical difficulties of standardization of such assays for fungi, particularly for molds (Johnson et al., 2011).

The necessity for susceptibility testing to antifungals arouse with the development during the last decades of new antifungal drugs and the emerging problem of appearance of resistant fungal species or fungal strains within a given species. The greater arsenal of antifungals, acting by different modes, led also to use of combinations of antifungals, which in turn led to susceptibility testing of antifungal drug combinations.

Most of the methods for in vitro susceptibility assays are based on the guidelines developed by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standarts Institute, 2008) in the United States (Johnson et al., 2011) and its European Equivalent—The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Cuenca-Estrella et al., 2003).

In terms of antifungal susceptibility, the CLSI developed methods for yeasts—the M27-A3 document and M38-A2—for molds, respectively. Both are based on use of the broth dilution method: the macro-dilution and micro-dilution. These methods enable establishment of minimal inhibitory concentrations (MICs) and in some instances breakpoints.

The broth dilution methods can be evaluated visually or by spectrophotometry (Clinical and Laboratory methods). The latter enables in addition to MIC determination, also 50% of MIC, or values in between.

A modification of the broth method which can also assess MIC values is the *E*-test (Espinel-Ingroff, 2001). The *E*-test is an agar-based technique in which a paper strip loaded with a concentration-gradient of an antimicrobial drug is placed on an agar plate seeded with the test microbe, allowing readings of the results by measuring an ellipse zone of inhibition.

An additional agar-based technique is the disk diffusion method which does not determine MIC but measures inhibition zones. The agar-based methods are evaluated visually.

Lee et al. (2009) compared the disk diffusion assay with the *E*-test in susceptibility tests of Candida and found a correlation between the two methods. The study of Salas et al. (2013) explored the predictive values of *in vitro* susceptibility testing of *A. fumigatus*, using both the micro-dilution and disk diffusion assays. These investigators concluded that the susceptibility data correlated with the *in vivo* data in an experimental animal model. An additional study of Lass-Flörl et al. (1998) have shown it in clinical cases of human aspergillosis, in patients with *A. fumigatus*, *A. flavus*, or *A. terreus* infection.

Reliable antifungal susceptibility testing requires standardization of the procedures, including medium, fungal inoculum, temperature of incubation, time of reading, and result interpretation (Johnson et al., 2011). It also needs adjustment as to the class of antifungals. Thus, establishing MICs of molds, such as *Aspergillus*, spp. to echinocandins is problematic, since no complete macroscopic growth inhibition is observed, but rather partial inhibition is noted, which is associated with abnormal hyphae formation. Hence, a different criterion for susceptibility to echinocandins was introduced: the minimal effective concentration (MEC), which defines the minimal antifungal concentration which causes the morphological changes (Kurtz et al., 1994).

TABLE 1 | Advantages and shortcomings of antifungal susceptibility testing of *Aspergillus* species.

Antifungal class	Broth dilution methods		Agar based methods	
	Macro-broth dilution	**Micro-broth dilution**	**Disk diffusion**	**E-Test**
Polyenes				
Advantages	Enable establishment of MIC*; Visual & spectrophotometric evaluation	Enable establishment of MIC; Visual & spectrophotometric evaluation	Simple, fast, affordable	Can assess MIC; Less laborious than micro-broth dilution
Shortcomings	Labor intensive and massive use of reagents and supplies.	Labor intensive, but lesser use of reagents and supplies	No MIC can be established	Difficult to determine end point
Azoles				
Advantages	Enable establishment of MIC*; Visual & Spectrophotometric evaluation	Enable establishment of MIC; Visual & spectrophotometric evaluation	Simple, fast, affordable	Can assess MIC; Less laborious than micro-broth dilution
Shortcomings	Enable establishment of MIC; Visual & spectrophotometric evaluation	Labor intensive and massive use of reagents and supplies	No MIC can be established	Maybe difficult to determine the end point
Echinocandins				
Advantages	Activity based on fungal morphological changes can be assessed by microscopic evaluation	Activity based on fungal morphological changes can be assessed by microscopic evaluation	Simple, fast, affordable	Less laborious than micro-broth dilution
Shortcomings	Only MEC** can be established and not MIC	Only MEC can be established and not MIC	Zone of inhibition maybe difficult to determine due to possibility of residual growth	Difficult to determine end point due to possibility of residual growth

**MIC, minimal inhibitory concentration; **MEC, minimal effective concentration.*

In addition to MICs or MECs, susceptibility testing enables also the determination of the drug's fungicidal activity, by defining the minimal drug concentration causing fungal death—minimal fungicidal concentration (MFC) (Pfaller et al., 2004).

The advantages and shortcomings of the different susceptibility test methods are summarized in **Table 1**.

Susceptibility Testing of Antifungals Against *Aspergillus*

Susceptibility testing of *Aspergillus* spp. is an established tool in clinical mycology in institutions dealing with invasive mycoses patients and also a tool for epidemiological studies.

Since triazoles are the major line of antifungal drugs in treatment of IA, many of the susceptibility studies on *Aspergillus* spp. focus on these drugs. An example of such studies is the investigation of Baddley et al. (2009) who assessed patterns of susceptibility of 274 clinical isolates of *Aspergillus* spp. in transplant recipients, using the M38-A2 broth dilution method. They assessed susceptibility to itraconazole, voriconazole, posaconazole, and ravuconazole and also amphotericin B. Interestingly, the authors report that no significant relationships of MIC and mortality were noted. A later study by Pfaller et al. (2011) examined a 9-year susceptibility trend (2001–2009) of *Aspergillus* spp. to triazoles (itraconazole, posaconazole, and voriconazole). These authors concluded that decreased susceptibility among *Aspergillus* spp. was observed; however, there was no consistent trend toward decreased susceptibility for any triazole in *A. fumigatus* or *A. flavus* over time.

In view of the increasing resistance to triazoles and concerns of cross resistance, Gregson et al. (2013) examined the susceptibility of *A. fumigatus* clinical isolates to isavuconazole in comparison to that of the older triazoles: itraconazole, voriconazole, and posaconazole. They found that isavuconazole MICs were higher in strains with reduced susceptibilities to other triazoles.

A recent publication by Sanguinetti and Posteraro (2017) points to the importance of introduction of newer technologies for antifungal susceptibility testing in view of the rising problem of resistance. Such technologies, which are based on detection of specific mutations in fungi, are not yet available in clinical settings for *Aspergillus* spp. susceptibility testing.

As indicated afore, susceptibility testing of molds to echinocandins may be problematic due to inability to establish clear-cut MIC values, but is evaluated by the MEC. A recent study in Brazil (Denardi et al., 2017) explored by the broth micro-dilution method of EUCAST and using the EUCAST-proposed breakpoints the *in vitro* susceptibility of 105 clinical and environmental strains of *A. fumigatus* and *A. flavus* to the antifungal drugs: amphotericin B, azoles, and echinocandins. They found that there were differences among the echinocandins (caspofungin, micafungin, and anidulafungin) as to activity and also variability as to susceptibility of the *Aspergillus* spp.

Susceptibility testing is of special importance regarding infections caused by *A. terreus*, known for its relative resistance. Lass-Florl et al. (2005) studied 67 cases of IA caused by *A. terreus* and non-*A. terreus* (32 vs. 35, respectively) regarding susceptibility to amphotericin B, voriconazole, and caspofungin. *In vitro*, *A. terreus* was found to be resistant to amphotericin B; the infections were associated with a lower response rate to amphotericin B therapy and had a poor outcome.

A relative recent aspect, consequence of the use of antifungal drugs, is the emergence of resistance in fungi to antifungal

drugs, particularly to triazoles. As triazoles are the mayor group of drugs in therapy of aspergillosis the issue of resistance is most relevant in this context. It is believed that the increase in resistant *Aspergillus* strains is associated with the massive use of azole compounds in agriculture (Meis et al., 2016).

Susceptibility Testing of Antifungal Drug Combinations—General Overview

As indicated earlier, with the increase in number of antifungal drugs of different classes enabling the use of drug combinations, it became necessary to evaluate the efficacy of such combinations *in vitro* and *in vivo* against the fungal pathogens. This led to susceptibility testing of antifungal drug combinations.

Susceptibility testing of antifungal drug combinations aims to determine, whether:

1. The combination may improve the management of the infection over that of each of the drugs in the combination, which would mean synergy.
2. The combination may worsen the management of the infection more than each of the drugs in the combination, meaning antagonism.
3. The combination does not affect the management of the infection vs. the treatment with each of the drugs in the combination, hence indicating indifference.

The preferred method for assessment of drug combinations is the checkerboard technique (Chiou et al., 2001; Semis et al., 2015), that is based on the micro-dilution broth assay.

A. Checkerboard Method

This technique determines the MIC of each drug in the combination and the activity of the combination. By using a formula which takes into account the mutual effects of each drug on the other component, it is possible to determine whether there is a synergistic or antagonistic effect by using the two drugs together, or no effect at all compared to the single drug.

A concentration gradient of each drug in the combination is prepared in 96-well microtiter plates in a two-dimensional manner. One hundred milliliters of test fungal suspension at concentration of 1–5 $\times$ 10^4 conidia/ml is added to each well (end result 200 μl/well). The plates are incubated at 37°C for 48°h. The MICs are determined as the lowest drug concentration with no visible growth. For echinocandins the MEC, indicating the lowest concentration causing abnormal hyphal growth, is estimated by microscopy (inverted microscope).

Drug interactions are evaluated by an index—the fractional inhibitory concentration index (FICI), which takes into account the MIC of drug A (MICA) and MIC of drug B (MICB) in the combination and as single drugs. The FICI is obtained by calculating: MICA in combination/MICA alone + MICB in combination/MICB alone. FICI values of up to 0.5 are considered as synergy, 0.5 to <4 indicate indifference and FICIs of 4 or above suggest antagonism (Chiou et al., 2001; Odds, 2003).

B. Disk Diffusion Method

Disk diffusion method is an additional recognized technique to assess *in vitro* susceptibility of microorganisms to various drugs (Johnson et al., 2011).

This technique can be adapted also for assessment of drug combinations. Specifically, the test organism is spread on agar plates which contain in the medium one of the drugs in the combination and the second drug is incorporated in the paper-disks placed on the agar plates.

Following incubation (generally 24/48 days at 37°C) the zone of inhibition is measured. To determine synergy, antagonism or indifference the inhibition zone is compared to the inhibition zones around paper disks entailing the individual drugs of the combination placed on agar plates spread with the test fungus. Larger inhibition zones on the combination plate would indicate synergy, smaller inhibition zone antagonisms, and a similar inhibition zone indifference.

Susceptibility Testing of Antifungal Drug Combinations Against *Aspergillus*

Therapy of the major invasive human fungal infections by antifungal combinational therapy was summarized recently by Spitzer et al. (2017) in a comprehensive, thorough review article.

Antifungal combination therapy is known since some decades in the clinical setting of meningeal cryptococcosis, where combining amphotericin B and 5-fluorocytosin results in the improvement of the outcome (Bennett et al., 1979). This combination therapy is considered as gold standard for treatment of cryptococcal meningitis by WHO guidelines (Perfect and Bicanic, 2015; Maziarz and Perfect, 2016).

In addition, the combination of amphotericin B and fluconazole is efficacious also in treating *Candida meningitis* (Smego et al., 1984).

Combination treatment of IA is thus far considered in high risk patients, but not as a general recommendation (Cadena et al., 2016).

As to other mycoses no specific guidelines for clinical settings are available and most of the published literature includes experimental studies both *in vitro* and *in vivo*.

Sionov et al. (2005) investigated in an experimental *in vivo* model in mice infected with *A. fumigatus* the efficacy of therapy with polyenes in comparison to that of the combination of polyenes and echinocandins. Specifically, the infected mice were treated with:

(1) amphotericin B or with a lipid formulation of amphotericin B: amphotericin B-intralipid;
(2) with a combination of the polyenes (amphotericin B/ amphotericin B-intralipid) and the echinocandin, caspofungin.

These experiments showed that mice treated with the combination of the two drugs had higher survival rates, prolonged survival time, and lower fungal visceral colonization.

The authors concluded that a combination of drugs acting by different modes of activity, such as the polyenes, affecting the

fungal cytoplasmic membrane and the echinocandins, acting on the fungal cell wall, result in a better outcome.

A number of investigators used a similar approach in experimental infections. A few examples: Santos et al. (2017) reported on the efficacy of fluconazole and amphotericin B in controlling a *Cryptococcus gattii* infection in a murine model of cryptococcosis. The combination treatment revealed improvement in survival and reduced morbidity. Another study (Chen et al., 2013) reported that posaconazole exhibits *in vitro* and *in vivo* synergy with caspofungin against drug susceptible or resistant *C. albicans* strains. The authors indicate the potential therapeutic applicability of such combinations.

As to combinational antifungal therapy in experimental aspergillosis there are also several studies (Petraitis et al., 2009, 2017; Zhang et al., 2014). Petraitis et al. (2009) studied in rabbits the activity of the combination of the echinocandin–anidulafungin and the triazole–voriconazole against IPA. They found a synergetic effect expressed in reduction in several parameters of the pulmonary infection vs. treatment with each of the drugs alone. The same group of investigators reported in a recent publication (Petraitis et al., 2017), that by using the same concept and same model, with a different combination of antifungals, namely the newer triazole—isavuconazole and a different echinocandin—micafungin, similar results were noted. Thus, enforcing the validity of the concept.

Another interesting study by a different group of investigators (Zhang et al., 2014) explored the combination of voriconazole and caspofungin against invasive pulmonary infection in neutropenic rats caused by different *Aspergillus* species: *A. fumigatus*, *A. flavus*, or *A. niger*. The authors overall conclusions were that the combination had a synergistic effect against infection caused by *A. flavus* and *A. niger*, but only minor improvement in infection caused by *A. fumigatus*.

These studies emphasize the complexity and difficulty to draw clear-cut conclusions as to the concept of combinational antifungal therapy.

As to *in vitro* susceptibility testing of antifungal combinations against *Aspergillus* spp.—here too are a number of reports in the literature. A recent article by Denardi et al. (2017) reports on susceptibility testing of *A. fumigatus* to antifungal–drug combinations. The authors evaluated combinations of triazoles and echinocandins on itraconazole-resistant strains, using two methods: the checkerboard assay and the *E*-test. The data generated, MIC and MEC, at two different time readings (24 and 48 h) were compared. The analysis of the data showed that the correlation coefficient between the methods depended on the reading-time and the specific combination. The combinations of the azoles and echinocandins measured by the *E*-test showed synergy when readings were done after 24 and 48 h, albeit the effect was reduced at the later reading point, at which some combinations revealed indifference. This study points to the difficulty of getting clear-cut conclusions, as they may be influenced by the method.

An additional recent interesting study of Pfaller et al. (2009) reports on the susceptibility of azole-resistant *A. fumigatus* and other molds and yeasts, to the combination of azoles with Hos2 fungal histone deacetylase (HDAC) inhibitor MGCD290. The study showed that the activity of fluconazole plus MGCD290 was synergistic against 6/10 *Aspergillus* isolates.

A different combination of echinocandins with the investigative anti-chitin synthase compound, nikkomycin, was explored against *A. fumigatus* and other molds (Chiou et al., 2001). The investigators found synergy for *A. fumigatus*, and indifference for other *Aspergillus* species: *A. flavus*, *A. terreus*, and *A. niger*.

Aspergillus terreus causes systemic infections in immunocompromised patients. The infection is difficult to treat, as *A. terreus* can be resistant or less sensitive to amphotericin B (Lass-Florl et al., 2005). Semis et al. (2015) used the checkerboard assay to evaluate *in vitro* different antifungal drug combinations against *A. terreus*. Antifungal activity of combinations of nystatin/nystatin-intralipid with voriconazole, caspofungin, terbinafine, or 5-fluorocytosine was assessed. It was noted that combination of nystatin-intralipid with caspofungin exhibited better antifungal activity than each drug alone and resulted in synergy in three out of six tested strains of *A. terreus*, while this was not noted with nystatin and caspofungin. Nystatin intralipid or nystatin with voriconazole yielded indifferent interactions. Nystatin-intralipid and terbinafine showed a strong antagonism in all six *A. terreus* strains tested. Susceptibility of *A. terreus* strains to the drug-combinations nystatin or nystatin-intralipid and terbinafine or 5-flurocytosine was also tested by the disk diffusion assay. The results were comparable with those obtained by the checkerboard assay.

In summary, susceptibility of *Aspergillus* spp. to antifungals or antifungal combinations should be considered in management of aspergillosis. However, the complexity associated with the interpretation of the data, particularly in regard to antifungal combinations, still necessitates additional investigational efforts. These should involve studies to better define the predictive value of the data obtained in the susceptibility tests. Furthermore, if possible, susceptibility data should be matched with specific mutations in fungi in order to avoid resistant strains, as suggested by Sanguinetti and Posteraro (2017).

This review comprised of a combination of texts, which focus both on animal and human aspergillosis, is unique as such. Furthermore, the part dealing with susceptibility of antifungal drugs and combination of drugs, as part of diagnosis in human aspergillosis, is unique as well. These factors taken together make the manuscript relevant within the scope of the Research Topic: "Diagnostic Approaches for Aspergillus Infections."

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REVIEW
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Frequency and Geographic Distribution of *CARD9* Mutations in Patients With Severe Fungal Infections

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Autosomal recessive deficiency in the caspase recruitment domain containing protein 9 (CARD9) results in susceptibility to fungal infections. In the last decade, infections associated with CARD9 deficiency are more reported due to the advent of genome sequencing. The aim of this study was to evaluate the frequency, geographic distribution and nature of mutations in patients with CARD9 deficiency. We identified 60 patients with 24 mutations and different fungal infections. The presence of the homozygous (HMZ) p.Q295X (c.883C > T) and HMZ p.Q289X (c.865C > T) mutations were associated with an elevated risk of candidiasis (OR: 1.6; 95% CI: 1.18–2.15; $p = 0.004$) and dermatophytosis (OR: 1.85; 95% CI: 1.47–2.37; $p < 0.001$), respectively. The geographical distribution differed, showing that the main mutations in African patients were different Asian patients; HMZ p.Q289X (c.865C > T) and HMZ p.Q295X (c.865C > T) accounted for 75% and 37.9% of the African and Asian cases, respectively. The spectrum of *CARD9* mutations in Asian patients was higher than in African. Asia is the most populous continent in the world and may have a greater genetic burden resulting in more patients with severe fungal infections. The presence of a high diversity of mutations revealing 24 distinct variations among 60 patients emphasize that the unique genetic alteration in *CARD9* gene may be associated with certain geographical areas.

Keywords: severe fungal infections, CARD9 deficiency, mutation, candidiasis, dermatophytosis

INTRODUCTION

Susceptibility to fungal infections in otherwise healthy individuals with Mendelian disorders are increasingly being recognized (Vinh, 2011) than before the widespread use of genome sequencing. Primary immunodeficiencies consist of various genetic defects that affect the innate and adaptive immune systems. In addition, evaluation of previously healthy, fungus infected patients, suspected of having a primary genetic immunodeficiency may give valuable insights on the role of specific proteins in the immune system for protection from these infections (Wang et al., 2014; Corvilain et al., 2018). Caspase recruitment domain containing protein 9 (CARD9) is a central regulator of innate immunity that is highly expressed in neutrophils, macrophages, dendritic cells, and during cell apoptosis in low-serum conditions (Bertin et al., 2000; Liang et al., 2015). Mutations in several proteins involved in the CARD9 signaling protein have been demonstrated to cause primary immunodeficiencies in humans. These mutations cause a decreased production of cytokines from innate immune cells, leading to deficiencies of TH17 and accordingly predispose patients to severe disseminated infections (Conti and Gaffen, 2015). Severe fungal infections in healthy patients have recently been reported from a few countries, i.e., Algeria, Brazil, France, China, Iran, Morocco and Tunisia (Glocker et al., 2009; Drewniak et al., 2013; Lanternier et al., 2013; Wang et al., 2014; Grumach et al., 2015) and linked to autosomal recessive CARD9 deficiency. The species involved in these infections are *Trichophyton violaceum, Trichophyton rubrum*, *Candida* species, *Exophiala* species, *Phialophora verrucosa*, *Aspergillus fumigatus, Prototheca zopfii, and Mucor irregularis.* Some of those etiological agents are plant pathogens, which rarely have been associated with human infection. Highly diverse clinical manifestations from cutaneous to disseminated and progressive infections are observed (Boudghène-Stambouli and Mérad-Boudia, 1991; Boudghène-Stambouli et al., 1992; Pruszkowski et al., 1995). Our aim was to evaluate the global frequency, geographic distribution and nature of mutations in patients with CARD9 deficiency associated with fungal infections.

MATERIALS AND METHODS

The review process involved study of existing published literature of all reported cases with fungal infection due to CARD9 deficiency. To search the published literature, Medline database through PubMed, Embase through Scopus, ISI Web of Science, Science Direct and Google Scholar were used to explore the published literature of patients with severe fungal infection and CARD9 deficiency using the key words "caspase recruitment domain deficiency," "CARD9 deficiency," "autosomal recessive CARD9 deficiency," "primary immunodeficiency," "mutations," "fungal infection" or "invasive fungal diseases," "candidiasis," "deep dermatophytosis," "disseminated phaeohyphomycosis," and "chronic mucocutaneous candidiasis" in different combinations. A total of 21 relevant articles were found using these key words. The extracted data were analyzed using R software version 3.4.1. The chi-square test was utilized to evaluate associations between nominal variables and the *p*-value was estimated using the Monte Carlo method. To compare the differential prevalence of *CARD9* mutations and determine differences in causative agents of fungal infections, odds ratios (ORs) were used. The significance of all ORs, using a 95% Bayesian credible interval (CI), was calculated using Bayesian logistic regression.

RESULTS

The Burden of CARD9 Deficiency Is Positively Correlated With Fungal Infection

To analyze the role of CARD9 deficiency in fungal infection, we reviewed the literature and identified 60 cases until 2018. The total number of patients with severe fungal infection related to CARD9 deficiency has been summarized in **Tables 1A,B** (Boudghène-Stambouli and Mérad-Boudia, 1989, 1991, 1998; Pruszkowski et al., 1995; Glocker et al., 2009; Drewniak et al., 2013; Gavino et al., 2014; Wang et al., 2014; Drummond et al., 2015; Grumach et al., 2015; Herbst et al., 2015; Jachiet et al., 2015; Lanternier et al., 2015a,b; Alves de Medeiros et al., 2016; Gavino et al., 2016; Jones et al., 2016; Rieber et al., 2016; Yan et al., 2016; Boudghene-Stambouli et al., 2017; Gavino et al., 2018; Sari et al., 2018; Vaezi et al., 2018; Wang et al., 2018a,b). The age at the time of diagnosis ranged from 4 to 91 years (mean 34.3 $\pm$ 17.9 years). Since 1989, a total of 14 countries reported cases of fungal infections associated with CARD9 deficiency (**Figure 1**). Although most cases originate from Algeria (North Africa) [n = 12 (21.1%)], the majority of cases were from several countries in the Asian continent (n = 29, 48.3%), with Iran reporting the majority (n = 10/29, 34.5%). The main fungal infection associated with CARD9 deficiency was candidiasis (40.3%) followed by deep dermatophytosis (37.3%), phaeohyphomycosis (16.4%) and invasive aspergillosis (3.0%). *T. violaceum*, *T. rubrum,* and *Trichophyton mentagrophytes* were observed as etiological agents of dermatophytosis. *Candida* infections were caused by *C. albicans* and non-*albicans Candida* species in 70.8% and 29.2% of the cases, respectively. *P. verrucosa* (36.4%) represented the major species of phaeohyphomycosis and were only reported from China. Neurological infection (40.5%) was the predominant clinical presentation in *Candida* infected patients followed by chronic mucosal and cutaneous candidiasis (29.7%). The outcome was recorded in 45 cases and 11 (24.4%) expired.

Associations Among Mutations of the *CARD9* Gene and Infection Status With Fungal Pathogens

Overall, 24 different genetic alterations in *CARD9* were described in the 60 patients. Three of those were identified

most frequently: homozygous (HMZ) p.Q289X (c.865C > T), HMZ p.Q295X (c.883C > T) and HMZ p.D274fsX60 (c.819-820insG), which accounted for 25.8%, 17.7%, and 8.1% of the patients, respectively. Multiple variations in *CARD9* were identified in 8.7% of all cases. The correlation between mutations and fungal infection is shown in **Figure 2**. The presence of the HMZ p.Q295X (c.883C > T) and HMZ p.Q289X (c.865C > T) mutation was associated with an elevated risk of candidiasis (OR: 1.6; 95% CI: 1.18–2.15; $p = 0.004$) and dermatophytosis (OR: 1.85; 95% CI: 1.47–2.37; $p < 0.001$), respectively. Also a strong association was evident between the presence of HMZ p.D274fsX60 (c.819-820insG)

TABLE 1A | Prevalence of fungal infections, duration of infections and causative pathogens in patients with CARD9 deficiency.

Fungal infection	Duration of infection, mean (± SD), year	Nr of cases (%)	Causative agent	Nr of cases (%)
Dermatophytosis	37.8 ± 18.7	25 (37.3)	*Trichophyton rubrum*	7 (13.0)
Phaeohyphomycosis	8.5 ± 6.6	11 (16.4)	*Trichophyton violaceum*	8 (14.8)
Invasive aspergillosis	–	2 (3.0)	*Trichophyton mentagrophytes*	1 (1.9)
Mucormycosis	–	1 (1.5)	*Candida* spp	5 (9.3)
Protothecosis	–	1 (1.5)	*Candida albicans*	17 (31.5)
Candidiasis	8.5 ± 10.8	27 (40.3)	*Candida dubliniensis*	1 (1.9)
Mucosal and cutaneous candidiasis	11.5 ± 15.5	11 (29.7)	*Candida glabrata*	1 (1.9)
Neurologic infection	5.3 ± 5.6	15 (40.5)	*Phialophora verrucosa*	4 (7.4)
Chronic candidiasis	6.5 ± 7.7	4 (10.8)	*Exophiala dermatitidis*	1 (1.9)
Osteomyelitis	3.3 ± 0.5	3 (8.1)	*Exophiala spinifera*	2 (3.7)
Endophthalmitis	2.3 ± 1.1	3 (8.1)	*Aspergillus fumigatus*	2 (3.7)
Colitis	–	1 (2.7)	*Corynespora cassiicola*	2 (3.7)
			Ochroconis musae	1 (1.9)
			Mucor irregularis	1 (1.9)
			Prototheca zopfii	1 (1.9)

Neurologic infection includes meningoencephalitis, meningitis, and brain abscesses.

TABLE 1B | Overview of patient demographics and mutations.

Condition	Nr of cases (%)	Mutation	Nucleotide change	Domain	Nr of cases (%)
Age (year)		HMZ Q289X	c.865C>T	CCD	16 (25.8)
<20	16 (26.7)	HMZ Q295X	c.883C>T	CCD	11 (17.7)
21–60	39 (65)	HMZ D274fsX60	c.819-820insG	CCD	5 (8.1)
>60	5 (8.3)	HMZ R70W	c.208C>T	CARD	4 (6.5)
Male/female	30(50)/30(50)	HMZ Y91H	c.271T>C	CARD	4 (6.5)
Country		HTZ L64fsX59	c.191–192insTGCT	CARD	3 (4.8)
Algeria	12 (21.1)	HMZ R101C	c.C301T	CARD	2 (3.2)
Angola	1 (1.7)	HTZ Q158X	c.472C>T	CCD	1 (1.6)
Brazil	1 (1.7)	HTZ G72S	c.214G>A	CARD	1 (1.6)
China	9 (15.8)	HTZ R373P	c.1118G>C	CCD	1 (1.6)
Egypt	1 (1.7)	HMZ R35Q	c.104G>A	CARD	1 (1.6)
France	4 (7.0)	HMZ R18W	c.52C>T	CARD	1 (1.6)
Iran	10 (17.5)	HMZ E323del	c.GAG967-969del	CCD	1 (1.6)
Korea	1 (1.7)	HMZ R101L	c.302G>T	CARD	1 (1.6)
Morocco	3 (5.3)	HMZ R57H	c.170G>A	CARD	1 (1.6)
Pakistan	1 (1.7)	HMZ M1I	c.3G>C	CARD	1 (1.6)
Tunisia	4 (7.0)	HTZ A380P	c.1138G>C	CCD	1 (1.6)
Turkey	8 (14.0)	HTZ R317R	c.951G>A	CCD	1 (1.6)
United Kingdom	1 (1.7)	HTZ S23X	c.68C>A	CARD	1 (1.6)
United States	1 (1.7)	HMZ V261fs	c.781delG	CCD	1 (1.6)
		HTZ G62fs	c.184G>A	CARD	1 (1.6)
		HTZ G96del36	c.288C>T	CARD	1 (1.6)
		HTZ T231M	c.692C>T	CCD	1 (1.6)
		HTZ F302del	c.905_907delTCT	CCD	1 (1.6)

HMZ, homozygous; HTZ, heterozygous; CCD, coiled-coiled domain of CARD9 protein; CARD, CARD domain of CARD9 protein.

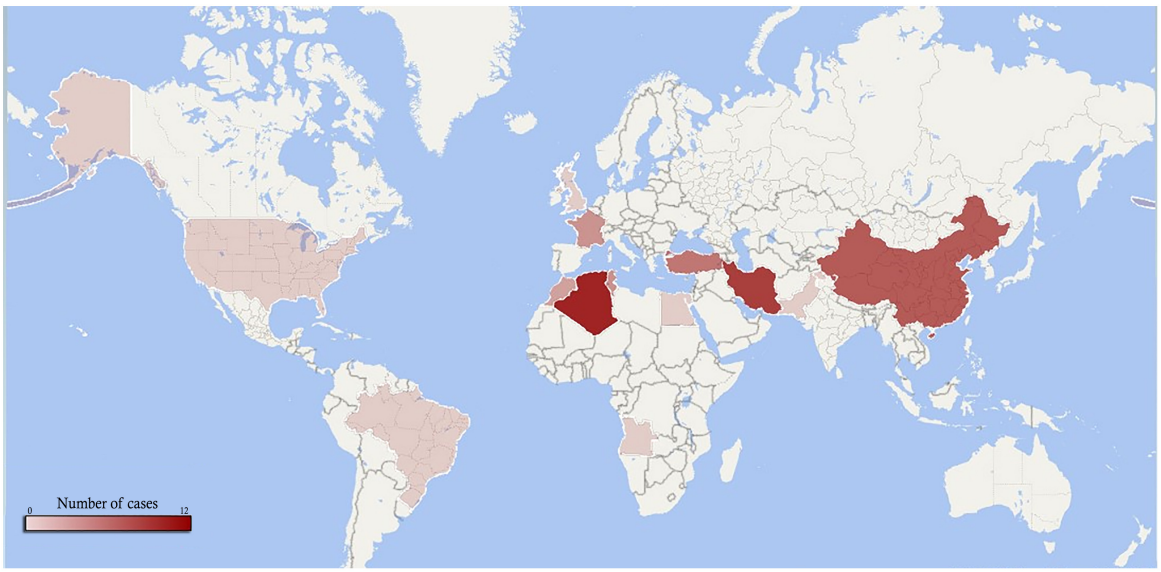

FIGURE 1 | The worldwide distributions of fungal infection cases with CARD9 deficiency.

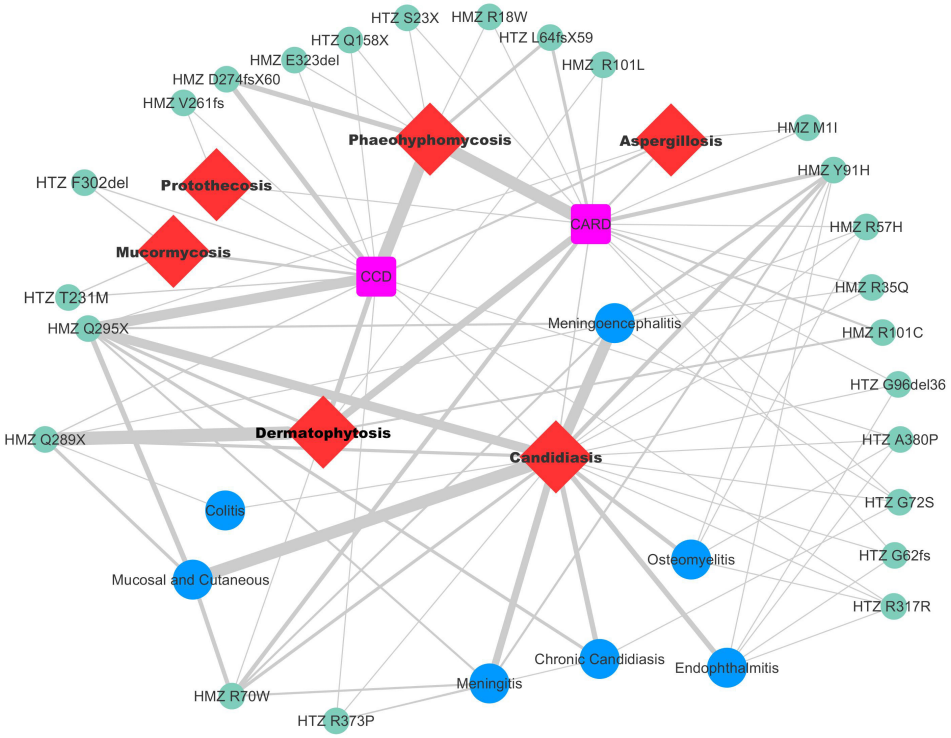

FIGURE 2 | Relation between the types of mutations and different clinical forms. Thick, short, and bold line indicates more reported cases with a mutation and clinical form; and less cases shown with thin and lane lines.

and disseminated phaeohyphomycosis; 2.42 (95% CI 1.84–3.2, $p < 0.001$). This study demonstrated that the HMZ p.Q289X (c.865C > T) mutation had a more than two-fold increased risk of dermatophytosis compared with HMZ p.Q295X (c.883C > T), $p < 0.001$. Similarly, HMZ p.Q295X (c.883C > T) alteration increased by two times the risk of developing candidiasis [OR: 1.95 (95% CI 1.42-2.69, $p < 0{\cdot}001$)] versus dermatophytosis (**Table 2**). *T. violaceum* infected patients carried a marginally higher frequency of HMZ p.Q289X (c.865C > T) compared to non-*T. violaceum* dermatophytosis cases (43 vs. 56%).

TABLE 2 | Analysis of 24 reported mutations among 60 patients with fungal infections.

			Dermatophytosis		Phaeohyphomycosis		Invasive aspergillosis		Candidiasis		Mucormycosis		Protothecosis	
Model type	**Factor**		**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**	**OR (95% CI)**	***P*-value**
Crude analysis	Mutation	HMZ p.Q289X*	1	–	1	–	1	–	1	–	1	–	1	–
		HMZ p.Q295X	0.53 (0.42–0.67)	<0.001	1.09 (0.84–1.43)	0.506	1.09 (0.96–1.25)	0.19	1.95 (1.41–2.67)	<0.001	1 (0.91–1.1)	0.998	1 (0.91–1.1)	0.998
		Other	0.44 (0.36–0.52)	<0.001	1.45 (1.18–1.78)	0.001	1.04 (0.93–1.15)	0.494	1.4 (1.09–1.78)	0.012	1.04 (0.96–1.12)	0.337	1.04 (0.96–1.12)	0.337
	Domain	CCD*	1	–	1	–	1	–	1	–	1	–	1	–
		CARD	0.64 (0.5–0.83)	0.001	1.11 (0.89–1.38)	0.342	1.03 (0.93–1.15)	0.54	1.37 (1.04–1.81)	0.031	0.98 (0.9–1.05)	0.509	0.97 (0.9–1.05)	0.509
Multivariate analysis**	Mutation	HMZ p.Q289X*	1	–	1	–	1	–	1	–	1	–	1	–
		HMZ p.Q295X	0.52 (0.4–0.66)	<0.001	1.15 (0.84–1.57)	0.412	1.11 (0.94–1.3)	0.197	2.09 (1.43–3.07)	<0.001	1.02 (0.91–1.15)	0.728	0.96 (0.85–1.08)	0.477
		Other	0.48 (0.4–0.58)	<0.001	1.45 (1.15–1.83)	0.003	1.05 (0.93–1.18)	0.429	1.4 (1.06–1.85)	0.022	1.05 (0.96–1.14)	0.294	1 (0.92–1.09)	0.908
	Domain	CCD*	1	–	1	–	1	–	1	–	1	–	1	–
		CARD	0.72 (0.57–0.91)	0.007	1.09 (0.87–1.36)	0.468	1.03 (0.93–1.15)	0.582	1.33 (1–1.76)	0.053	0.97 (0.9–1.05)	0.486	0.96 (0.89–1.04)	0.305

*Levels; **The results were adjusted for age and sex; *CCD and HMZ p.Q289X were reference levels in their categories; OR, Odds ratio; CI, Bayesian credible interval; HMZ, homozygote; CCD, coiled-coiled domain; CARD, CARD domain; Mutations in CCD, Q289X, Q295X, R373P, Q158X, D274fsX60, E323del, A380P, R317R, V261fs, T231M, F302del; Mutations in CARD, Y91H, R70W, R35Q, R18W, G62fs, G96del36, R101C, G72S, L64fsX59, R101L, R57H, M11, S23X; Other mutations, Q289X, Q295X are not included.*

A Relationship Between *CARD9* Gene Mutations and Specific Geographic Distribution

The pattern of distribution was differed by geographical region in reported cases with *CARD9* mutations. The main mutations in African patients were different from those in Asians; HMZ p.Q289X (c.865C > T) and HMZ p.R101C (c.C301T), accounting for 75% and 10%, respectively, were the common mutations in Africa. The three most common mutations in Asia were HMZ p.Q295X (c.883C > T), HMZ p.D274fsX60 (c.819-820insG), and HMZ p.R70W (c.208C > T), which accounted for 34.5%, 17.2%, and 13.8% of the Asian cases, respectively. Notably, HMZ p.Q289X (c.865C > T) was the most common mutation observed in 75% of the Algerian patients (9 out of 12), while the HMZ p.Q295X (c.883C > T) mutation was reported in 8 out of 10 Iranian patients (80%). This finding is important as it provides a relationship between mutation and specific geographic occurrence in these patients.

DISCUSSION

CARD9 deficiency is inherited in an autosomal recessive manner. CARD9 plays an important role in the activation of antifungal mechanisms leading to expression of gene products that initiate the inflammatory cascade (Liang et al., 2015; Drummond and Lionakis, 2016). The importance of the CARD9 signaling protein in host defense has been demonstrated in a murine CARD9−/− model with targeted disruptions of innate signaling from the antifungal pattern-recognition receptor, dectin-1, that identifies the β-glucan component of the fungal cell (Taylor et al., 2007). Defective antifungal clearance and latently infected cells could be the result of impaired CARD9 function (Yamamoto et al., 2014; Drummond and Lionakis, 2016). We analyzed the characteristics, distribution, frequency, and relationship between the genotype of the *CARD9* gene mutations and fungal infections among the reported cases. Since the first mutation described in 1989 from Algeria (Boudghène-Stambouli and Mérad-Boudia, 1989), several mutations have been reported from Africa. However, only few reports are from Europe and America. Glocker et al. (2009), reported a novel *CARD9* mutation, HMZ p.Q289X (c.883C > T), in seven Iranian patients. In this review, the spectrum of *CARD9* mutations in Asian patients is higher than in African patients. So far, more than 24 mutations in the *CARD9* gene have been reported associated with severe fungal infections. Among these mutations, HMZ p.Q289X (c.865C > T) was the most common, indicating it is a hot spot in Africa. Infections caused by *T. violaceum* and *C. albicans* dominate, but frequency differ by region. We found a remarkably low prevalence of dermatophyte infection in Asian CARD9 deficiency patients. However, we demonstrate that *Candida* species infection is also uncommon in African patients. Our review showed that the two mutations [HMZ p.Q289X (c.865C > T) and HMZ p.Q295X (c.883C > T)] are present in 44.3% of the patients. Dermatophytosis due to the HMZ p.Q289X (c.865C > T) mutation encompass 75% of African cases and 34.5% of Asian patients have candidiasis associated with HMZ p.Q295X (c.883C > T). However, mutations such as HMZ p.R57H (c.170G > A), heterozygous (HTZ) p.A380P (c.1138G > C) and HMZ p.R70W (c.208C > T) are only found in the United States, United Kingdom, and Turkey, respectively, which suggests that mutations may be specific in particular populations or geographic regions. Another possible explanation is the high rate of consanguinity in many closed groups. Although this autosomal recessive disorder which is rare on a world-wide scale, it may not be rare in some countries. The variations in the gene, which are associated with a specific fungal infection, remain unknown. Asia is the most populous continent in the world and may have a greater genetic burden resulting in more patients with severe fungal infections. Although we cannot exclude other causative factors, our data support the notion that some *CARD9* mutations, circulating in specific geographic regions, could be the contributing factor for fungal infections. However, because of the small sample size, future screening should be conducted to confirm these conclusions. Studying the impact of genetic variation on severe fungal infection will improve our understanding of pathogenesis and may ultimately aid future interventions. CARD9 deficiency should be considered in patients with unexplained progressive fungal infection, as it may allow early initiation of appropriate antifungal treatment. Regular medical follow-up and identification of patients with CARD9 deficiencies is recommended including family members.

CONCLUSION

In recent years, interest in primary immunodeficiency disorders and opportunistic infections has grown. The current study reviewed 60 reported cases with *CARD9* mutations and severe fungal infections, which may provide more information about the relationship between these mutations, the specific geographic presence and the unique predisposition to a particular fungal disease.

AUTHOR CONTRIBUTIONS

AV, HB, and JM conceptualized the study, gathered resources, and wrote, reviewed, and edited the manuscript. AV, HF, ZA, MG, SK, and AA curated the data. AV, HB, and AA performed the formal analysis of the study. HB contributed to funding acquisition, project administration, and data validation. AV and HF investigated the data. AV, HF, ZA, MG, and SK provided methodology for this study. HB and JM supervised the study. AV, HF, ZA, MG, SK, AA, JM, and HB wrote the original draft of the manuscript.

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Azole-Resistance in *Aspergillus terreus* and Related Species: An Emerging Problem or a Rare Phenomenon?

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Objectives: Invasive mold infections associated with *Aspergillus* species are a significant cause of mortality in immunocompromised patients. The most frequently occurring aetiological pathogens are members of the *Aspergillus* section *Fumigati* followed by members of the section *Terrei*. The frequency of *Aspergillus terreus* and related (cryptic) species in clinical specimens, as well as the percentage of azole-resistant strains remains to be studied.

Methods: A global set ($n = 498$) of *A. terreus* and phenotypically related isolates was molecularly identified (beta-tubulin), tested for antifungal susceptibility against posaconazole, voriconazole, and itraconazole, and resistant phenotypes were correlated with point mutations in the *cyp51A* gene.

Results: The majority of isolates was identified as *A. terreus* (86.8%), followed by *A. citrinoterreus* (8.4%), *A. hortai* (2.6%), *A. alabamensis* (1.6%), *A. neoafricanus* (0.2%), and *A. floccosus* (0.2%). One isolate failed to match a known *Aspergillus* sp., but was found most closely related to *A. alabamensis*. According to EUCAST clinical breakpoints azole resistance was detected in 5.4% of all tested isolates, 6.2% of *A. terreus sensu stricto (s.s.)* were posaconazole-resistant. Posaconazole resistance differed geographically and ranged from 0% in the Czech Republic, Greece, and Turkey to 13.7% in Germany. In contrast, azole resistance among cryptic species was rare 2 out of 66 isolates and was observed only in one *A. citrinoterreus* and one *A. alabamensis* isolate. The most affected amino acid position of the *Cyp51A* gene correlating with the posaconazole resistant phenotype was M217, which was found in the variation M217T and M217V.

Conclusions: *Aspergillus terreus* was most prevalent, followed by *A. citrinoterreus*. Posaconazole was the most potent drug against *A. terreus,* but 5.4% of *A. terreus sensu stricto* showed resistance against this azole. In Austria, Germany, and the United Kingdom posaconazole-resistance in all *A. terreus* isolates was higher than 10%, resistance against voriconazole was rare and absent for itraconazole.

Keywords: cryptic species, *Aspergillus* section *Terrei*, susceptibility profiles, azoles, *Cyp51A* alterations

INTRODUCTION

In the last decade, the taxonomy and nomenclature of the previously morphologically defined genus *Aspergillus* changed, mainly due to comprehensive molecular phylogenetic studies and the introduction of the single name nomenclature (Samson et al., 2011, 2014; Alastruey-Izquierdo et al., 2013). With the introduction of molecular identification methods morphologically similar species were split into several cryptic species (Balajee et al., 2009a,b; Samson et al., 2011; Gautier et al., 2014). Samson et al. (2011) recognized 13 species in section *Terrei*: *A. terreus sensu stricto (s.s.), A. alabamensis*, *A. allahabadii*, *A. ambiguus*, *A. aureoterreus*, *A. carneus*, *A. floccosus*, *A. hortai*, *A. microcysticus*, *A. neoafricanus*, *A. neoindicus*, *A. niveus,* and *A. pseudoterreus*. In 2015, Guinea et al. (2015) described *A. citrinoterreus* as a new

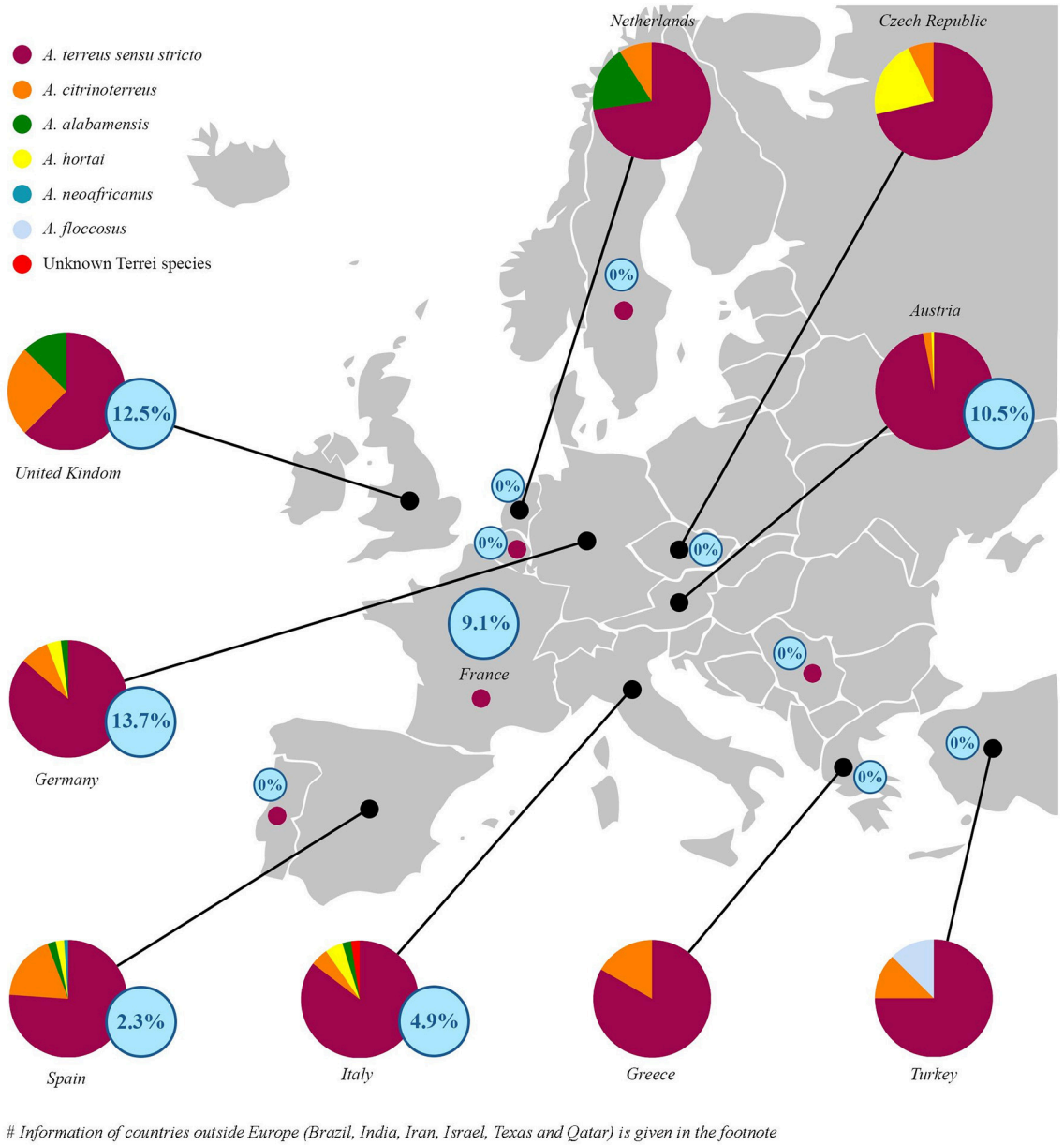

FIGURE 1 | Epidemiological distribution of species (circles) and relative percentage of posaconazole resistance (according to EUCAST clinical breakpoints, see **Table 2**) isolates per country (blue numbers in blue circles) in respect to all investigated isolates. In France, Portugal, Serbia, and Sweden all collected isolates were identified as *A. terreus* sensu stricto (small dots in magenta). Azole-resistance percentage per countries are given in blue circled numbers. Species distribution in non-EU countries were as follows: India 100% *A. terreus s.s.*; Israel 84.85% *A. terreus s.s.* 12.12% *A. citrinoterreus* 3.03% *A. hortai*; Texas 80% *A. terreus s.s.* 10% *A. alabamensis* 10% *A. hortai*; Qatar: 83.34% *A. terreus s.s.* 16.66% *A. citrinoterreus*; Iran 63.64% *A. terreus s.s.* 36.36% *A. citrinoterreus*; and Brazil 85.71% *A. terreus s.s.*, 14.29% *A. hortai*. All isolates from Iran, Israel, India, Brazil, Texas, and Qatar were susceptible to all azoles tested. For detailed information see **Table 4**.

species of the section *Terrei* and subsequently *A. bicephalus* and *A. iranicus* were introduced (Arzanlou et al., 2016; Crous et al., 2016), resulting in a total of 16 accepted species.

Aspergillus terreus s.s., an important cause of fungal infections in immunocompromised patients, is reported as second or third most common pathogen of invasive aspergillosis (Baddley et al., 2003; Lass-Flörl et al., 2005; Blum et al., 2008). Treatment of infections caused by *A. terreus s.s.* and other section *Terrei* species (Walsh et al., 2003; Risslegger et al., 2017) may be difficult because of intrinsic amphotericin B resistance (Sutton et al., 1999; Escribano et al., 2012; Hachem et al., 2014; Risslegger et al., 2017). In addition, the emergence of *A. terreus sensu lato* (*s.l.*) isolates with reduced azole-susceptibility was reported (Arendrup et al., 2012; Won et al., 2017). Azole resistance in *A. terreus s.s.* and *A. fumigatus* is associated with mutations

TABLE 1 | Clinical breakpoints according to EUCAST[1].

Antifungal agent	MIC (mg/L)	
	S	R
Posaconazole	≤0.125	>0.250
Voriconazole*	≤1.000	>2.000
Itraconazole	≤1.000	>2.000

[1] *http://www.eucast.org/clinical_breakpoints/*

*MIC, minimum inhibitory concentration; *CBPs are only available for Aspergillus fumigatus.*

and alterations of the lanosterol-14-α steroldemethylase gene (*Cyp51A*), a key protein in the ergosterol biosynthesis pathway (Chowdhary et al., 2015, 2017). However, aside from mutations in the primary target gene, also other less known mechanisms (e.g., efflux pumps, overexpression of *cyp51*) were found to be involved in azole resistance (Arendrup, 2014; Rivero-Menendez et al., 2016).

The aim of this study was to evaluate the frequency of *A. terreus s.s.* and phenotypically similar (cryptic) species in a global set of clinical isolates and to screen for the presence of azole resistance.

MATERIALS AND METHODS

Fungal Isolates

During an international *A. terreus* survey (Risslegger et al., 2017) various *A. terreus sensu lato (s.l.)* isolates were sent to and collected at the Medical University of Innsbruck by members of the ISHAM-ECMM-EFISG *TerrNet Study group* (www.isham.org/working-groups/aspergillus-terreus). Isolates were from Europe ($n = 390$), Middle East ($n = 70$), South America ($n = 10$), North America ($n = 7$), and South Asia ($n = 19$). A total of 498 strains, including isolates collected in Innsbruck within the last years, were analyzed (Supplementary Figure S1 and Supplementary Table S1), 495 were of clinical and 3 of environmental origin. For two isolates, the source is unknown. Isolates were cultured on Sabouraud's agar (Becton Dickinson, France), incubated at 37°C and stored in Sabouraud's broth with glycerin at −20°C.

Antifungal Susceptibility Testing

Susceptibility to itraconazole, posaconazole, and voriconazole was determined by using reference broth microdilution according to EUCAST (www.EUCAST.org) and ETest® (bioMérieux, France). ETest® MICs were rounded to the next higher EUCAST concentrations and isolates displaying high MICs (≥0.25 mg/L for posaconazole, ≥2.0 mg/L for each, voriconazole and itraconazole) with ETest® were evaluated according to EUCAST. MIC_{50} and MIC_{90} were calculated for all studied section *Terrei* strains and each individual species. EUCAST clinical breakpoints (CBP) for *Aspergillus fumigatus* (see **Table 3**) were applied for wild typ and non-wildtyp categorization, as CBP for *Aspergillus terreus* are not available.

Molecular Identification

Genomic DNA was extracted by a method using CTAB (Lackner et al., 2012), and partial β-tubulin gene was amplified using bt2a/bt2b as previously described (Balajee et al., 2009a; Kathuria et al., 2015). KAPA2G Robust HotStart ReadyMix PCR Kit (Kapa Biosystems, USA) was used as master mix and PCR products were cleaned with ExoSAP-IT. For sequencing the BigDye XTerminator purification kit (Applied Biosystems, USA) was used. Sequencing was performed with the 3500 Genetic Analyzer (Applied Biosystems, USA) and data were analyzed with Bionumerics 6.6. Software (Applied Maths, Belgium). Generated sequences were compared with an in-house database of the Westerdijk Institute containing all available *Aspergillus* reference sequences.

Sequencing of Lanosterol 14-α Sterol Demethylase Gene (*cyp51A*)

Azole-resistant isolates (**Table 3**) and a control set of susceptible isolates (Supplementary Table S2) underwent *Cyp51A* sequencing. *Cyp51A* genes were amplified by PCR, using KAPA2G Robust HotStart ReadyMix PCR Kit (Kapa Biosystems, USA) and in-house designed primers described by Arendrup et al. (2012). In short, PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 2 min 30 s, and a final elongation step of 72°C for 10 min. Primers used for *Cyp51A* sequencing are provided in Supplementary Table S3. PCR products were cleaned with ExoSAP-IT and for sequencing the BigDye XTerminator purification kit was used. Sequencing was performed with the 3500 Genetic Analyzer and data were analyzed with Bionumerics 6.6. Software and Geneious 8 (Biomatters Limited).

RESULTS AND DISCUSSION

Epidemiology of Cryptic Species

Reports on cryptic species within the genus *Aspergillus* are on the rise (Balajee et al., 2009b; Alastruey-Izquierdo et al., 2013; Negri et al., 2014; Masih et al., 2016) and display variabilities in antifungal susceptibility (Risslegger et al., 2017). Negri et al. (2014) observed an increase of cryptic *Aspergillus* species causing fungal infections, and others calculated a prevalence of 10–15% of cryptic *Aspergillus* species in clinical samples (Balajee et al., 2009b; Alastruey-Izquierdo et al., 2013).

The present study analyzed a large number of isolates ($n = 498$) collected from Europe, Middle East, South America, North America, and South Asia (Supplementary Table S1 and Supplementary Figure S2) and identified *A. terreus* ($n = 432$), *A. citrinoterreus* ($n = 42$), *A. alabamensis* ($n = 8$), *A. hortai* ($n = 13$), *A. floccosus* ($n = 1$), and *A. neoafricanus* ($n = 1$). As previously reported (Risslegger et al., 2017) one isolate failed to be associated with any existing species, but clustered most closely to *A. alabamensis* (Supplementary Figure S1).

Our study showed limitations due to the unknown source and date of some clinical isolates. A differentiation between isolates from superficial and deep seeded infections was not made, therefore, source-variable resistance rates cannot be excluded. Number of studied isolates varied per country and might also introduce a bias to resistance rates.

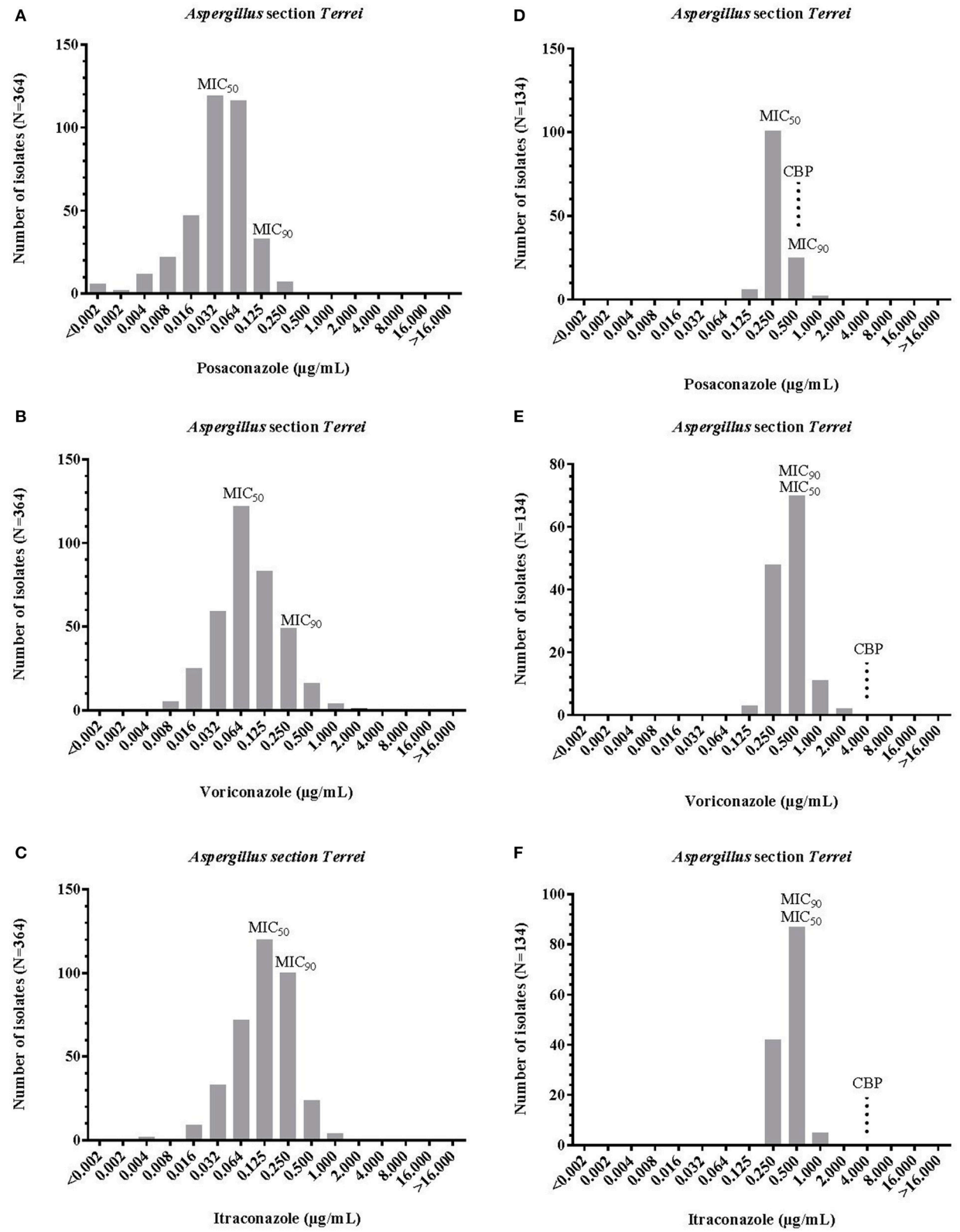

FIGURE 2 | MIC distribution of posaconazole, itraconazole, voriconazole, and posaconazoleintraconazole against Aspergillus section Terrei, obtained by ETest® **(A-C)** and EUCAST method **(D-F)**. MIC, minimum inhibitory concentration; MIC_{50} and MIC_{90}, MIC for 50 and 90% of tested population; CBP EUCAST clinical breakpoint (see **Table 2**).

TABLE 2 | Antifungal susceptibility of *A. terreus s.s.* and related (cryptic) species (Balajee et al., 2009a,b; Samson et al., 2011; Gautier et al., 2014).

Species	PSC (mg/L)			VRC (mg/L)			ITC (mg/L)		
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
***A. terreus sensu stricto* ($n = 432$)**									
Etest® ($n = 315$)	<0.002–0.500	0.032	0.125	0.008–4.000	0.064	0.250	0.016–2.000	0.125	0.250
EUCAST ($n = 117$)	0.125–0.500	0.250	0.500	0.125–1.000	0.500	0.500	0.250–1.000	0.500	0.500
Cryptic species ($n = 66$)									
Etest® ($n = 55$)	<0.002–0.190	0.032	0.064	0.012–4.000	0.064	0.500	0.003–0.380	0.064	0.250
EUCAST ($n = 11$)	0.125–0.250	NA	NA	0.125–2.000	NA	NA	0.125–0.250	NA	NA

Minimum inhibitory concentrations (MICs) of posaconazole, voriconazole, and itraconazole were obtained by ETest® and EUCAST method.
MIC, minimum inhibitory concentration; MIC_{50} and MIC_{90}, MIC for 50 and 90% of tested population; ITC, itraconazole; VRC, voriconazole; POS, posaconazole; EUCAST, European Committee for Antimicrobial Susceptibility Testing; NA, not applicable; N, number of tested isolates.

Aspergillus terreus s.s. was the most prevalent species (86.8%), followed by *A. citrinoterreus* (8.4%), *A. hortai* (2.6%), and *A. alabamensis* (1.6%). This is in agreement with other authors (Balajee et al., 2009a; Neal et al., 2011; Escribano et al., 2012; Kathuria et al., 2015) showing that *A. terreus s.s.* is the most common species of section *Terrei* in clinical and environmental samples. In addition, we detected *A. floccosus* and *A. neoafricanus*. We did not identify *A. allahabadii*, *A. ambiguus*, *A. aureoterreus*, *A. bicephalus*, *A. carneus*, *A. iranicus*, *A. microcysticus*, *A. neoindicus*, *A. niveus,* and *A. pseudoterreus*. The reason for this might be that these species are less common in clinical samples and the environment. Our species distribution is in line with Kathuria et al. (2015), who reported for the first time a probable invasive aspergillosis and aspergilloma case due to *A. hortai,* which was found to occur in a prevalance of 1.4% of all section *Terrei* isolates. A multicenter study by Balajee et al. (2009a) observed a high frequency (33% of all clinical *A. terreus s.l.* isolates were *A. alabamensis*) of *A. alabamensis.* Other studies (Neal et al., 2011; Gautier et al., 2014; Risslegger et al., 2017) reported a lower prevalence of *A. alabamensis* isolates (up to 4.3%).

Little is known about the geographical distribution of cryptic species of section *Terrei* in clinical specimens. *A. terreus s.s.* was exclusively found in France, Portugal, Serbia, India, and Sweden (Supplementary Table S1). Spain, Italy, Texas and Germany showed highest species diversity (**Figure 1** and Supplementary Table S1). In Spain, the prevalent cryptic species were *A. citrinoterreus* (18.2%), *A. alabamensis* (2.3%), *A. hortai* (2.3%), and *A. neoafricanus* (1.1%), in Italy *A. citrinoterreus* and *A. hortai* (4.9%), together with one *A. alabamensis* (2.4%) and one unknown *Terrei* species (2.4%). In Germany *A. citrinoterreus* (7.8%) was followed by *A. hortai* (3.9%), and *A. alabamensis* (2.0%). In Texas 80.0% were *A. terreus s.s.* followed by 10% *A. alabamensis* and 10.0% *A. hortai*. Percentage of *A. citrinoterreus* was highest in Iran accounting 36.36% of all isolates (**Figure 1**).

Azole Resistance Among Studied Section *Terrei* Isolates

Proposed epidemiological cut off values (ECOFF) values by EUCAST for *A. terreus s.s.* were 0.25 μg/mL for posaconazole, 2 μg/mL each for voriconazole and itraconazole. Antifungal susceptibility results (MICs) for *A. terreus s.s.* and cryptic species of the section *Terrei* are reported in **Table 1** and **Figure 2**. Posaconazole had the lowest MICs for section *Terrei* isolates (MIC_{50}, 0.032 μg/mL Etest® and 0.250 μg/mL EUCAST), followed by itraconazole (MIC_{50}, 0.125 μg/mL Etest® and 0.500 μg/mL EUCAST), and voriconazole (MIC_{50}, 0.064 μg/mL Etest® and 0.500 μg/mL EUCAST) (**Figure 2**). Lass-Flörl et al. (2009) observed similar MIC values for posaconazole among clinical isolates of *A. terreus s.l.* Astvad et al. (2017) tested *A. terreus* species complex isolates against voriconazole and observed slightly higher MIC ranges of 0.250–8.000 μg/mL.

No major differences in azole susceptibility profiles for *A. terreus s.s.* and cryptic species were observed (**Table 2**). Posaconazole and itraconazole MIC ranges for *A. terreus* were only slightly higher when compared to cryptic species. As shown in **Table 2**, $MICs_{50}$ obtained with Etest® are equal among *A. terreus s.s.* isolates and cryptic species for posaconazole (0.032 μg/mL) and voriconazole (0.064 μg/mL). No significant differences in MIC_{90} values were observed among *A. terreus s.s.* isolates and cryptic species for itraconazole and posaconazole. Voriconazole $MICs_{90}$ were somewhat higher among cryptic species (0.500 μg/mL) when compared to *A. terreus s.s.* (0.250 μg/mL). In general, all cryptic *A. terreus* species were per trend more susceptible to posaconazole and itraconazole than *A. terreus s.s.* The two most common cryptic species in our study, *A. citrinoterreus,* and *A. alabamensis*, showed highest MICs for voriconazole (range: 0.016–2.000 and 0.023–2.000 μg/mL).

According to EUCAST breakpoints 5.4% of all section *Terrei* isolates are posaconazole resistant. This is a relatively high frequency in comparison to *A. fumigatus.* A prospective multicenter international surveillance study (van der Linden et al., 2015) showed a prevalence of azole-resistance of 3.2% in *A. fumigatus*. As shown in **Table 3**, only mono-azole resistance was observed (posaconazole, MICs ranged from 0.500 to 1.000 μg/mL). Azole resistance was more frequently observed among *A. terreus s.s.* isolates and was rare among cryptic species. One *A. citrinoterreus* isolate was resistant against posaconazole (0.500 μg/mL). Posaconazole resistant strains were detected from Germany (13.7%) followed by the United Kingdom

TABLE 3 | Summary of mutations detected in azole-resistant *A. terreus and A. citrinoterreus*.

Species	Isolate	EUCAST MIC(mg/L)			Mutation (NA)	Substitution (AA)
		VRC	ITC	POS		
A. terreus sensu stricto						
(*n* = 26)	51	0.500	2.000	0.500	M217T	T650C
	10	0.500	0.250	0.500	No mutation	
	138	1.000	0.500	1.000	M217V, D344N	A649G, G1030A
	368	1.000	0.500	1.000	No mutation	
	T104	0.500	1.000	0.500	No mutation	
	T112	0.500	0.500	0.500	E319G	A956G
	T13	0.500	0.500	0.500	No mutation	
	T136	0.500	0.500	0.500	No mutation	
	T15	0.500	1.000	0.250	No mutation	
	T152	0.500	0.500	0.500	No mutation	
	T153	0.500	0.500	0.500	A221V	C662T
	T156	0.500	0.500	0.500	No mutation	
	T157	0.500	0.500	0.500	No mutation	
	T159	0.500	0.500	0.500	No mutation	
	T160	0.500	0.500	0.500	No mutation	
	T55	0.500	0.500	0.500	No mutation	
	T59	0.500	0.250	0.500	No mutation	
	T61	0.500	0.500	0.500	No mutation	
	T65	0.500	0.500	0.500	No mutation	
	T67	0.500	0.500	0.500	No mutation	
	T68	0.500	0.500	0.500	No mutation	
	T80	0.500	0.500	0.500	No mutation	
	T9	0.500	0.500	0.250	No mutation	
	T91	0.500	0.500	0.500	No mutation	
	T98	0.500	0.500	0.500	No mutation	
	16	0.500	1.000	1.000	No mutation	
A. citrinoterreus						
(*n* = 1)	150	0.500	0.500	1.000	I23T, R163H, E202D, Q270R	T69C, G489A, G607C, A810G

*Susceptibily was determined by EUCAST and resistance categorization was based on EUCAST clinical breakpoints (see **Table 1**).*
MIC, minimum inhibitory concentration; NA, nucleic acid; AA, Amino acid; ITC, itraconazole; VRC, voriconazole; POS, posaconazole: resistant strains based on the EUCAST Antifungal Clinical Breakpoints. EUCAST. European Committee for Antimicrobial Susceptibility Testing.

TABLE 4 | Posaconazole resistance per country relative to (1) all studied isolates and (2) *A. terreus s.s.* only (also see **Figure 1**).

Country	All isolates studied (%)	*A. terreus sensu stricto* (%)
Austria	10.5	10.9
France	9.1	9.1
Germany	13.7	15.9
Italy	4.9	5.7
Spain	2.3	1.5
UK	12.5	12.5
Iran	0.0	0.0
Israel	0.0	0.0
India	0.0	0.0
Brazil	0.0	0.0
Texas	0.0	0.0
Qatar	0.0	0.0

(12.5%), Austria (10.5%), France (9.1%), Italy (4.9%), and Spain (2.3%) (**Tables 3**, **4** and **Figure 1**). In Turkey, Greece, Serbia, Iran, Israel, India, Brazil, Texas, and Qatar all isolates were susceptible against all azoles tested. However, resistance rates per countries might be influenced by multiple factors such as specimen handling and sampling, and investigated patient cohorts.

Posaconazole showed to be the most effective azole against *A. terreus s.s.* and related (cryptic) species. However, a high frequency of posaconazole resistant isolates was detected and it was shown that the occurrence of azole resistance differed geographically. Posaconazole resistance among cryptic species was rare when compared to *A. terreus s.s.*.

SNPs in the *Cyp51A* Gene

Mutations at the position M217 were reported to be associated with reduced susceptibility against itraconazole (MICs of 1.0–2.0 μg/mL), voriconazole (MICs of 1.0–4.0 μg/mL), and posaconazole (MICs of 0.25–0.5 μg/mL) (Arendrup et al., 2012), however the substituting amino acids varied from the one found in our study. Our isolates carried the mutations M217T (nucleic acid change T650C) or M217V (nucleic acid change A649G) (**Table 3**) and were exclusively resistant against posaconazole, when applying the EUCAST clinical breakpoints. Strains carrying the point mutation M217I in the study from Arendrup et al. (2012) were isolated from cystic fibrosis patients receiving long-term azole therapy and showed a pan-azole resistant phenotype. Another posaconazole resistant isolate (T153) carried an amino acid substitution at position A221V, a mutation, which was also previously reported by Arendrup et al. (2010), but was not associated with posaconazole resistance. Hence, functional studies in mutant strains are needed to evaluate the role of the mutations M217V, M217I, M217T, and A221V, which are all located in close proximity to the hot spot mutation M220I of *A. fumigatus*. Understanding the impact of mutations at the position M217 on the protein folding pattern and subsequently on binding capacities of azoles is the key to evaluate its role as azole-resistance markers. Other hotspot mutations, which were linked to acquired azole-resistance in *A. fumigatus,* are G54, L98, and M220 (Arendrup et al., 2010). None of them were found in our resistant isolates, suggesting different mechanisms of acquired azole-resistance than in *A. fumigatus*. The role of the other coding mutations within *A. terreus s.s.* isolates E19G (nucleic acid substitution A956G) and D344N (nucleic acid substitution C662T) remains to be studied. Voriconazole resistant *A. citrinoterreus* carried the amino acid changes I23T,

R163H, E202D, Q270R (**Table 3**), which need to be analyzed in detail.

CONCLUSIONS

Aspergillus terreus s.s. was most prevalent, followed by *A. citrinoterreus*. Posaconazole was the most potent azole against the investigated isolates and species. Approximately 5% of all tested *A. terreus s.s.* isolates were resistant against posaconazole *in vitro*. In Austria, Germany and the UK posaconazole resistance was higher than 10% in all *A. terreus s.s.* isolates. Resistance against itraconazole and voriconazole was rare.

AUTHOR CONTRIBUTIONS

TZ: manuscript writing, Etest susceptibility testing, data analysis and interpretation, discussion of results, DNA extraction, sequencing; BS: wrote parts of the manuscript (M&M), DNA extraction, sequencing, nucleic acid alignments, and amino acid alignments; LS: EUCAST susceptibility testing, DNA extraction; JH: BLAST comparison of sequences, molecular species identification; BR: culturing of isolates, subcultivation of isolates, morphological identification, data management; CL-F: manuscript writing, discussion of results, clinical background, funding, coordination of the TerrNet study group, isolate recruitment; ML: manuscript writing, data analysis, study design, supervising TZ, BS, and LS; MA, FS-R, AR, AnC, ST-A, MA, SO, DK, AA-I, KL, GL, JM, WB, CF, MD-A, AG, AT, BW, AH, EJ, LK, VA-A, OC, JM, WP, VT, J-JV, LT, RL, ES, P-MR, PH, MR-I, ER, SA-A, ArC, ALC, MF, MM-G, HB, GP, NK, SH, OU, MR, SdlF are members of the EFISG-ISHAM-ECMM TerrNet Study group: providing strains and data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00516/full#supplementary-material

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Determination of the Prevalence of Triazole Resistance in Environmental *Aspergillus fumigatus* Strains Isolated in South Wales, UK

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Background/Objectives: Azole resistance in *Aspergillus fumigatus* associated with the TR34/L98H mutations in the *cyp51A* gene have been increasingly reported. Determining the environmental resistance rate has been deemed important when considering front-line therapy for invasive aspergillosis. The aim of the study was to determine prevalence of azole resistance in environmental *A. fumigatus* isolates across South Wales.

Methods: Over 5 months in 2015, 513 *A. fumigatus* isolates were cultured from 671 soil and 44 air samples and were screened for azole resistance using VIPcheck™ agar plates containing itraconazole, voriconazole and posaconazole. Resistance was confirmed by the CLSI M38-A2 methodology. The mechanism of resistance was investigated using the PathoNostics AsperGenius® Assay.

Results: Screening by VIPcheck™ plate identified azole-resistance in 30 isolates, most of which (28/30) harbored the TR34/L98H mutation, generating a prevalence of 6.0%. Twenty-five isolates had a MIC of ≥2 mg/L with itraconazole, 23 isolates had a MIC of ≥2 mg/L with voriconazole and seven isolates had a MIC ≥0.25 mg/L with posaconazole. All isolates deemed resistant by VIPcheck™ plates were resistant to at least one azole by reference methodology.

Conclusions: There is significant environmental azole resistance (6%) in South Wales, in close proximity to patients susceptible to aspergillosis. Given this environmental reservoir, azole resistance should be routinely screened for in clinical practice and environmental monitoring continued.

Keywords: *A. fumigatus*, azole resistance, TR34, L98H, environmental prevalence

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INTRODUCTION

Aspergillus fumigatus causes a variety of diseases in humans ranging from allergic bronchopulmonary aspergillosis (ABPA), through chronic pulmonary aspergillosis and aspergilloma, to invasive aspergillosis (IA) (Latge, 1999). Triazole therapy has become the established treatment for these infections, based on their proven efficacy and ease of administration, but patients often require prolonged therapy (Patterson et al., 2016). Monitoring for resistance to these drugs is therefore imperative, as treatment failure, or delayed appropriate therapy will increase mortality.

Current data suggests that resistance can develop by two routes; *in situ* within the lungs of a chronically infected patient as a result of prolonged exposure, or by acquisition of a resistant *A. fumigatus* strain from the environment, potentially driven by agricultural use of azole compounds (Verweij et al., 2009; Denning et al., 2013). The prevalence of azole resistant *A. fumigatus* in most countries is still not clearly determined as routine resistance testing is not common practice. A recent report from the European Centre for Disease Prevention and Control acknowledges that resistant *A. fumigatus* strains are spreading in many European countries and efforts to monitor resistance rates need to be undertaken (Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species., 2013). Environmental surveillance studies of azole resistance have been undertaken in many countries including Italy, Austria, India, Thailand and the USA (Mortensen et al., 2010; Chowdhary, 2012; Prigitano et al., 2014; Hurst et al., 2017; Tangwattanachuleeporn, 2017). Resistant isolates harboring either TR34/L98H or TR46/Y121F/T289A mutations have been found in environmental and clinical samples from several countries, raising concern that azole resistance could become a global public health threat with fungal spores able to disperse great distances on air currents (Bader, 2015; Verweij et al., 2015a).

Data is also lacking in the UK. Research performed by the Centre for Aspergillosis in Manchester showed resistance rates in clinical isolates collected between 1992 and 2007 was approximately 6%. A great variability of mutations in the *cyp51*A gene (18 in total, including two TR34/L98H mutations) was found with no prevalent mutation, indicating a clinically driven route of resistance (Howard et al., 2009). In the corresponding study of several hundred environmental isolates, from both rural and urban areas, only one isolate with azole resistance driven by the TR34/L98H mutation was found, originating from the rural environment (Bromley et al., 2014).

The aim of the present study was to investigate the prevalence of azole resistance in environmental *A. fumigatus* isolates, collected primarily in South Wales, and to establish an appropriate method for screening for azole resistance. One such method which utilizes agar with set concentrations of three different azoles (VIPcheck™ plates) was devised in the Netherlands and showed promising results (Van der Linden et al., 2009; Buil et al., 2017). It is a cheap, fast and simple method requiring no specialist skill or equipment to screen a high number of specimens. If accurate it would be ideal as a screening tool suitable for use outside of reference settings.

MATERIALS AND METHODS

Environmental Sampling

Soil Samples

From June to November 2015, 671 soil samples were collected from urban and rural locations in South Wales (**Table 1** and **Figure 1**). The samples were treated as previously described to optimize recovery of *A. fumigatus*, with minor modifications (Snelders et al., 2009). Briefly, 2 g of soil or compost was dissolved in 8 mL of sterile distilled water with 1% Tween 20 (Sigma, Haverhill, UK). After mixing it was left to sediment for 30–60 min at room temperature and 100 μL of the supernatant was used to inoculate two plates of Sabouraud dextrose agar supplemented with chloramphenicol (E&O Laboratories Limited, Bonnybridge, UK). The plates were incubated at 37° and 42°C to maximize the selective yield of *A. fumigatus* isolates, and examined after 48 h of incubation. *A. fumigatus* isolates were phenotypically identified by observation of macroscopic and microscopic morphology.

Air Samples

During the same time period 44 air samples were collected from clinical sites in three major hospitals in the Cardiff and Newport area. Air samples were collected with single-headed SAS super 100 Air sampler (Cherwell Laboratories, Bicester, UK). Five hundred liters of air was directed onto each contact Sabouraud plate supplemented with chloramphenicol. The plates were then incubated and any *A. fumigatus* isolates were confirmed as previously described.

Screening for Azole Resistance

Primary screening for azole resistance was performed using the commercially available VIPcheck™ plates containing azoles (Balis Laboratorium VOF, Leeuwen, Netherlands). The four well screening plates contained RPMI-1640 agar medium supplemented with 4 mg/L itraconazole, 2 mg/L voriconazole and 0.5 mg/L posaconazole and a fourth control well (**Figure 2**). Using a dry cotton wool swab, conidia from fresh sporulating colonies were suspended in sterile water to produce a suspension equivalent to 0.5–2 McFarland standard. All the wells were inoculated with 25 μL of the suspension. To enhance efficiency and reduce costs of screening, conidia from more than one colony were combined to prepare the suspensions. However, when multi-azole resistance was noted each well exhibiting growth of *A. fumigatus* was individually sub-cultured and VIPcheck™ plates inoculated to determine if there was a single multi-azole resistant strain or if there were individual strains with different patterns of azole resistance. The screening plates were then incubated at 37°C for 48 h and visually inspected, with growth quantified as good/confluent (+), sporadic (±) or no growth (–). Resistance was determined by good/confluent growth on at least one azole containing well, with or without growth on the other two azole containing wells. Where confluent growth was not present on at least one azole containing well but sporadic growth was observed on at least one azole containing well the isolate was classified as indeterminate.

Any resistant isolates were subsequently confirmed by comparison to the reference CLSI broth micro-dilution (CLSI) M38-A2 method, performed at the Public Health England Mycology Reference Laboratory in Bristol. Resistance to itraconazole and voriconazole in *A. fumigatus* was determined using epidemiological cut-offs (1 mg/L) as defined in the CLSI M59 ED1E document (CLSI: M59-ED1., 2016). As an epidemiological cut-off for *A. fumigatus* to posaconazole is not available in the CLSI M59 document EUCAST antifungal clinical breakpoint (Version 8.0) of >0.25 mg/L was used (EUCAST, 2015).

TABLE 1 | Soil sampling areas and rates of *A. fumigatus* recovery, with azole resistance determined by VIPcheck™ plate.

Sampling site	Samples collected (*n*)	Number of *A. fumigatus* isolates recovered (%)	Azole susceptibility[a]			Resistance rates (95% CI)
			Resistant (*n*)	Indeterminate (*n*)	Sensitive (*n*)	
AGRICULTURE						
Overall	419	288 (68.7)	15	49	224	5.2% (3.2–8.4)
Beans fields	37	29 (78.4)	1	8	20	3.4% (0.6–17.2)
Cereal fields	171	120 (70.2)	4	21	95	3.3% (1.3–8.3)
Clover fields	11	6 (54.5)	0	0	6	0% (0.0–3.9)
Compost	6	4 (66.6)	0	1	3	0% (0.0–4.9)
Corn fields	37	26 (70.3)	4	7	15	15.4% (6.2–33.5)
Environmental strips	2	2 (100)	0	0	2	0% (0.0–6.6)
Grass fields	7	2 (28.6)	1	0	1	50.0% (9.5–90.6)
Potato fields	83	46 (55.4)	1	5	40	2.2% (0.4–11.3)
Rapeseed fields	65	53 (81.5)	4	7	42	7.5% (3.0–17.9)
NONPUBLIC AREAS						
Overall	35	28 (80.0)	0	9	19	0% (0.0–11.4)
Private allotments	2	2 (100)	0	1	1	0% (0.0–65.8)
Private gardens	33	26 (78.8)	0	8	18	0% (0.0–12.1)
HORTICULTURAL NURSERY						
Compost	1	1 (100)	0	0	1	0% (0.0–79.4)
PUBLIC AREAS						
Overall	216	179 (91.2)	15	32	132	8.4% (5.1–13.4)
Botanical gardens	31	27 (87.1)	7	7	13	25.9% (13.2–44.7)
Compost	5	5 (100)	0	1	4	0% (0.0–43.5)
Hospital grounds	26	24 (92.3)	1	5	18	4.2% (0.7–20.2)
Parks	35	22 (62.9)	0	0	22	0% (0.0–14.9)
Public gardens	119	101 (84.9)	7	19	75	6.9% (3.4–13.6)
(Hospital gardens)[b]	(27)	[22 (81.5)]	(3)	(6)	(13)	[13.6% (4.8–33.3)]
COMBINED TOTAL	671	496 (73.9)	30	90	376	6.0% (4.3–8.5)

[a] *Resistance determined by confluent growth in the presence of at least one azole antifungal drug.*

[b] *Data for Hospital Gardens is accounted for in the data for Public gardens and to avoid duplication has not been included separately in the overall counts.*

Molecular Determination of the Azole Resistance Mechanisms

All resistant strains, 30 indeterminate and 20 sensitive strains had DNA extracted from conidia by bead-beating and automated nucleic acid extraction using the EZ1 Advance XL tissue kit (Qiagen, Crawley, UK). Conidia from sporulating cultures were mechanically disrupted for 30 s using the equivalent of 20–30 μL of MagNA Lyser green ceramic beads (Roche, Burgess Hill, UK) and a Mini bead-beater (Biospec Products, Bartlesville, Oklahoma, USA). The beads were then washed with 190 μL of G2 buffer (from EZ1 Tissue kit) and the extraction was completed following the manufacturer's protocol. Nucleic acid was eluted in 100 ul, with a further incubation at 70°C for 10 min to remove excess ethanol.

Extracted DNA was subsequently tested using a commercially available AsperGenius® real-time PCR assay (PathoNostics, Maastricht, Netherlands). The AsperGenius® assay is a multiplex real-time PCR assay that can detect *A. fumigatus*, *A. terreus*, *Aspergillus* species (Chong, 2015). In a second multiplex reaction, the AsperGenius® resistance assay has the ability to detect the four mutations (TR34, L98H, Y121F, and T289A), representing the most prevalent mutations L98H/TR34 and TR46/Y121F/T289A in the *cyp*51A gene of *A. fumigatus* associated with azole resistance. The successful amplification of the region of *cyp*51A gene also confirmed the isolate to be *A. fumigatus*. PCR was performed in a 12.5 μL volume containing 5 μL AsperGenius® Resistance mastermix, 1 μL Taq polymerase, 4 μL dilution buffer and 2.5 μL DNA extract. PCR was performed on a Rotorgene Q high-resolution melt instrument (Qiagen, Crawley, UK) following the manufacturer's instructions.

Statistical Analysis

Ninety-five percent confidence intervals (95% CI) were generated for each proportionate value and, where required, two-tailed P values were calculated using Fishers exact test; (P: 0.05) to determine the significance between rates. Observed agreement and Kappa statistic between the different methods were calculated using 2 × 2 tables, with the concentration of individual azole in the VIPcheck™ plate as the defining factor. For example, for posaconazole 0.5 mg/L is present

FIGURE 1 | Soil sampling distribution and intensity across South Wales.

in the VIPcheck™ plate, agreement between methods was confirmed when confluent growth was observed on the VIPcheck™ plate and the MIC by the reference method was ≥0.5 mg/L, or sporadic/no growth was observed on the VIPcheck™ plate and the MIC by the reference method was <0.5 mg/L.

RESULTS

Resistance Rates in Environmental *Aspergillus fumigatus* Isolates

Seven hundred and fifteen environmental (soil and air) samples were collected. *A. fumigatus* isolates grew in 496/671 soil samples (73.9%) and in 17/44 air samples (38.6%) (**Figure 3**). Thirty soil isolates showed resistance to azoles, while 376 (75.8%) soil isolates were susceptible by screening on VIPcheck™ plates. Ninety (18.1%) soil isolates demonstrated indeterminate growth in the presence of at least one azole (See Wells 2 and 3, **Figure 2C**). All 17 air isolates, were susceptible to azoles.

The prevalence of azole resistance across South Wales was 6.0% (95% CI: 4.3–8.5). **Table 1** and **Figure 1** show the sampling sites, population density, sampling intensity and location of resistant isolates across the testing region. The lowest resistance rates were found in south central regions [3.9% (95% CI: 1.7–8.8), 5/128 *A. fumigatus* isolates], while the highest rates were associated with the Gwent/Monmouthshire region (10.5% (95% CI: 4.2–24.1), 4/38 isolates). The rate of resistance in Cardiff and the surrounding rural areas was 6.8% (95% CI: 4.3–10.7, 17/249 isolates), whereas the rate in South West Wales was 5.2% (95% CI: 2.0–12.6, 4/77 isolates). The four isolates from South West England were sensitive to azole antifungal drugs. In seven locations, spread across the testing area, the rate of azole resistance was >10% (Range 11.1–44.4%) (**Figure 1**). The highest rate (4/9 isolates) was associated with a botanical garden in a city center park that was within one mile of a tertiary referral hospital caring for patients who would be at risk of IA. A rate of 13.6% (95% CI: 4.8–33.3, 3/22 isolates) was also associated with isolates cultured from a public garden within the grounds of a district general hospital.

The rate of azole resistance in agricultural areas was 5.2% (95% CI: 3.2–8.4), compared to 8.4% (95% CI: 5.1–13.4) in public areas, many within urban settings. The difference between rates of resistance in these settings were not statistically different (Difference: 3.2%, 95% CI: −1.4 to 8.6, *P*: 0.1799). Disturbingly, four resistant isolates were recovered from the grounds/gardens across two hospital sites (resistance rate 8.7%, 95% CI: 3.4–20.3). The rate of resistance in botanical gardens (7/27; 25.9%) was significantly greater than other areas combined (23/469; 4.9%) (Difference 21.0%, 95% CI: 8.1–39.9, *P*: 0.0005). No resistant isolates were cultured from private gardens/allotments or compost heaps, although sampling of such areas was limited. and indeterminate growth was evident (32.1%).

All 30 *A. fumigatus* isolates exhibiting azole resistance by VIPcheck™ plate screening were resistant to itraconazole, with 24 and 19 isolates exhibiting at least sporadic growth on the voriconazole and posaconazole wells. Four isolates only showed resistance to itraconazole, with two showing growth on itraconazole and sporadic growth on posaconazole, while seven showed growth on itraconazole and sporadic growth on voriconazole. Seventeen isolates potentially showed pan-azole resistance, when considering sporadic growth as significant. Resistance to itraconazole was always exhibited by confluent growth (30/30), whereas 21/24 and only 2/19 isolates showed confluent growth on voriconazole and posaconazole-containing wells, respectively.

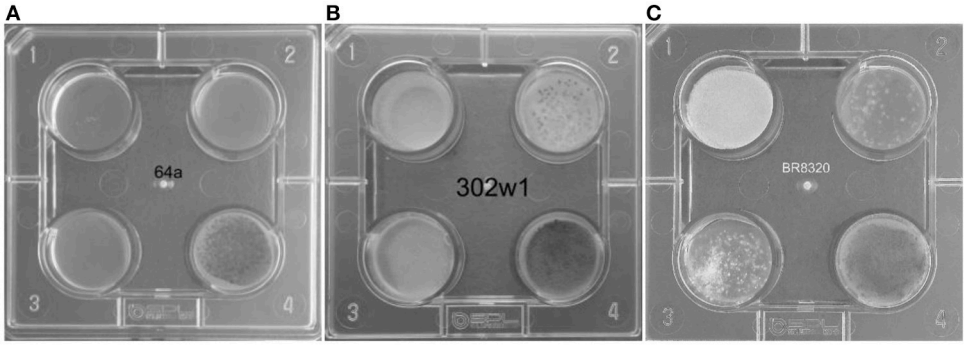

FIGURE 2 | VIPcheck™ plates containing itraconazole at concentration of 4 mg/l (Well 1; top left), voriconazole at a concentration of 2 mg/l (Well 2; top right), posaconazole at a concentration of 0.5 mg/l (Well 3; bottom left) and a control well containing only RPMI agar (Well 4; bottom right) for **(A)** a fully azole sensitive environmental strain of *A. fumigatus* (64a); **(B)** a pan-azole resistant environmental strain of *A. fumigatus* (302w1) and **(C)** an itraconazole resistant environmental strain of *A. fumigatus* (BR8320) showing sporadic growth in the presence of voriconazole and posaconazole.

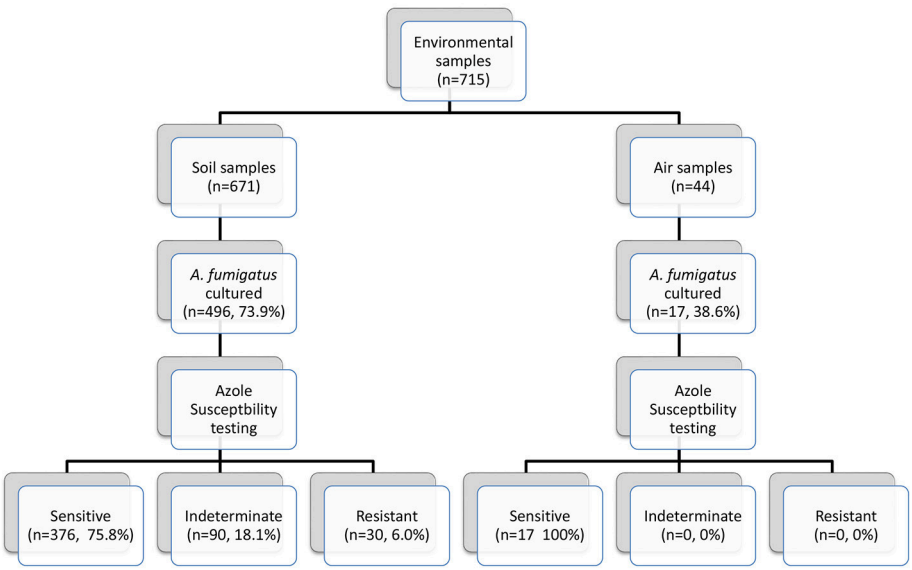

FIGURE 3 | Environmental sampling scheme and breakdown of azole susceptibility testing.

Confirmation of Azole Resistance by CLSI M38-A2 Broth Microdilution Testing

When testing for itraconazole resistance by the reference method, 25/30 (83.3%, 95% CI: 66.4–92.7) VIPcheck™ itraconazole resistant isolates generated resistant minimum inhibitory concentrations (MIC). Twenty-one had a MIC of >16 mg/L, three with 4 mg/L and one with 2 mg/L. Of the five VIPcheck™ itraconazole resistant/CLSI susceptible isolates four had a MIC of 1 mg/L and one 0.5 mg/L. Agreement with VIPcheck™ testing was 80% (95% CI: 62.7–90.5), but it was not possible to calculate a Kappa statistic.

When testing for voriconazole resistance by the reference method 23/30 (76.7%, 95% CI: 59.1–88.2) generated resistant MIC values. Sixteen had resistant MIC of 2 mg/L and seven of 4 mg/L. Of the seven CLSI voriconazole susceptible isolates five had a MIC of 1 mg/L, one of 0.5 mg/L and one of 0.25 mg/L. When comparing voriconazole resistance as determined by the VIPcheck™ plate with the CLSI method 19 were deemed resistant by both, 5 were susceptible by both, whereas two were resistant by VIPcheck™ plate only (CLSI method MIC 1 mg/L) and four resistant by CLSI method only (MIC 2 mg/L). This generated an observed categorical agreement of 80.0% (95% CI: 62.7–90.5) and a Kappa statistic of 0.49, representing fair/good agreement.

When testing for posaconazole resistance by the CLSI method 7/30 showed resistance (23.3%, 95% CI: 11.8–40.9), six generating resistant MIC values of 0.5 mg/L and one of 1 mg/L. A further 21/30 had an intermediate MIC of 0.25 mg/L (70.0%, 95% CI: 52.1–83.3). The MIC of the two susceptible isolates were 0.12 mg/L and 0.06 mg/L, respectively. Both isolates showing

confluent growth on the VIPcheck™ posaconazole well (Well 3 **Figure 2B**) were resistant by the CLSI method. For 17 isolates with sporadic growth on VIPcheck™ plates (Well 3 **Figure 2C**) 11, five and one had a MIC of 0.25, 0.5, and 0.06 mg/L, respectively. Of the 11 VIPcheck™ posaconazole susceptible isolates 10 had a MIC of 0.25 mg/L and one had a MIC of 0.12 mg/L by the CLSI method. This generated an observed categorical agreement of 83.3% (95% CI: 66.4–92.7) and a Kappa statistic of 0.38, representing fair agreement.

Determining the Molecular Mechanism of Azole Resistance Using the PathoNostics AsperGenius® Resistance Assay

The AsperGenius® PCR results showed that 28/30 96.7% (95% CI: 83.3–99.4) azole resistant strains had the TR34/L98H mutations. For one isolate the results were unclear, with the presence of TR34 indicated, but both wild-type and mutation indicated for L98H and TR46/Y121F/T289A (**Figure 4**). Retesting a colony from the VIPcheck™ plate confirmed the presence of the TR34/L98H mutations. However, retesting a colony from a purity plate confirmed the presence of the TR46/Y121F/T289A mutations, but the TR34/L98H mutations were absent, indicating that more than one resistant strain was present. One resistant isolate did not possess any of the targeted mutations. Twenty azole-sensitive and 30 indeterminate strains did not possess any of the mutations targeted.

DISCUSSION

The study showed that the overall prevalence of azole resistant *A. fumigatus* in the environment is 6%, ranging from 3.9% in central regions to 10.5% in eastern regions. The resistance rate was 5.2% in agricultural areas compared to 8.4% in urban areas, including public gardens (6.9%) and hospital grounds (8.7%). One concerning discovery was a significantly greater rate of azole resistance associated with botanical gardens (25.9%). This is a marked difference when compared to the only other UK-based environmental prevalence study performed in Manchester, where environmental resistance was detected in 4/231 rural samples (prevalence of 1.7%) and no resistant isolates were found in the urban environment (Bromley et al., 2014). One explanation, are the differences in city size and local geography. Cardiff is significantly smaller than Greater Manchester and is largely surrounded by agriculture. Although there is significant agriculture around Manchester it is flanked by the Pennines and several other large towns and cities. The heavy fungicidal compound use around Cardiff potentially drives the rural resistance that easily disperses, via coastal air currents, into the urban area due to its immediate vicinity and smaller city size. PCR also confirmed an agricultural source with the vast majority of the resistant isolates (28/30) containing the TR34/L98H mutation (Snelders et al., 2009). However, in one isolate with confirmed azole resistance the mechanism of resistance has not been identified. Interestingly, it has very high MIC for (>16 mg/L) itraconazole and posaconazole (>1 mg/L), but it appears to be sensitive to voriconazole (0.12 mg/L). The exact mechanism of resistance for this isolate is being investigated by whole genome sequencing.

Of concern, is that the highest rates of resistance have been found in highly populated urban areas, where the population density of highly susceptible patients is greatest (**Figure 1**). Understanding local rates of environmental resistance is critical to efficient patient management and it has been proposed that the level of environmental *A. fumigatus* azole resistance should be used to guide treatment of *Aspergillus* infections (Snelders et al., 2011). It has been advocated that in areas with a high environmental azole resistance rate (designated as >10%), the use of voriconazole monotherapy for primary treatment of clinical aspergillosis should be reconsidered (Verweij et al., 2015a). The current environmental resistance rate (6%) is of concern, but opinions are divided over whether to change the primary therapy in the clinic (Verweij et al., 2015b). With the availability of highly sensitive and rapid diagnostics with the capacity to detect the most frequent mutations a strategic approach incorporating enhanced resistance screening is justified.

In this study, the VIPcheck™ plate method was assessed as a frontline screen for resistance and its accuracy corroborated by the CLSI M38-A2 reference method. All isolates with resistance determined using the VIPcheck™ plate were resistant to itraconazole and 83% of these were confirmed by the CLSI method, although one of the VIPcheck™ itraconazole resistant isolates had a CLSI MIC of 2 mg/L below the concentration in the plate. Eighty percent (4/5) that were itraconazole sensitive by the CLSI method had MIC values on the epidemiological cut-off (1 mg/L). Similar concordance (80%) was obtained for voriconazole resistance with most discrepancies showing MIC values around the cut-off. Of the three isolates showing sporadic growth on the voriconazole well two had a resistant MIC (2 mg/L). For posaconazole categorical agreement between the VIPcheck™ and CLSI method was also 83%. Five isolates that were resistant by the CLSI method (MIC: 0.5 mg/L) showed sporadic growth on VIPcheck™ plates. Conversely, 12 isolates deemed indeterminate by the CLSI method (MIC: 0.25 mg/L) showed sporadic growth on the VIPcheck™ plate, and only a single isolate with sporadic growth was genuinely susceptible (MIC: 0.06 mg/L). The presence of sporadic growth on the posaconazole well accompanied with confluent growth in at least one of the other azole containing wells likely represents a raised MIC, but also highlights the difficulty in interpreting MIC values when a gray zone exists (posaconazole breakpoints: $S < 0.125$ mg/L; $R > 0.25$ mg/L). With the VIPcheck™ plates containing posaconazole at a concentration of 0.5 mg/L, isolates with intermediate resistance may fail to grow. Nevertheless, it is widely accepted that itraconazole is a good marker for resistance derived from mutations in the *cyp51A* gene (Stensvold et al., 2012). All isolates that were resistant to itraconazole by VIPcheck™ plate were resistant to at least one azole by the CLSI method. Over 90% of resistant isolates had the TR34/L98H mutation and this is said to confer pan-azole resistance (Stensvold et al., 2012). Only two and seven

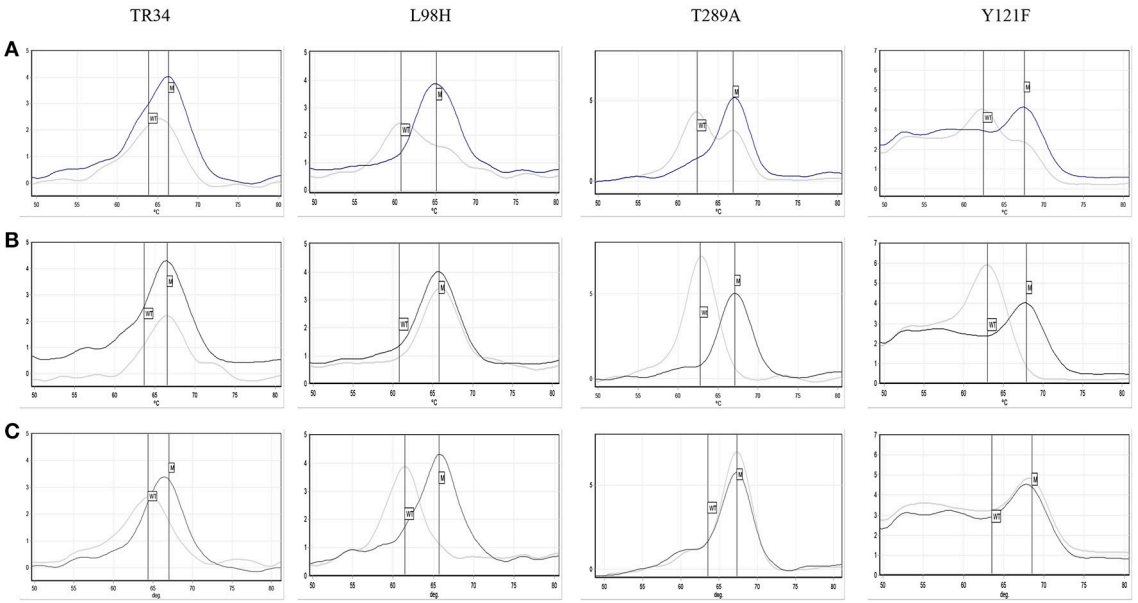

FIGURE 4 | Pathonostics Aspergenius® melt-curve analysis for the TR34/L98H and T289A/Y121F mutations, with typical melting temperatures for wild-type (WT) and mutations (M) indicated by the respective lines with initial testing direct from VIPcheck™ plate showing ambiguous results **(A)**, repeat testing from VIPcheck™ plate showing the TR34/L98H mutation **(B)** and repeat testing from purity plate showing the T289A/Y121F mutation **(C)**. The control is represented by the black line and the isolate is represented by the gray line.

of the isolates were shown to be pan-azole resistant by the VIPcheck™ and CLSI methods, respectively. However, a further 17 isolates would have been considered pan-azole resistant on VIPcheck™ plates if sporadic growth on posaconazole was considered significant and 21 isolates had a CLSI MIC to posaconazole of 0.25 mg/L. Next generation sequencing is currently being performed on these isolates to confirm the presence of the TR34/L98H mutation and to possibly identify other mutations.

There were 90 isolates that were deemed indeterminate as sporadic growth was observed in at least one of the azole wells of the VIPcheck™ plate, but in the absence of any confluent growth. A subset of 30 isolates was tested by the pathonostics assay but no mutations (TR34/L98H or TR46/Y121F/T289A) were present. Unfortunately, due to cost limitations MIC testing and next generation sequencing of these isolates has not been performed to confirm or refute presence of resistance.

The major limitation of the study is that as VIPcheck™ plates were used for the front-line screen it is possible that resistant strains may have been detected using other methods, although a small subset of sensitive isolates that were subjected to confirmatory susceptibility testing remained sensitive to all azoles (results not shown). In addition, as confirmatory susceptibility testing was focused mainly on resistant isolates observed agreement is likely to be under-estimated. A further limitation coincides with the screening mechanism employed, which enhanced the opportunity for detection of resistance but in doing so also allows for more than one resistant strain to be present.

This study suggests that screening for azole resistance in environmental *A. fumigatus* isolates is needed for all centers caring for patients at risk of aspergillosis. The overall rate of environmental azole resistance (6%) dictates that monitoring for resistant disease and environmental surveillance is essential, but this study shows that resistance rates can vary significantly between geographically close regions and it is essential that a single resistance rate is not universally applied. The VIPcheck™ plates provided a simplistic and cost effective method for determining azole resistance based on the itraconazole susceptibility profile. The level of the TR34/L98H mutation was high, indicating that the primary source of azole resistant *Aspergillus* in the region will be attributed to agricultural azole use. The AsperGenius® assay provides a robust means for screening for the most common resistance mechanisms. All resistant isolates will be subjected to next generation sequencing as part of an international study into azole resistance in *A. fumigatus* and this will provide essential and evolutionary information, confirm and identify mechanisms of resistance and possibly establish links between clinical and environmental strains.

AUTHOR CONTRIBUTIONS

AT and RP performed environmental sampling, laboratory testing and contributed significantly to the writing of the manuscript. LV and EJ performed laboratory testing, scientific advice and contributed to the writing of the manuscript. SB performed environmental sampling,

laboratory testing and contributed to the writing of the manuscript. PW was responsible for study design, securing funding and contributed significantly to the writing of the manuscript.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
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Overview of Commercially Available PCR Assays for the Detection of *Aspergillus* spp. DNA in Patient Samples

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Invasive aspergillosis (IA) is a life-threatening infection in immunocompromised patients. Early diagnosis is essential to improve survival. Since the 1990s, attempts for PCR-based diagnosis of IA were made. Progress in the standardization of methods enabled the development of commercially available *Aspergillus* PCR assays in the last few years. Up to now, the clinical value of only a few commercial assays was investigated more extensively in large cohort studies. Most often, respiratory secretions such as bronchoalveolar lavage (BAL) were investigated, but some studies also included serum samples from high-risk patients. The data indicate that *Aspergillus* PCR, most likely in combination with galactomannan detection, has the potential for early and reliable diagnosis of IA including azole resistance markers. With the broad implementation of this technique in routine diagnosis and incorporation into patient care pathways, it is conceivable that an improvement in management of IA and subsequently patient outcome could occur.

Keywords: *Aspergillus*, aspergillosis, PCR, laboratory diagnosis, azole resistance

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INTRODUCTION

Aspergillosis, which is defined as an infection or disease caused by fungi of the genus *Aspergillus*, mainly affects immunocompromised and critically ill patients and invasive disease is associated with high mortality. Conventional diagnosis is difficult and there is a widespread use of prophylaxis and empirical antifungal treatment in the management of invasive disease despite a relatively low incidence. Furthermore, the global emergence of infections with azole-resistant *Aspergillus fumigatus* (Steinmann et al., 2015; Verweij et al., 2016), associated with therapeutic failure, strengthened the need for rapid and reliable diagnostic methods.

The conventional diagnosis of *Aspergillus* infections is based on the presence of risk factors, radiological features, and microbiological results, i.e., histopathology and/or culture of *Aspergillus* spp. A milestone was the standardization of the classification of disease based on probability of aspergillosis [i.e., proven, probable, or possible invasive fungal disease according to the definitions of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study

Group (EORTC/MSG; De Pauw et al., 2008)]. These criteria are the basis for determining drug efficacy, validation of diagnostic tests, and epidemiological analyses. Primarily established for the classifications of patients in clinical and laboratory studies, these definitions can also be very useful in clinical settings. However, they were established for hemato-oncological settings and modifications were necessary to fit also other patients groups like intensive care unit (ICU) patients (Meersseman et al., 2004; Blot et al., 2012). The microbiological criteria are a positive histopathology, microscopy, or culture of primarily sterile samples, defining a proven infection, and the detection of the antigens galactomannan and (1-3)-β-D-glucan in serum or other body fluids such as bronchoalveolar lavage (BAL; De Pauw et al., 2008). PCR assays were not included in 2008, but will be included in the next/current version. In recent years, *Aspergillus* PCR has been shown, in combination with other biomarkers, to be a promising tool in diagnostic algorithms as reviewed recently, for example, by White et al. (2015b), Buchheidt et al. (2017), and Lamoth and Calandra (2017).

Since the 1990s, several studies were published on in-house PCR assays to detect *Aspergillus* DNA in clinical samples. These studies showed in nearly all cases a higher sensitivity than in cultures, particularly in immunocompromised patients. However, the assays differed markedly in sample preparation, target, platforms (Buchheidt et al., 2017), and consequently in sensitivity and specificity (Arvanitis et al., 2015; White et al., 2015b). Since 2006, major progress was made in the standardization of PCR assays for the detection of *Aspergillus*-DNA in clinical samples by the European *Aspergillus* PCR Initiative (EAPCRI) Working Group of the International Society of Human and Animal Mycoses (ISHAM; White et al., 2011a). A meta-analysis reported a higher accuracy to diagnose invasive aspergillosis (IA) for those PCR studies, compliant with the EAPCRI recommendations (Arvanitis et al., 2014). The advances in the standardization of clinical criteria and laboratory procedures show that PCR is now sufficiently robust for routine *Aspergillus* diagnostics. Consequently, commercial PCR assays such as AsperGenius® (PathoNostics, Maastricht, Netherlands), MycAssay *Aspergillus*® (Myconostica Ltd., Cambridge, United Kingdom), MycoReal *Aspergillus*® (ingenetix GmbH, Austria), RenDX Fungiplex® (Renishaw Diagnostics Ltd., Glasgow, United Kingdom), MycoGenie® (Ademtech, Pessac, France), LightCycler SeptiFast® (Roche Molecular Diagnostics, Penzberg, Germany), GeneProof *Aspergillus* PCR® (Brno, Czechia), *Aspergillus* spp. Alert Kit® (Nanogen, now ELITechGroup, Turin, Italy), *Aspergillus* Real-time PCR Panel® (Viracor Eurofins, Framingham, MA, United States), *A. fumigatus*® Bio-Evolution (Bio-Evolution, Bry-sur-Marne, France), and Fungiplex *Aspergillus*® (Bruker Daltonik GmbH, Bremen, Germany) became available in recent years. For only some of these assays data on the sensitivity and specificity for diagnosing IA are currently available. The performances of the PCR assays will be discussed in the following. In **Table 1** an overview on the performance of the assays in prospective studies is given.

MycAssay *Aspergillus*®

The MycAssay *Aspergillus*®, developed by Myconostica Ltd. (Cambridge, United Kingdom), now Microgen Bioproducts Ltd. (Camberley, United Kingdom), is a real-time PCR it for use on the Cepheid SmartCycler, Roche LightCycler 2.0, Stratagene Mx3000, AB 7500, or BioRad CFX96 platforms. The target is the 18S rDNA and the detection limit is less than 50 target copies. The assay detects different *Aspergillus* spp. including *A. fumigatus*. Torelli et al. (2011) investigated the usefulness of the MycAssay *Aspergillus*® in comparison with an in-house PCR and galactomannan detection in hematological ($N = 68$) and non-hematological ($N = 90$) patients using BAL samples. Most of the patients (89.9%) were neutropenic and were classified as proven, probable, or possible aspergillosis using the EORTC/MSG criteria or the modifications suggested by Meersseman et al. (2004). Two proven and 15 probable cases were found. The control group consisted of 141 patients. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 94.1%, 98.6%, 88.9%, and 99.3%. Nearly identical data were found by using the galactomannan assay (index cutoff of ≥1).

Guinea et al. (2013) investigated 322 respiratory samples from 175 patients without hemato-oncological diseases, of which 15 had probable IA. Sensitivity, specificity, PPV, and NPV were 86.7%, 87.6%, 34.1% and 92.2%. Culture was positive in 65 samples of 35 patients.

Orsi et al. (2012) evaluated the performance of the MycAssay *Aspergillus*® in BAL samples of 20 hematological and non-hematological immunosuppressed patients, including seven suffering from IA. All patients with proven or probable aspergillosis were positive by PCR (100% sensitivity).

In a larger study (Orsi et al., 2015), the MycAssay *Aspergillus*® was compared with galactomannan and culture in BAL samples from 41 patients (44 samples) at risk for infection along with assays for the detection of *Pneumocystis jirovecii*. Patients were in part hemato-oncological patients, for which the EORTC/MSG criteria were used (De Pauw et al., 2008), for patients with other underlying diseases additional host factors, such as steroid treatment, chronic obstructive pulmonary disease, lower respiratory infections, and new infiltrates were applied. Ten patients were diagnosed as probable infected with molds. Culture of BAL was positive in 6/10 patients and all had a positive galactomannan in BAL. PCR was positive in 8/10 patients, and in 1/34 of control patients. The authors calculated for the PCR assay a sensitivity of 80%, a specificity of 97.1%, a PPV of 88.9%, and a NPV of 94.3%.

White et al. (2011b) compared the sensitivity and specificity of the assay and an in-house assay using 170 serum samples from patients (104 samples of 10 patients with proven or probable IA and 66 samples from 21 patients at risk for IA). Seven of the 10 patients with proven/probable IA were positive in at least one sample. The MycAssay *Aspergillus*® showed a sensitivity of 60 to 70% and a specificity of 90.5% to 100%, which was comparable to the in-house assay.

Pini et al. (2015) compared the MycAssay *Aspergillus*® in serum with the galactomannan assay in 71 episodes of 64 hematological and non-hematological patients (30

TABLE 1 | Overview of published prospective studies on commercial available PCR assays for *Aspergillus* detection.

Assay (Detected species)	Sample type (no. of samples/patients)	Sensitivity	Specificity	Patients	No. of patients with proven/probable IA	Reference
MycAssay	BAL (158/158)	94.1%	98.6%	Mixed	17	Torelli et al., 2011
Aspergillus (*Aspergillus* spp., *Penicilliurn* spp.)	Resp. samples (322/175)	86.7%	87.6%	Non-hematology	15	Guinea et al., 2013
	BAL (44/41)	80%	97.1%	Mixed	10	Orsi et al., 2015
	Serum (71/64)	46.7%	97.6%	Mixed	30 episodes	Pini et al., 2015
	Serum (358/78)	75%	Not given	Hematology	18	Oz et al., 2016
AsperGenius	BAL (201/201)	84%	80%	Hematology	52	Chong et al., 2016
(*A. fumigatus* complex, *A. terreus, Aspergillus* spp.; TR34, L98H, Y121F, T289A)	BAL (387/387)	68.4%	>92%	"at high risk"	38	Guegan et al., 2018
MycoGenie (*A. fumigatus*; TR34/L98H)	BAL (387/387)	71.1%	>92%	"at high risk"	38	Guegan et al., 2018

episodes were classified as IA). Classification was done based on the EORTC/MSG criteria for the hematological patients and on the criteria proposed by Meersseman et al. (2004) for non-hematological patients. Sensitivity, specificity, PPV and NPV for the MycAssay *Aspergillus*® were 46.7%, 97.6%, 93.3%, and 71.4%. These numbers were similar to those for the galactomannan assay. When considering hematological patients only, the sensitivity increased to 60%.

Danylo et al. (2014) compared the MycAssay *Aspergillus*® in serum with galactomannan using 146 serum samples of 35 patients with hematological malignancies. Four patients suffered from a proven infection, 12 patients from a probable infection, and 13 patients were not infected. Sensitivity, specificity, PPV, and NPV were 25%, 83.3%, 39.3%, and 72%. The relative low sensitivity might be a result of a high proportion of patients under antifungal treatment in the proven/probable group (14/16 patients). The galactomannan assay showed a higher specificity (93.1%).

Another recent study also tested the MycAssay *Aspergillus*® in comparison with an in-house PCR and galactomannan in 358 sera from 78 febrile neutropenic episodes in patients with IA and 83 episodes in patients with no IA (Oz et al., 2016). The topic of this study was the investigation of factors that can influence test results. The hospitalization period, duration of neutropenia, and T-cell suppression were significantly higher in the IA group. Even though there were no significant differences in test performances, the use of larger volumes (>500 μl) of sera improved the performance of the commercial PCR assay.

Taken together, the MycAssay *Aspergillus*® showed a very good performance in BAL samples, especially for patients with hematological malignancies. The accuracy in the serum was not better than galactomannan.

Aquino et al. (2012) compared the performance of two commercial real-time PCR assays and galactomannan in BAL of 47 ventilated patients with chronic obstructive pulmonary disease. The included assays were the MycAssay *Aspergillus*® and the *Aspergillus* spp. q-PCR Alert® Kit (ELITechGroup, Turin, Italy). The *Aspergillus* spp. q-PCR kit, which is a quantitative real-time PCR with a target within the 28S rDNA, detected one of two patients with positive *Aspergillus* culture and positive galactomannan. The MycAssay *Aspergillus*® was positive in 10 patients including the two with positive culture.

AsperGenius®

The AsperGenius® assay is a multiplex real-time PCR assay developed by PathoNostics (Maastricht, Netherlands). It runs on LightCycler 480, Rotor-Gene 6000 (Corbett) and Rotor-Gene Q (Qiagen). Two PCR assays for respiratory secretions are available, one for the detection and differentiation of *Aspergillus* spp., targeting the 28Sr DNA [AsperGenius Species multiplex® with a probe for the *A. fumigatus* complex (including *A. fumigatus*, *A. lentulus*, *A. udagawae*, and *A. viridinutans*), a probe for *Aspergillus* spp. (including *A. fumigatus* complex, *A. terreus*, *A. flavus*, and *A. niger*), and one (AsperGenius Resistance multiplex®)] for the detection of four azole resistance markers within the Cyp51A gene of *A. fumigatus* (L98H, TR34, T289A, Y121F).

Chong et al. (2015) investigated BALs from 10 hematologic and 12 non-neutropenic patients. For neutropenic patients the EORTC/MSG criteria were used, and most of the non-neutropenic patients and some hematological patients were classified as non-classifiable. There was one proven IA case. Sensitivity, specificity, PPV, and NPV were 84.2%, 91.4%, 76.2%, 96.6%, respectively. In two cases azole-resistant strains were found.

A multicenter study from 2016 aimed to validate the assay by using stored BAL samples from patients with hematological diseases with suspected IA (Chong et al., 2016). From a total of 201 included samples, 88 were positive and 113 were negative controls. A cut-off value of <38 cycles was used. The diagnostic performance was the following: sensitivity 84%, specificity 80%, PPV 76%, and NPV 87%. In eight samples, a resistant mutation was detected. Mortality was 2.7 times higher in patients with isolates with azole-resistant mutations.

The assay was also tested by Schauwvlieghe et al. (2017) using BAL samples from 91 patients from Netherlands with positive galactomannan in BAL and suspected invasive *Aspergillus* infection. In 79% DNA of *A. fumigatus* or *Aspergillus* spp. was detected. A mutation associated with azole resistance was

detected in eight cases (TR_{34}/L98H) and in three patients, T289A/Y121F alterations were found.

Montesinos et al. (2017) evaluated the assay by using BAL from 100 patients including 29 patients (cystic fibrosis, lung transplant recipients) with proven or probable aspergillosis. Twenty-seven patients were positive for *Aspergillus* spp. DNA and 20 patients for *A. fumigatus* DNA. Three of them harbored mutations in the Cyp51A gene (one patient with colonization only).

In a retrospective study White et al. (2015a) investigated 124 serum samples of 49 hemato-oncological patients (14 proven/probable cases, 33 control patients). Sensitivity was 78.6% and specificity 100%. No resistance markers were found.

In a larger study, the same group (White et al., 2017) investigated 211 plasma samples from 10 patients with proven or probable IA, two possible cases and 27 controls. Sensitivity and specificity was 80% and 77.8%. If more than one positive sample was required for being relevant, the specificity increased to 100%, but sensitivity dropped to 50%.

Based on the existing studies, the AsperGenius® assay had a good diagnostic utility. An innovative advantage of this of the assay is the ability to detect azole-resistant mutations.

MycoGenie®

The MycoGenie® assay, developed by AdemTech (Pessac, France) detects *A. fumigatus* based on the 28S rDNA, and the TR34/L98H mutation. Recommended materials are biopsies and respiratory and serum samples. The platforms are CFX96, ABI 7500, LightCycler 480, SmartCycler, MX3000, and the Rotor-Gene. Recently Dannaoui et al. (2017) validated the assay using 88 respiratory samples (59 culture positive) and 69 serum samples (from 16 patients) with proven or probable aspergillosis. Sensitivity and specificity was 92.9% and 90.1% for the respiratory samples, and 100% and 84.6%, respectively, for serum samples, when analyzing samples at the time of clinical diagnosis.

A large study by Guegan et al. (2018) from France compared the MycoGenie® assay with the AspergGenius® assay and two in-house assays included BAL samples from 387 hematological and non-hematological patients. One patient suffered from a proven infection, 37 patient cases were classified as probable, and 23 as possible IA. The MycoGenie® assay showed a sensitivity of 53.7% in patients ($n = 41$) with hemato-oncological diseases, and a sensitivity of 75% in the 20 patients with non-hematological diseases. The AspergGenius® assay showed a lower sensitivity of 41.5% in the hematological patients compared to 60% in the non-hematological patients. Not surprisingly, the sensitivity of all assays was lower in patients with antifungal prophylaxis (36–45% vs. 50–62%) or treatment (35–40% vs. 53–70%). Due to the study design, data on the specificity of the assays were not collected (analysis samples of patients with IA only). Interestingly, resistance markers were not found.

The assay was also used for molecular detection of *Aspergillus* in French patients with clinically diagnosed fungal rhinosinusitis and positive microscopy ($N = 137$), most of them with fungal ball as clinical manifestation (Morio et al., 2018). In 77.4% of the patients the assay gave positive results compared with 32.1% culture positivity. Again, no resistance markers were found.

In a recently published study, the MycoGenie® assay was compared with the *A. fumigatus* Bio-Evolution® assay (Bio-Evolution®, Bry-sur-Marne, France). The *A. fumigatus* Bio-Evolution assay uses the ITS1 region for specific detection of *A. fumigatus* DNA. In the retrospective study by Denis et al. (2018), 73 BAL samples from hematological and non-hematological patients, previously used for *P. jirovecii* PCR, were retrospectively analyzed. Thirty-one patients were classified as probable IA, 11 as probable IA, and 31 patients as control group. Both assays showed a 100% specificity and a sensitivity of 81% (*A. fumigatus* Bio-Evolution assay) and 71% (MycoGenie® assay). Interestingly, antifungal treatment had no influence on sensitivities. No resistance markers were found.

In summary, the diagnostic performance of this new assay is promising; also the possibility to detected one of the most common azole resistant mutation is advantageous. More data is needed to give a final rating about the utility in the clinical routine.

SeptiFast®

The SeptiFast® assay is a multiplex-real-time PCR for the detection of a spectrum of bacterial and fungal pathogens in whole blood. Besides *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida glabrata*, it also detects *A. fumigatus*. With respect to bacterial pathogens, the system showed a sensitivity and specificity of 68% and 86% when compared to blood culture (Dark et al., 2015).

A retrospective study by Steinmann et al. (2016) analyzed the diagnostic performance for *A. fumigatus* detection and the relevance of the assay in whole blood from critically ill ICU patients with probable or proven IA ($N = 38$) and without IA ($N = 100$). Modifications of the EORTC/MSG criteria (additional risk factor: an ICU stay longer than 4 days) were used to classify the patients. The assay showed in these patients a sensitivity of 66% and a specificity of 98%, a PPV of 93%, and an NPV of 88%. One important result was the high mortality in patients with positive PCR.

The accuracy of the SeptiFast® assay for whole blood was very good. However, the disadvantages of this assay are the laborious workflow and the higher costs compared to other species-specific PCR tests.

RenDx Fungiplex®

The RenDX Fungiplex® assay (Renishaw Diagnostics Ltd Glasgow, United Kingdom) is based on a PCR targeting sequences of the 18S rDNA and 28S rDNA, followed by a surface-enhanced Raman scattering (SERS) for the sensitive detection of pathogen-specific oligonucleotides. It used an *Aspergillus* probe in combination with a broad-range *Saccharomycetales* probe to simultaneously detect *Aspergillus* and *Candida* spp. White et al. (2014) validated this assay, using plasma samples from 14 mostly hemato-oncological patients with proven/probable IA and 80

patients without IA. Sensitivity was 82.2% and specificity 87.5%. The RenDX Fungiplex® has been reformatted to utilize real-time PCR technology. Results concerning the clinically utility of the Fungiplex *Aspergillus* PCR® assay (Bruker Daltonik GmbH, Bremen, Germany) are so far not available. However, first results with spiked samples showed an excellent analytical performance in the detection of DNA of *A. fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* (Green and Dougan, 2017).

CONCLUSION

A number of commercial tests to detect *Aspergillus* spp. in clinical samples are available, but only a few have been currently (March 2018) validated independently from the manufacturers. Nevertheless, based on the published data so far, the mentioned assays seem to have comparable sensitivities and specificities. Overall, sensitivity is significantly lower in serum samples than in respiratory specimens. Some data indicate a lower sensitivity in patients with antifungal prophylaxis or treatment.

At the moment, the MycAssay *Aspergillus*® and the AsperGenius® assay can be recommended for routine PCR-detection of *Aspergillus* spp. DNA in respiratory samples. It is expectable that more PCR assays will be commercially available in the near future and thus, evaluating (multicenter) studies should also focus on the comparison of different PCR assays. Furthermore, other clinical patient specimens than serum or respiratory secretions should be included in future studies. Cerebrospinal fluid, biopsies, and pleura infusion for example are also relevant specimens for *Aspergillus* diagnostic.

AUTHOR CONTRIBUTIONS

P-MR and JS contributed equally by checking the literature and writing the manuscript.

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MINI REVIEW
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Molecular Detection of Azole-Resistant *Aspergillus fumigatus* in Clinical Samples

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Aspergillus diseases are often caused by *Aspergillus fumigatus*. Azoles are the mainstay of therapy, but the management of aspergillosis is hampered by the emergence of azole resistance. Rapid detection of azole resistance might benefit treatment outcome by early treatment modifications. However, the yield of fungal culture in invasive aspergillosis is low and susceptibility testing requires several days to be completed. To overcome the low yield of fungal cultures and slow detection of resistance, it is possible to use molecular tools directly on clinical specimens in order to rapidly detect molecular markers of azole resistance. Molecular tools to detect resistant markers in the *Cyp51A* gene can be expected to be less sensitive compared to molecular tools to detect *Aspergillus* DNA as the *Cyp51A* gene is a single copy gene and the target for *Aspergillus* DNA is often a multi-copy gene. In this mini-review, we summarize the current molecular tools for detection of azole-resistant *A. fumigatus* directly in clinical material. Several in-house PCR assays have been applied directly on clinical material. Furthermore, two assays are commercial available; the AsperGenius and MycoGENIE. The amplification of resistance markers was successful in 70–100% of samples that were positive for *Aspergillus* DNA in BAL samples using the AsperGenius assay. Despite using several samples per patient, amplification of resistance markers was only successful in 33–57% of patients with *Aspergillus* DNA in blood. Furthermore, several sequence based methods have been applied with the benefit of the ability to detect other *Cyp51A* gene alterations.

Keywords: azole resistance, *Aspergillus fumigatus*, polymerase chain reaction, azole, antifungal, diagnostics

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INTRODUCTION

Aspergillus species cause a wide spectrum of disease, including invasive aspergillosis (IA), chronic pulmonary aspergillosis (CPA), *Aspergillus* bronchitis, and allergic bronchopulmonary aspergillosis (ABPA). The most common species involved in aspergillus diseases is *Aspergillus fumigatus*. If antifungal treatment is required, azoles are the first line of treatment, independently of the manifestation of disease (Kosmidis and Denning, 2015). However, the management of aspergillosis is hampered by the emergence of azole resistance (Verweij et al., 2016). Several case series indicate that detection of azole resistance is associated with treatment failure (van der Linden et al., 2011; Chong et al., 2016).

The detection of azole resistance in IA is challenging as the yield of fungal cultures is generally low and phenotypical evaluation of azole susceptibility is therefore often impossible. Furthermore, even if a culture is positive for *A. fumigatus*, susceptibility testing requires several days before results

are available. A mature culture is recommended for MIC-testing, which requires up to 7 days of incubation and MICs can be read after an additional 48 h. As early treatment of patients with IA with an appropriate drug improves outcome (Hauggaard et al., 2002), inappropriate therapy due to azole resistance may increase treatment failure. Nowadays it is possible to use molecular tools directly on clinical specimens in order to rapidly detect resistance markers. In this review, we summarize the current molecular tools of azole resistance detection in *A. fumigatus* directly in clinical material.

MOLECULAR MARKERS OF AZOLE RESISTANCE IN *A. fumigatus*

The hot spot for resistance mutations in *A. fumigatus* is the *Cyp51A* gene, a gene important for the ergosterol biosynthesis pathway. Generally, two groups of resistance mutations can be acknowledged; the environmental resistance mutations and the resistance mutations due to long-term azole therapy. TR_{34}/L98H and TR_{46}/Y121F/T289A are the most common environmental resistance mutations which are now recovered globally. Furthermore, several less prevalent environmental resistance mutations have been reported including TR_{53}, $TR_{46}{}^{3}$, and $TR_{46}{}^{4}$ (three and four repeats of TR_{46}, respectively) (Zhang et al., 2017). The other group of resistance mutations consists of point mutations in the *Cyp51A* gene at various positions including G54, M220, P216, G138, and G448 with mutations at locus G54 and M220 leading to the most common amino acid substitutions (Meis et al., 2016; Verweij et al., 2016).

It is estimated that 10% of azole resistance is not *Cyp51A* mediated (Meis et al., 2016). Several other resistance mechanisms have been described including increased efflux pump activity and a substitution in the *HapE* gene (Camps et al., 2012; Fraczek et al., 2013). Moreover, it seems likely that several other, yet uncharacterized, mechanisms are in involved in azole resistance in *A. fumigatus*.

MOLECULAR TOOLS TO DETECT AZOLE RESISTANCE DIRECTLY IN CLINICAL SAMPLES

Real-Time Resistance-PCR Tests

In-House Assays

The first report of the use of a qPCR for the detection of resistance mutations on clinical samples was in 2010 in a case of pulmonary and cerebral aspergillosis (van der Linden et al., 2010). A pulmonary isolate was phenotypically resistant to azoles. Brain biopsy showed septate hyphae but fungal cultures remained negative. Sequence-based analysis of the pulmonary isolates showed the TR_{34}/L98H mutation and a real-time PCR was applied on the brain tissue. For detection of the L98H mutation, a 122-bp fragment was amplified, using a hybridization probe to detect the L98H mutation. Furthermore, a second amplification of a 110-bp fragment was used to detect the 34-bp insertion (TR_{34}) using a TaqMan probe that was designed to bind to the last 13-bp and the first 9-bp of the 34-bp insertion. The real-time PCR revealed the presence of a L98H mutation as well as the 34-bp insertion (van der Linden et al., 2010). Further details of the published papers evaluating qPCR resistance detection can be found in **Table 1**.

Another in-house PCR assay was used on 29 *Aspergillus* DNA positive sputum samples from patients with CPA and ABPA. In this series a nested-PCR approach was used. The *Cyp51A* gene was amplified in two fractions and a qPCR with allele-specific molecular beacons was used to detect mutations at locus G54, L98, G138, and M220 and to detect the 34-bp insertion. *Cyp51A* was successfully amplified in all 29 *Aspergillus* PCR positive samples. Four samples had a mutation at M220, 27 of 29 had a L98H mutation of which 16 also had TR_{34}. TR_{34} without L98H was found in two patients. In two patients with a M220R mutation, TR_{34} and L98H were also detected. Only four samples were culture positive, in three the molecular results did not correspond with the culture results (Denning et al., 2011). The detection of either TR_{34} or L98H alone and a TR_{34}/L98H with an additional M220 mutation has not been reported in cultured isolates, to the best of our knowledge. It seems likely that due to fact that two genomic regions are amplificated independently for the detection of TR_{34} and L98H, the amplicons were from different strains, (e.g., a susceptible and resistant strain), resulting in the single detection of either TR_{34} or L98H. The frequency of resistance markers seems very high in this series and these results have not yet been confirmed by other studies.

The same nested PCR-assay, with the addition of a new molecular beacon for G448, was used to analyze 94 BAL samples. Sixty-one of 71 (86%) samples positive for a pan-*Aspergillus* PCR had a successful amplification of the *Cyp51A* gene. Four of the *Cyp51A* negative samples were culture positive for *A. flavus*. One sample contained M220V and another P216L. The authors did not describe the use of a molecular beacon for P216L and the mutation was thus presumably detected by subsequent sequence analysis (Zhao et al., 2013). The high rate of TR_{34}/L98H mutations observed by Denning et al. (2011) was not observed in the latter study from Denmark.

Commercial Available Assays

Results from the first commercial available resistance PCR assay, the AsperGenius (PathoNostics, Maastricht, Netherlands) were published in 2015. The qPCR has the ability to detect *Aspergillus* DNA, and differentiates *A. fumigatus* and *A. terreus*. In addition, four mutations (TR_{34} and L98H to detect TR_{34}/L98H, Y121F, and T289A to detect TR_{46}/Y121F/T289A) can be detected using melting-curve analyses. The PCR was validated on stored BAL fluid samples from hematological and ICU patients. Of 19 BAL fluids, 16 were positive for *Aspergillus* DNA, whereas 14 of 16 were positive for *A. fumigatus*. All 14 samples were also positive for resistance targets and mutation analyses revealed 12 samples with WT *Cyp51A* DNA, one TR_{34}/L98H and one TR_{46}/Y121F/T289A (Chong et al., 2015).

The assay was validated prospectively in a multicenter study in 201 patients with hematological disease suspected for IA.

TABLE 1 | Overview of studies that applied real-time PCR assays directly on clinical specimen to detect azole resistance markers in *Aspergillus fumigatus*.

Country	Patients samples	Resistance markers	PCR system	Remarks	Positivity	Reference
Netherlands	Tissue (FFPE) from single case	TR_{34}, L98H	In-house		1 *Aspergillus* DNA positive 1 *Cyp51A* profiling successful 1 R	van der Linden et al., 2010
United Kingdom	Selection of 29 *Aspergillus* PCR positive sputa	TR_{34}, L98H, G54, G138C, M220	In-house	Nested amplification of target with qPCR with allele specific molecular beacons. Primer and probe sequences not published	29 *Aspergillus* DNA positive: 29 *Cyp51A* profiling successful. 27 L98H, 4 M220, 18 TR_{34}	Denning et al., 2011
Denmark	94 BAL samples from 87 patients	TR_{34}, L98H, G54, G138C, M220, G448S	In-house	Assay described by Denning et al. (2011) with additional molecular beacon for G448S	41 *Aspergillus* DNA positive: 36 *Cyp51A* profiling successful in 44 BAL samples GM > 3. 21 *Aspergillus* DNA positive and 18 *Cyp51A* profiling successful in 32 BAL samples GM 0.5–3. 9 *Aspergillus* DNA positive and 7 *Cyp51A* profiling successful in 18 GM negative BAL samples.	Zhao et al., 2013
Netherlands	BAL	TR_{34}, L98H, Y121F, T289A	AsperGenius	LoD species at <36 *Ct*	16 *Aspergillus* DNA (<36 *Ct*) positive. 14 Cyp51A profiling successful 12 WT 2 R	Chong et al., 2015
United Kingdom	Serum	TR_{34}, L98H, Y121F, T289A	AsperGenius	LoD of species and resistance assay was 10 and >75 genomes	25 *Aspergillus* DNA positive, 12 *Cyp51A* profiling successful 5 WT/7 R	White et al., 2015b
Netherlands	BAL	TR_{34}, L98H, Y121F, T289A	AsperGenius	LoD species at <38 *Ct*	97 *Aspergillus* DNA positive (<38 *Ct*) 68 *Cyp51A* profiling successful 57 WT/11R	Chong et al., 2016
Belgium	BAL samples of which 45 GM positive	TR_{34}, L98H, Y121F, T289A	AsperGenius	LoD species at <36 *Ct*	20 *A. fumigatus* DNA positive (<36 Ct) 20 *Cyp51A* profiling successful 17WT/3 R	Montesinos et al., 2017
United Kingdom	Plasma of 12 patients	TR_{34}, L98H, Y121F, T289A	AsperGenius	LoD of species and resistance assay was 10 and 50 genomes. Meanly 7 samples per patient.	6 patients *A. fumigatus* DNA positive, 2 *Cyp51A* profiling successful 2 WT	White et al., 2017

(Continued)

TABLE 1 | Continued

Country	Patients samples	Resistance markers	PCR system	Remarks	Positivity	Reference
France	Respiratory + Serum	TR_{34}, L98H	MycoGENIE	LoD of species and resistance assay was 1 and 6 genomes.	Respiratory: 55 *A. fumigatus* DNA positive Serum: 16 *A. fumigatus* DNA positive 0 Cyp51A R	Dannaoui et al., 2017
France	Respiratory	TR_{34}	MycoGENIE	No WT *Cyp51A* gene – control in assay.	106/136 *A. fumigatus* DNA positive. 0 *Cyp51A* R.	Morio et al., 2018
Netherlands	Respiratory samples. 91 samples tested. Three cases with co-infection described	TR_{34}, L98H, Y121F, T289A	AsperGenius	In one case both resistant and susceptible isolates cultured. No sequence information of isolates available.	72 *Aspergillus* DNA positive, 45 *Cyp51A* profiling successful 3 samples showed presence of both WT and resistant peaks	Schauwvlieghe et al., 2017

FFPE, formalin-fixed paraffin-embedded; BAL, bronchoalveolar lavage; LoD, limit of detection.

The *Aspergillus* PCR was positive in 97 samples (74 samples with either galactomannan (GM)-index >1 or with a positive culture) and the resistance PCR was successful in 68 of 97 (70%) samples. In 7 samples TR_{34}/L98H was detected, in one sample TR_{46}/Y121F/T289A, while a combination of TR_{34}/L98H and WT *A. fumigatus* was observed in three samples. Importantly, this study showed that the detection of resistance markers was associated with azole treatment failure; even in culture-negative cases (Chong et al., 2016).

Another study used the assay on 45 GM-positive BAL samples and 55 GM-negative BAL samples. Twenty-seven of 100 patients were positive for *Aspergillus* DNA and 20 for *A. fumigatus* DNA. The amplification of resistance markers appeared to be successful in all *A. fumigatus* positive samples and evidence for azole resistance was found in three (Montesinos et al., 2017).

Other studies have evaluated the AsperGenius assay directly on blood samples, for early detection of *Aspergillus* disease in patients at risk for IA (White et al., 2015a). The assay was evaluated on serum samples from 14 patients with proven or probable IA (total 54 extracts). *A. fumigatus* DNA was detected in 25 of 54 samples. The amount of DNA in the serum, however, was low and only 12 samples of 7 cases had sufficient DNA-levels for the resistance PCR to be performed reflecting the lower sensitivity of the resistance PCR compared to the *Aspergillus* DNA assay. Samples from four patients (57%) had 3–4 loci (TR_{34}, L98H, T289A, or Y121F) amplified and 50% of all patients had at least one loci amplified. Mutation analyses showed no mutations in these patients. Thus despite using multiple samples per patient; genotyping both TR_{34}/L98H and TR_{46}/Y121F/T289A was possible in only four of 14 patients with proven/possible IA. For successful amplification of resistance markers, a *C*t value of <33 was needed in the *A. fumigatus* assay (White et al., 2015b).

As the performance of *Aspergillus*-PCR was shown to be superior on plasma compared to serum, it was hypothesized that plasma was also superior for the detection of resistance markers (White et al., 2015a). Eighty-six plasma samples from 10 patients with proven/probable IA and two patients with possible IA were tested. Eleven samples from six patients were positive for *A. fumigatus*. Amplification of resistance markers was successful in only two of six (33%) patients. Thus, using a mean of seven samples per patient, the resistance markers were successfully amplified in only two of 12 (17%) patients with IA. However, as the *A. fumigatus* PCR was only positive in six of 12 patients and all culture results were negative, it is not known whether the IA was due to *A. fumigatus* in the other patients (White et al., 2017). As the target for azole resistance detection is a single copy gene, the sensitivity can be expected to be lower than the *Aspergillus* DNA assay for which a multi-copy gene is targeted. The results of PCR used on blood samples clearly reflects this lower sensitivity and due to the low amount of DNA in blood samples, clinical utility in routine diagnostics is questionable.

An interesting advantage of the AsperGenius PCR is the simultaneous detection of both WT and mutant *Cyp51A* DNA. As the detection of resistance markers is based on melting curve analysis, mixed infections with both WT as well as DNA from resistant strains will result in two peaks in the assay. This was

shown in three cases were evidence for both WT and L98H was found (Schauwvlieghe et al., 2017).

A report of a second commercial available assay, the MycoGENIE (Ademtech, Pessac, France), was published in 2017. The MycoGENIE is a multiplex assay detecting *A. fumigatus* DNA, the L98H mutation and TR_{34}. The PCR was tested on a total of 55 serum samples from 16 patients and 88 respiratory samples from 62 patients. Fifty-Five respiratory and 16 serum samples were positive for *A. fumigatus* (Dannaoui et al., 2017). The same MycoGENIE was then used on stored clinical samples from 137 patients with fungal rhinosinusitis with a positive microscopy for fungal hyphae. The PCR was positive for *A. fumigatus* in 116 of 147 samples (78.9%), whereas 47 of 48 *A. fumigatus* culture positive samples were positive in the assay (Morio et al., 2018). In both studies no samples were positive for either TR_{34} or L98H.

An important limitation of the MycoGENIE is the absence of WT probes for the resistance marker. As a consequence, non-positivity in the resistance markers might be caused a WT genotype but might also be due to a missed mutation due to limited sensitivity compared to the probe for detection of *A. fumigatus* DNA. Furthermore, the less prevalent environmental TR_{46}/Y121F/T289A mutation is not detected by the MycoGENIE.

Sequence Based Assays

In addition to direct qPCR assays, several other methods have been applied directly to clinical specimen (**Table 2**). Amplification with subsequent pyrosequencing was applied directly on blood samples to detect G54 mutations in the *Cyp51A* gene. *A. fumigatus* DNA was detected in two of 56 whole-blood samples targeting a 269-p *Cyp51A* gene region and *Cyp51A* sequence of locus G54 was determined. The study showed the utility of amplification and subsequent sequencing of clinical material for detection of *Cyp51A*-mutations (Trama et al., 2005). Most other loci were not covered by the targeted region but this can be overcome by sequencing of additional *Cyp51A* regions involved in azole resistance.

Other authors generated additional primers for the specific amplification and subsequent sequencing of the *A. fumigatus Cyp51A*-gene. Three different primer sets were used for the amplification of the *Cyp51A* promoter region, L98 and M220 regions. Eight stored clinical samples were tested and TR_{34}/L98H was successfully detected in a brain abscess. Interestingly, in one BAL fluid, L98H was found without the TR_{34} (Spiess et al., 2012). Using the same method with the addition of another target region for the detection of TR_{46}, stored clinical samples of 155 immunocompromised patients previously tested positive for *Aspergillus* DNA were evaluated. In 75 of 181 (41%) investigated samples, the assay was positive for at least one region. The L98H region alone was successfully amplified and sequenced in 26 (14%), the regions for L98H and TR_{34} in 17 (9%); both revealed no mutations. In 22 of 181 (12%) samples the L98H, TR_{34} as well as the M220 PCR targets were successfully amplificated, showing two samples with both the TR_{34} and L98H mutations. One sample had L98H but not TR_{34} (Spiess et al., 2014). Higher rate of successful amplification was achieved by a nested approach.

TABLE 2 | Overview of studies that applied non-real-time molecular tools directly on clinical specimen to detect azole resistance markers in *A. fumigatus*.

Country	Patients samples	Resistance markers	PCR system	Remarks	Positivity	Reference
United States	56 whole blood samples	G54	Pyrosequencing	No control was available for sequence results	2 *Cyp51A* DNA positive 2 *Cyp51A* profiling successful 2 WT	Trama et al., 2005
Germany	BAL fluid and tissue samples from 8 patients positive for *A. fumigatus* DNA	TR_{34}, L98H, M220	Sequencing		8 *A. fumigatus* DNA positive 8 *Cyp51A* profiling successful	Spiess et al., 2012
Germany	Serum, BAL, tissue from 155 patients	TR_{34}/L98H/TR_{46}/M220	TR34 M220 TR46 nested sequenced based	LoD: L98H, 300fg. TR46, 300fg. Other assays: Spiess et al. (2012)	181 *Aspergillus* DNA positive, 26 L98H profiling successful, 17 L98H and TR_{34} profiling successful, 32 L98H, TR_{34} and TR_{46} profiling successful	Spiess et al., 2014
France	97 respiratory samples	TR_{34}, L98H, G54, G138C, M220	Sequencing		44 *Aspergillus* DNA positive, 38 *Cyp51A* profiling successful	Zhao et al., 2016

LoD, limit of detection.

The sequence method described by Denning et al. (2011) was used but without the molecular beacons. The *Cyp51A* was successfully amplified in 38 of 44 (86%) pan-*Aspergillus* PCR positive samples. Using this technique, the authors found a sputum sample harboring TR_{34}/L98H, which was confirmed by culture (Zhao et al., 2016).

CONCLUSION AND PERSPECTIVES

The application of molecular tools has been shown to increase the sensitivity of detection of azole resistance in *A. fumigatus* compared to culture. Furthermore, the detection of resistance markers in BAL samples using the AsperGenius was associated with increased probability of azole treatment failure. The commercial available assays target TR_{34}/L98H and TR_{46}/Y121F/T289A mutations and are able to detect most of azole-resistant *A. fumigatus* in patients with acute IA. However, the clinical utility depends on regional resistance epidemiology. In patients with prolonged azole therapy, a greater diversity of resistance mutations should be taken into consideration. Detection of resistance markers in blood samples remains problematic; nonetheless if very low Ct value in the *Aspergillus* PCR is observed, resistance markers might be successfully detected.

Direct amplification and subsequent sequencing of the *Cyp51A*-gene showed the potential to cover most *Cyp51A*-mediated resistance mutations. However, compared to real-time assays, sequencing is relatively time-consuming, expensive and laborious, and might lack sensitivity due to low amounts of DNA in most clinical samples. Furthermore, compared to the commercial qPCR assay for detection of azole resistance, clinical validation studies are currently lacking.

New molecular assays should aim to increase sensitivity and extend the coverage of resistance markers to include both environmental as other azole resistance mutations. Sequencers have become portable, inexpensive and can be used in real time. When the capacity of whole genome sequencing increases in the near future, it seems likely that whole genome sequencing can be applied directly on clinical specimens to detect not only the pathogen, but also a wide range of resistance mechanisms.

AUTHOR CONTRIBUTIONS

WM, JZ, and JB reviewed the available papers. JB wrote the first draft manuscript. WM and PV edited the manuscript. All authors gave their final approval.

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ORIGINAL RESEARCH
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Performance of Molecular Approaches for *Aspergillus* Detection and Azole Resistance Surveillance in Cystic Fibrosis

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Aspergillus fumigatus triazole resistance is an emerging concern for treating chronically infected/colonized patients. This study sought to evaluate the performance of PCR assays to detect *Aspergillus* fungi together with azole resistance in sputum samples from cystic fibrosis (CF) patients. In total, 119 sputum samples from 87 CF patients were prospectively processed for *Aspergillus* detection by means of mycological culture and four qPCR assays, 2 in-house methods and two commercial multiplex real-time PCR assays simultaneously detecting *Aspergillus* and the most relevant *cyp51A* gene mutations (MycoGENIE® and AsperGenius®). Azole susceptibility of *A. fumigatus* isolates was assessed using Etest® method and *cyp51A* gene mutation were characterized by sequencing. The overall rate of *Aspergillus* detection with the four qPCR assays ranged from 47.9 to 57.1%, contrasting with 42/119 (35.3%) positive cultures with *A. fumigatus*. The high sensitivity of PCR on sputum could then contribute to more effective grading of *Aspergillus* disease in CF patients. Five out of 41 isolated strains (12.2%) exhibited azole-resistant MIC patterns, three of which harbored *cyp51A* mutations and only 1/3 with the sequence TR_{34}/L98H. Combined with culture, PCR assay achieved high sensitivity *Aspergillus* screening in CF samples. However, *cyp51A* targeting was only moderately effective for azole resistance monitoring, while *Aspergillus* resistance remains of great concern.

Keywords: *Aspergillus*, aspergillosis, cystic fibrosis, quantitative real-time PCR, azole resistance, sputum

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INTRODUCTION

Aspergillus fumigatus is responsible for severe asthma or allergic bronchopulmonary aspergillosis (ABPA) in up to 15% of cystic fibrosis (CF) patients (Stevens et al., 2003; Pihet et al., 2009), though the significance of its detection is often questioned when there are no accompanying clinical signs. *A. fumigatus* is usually susceptible to triazole antifungal drugs (with the exception of fluconazole), which have proved beneficial in treating chronic pulmonary aspergillosis (CPA). During ABPA and chronic colonization, however, their use is still controversial (Stevens et al., 2003; Pihet et al., 2009).

Over the last decade, *A. fumigatus* resistant isolates to triazole have been increasingly reported, and proven associated with a markedly higher mortality rate (van der Linden et al., 2011). This decreased susceptibility is primarily due to mutations in the *cyp51A* gene encoding lanosterol 14α-demethylase, the enzyme involved in ergosterol biosynthesis (Mellado et al., 2001; Alcazar-Fuoli and Mellado, 2013). While TR_{34}/L98H and TR_{46}/Y121F/T289A alterations account for the majority of azole resistance cases, a large diversity of *cyp51A* mutations has also been associated with resistance (Verweij et al., 2016). Mutations have been thought to arise during prolonged antifungal therapy or prophylaxis in individual patients, yet a number of these mutated strains were cultured from patients with no previous azole exposure. Recent data suggest that the proliferating resistance is also caused by intensive use of azole fungicides in agriculture (Verweij et al., 2016). In France, the prevalence of resistant *A. fumigatus* strains grown from sputum samples of CF patients is particularly high, ranging from 4.6 to 10.6% (Burgel et al., 2012; Morio et al., 2012). On the other hand, the current prevalence of triazole resistance in immunocompromised patients with invasive aspergillosis (IA) still remains low, estimated at ≤1% in France (Alanio et al., 2011; Guegan et al., 2018).

In vitro antifungal susceptibility testing is thus essential for patient management. In routine practice, the detection of azole resistance is primarily based on the *in vitro* determination of minimum inhibitory concentration (MIC) from isolates. However, the data on *A. fumigatus* resistance are scarce as susceptibility testing is not always routinely performed and sputum culture lacks sensitivity. To overcome these limitations in resistance screening, molecular methods have recently been developed to detect *A. fumigatus cyp51A* gene mutations, primarily in clinical specimens. Several nested PCR assays (van der Linden et al., 2010; Denning et al., 2011; Spiess et al., 2012, 2014) and commercial kits have previously been used, such as Aspergenius® (Chong et al., 2015; White et al., 2015) and Mycogenie® (Dannaoui et al., 2017) targeting both *Aspergillus* and key *cyp51A* alterations associated with azole resistance, though they have yet to be evaluated in field studies, particularly involving patients with chronic infection.

In this study, we prospectively investigated the prevalence of *Aspergillus* and the triazole resistance of *A. fumigatus* in sputum samples from CF patients using both phenotypic and molecular approaches. The efficacy of two commercial multiplex PCR assays AsperGenius® (PathoNostics, Maastricht, Netherlands) and Mycogenie® (Ademtech, Pessac, France) were compared with two real-time in-house *Aspergillus* assays as well as cultures. The ability of these PCR assays to detect *Aspergillus* and resistance markers was compared with culture-based susceptibility testing and subsequent *cyp51A* gene sequencing.

MATERIALS AND METHODS

Population

Over a 6-month period, (December 2015–May 2016), all expectorated sputum samples collected from CF patients as part of their routine quarterly follow-up at the *Centre de Ressources et de Compétences de la Mucoviscidose* at Rennes University Hospital (France) were included. The samples were processed for all methods each time the minimum sample volume (≥1 ml) was available.

Sputum Culture

On reception, 1–2 mL of sputum were digested with 1X Digest-EUR®, Eurobio (ratio: 1:1), and homogenized for 30 min at room temperature. Samples were divided into two aliquots to perform fungal culture and PCR assays, respectively.

For the cultures, 100 μL of pellets were inoculated in two plates of fungal media (Sabouraud dextrose agar supplemented with 0.5% chloramphenicol). One was incubated at 30°C and the other at 37°C for 7 days (Borman et al., 2010). Mold types isolated from the cultures were identified by microscope.

DNA Extraction From Sputum Samples

We extracted 1mL of digested sputum for centrifugation, and DNA was extracted from a 200 μL pellet using the QIAamp® DNA Mini Kit (Qiagen) following overnight incubation with proteinase K, according to the manufacturer's instructions. As an extraction control, 10 μL of viral DNA (DiaControlDNA, Diagenode) were added. Elution was performed in 100 μL, and the extracted DNA was stored at −20°C until PCR testing.

Susceptibility Testing

Each *A. fumigatus* isolate recovered from Sabouraud slants was tested as an individual isolate using itraconazole (ITC) and voriconazole (VRC) Etest® strips (Biomérieux, Marcy-L'Etoile, France). MICs were determined following 48 h incubation at 37°C. Posaconazole (POS) susceptibility was also tested using Etest® method, only for strains with decreased susceptibility to ITC or VRC. Strains with MIC >2 mg/L for ITC and VRC, and >0.25 mg/L for POS, were considered resistant, according to recent EUCAST breakpoints for fungi (European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2017).

In-House *Aspergillus* PCR Assays

Sputum DNA extracts were tested using two real-time "in-house" PCR assays for *Aspergillus* detection.

The first PCR assay ("Af-mito") amplified a 196 bp-sequence of *A. fumigatus* mitochondrial gene, as previously described (F: GAAAGGTCAGGTGTTCGAGTCA; R: CATC ATGAGTGGTCCGCTTTAC; 5′FAM and 3′TAMRA-labeled probe 5′-ATCCCTAAACCCGCAACCAAAGGC) (Bretagne et al., 1995).

The second PCR assay ("28S") targeted a 67 bp-fragment of the *A. fumigatus 28S rRNA* gene, employing the primers and probe used by Challier et al. (2004) (28S-466: CTCG GAATGTATCACCTCTCGG; 28S-533: TCCTCGGTCCAGG CAGG; 28S-490: FAM-TGTCTTATAGCCGAGGGTGCAATGC G-TAMRA).

Each amplification was performed in a 25 μL final volume containing 1X TaqMan® Universal PCR MasterMix, 0.5 μM

of each primer, 0.2 μM of probe, 2.5 μL of mix for internal control amplification (DiaControlDNA, Diagenode), and 5 μL of sampled DNA.

Amplification was performed in the following thermal conditions: 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C, then 1 min at 60°C, all on a StepOne Plus® instrument (Applied Biosystems).

Commercial *Aspergillus* PCR Assays

Sputum specimens were analyzed by means of two commercial multiplex real-time PCR assays simultaneously detecting *Aspergillus* and the most relevant *cyp51A* gene mutations.

MycoGENIE® (AdemTech, Pessac, France) is a quadruplex real-time PCR assay which targets *A. fumigatus* (*28SrRNA* multicopy gene) in the TR_{34} and L98H regions of the single-copy *cyp51A* gene, including an internal control to monitor for sample inhibition. Amplification was performed over 45 cycles, using an LC480 PCR device (Roche, Meylan, France). When the *A. fumigatus* target was negative and the *C*t value >35 for IC amplification, or when results contrasted with those of the in-house PCRs, the samples were retested, diluted to 1:10, 1:20, and 1:50.

AsperGenius® assay (PathoNostics, Maastricht, Netherlands) provides two different real-time quadruplex amplification mixtures, one for the detection of *Aspergillus* species, and the other one for identifying prevalent resistance mutations. The species multiplex assay enables specific detection of the *A. fumigatus* complex (Af), *A. terreus*, and *Aspergillus* sp. (*Asp* sp.), by targeting the *28S rRNA* multicopy gene. Samples were retested diluted to 1:10 if the internal control *C*t was >36. The resistance multiplex assay targets the single-copy *cyp51A* gene of *A. fumigatus*, and can detect the TR_{34}, L98H, Y121F, and T289A regions. Distinction between wild-type and mutant *A. fumigatus* strains was performed by melting curve analysis. The differences in fusion temperatures necessary to assign resistance were interpreted following the manufacturer's instructions.

Amplification was performed over 45 cycles, according to the manufacturer's instructions, on a LightCycler® 480 instrument (Roche). Analysis was performed on the LightCycler® 480 software, using the second derivative function. The horizontal threshold was fixed above background noise and a positive result was defined by a signal detection with a *C*t value <45 cycles.

DNA Extraction From Resistant *A. fumigatus* Strains

DNA was extracted from *A. fumigatus* cultures a minimum of 4 days old. A conidial suspension was created in MagNA® Pure Bacteria Lysis Buffer (Roche) and transferred to a MagNa® Lyser Green Beads tube (Roche) for homogenization with the MagNa® Lyser Instrument (Roche). We then extracted 400 μL of supernatant using the MagNa® Pure Compact Nucleic Acid Isolation Kit and a MagNa® Pure Extraction Instrument (Roche Diagnostics), according to the manufacturer's instructions.

Molecular Identification of Resistant Strains

Identification of *A. fumigatus* isolates with ITC MIC >2 mg/L was further confirmed by *beta-tubulin* gene sequencing.

PCR amplification was performed using *Bt2a* and *Bt2b* primers (Glass and Donaldson, 1995). Amplification reaction and subsequent sequencing were performed as previously described (Comacle et al., 2016). Sequencing was performed using an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). Bidirectional sequences were analyzed using Seqscape® software v.2.5 and assessed within the GenBank public database using the BLAST Search program for comparison and species identification.

Cyp51A Gene Typing

The whole *cyp51A* gene and its promoter were sequenced in both strands from all *A. fumigatus* strains with elevated MIC values, using five sets of primers: PA5 and PA7 (Mellado et al., 2001), AF306F and AF855R, AF766F and AF1330R, AF1179F and AF1709R, and AF1426F and AF2025R (Alanio et al., 2011).

The PCR mixture contained 5 μL of DNA extract and 20 μL of mix composed of 0.625 U of GoTaq® Hot Start Polymerase (Promega), 1x Colorless GoTaq®Flexi Buffer (Promega), 2 mM of $MgCl_2$ (Promega), 0.8mM of dNTP mix (Eurobio), and 0.2 μM of each primer. The amplification program consisted of 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C, followed by a final step of 10 min at 72°C.

Following purification and sequencing as described above, sequences of resistant strains were compared to the wild-type *A. fumigatus* sequence CM 237 (GenBank accession number AF338659), at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Statistical Analysis

Data analysis was performed using GraphPad PRISM® v.5.02 software. For continuous variables, the Mann–Whitney test was used. A *p*-value of 0.05 was considered statistically significant.

RESULTS

Study Population

One hundred and nineteen specimens were collected in the context of routine clinical follow-up from 87 CF patients (sex ratio: 1.1), aged 4–59 years old (mean age: 26 ± 13 years), and included for both culture and PCR assays. Of the 87 patients, 37 (42.5%) were receiving triazole therapy at the time of sampling, specifically itraconazole (n = 25), posaconazole (n = 11), or voriconazole (n = 1).

Detection of *Aspergillus* in Sputum Samples

As depicted in **Table 1**, *A. fumigatus* grew in 42 of the 119 sputum samples (35.3%), corresponding to 33 distinct patients. The two in-house Af-mito and 28S PCRs were positive in 65 (54.6%) and 68 samples (57.1%), respectively. Only 37 and 38 of the 42 sputum samples grown with *A. fumigatus* yielded positive PCR results

TABLE 1 | *Aspergillus* PCR results according to sputum culture results (*n* = 119).

Culture results	Positive *Aspergillus* PCR assay results *n* (%)				
	Af mito PCR	28S PCR	Mycogenie® PCR	Aspergenius® Af PCR	Aspergenius® *Asp* sp. PCR
All (*n* = 119)	65 (54.6)	68 (57.1)	64 (53.8)	57 (47.9)	64 (53.8)
Positive culture for *Aspergillus*					
A. fumigatus (*n* = 42)	37 (88.1)	38 (90.5)	31 (73.8)	33 (78.6)	35 (83.3)
Non-*fumigatus Aspergillus* species[a] (*n* = 5)	2[b] (40.0)	2[b] (40.0)	2[b] (40.0)	2[b] (40.0)	3[b,c] (60.0)
Positive culture for other molds[d] (*n* = 19)	2[e] (10.5)	3[e] (15.8)	3[e] (15.8)	1[e] (5.3)	2[f] (10.5)
Negative culture [g] (*n* = 55)	26 (47.3)	27 (49.1)	30 (53.6)	23 (41.8)	26 (43.3)

[a]*A. nidulans (n = 1), A. versicolor (n = 1), Aspergillus sp. (n = 1), A. terreus (n = 1), A. flavus (n = 1).* [b]*Samples grown simultaneously with A. fumigatus and flavus (n = 1), A. fumigatus and A. terreus (n = 1).* [c]*A. nidulans (n = 1).* [d]*Scedosporium sp. (n = 6); Penicillium sp. (n = 8); Rasamsonia argillacea (n = 2); dematiaceous molds (n = 2); unidentified mold (n = 1).* [e]*All were Scedosporium sp..* [f]*Scedosporium sp. (n = 1), Penicillium (n = 1)* [g]*Yeasts were not taken into account. PCR, polymerase chain reaction.*

with Af-mito and 28S PCR, respectively. Roughly similar results were recorded with the two commercial PCRs (Mycogenie® and Aspergenius®), which amplified 64 (53.8%) and 57 (47.9%) of the total 119 samples, respectively.

Of note, the use of internal control for PCR inhibitors monitoring showed the presence of inhibitors to be prevalent in sputum samples. For in house-PCRs, 28 out of 119 (20%) samples yielded a positive signal for *Aspergillus* detection after dilutions (up to 1:50).

Interestingly, all PCRs detected *A. fumigatus* in a large number of samples that were negative in culture. As many as 41.8% to 53.6% of the 55 negative culture samples were, in fact, positive with at least one of the four PCRs. Mycogenie® achieved the highest sensitivity in these samples, detecting 30/55 positive specimens (53.6%), whereas the AsperGenius® PCR targeting *A. fumigatus* (Af) offered the lowest sensitivity (23/55, 41.8%). The AsperGenius® *Asp* sp. assay was positive in 26/55 negative culture samples (43.3%), two of which tested positive exclusively for this target, suggesting that non-*Aspergillus* DNA was present in these samples.

None of the three sputum specimens grown with only non-*A. fumigatus* species yielded positive results with any of the four PCRs specifically targeting *A. fumigatus*. Conversely, Aspergenius® *Asp* sp. correctly detected *A. nidulans*, yet was negative for the sample grown with *A. versicolor*. Finally, as observed previously by our team, all the PCRs yielded cross-reaction, with three out of six specimens detected as positive for *Scedosporium* (Guegan et al., 2018).

Resistance Screening Using Commercial PCR Assays

As shown in **Table 2**, no *cyp51A* gene alterations were detected by Mycogenie® and Aspergenius® assay in the 57 and 64 sputum samples positive for *A. fumigatus*, respectively. However, Aspergenius® assay correctly amplified the four regions of the *cyp51A* gene in only 20 of the 57 assessable specimens (35%). Amplification success was variable and depended on the *cyp51A* target, with detection rates ranging from 38.6% (L98H region) to 56.1% (T289A region) (**Table 2**). This finding was probably linked to the low fungal burden in samples with incomplete *cyp51A* typing, as shown by the mean *C*t values of Af target, which were significantly higher in samples with failed amplification compared to others (32.7 ± 1.6 vs. 34.1 ± 1.6, $p = 0.005$).

Resistance Screening Using MIC Determination

We were able to assess susceptibility to triazole drugs for 41/42 *A. fumigatus* isolates. As presented in **Figure 1**, five isolates presented a resistance profile (12.2%) with high ITC MIC (≥32 mg/L). Of these five, collected from five distinct patients, three displayed cross-resistance to VRC, with high MICs ranging from 8 to >32 mg/L. Susceptibility to POS was also reduced in four of the five strains (MICs from 4 to >32 mg/L).

Cyp51A Gene Sequencing of Resistant *A. fumigatus* Isolates and Occurrence During Patient Follow Up

The whole *cyp51A* gene of the five resistant isolates was further sequenced to identify the mechanism responsible for resistance. Three of the five displayed *cyp51A* gene alterations (**Table 3**). One L98H mutation (associated with a 34 bp tandem repeat in the promoter) was found in a pan-azole-resistant isolate. Surprisingly, the sputum sample from which this strain was cultured did not yield a detection of *cyp51A* mutation with any PCR assay (Mycogenie®, Aspergenius®). M220K and G54R mutations were both observed in strains with ITC and POS combined resistance.

The characteristics of patients colonized with resistant strains are depicted in **Figure 2**. All five had been receiving long-term itraconazole therapy for periods ranging from 11 months (Patient 1) to over 10 years (Patient 4) prior to sampling. No correlation was found between patient age or duration of triazole exposure and *cyp51A* genotype.

DISCUSSION

The burden of *Aspergillus* infections and sensitization is high among fungal diseases, particularly in the context of chronic pulmonary diseases (Gangneux et al., 2016). The emergence of triazole resistance in *A. fumigatus* has fuelled interest in

TABLE 2 | Detection of *cyp51A* mutation using Mycogenie® and Aspergenius® assays.

PCR assay		Number of positive samples (%) for				
		A. fumigatus	**TR_{34}**	**L98H**	**Y121F**	**T289A**
AsperGenius®	Target amplification[a]	57 (47.9)	25/57 (43.9)	22/57 (38.6)	30/57 (52.6)	32/57 (56.1)
	Detection of mutated allele[b]	NA	0/25	0/21	0/30	0/32
Mycogenie®	Detection of mutated allele	64 (53.8)	0/64	0/64	NA	NA

NA, not applicable. [a]*Successful detection of regions carrying targeted mutations.* [b]*Mutated cyp51A allele detection achieved by determining melting temperature values from TR_{34}, L98H, Y121F, and T289A region amplicons.*

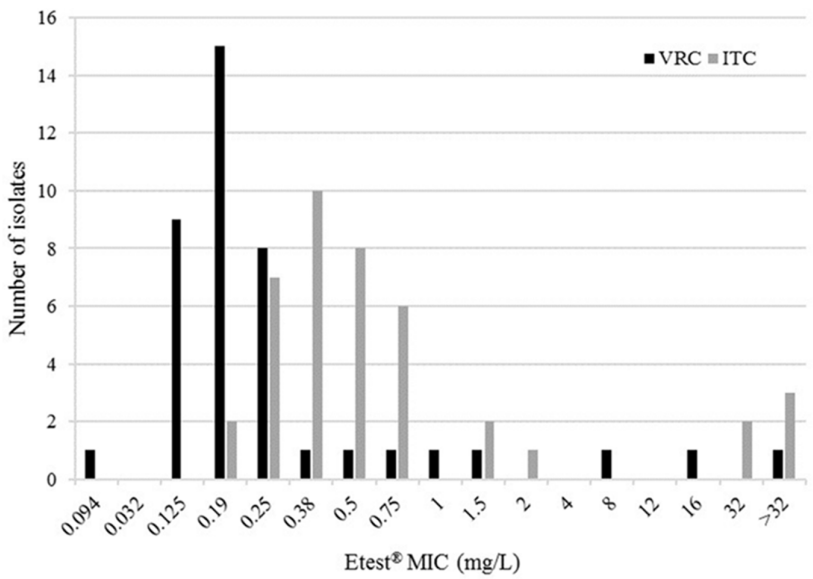

FIGURE 1 | Distribution of itraconazole (ITC) and voriconazole (VRC) minimum inhibitory concentrations (MICs) of 41 *Aspergillus fumigatus* strains using Etest®.

molecular screening of clinical specimens. Here, we investigated prospectively whether using two commercial PCR assays was effective in (i) detecting *Aspergillus* in sputum samples from CF patients compared to culture or in-house PCRs, and (ii) in typing azole resistance.

Aspergillus fumigatus was the most frequent fungus isolated from CF sputum, with 35.3% of samples testing positive. PCR offered much higher sensitivity than culture, with over 40% of negative cultures yielding positive results with at least one PCR assay. There is very little data on PCR efficacy in CF sputum analyses in the literature, and our results reinforced the findings of a previous study that revealed considerable discrepancies between culture and PCR sensitivity (33% vs. 73%, respectively) (Baxter et al., 2011). We performed a standardized sputum homogenization, as this process has been shown to be crucial for these analyses (Baxter et al., 2011). The viscous nature and complex matrix of CF sputum can be responsible for inhibition and impaired detection. We thus introduced internal checks to monitor sample inhibition and systematically performed sample dilutions for in-house PCRs to minimize this phenomenon. More than 20% were found to contain PCR inhibitors. Exposure to antifungals may also contribute to limited performances of culturing but also molecular detection of *Aspergillus*. In the context of invasive aspergillosis, we previously showed the worth of molecular detection of *Aspergillus* in intensive care unit patients compared to patients that benefit from antifungal prophylaxis in hematology units (Guegan et al., 2018).

Scedosporium is usually considered the second most prevalent filamentous fungus in CF. In our study, we recorded a significant incidence of *Scedosporium* isolates (6/119), producing three positive PCR results. These results question an apparent lack of

TABLE 3 | Minimum inhibitory concentration (MIC) and *cyp51A* sequencing of azole-resistant isolates.

Patient *n*°	***cyp51a* mutation (typing)**		**MIC Etest® (mg/L)**		
	Nucleotide sequence	**Amino acid sequence**	**VRC**	**ITC**	**POS**
1	None	None	16	32	0.094
2	t293a	TR_{34}/L98H	>32	>32	>32
3	t659a	M220K	1.5	>32	>32
4	g160a	G54R	0.25	>32	>32
5	None	None	8	32	4

MIC, minimum inhibitory concentration; VRC, voriconazole; ITC, itraconazole; POS, posaconazole.

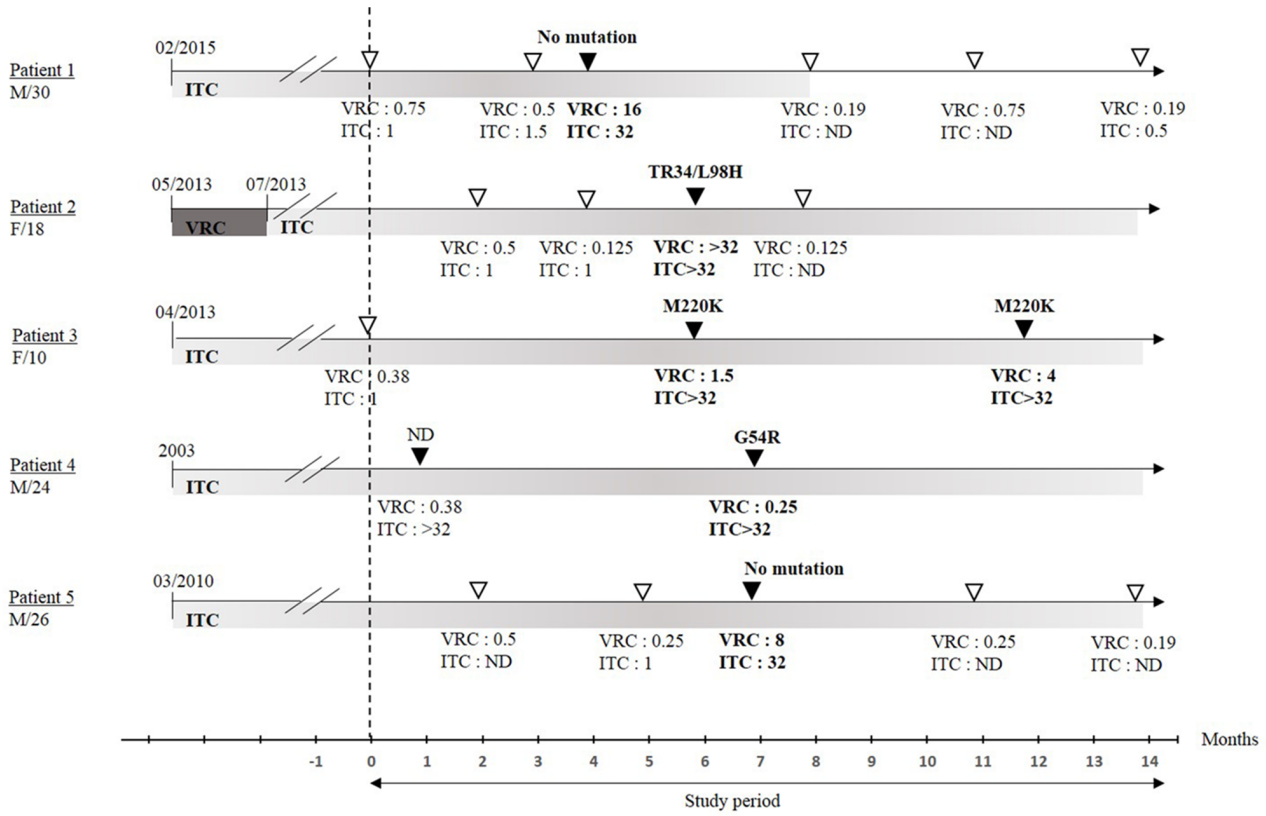

FIGURE 2 | Date of treatment onset and time frame of sample collection over the study period. Isolates susceptible to azole are represented by open triangles, isolates resistant to at least one azole are represented by black triangles. Respective MIC values (mg/L) are indicated. MIC, minimum inhibitory concentration; ITC, itraconazole; VRC, voriconazole; ND, not determined; F, female; M, male; /*n*, age in years. Susceptibility testing was performed only from 09/2015.

specificity of the *Aspergillus* PCR assay regarding *Scedosporium* species. Three isolates were sequenced using the *ITS1-5.8S-ITS2* gene. No species relationship was confirmed, as all strains were identified as part of *Scedosporium apiospermum complex* (data not shown).

While some studies have identified associations between *Aspergillus* isolation in sputum samples and the risk of pulmonary exacerbations or deteriorating respiratory function (Amin et al., 2010), the clinical relevance of PCRs in these specimens has not yet been assessed. The question remains, for example, of whether DNA fungal detection is associated with respiratory disease or is only a marker of colonization (Jones et al., 2014). In our cohort, the detection of serological markers was completed within a 3-month period surrounding sputum sampling for 26 of the 27 patients producing positive PCRs but negative cultures. *Aspergillus* antibodies or precipitins were detected in nine of them (34.6%, data not shown), indicating chronic exposure. Overall, the high sensitivity of PCR on sputum could contribute to more effective grading of *Aspergillus* disease in CF patients together with clinical signs and other biological markers, as proposed by Baxter et al. (2013).

The optimal therapeutic management of CF patients chronically colonized with *Aspergillus* is still a matter of debate. There is no consensus on the use of triazole components when *A. fumigatus* is detected in respiratory samples (Burgel et al., 2016). However, the emergence of *A. fumigatus* strains resistant to azole drugs has to be taken into account for clinical management. Here we observed reduced susceptibility to itraconazole in 5/41 *A. fumigatus* isolates (12.2%) similar to that already reported (Morio et al., 2012). The growing incidence of resistant fungi in clinical specimens is currently driving the development of new molecular approaches for rapid resistance screening directly on respiratory samples. As far as we know, this study was the first prospective evaluation of commercial multiplex PCRs to monitor triazole resistance in CF samples. These assays did not enable us to detect any hotspot mutation markers in the 57 and 64 sputum samples amplified with Aspergenius® Af PCR and Mycogenie® PCR, respectively. It should be noted that, in the respiratory samples from British patients with CPA, high azole resistance rates was reported using PCR (around 50%), while the cultures remained negative (Denning et al., 2011).

Globally, the sensitivity of marketed *cyp51A* PCR assays was low, primarily due to there being only a single copy of the gene. In contrast, culture isolation of resistant *Aspergillus* enabled us to sequence *cyp51A*. Of the five resistant isolates, three displayed TR_{34}/L98H, M220 or G54 mutations that have already been linked to azole resistance in CF patients. Moreover,

strains without any *cyp51A* alterations have been shown to be an emerging concern, suggesting the possibility there are other mechanisms responsible for triazole resistance (Rivero-Menendez et al., 2016).

Finally, PCR achieved much higher positivity in its detection of *Aspergillus* than culture, regardless of the method used (in-house or marketed). As for multiplex PCR, also able to detect *cyp51A* mutations, the presence of this gene in a single copy limits the sensitivity of a molecular approach compared to culture. There are, however, two potential limits to the resistance screening method based on MIC determination that should be discussed. Firstly, MIC is usually determined using a single colony in routine practice, yet there has been biodiversity reported within the same sputum. For example, a previous study reported up to 28% of CF patients presented complex colonization patterns containing various genotypes which succeeded each other, regardless of their antifungal susceptibility (de Valk et al., 2009). In our study, of the five patients colonized with resistant strains, only two produced several consecutive cultures growing with resistant strains (patients 3 and 4, **Figure 2**), despite no drug switches being performed between samplings. To overcome this potential inaccuracy, the use of a selective medium supplemented with antifungals for resistance detection from primary culture is currently spreading in routine practice, although there are no studies as yet reporting their relevance in CF samples (Hamprecht et al., 2017). Secondly, while there are few strains that show low MICs to itraconazole and resistance to voriconazole, itraconazole should be the preferred drug for measuring *in vitro* MIC due to the discrepancies between resistance expression against the various azoles. However, this procedure may miss some exceptional strains resistant to voriconazole despite low itraconazole MIC.

ETHICS STATEMENT

This study was conducted in accordance with the Declaration of Helsinki and national and institutional standards. It was approved by the local 'Rennes Ethics Committee'. No supplementary samples were drawn and investigations were considered part of routine clinical practice. However, patients or next of kin was informed of their inclusion in this study and could refuse to participate.

AUTHOR CONTRIBUTIONS

J-PG and FR-G designed, performed, and wrote the manuscript. HG and SC performed the analysis and wrote the manuscript. CB and ED contributed to the analysis of data.

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Comparison of Two Molecular Assays for Detection and Characterization of *Aspergillus fumigatus* Triazole Resistance and *Cyp51A* Mutations in Clinical Isolates and Primary Clinical Samples of Immunocompromised Patients

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In hematological patients, the incidence of invasive aspergillosis (IA) caused by azole resistant *Aspergillus fumigatus* (ARAf) is rising. As the diagnosis of IA is rarely based on positive culture in this group of patients, molecular detection of resistance mutations directly from clinical samples is crucial. In addition to the in-house azole resistance ARAf polymerase chain reaction (PCR) assays detecting the frequent mutation combinations TR34/L98H, TR46/Y121F/T289A, and M220 in the *Aspergillus fumigatus* (*A. fumigatus*) *Cyp51A* gene by subsequent DNA sequence analysis, we investigated in parallel the commercially available AsperGenius® real time PCR system in detecting the *Cyp51A* alterations TR34/L98H and Y121F/T289A directly from 52 clinical samples (15 biopsies, 22 bronchoalveolar lavage (BAL), 15 cerebrospinal fluid (CSF) samples) and ARAf isolates ($n = 3$) of immunocompromised patients. We analyzed DNA aliquots and compared both methods concerning amplification and detection of *Aspergillus* DNA and *Cyp51A* alterations. As positive control for the feasibility of our novel Y121F and T289A PCR assays, we used two *A. fumigatus* isolates with the TR46/Y121F/T289A mutation combination isolated from hematological patients with known *Cyp51A* alterations and a lung biopsy sample of a patient with acute myeloid leukemia (AML). The rate of positive ARAf PCR results plus successful sequencing using the ARAf PCR assays was 61% in biopsies, 29% in CSF, 67% in BAL samples and 100% in isolates. In comparison the amount of positive PCRs using the AsperGenius® assays was 47% in biopsies, 42%

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in CSF, 59% in BAL samples and 100% in isolates. Altogether 17 *Cyp51A* alterations were detected using our ARAf PCRs plus DNA sequencing and therefrom 10 alterations also by the AsperGenius® system. The comparative evaluation of our data revealed that our conventional PCR assays are more sensitive in detecting ARAf in BAL and biopsy samples, whereby differences were not significant. The advantage of the AsperGenius® system is the time saving aspect. We consider non-culture based molecular detection of *Aspergillus* triazole resistance to be of high epidemiological and clinical relevance in patients with hematological malignancies.

Keywords: invasive aspergillosis, triazole resistance, PCR, clinical samples, melting curve analysis

INTRODUCTION

Aspergillus fumigatus (*A. fumigatus*) is one of the major live-threatening fungal pathogens (Brown et al., 2012). It is estimated that more than 200,000 severe infections occur worldwide annually (Brown et al., 2012). Due to an increase in immunocompromised patients more people are at risk to suffer from invasive aspergillosis (IA) (Kim, 2016) which is associated with high mortality rates, especially in patients with malignant hematological diseases (Kontoyiannis et al., 2010; Perfect et al., 2014; Koehler et al., 2017). Triazoles are the main stay in the prophylaxis and treatment of IA.

The situation is worsened by an increasing prevalence of triazole resistant *Aspergillus* infections (Steinmann et al., 2015; van der Linden et al., 2015; Verweij et al., 2016; Garcia-Rubio et al., 2017) which is associated with a much higher mortality rate (van der Linden et al., 2011; Steinmann et al., 2015; Verweij et al., 2015; Chong et al., 2016; Meis et al., 2016). Triazole treatment failure was observed in 6/8 patients with a resistance associated mutation (RAM) compared with 12/45 patients without RAMs ($p = 0.01$). Six week mortality was 2.7 times higher in patients with RAMs (50 vs. 19%; $p = 0.07$) (Chong et al., 2016). About 50–80% of triazole resistance in *A. fumigatus* is caused by mutations in the *Cyp51A* gene (Dudakova et al., 2017). The 14-alpha-sterol-demethylase, the product of the *Cyp51A* gene, plays a major role in the ergosterol biosynthesis (Mellado et al., 2001), whereby triazoles act through inhibiting the 14-alpha-sterol-demethylase. The most frequent *Cyp51A* mutation combination found is the TR34/L98H gene alteration (Dudakova et al., 2017). In 2015 van der Linden et al. described the TR46/Y121F/T289A mutation combination as the second most frequent resistance-mechanism causing high level triazole resistance (van der Linden et al., 2015; van Ingen et al., 2015).

Due to mostly negative *Aspergillus* cultures from clinical material of hematological patients in microbiological diagnostics (De Pauw et al., 2008; Ruhnke et al., 2012; Morrissey et al., 2013; Koehler et al., 2017) and due to the higher mortality rates caused by azole resistant *Aspergillus fumigatus* (ARAf) infections, it is of high clinical impact to achieve sensitive and early detection of *A. fumigatus* including a triazole resistance. Therefore, molecular methods are required especially in hematological patients. Several polymerase chain reactions (PCR) assays for the detection of *A. fumigatus* and its *Cyp51A* mutations from clinical isolates have been published (Dudakova et al., 2017). Concerning the detection of *Aspergillus* DNA and triazole resistance mutations directly from primary clinical samples, the commercial real time PCR kit system AsperGenius® (Pathonostics; Maastricht, The Netherlands) is described to detect *Aspergillus* and four resistance-related mutations validated for bronchoalveolar lavage (BAL) and serum specimens (Chong et al., 2015, 2016; White et al., 2015b). The novel MycoGENIE® (Ademtech, Pessac, France) real time PCR kit is also able to identify *Aspergillus* DNA, but only the TR34/L98H mutation combination in serum and respiratory samples (Dannaoui et al., 2017).

Our group established TR34/L98H, TR46, and M220 ARAf PCR assays with subsequent DNA sequence analysis for the detection of the most common *Cyp51A* mutations that are correlated with triazole resistance in *A. fumigatus* directly from primary clinical samples. In addition, we recently developed two PCR assays for the detection of the Y121F and the T289A mutations from clinical samples that are associated with the TR46 tandem repeat. Blood, BAL, biopsy, and cerebrospinal fluid (CSF) samples were previously investigated and there four TR34/L98H and one TR46/Y121F/T289A *Cyp51A* mutations were successfully identified in five BAL and biopsy samples (Hamprecht et al., 2012; Rath et al., 2012; Spiess et al., 2012, 2014; Rössler et al., 2017).

In this study we compared the AsperGenius® kit system to our six in-house ARAf PCR assays with subsequent DNA sequence analysis concerning the sensitivity of detection of *Aspergillus* DNA and present triazole resistance mutations investigating 22 BAL and 15 CSF samples, 15 biopsies and three clinical ARAf isolates of immunocompromised patients.

MATERIALS AND METHODS

Patients

For the determination of mutations in the *A. fumigatus Cyp51A* gene conferring triazole resistance (TR34/L98H; TR46/Y121F/T289A and M220 alterations), we investigated clinical samples (BAL, tissue biopsies, CSF) of 52 immunocompromised patients mainly with hematological malignancies [AML $n = 11$; ALL $n = 11$; CLL $n = 3$; MDS $n = 1$; NHL $n = 14$; Hodgkin lymphoma $n = 1$; solid tumor $n = 3$; autoimmune neutropenia $n = 2$; immunosuppression not otherwise specified (NOS, $n = 6$)]. All samples were previously tested positive for *Aspergillus* DNA using our in-house diagnostic

Aspergillus PCR assay (Skladny et al., 1999). Samples submitted to the scientific laboratory of the Department of Hematology and Oncology of the Mannheim University Hospital, Germany, for diagnosing IA were analyzed to elucidate PCR performance. Patients' data had been anonymized previously. Analyses were done according to Good Clinical Practice (GCP) guidelines as well as in concordance with the Declaration of Helsinki. The study was approved by the local Ethics Committee (Ethics Committee of the Faculty of Medicine Mannheim, University of Heidelberg, Germany; reference number 2011-280N-MA) and documented under ClinicalTrials.gov (identifier NCT01695512).

Clinical Samples

Bronchoscopy and BAL was performed according to standardized operating procedures as described elsewhere (Skladny et al., 1999), and BAL samples were obtained in a sterile vessel without conservation media. The mean sample volume was 10 mL. Tissue samples were obtained by needle biopsies (lung, liver, kidney) or surgical procedures (brain, other samples) under sterile conditions. Cerebrospinal fluid was gained and prepared as described (Hummel et al., 2006). We examined 55 specimens in total; these included 22 BAL specimens, 15 biopsies, 15 CSF specimens, and three ARAf isolates which served as positive controls.

Strains and Growth Conditions

A. fumigatus wildtype strain (DSM 819) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and the Institute of Medical Microbiology and Hygiene, Mannheim University Hospital, Mannheim, Germany.

One triazole-resistant TR34/L98H positive *A. fumigatus* clinical isolate was obtained from the University Hospital of Cologne, Cologne, Germany. The isolate showed the following MIC (minimum inhibitory concentration) values obtained by EUCAST reference microdilution method: voriconazole 2.0 mg/L, itraconazole >16.0 mg/L, posaconazole 0.5 mg/L (Hamprecht et al., 2012). The second triazole resistant TR46/Y121F/M172I/T289A positive *A. fumigatus* strain was from the Institute for Medical Microbiology, University Medical Center Göttingen, Göttingen, Germany (originated from the Institute of Medical Microbiology and Hygiene, Technische Universität Dresden, Dresden, Germany) and showed the following EUCAST MIC values: voriconazole >32 mg/L, itraconazole 1 mg/L, posaconazole 0.5 mg/L (Rössler et al., 2017). Characterization of the third multi-azole resistant TR46/Y121F/T289A positive clinical isolate (IMMi2107) from the Institute of Medical Microbiology, University Hospital Essen, Essen, Germany revealed the following EUCAST MIC values: itraconazole >16.0 mg/L, voriconazole 2 mg/L, posaconazole 0.5 mg/L (Steinmann et al., 2015).

DNA Extraction

DNA extraction from fungal cultures and from biopsy, CSF, and BAL samples was performed using the phenol/chloroform extraction method as previously described (Sambrook et al., 1989; Skladny et al., 1999). Tissue samples were processed additionally in liquid nitrogen for disruption. The tissue was sheared using a scalpel in a sterile petri dish under sterile conditions. The generated nuggets were transferred into a tissueTUBE^{TM} used for processing of the sample in a cryoPREP^{TM} workflow (Covaris; USA). The tissueTUBE^{TM} containing the tissue material was incubated in liquid nitrogen for 30–45 s until the sample was completely frozen. After freezing the tube was fitted into the cryoPREP^{TM} workflow, were the tissue was pestled. The frozen tissue pieces were transferred into a sterile 50 ml reaction tube and mixed with 1.5 ml 1x PBS buffer. The tissue/PBS mixture was transferred into a 1.5 ml reaction tube and centrifuged at 13,000 rmp for 10 min. Supernatant was discarded and the pellet was resuspended in 250 µl 1x PBS buffer.

ARAf PCR Assays

All 52 clinical specimens plus the 3 isolates were analyzed using our six in-house ARAf PCR assays. Our modified one-step L98H-PCR assay amplifies a 143 bp fragment and has been previously described in Spiess et al. (2014). The TR34 nested PCR assay, as well as the M220 one-step PCR-assay have been described in 2012, amplifying a 100 bp and a 173 bp DNA-fragment, respectively (Spiess et al., 2012). To complete our TR46 PCR assay (Spiess et al., 2014), we established new PCR assays to detect the corresponding Y121F and T289A mutations. Additionally, we slightly adjusted our established TR46 PCR assay (Spiess et al., 2014) by lowering the annealing temperature to 50°C instead of 52°C. The in-house ARAf PCR assays are summarized in **Table 1** and shown in **Figure 1**.

The *A. fumigatus Cyp51A* gene specific Y121F and T289A primer pairs were designed from the *A. fumigatus Cyp51A* DNA sequence (AF 338659.1) available in the GenBank database (http://www.ncbi.nlm.nih.gov/). To predict the potential cross reactivities of the *A. fumigatus Cyp51A* primer sequences with human genomic DNA sequences, additional database searches were performed by using the primer-BLAST service. The melting temperatures (T_ms) of the primers and possible secondary structures were calculated using also the NCBI primer designing tool primer-BLAST 2016 (http://www.ncbi.nlm.nih.gov/). The synthetic oligonucleotides were commercially synthesized (Sigma, Munich, Germany) and diluted to 100 µM in ddH_2O.

TABLE 1 | Summary of the six established in-house ARAf PCR assays.

Mutation	Fragment length	PCR assay	Sensitivity
L98H (Spiess et al., 2012)	143 bp	One-Step	6 pg
TR34 (Spiess et al., 2012)	1st step: 235 bp (WT) 2nd step:100 bp (WT)	Two-Step	600 fg
M220 (Spiess et al., 2012)	173 bp	One-Step	4 pg
L98H (Spiess et al., 2014)	143 bp	One-Step	300 fg
TR46 (Spiess et al., 2014)	1st step: 213 bp (WT) 2nd step:103 bp (WT)	Two-Step	300 fg
Y121F	121 bp	One-Step	300 fg
T289A	133 bp	One-Step	300 fg

WT, wild type.

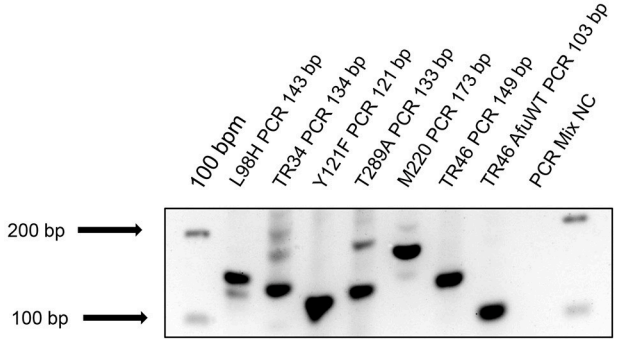

FIGURE 1 | Agarose gel electrophoresis of the six established in-houes ARAf PCR assays. L98H, TR34, Y121F, T289A, and M220 PCRs were performed using DNA (mixture of human and fungal DNA) of a TR34/L98H mutation positive BAL sample of a patient suffering from AML (Hamprecht et al., 2012). The TR46 PCR was performed using a TR46 positive clinical *A. fumigatus* isolate (lane 7) and *A. fumigatus* wildtype (WT) DNA (lane 8). NC, negative control, bpm, base pair marker.

Novel ARAf PCR Assays, Specificity and Sensitivity

The novel PCR assays were performed as one-step PCR assays. The generated PCR fragment for the Y121F assay was 121 bp in length using the primer pair *Cyp51A*-Y121F-s1 (5′-CATTG ACGACCCCCGTTTTC-3′) and *Cyp51A*-Y121F-as1 (5′-GGCA CATGAGACTCTAACGCA-3′). The generated PCR fragment for the T289A assay was 133 bp in length using the primer pair *Cyp51A*-T289A-s1 (5′-CACATACAAAAACGGCCAGCA-3′) and *Cyp51A*-T289A-as1 (5′-TTTTGGCTGTGAGGCCAGT C-3′). The following PCR conditions were used for both PCR reactions: total volume, 25 μl; 3 μl template DNA (∼100 ng human DNA plus an unknown amount of *A. fumigatus* DNA), 3 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate, 1 U of *Taq* polymerase (Invitrogen GmbH, Karlsruhe, Germany), 20 pmol of each primer; DNA thermal cycler, 5 min of initial denaturation at 94°C, 40 cycles of 94°C for 45 s, 54°C for 1 min, 72°C for 1 min, after the 40 cycles 94°C for 45 s, 54°C for 45 s, and final extension at 72°C for 10 min. Sensitivity of the novel PCR assays was determined using serially diluted *A. fumigatus* wildtype DNA as template. To test cross reactivity of the designed primer pairs with human genomic DNA, we investigated a sample adopted in the PCR assays containing a mixture of 100 ng human genomic DNA and 50 ng of *A. fumigatus* wildtype DNA. PCR products were analyzed by agarose gel analysis stained with GelStar (Bio-Rad GmbH, Munich, Germany). The detection threshold for both assays was 300 fg of genomic *A. fumigatus* DNA.

Control

The established PCR assays for the detection of Y121F/T289A directly from clinical samples (BAL, tissue biopsies, CSF) as a marker of the TR46/Y121F/T289A genotype were tested using DNA of a TR46/Y121F/T289A positive, multi-azole resistant clinical isolate (IMMi 2107).

Sequence Analysis

The PCR products were used directly for mandatory sequence analysis. The PCR products were purified using the MiniElute PCR purification kit (Qiagen, Hilden, Germany) and a minimum of 50 ng DNA was sequenced (Sequiserve, Vaterstetten, Germany). To detect potential mutations in the PCR products analyzed by DNA sequence analysis, the sequence of the products was compared to the sequence of the *A. fumigatus Cyp51A* wildtype sequence using the NCBI alignment service AlignSequenceNucleotideBlast (http://www.ncbi.nlm.nih.gov/) and the FunResDB-A (Weber et al., 2018).

AsperGenius® PCR Kit System

The AsperGenius® system is a real time multiplex PCR approach. The system was used for the identification of prevalent mutations conferring resistance against triazoles in clinical samples tested positive for *Aspergillus* DNA before using our in-house diagnostic *Aspergillus* PCR assay (Skladny et al., 1999).

The AsperGenius® system contains both a diagnostic *Aspergillus* DNA detection kit and a kit for detection of four triazole resistance mutations, namely TR34, L98H, Y121F, and T289A. The species multiplex assay allows the specific detection of *A. fumigatus* complex, *Aspergillus terreus*, and other *Aspergillus* species by targeting the 28S rRNA multicopy gene. 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*. The different *Cyp51*A alterations are detected by melting curve analysis in different fluorescence detection channels (450, 530, 598, and 645 nm) by a shift in the melting curves of the mutation-bearing DNA compared to wild type DNA. We tested the approach using the LightCycler 480 technology (Roche Diagnostics GmbH, Mannheim, Germany) and only analyzed the triazole resistance detection in clinical BAL, biopsy, and CSF samples. One representative analysis of a BAL sample for the detection of the L98H mutation by melting curve analysis is shown in **Figure 2**.

Statistics

Statistical calculations were performed using the Chi-square test.

RESULTS

Four established (Spiess et al., 2012, 2014) and two novel in-house ARAf PCR assays were compared to the commercially available AsperGenius® kit system concerning the detection of *A. fumigatus* DNA and *Cyp51A* key mutations directly from clinical samples of immunocompromised patients. Fifty-two clinical samples (15 biopsies, 22 BAL, 15 CSF samples) of 52 immunocompromised patients and three *A. fumigatus* isolates were investigated with both methods and results were compared.

Analyzing 22 BAL specimens with our six Mannheim ARAf PCR assays revealed the following results: 82% (18/22) of the samples showed a positive signal in the TR34 PCR assay, 73% (16/22) were sequenced successfully with one TR34 mutation detected. The AsperGenius® PCR kit system showed positive

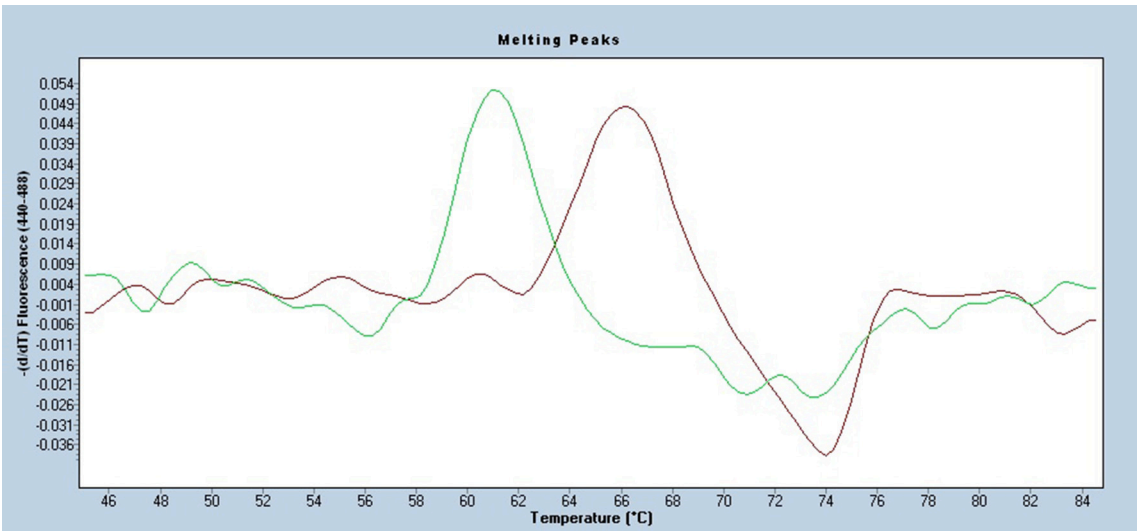

FIGURE 2 | AsperGenius® based melting curve analysis of a potentially present L98H mutation in a BAL sample containing *A. fumigatus* DNA. The sample contained *A. fumigatus* wild type DNA (green curve in the range 61.0–64.0°C) compared to the L98H positive control DNA (brown curve in the range 65.5–68.5°C).

signals in 64% (14/22) of the cases and discovered also the TR34 mutation. In the ARAf L98H PCR assay we received positive signals in 91% (20/22) of the cases, in 77% (17/22) the sequence analysis was effective and one L98H mutation was detected. This mutation was also detected by the AsperGenius® kit system, but only 31% (7/22) positive L98H PCR results were achieved using this system. Considering the in-house Y121F and T289A assays, positive signals were obtained in 68% (15/22) and 59% (13/22) of the cases, respectively. From 13/22 (59%) and 11/22 (50%) PCR reactions sequence analysis was also possible. No Y121F/T289A mutations were revealed. In 72% (16/22) of the samples, the Y121F PCR showed positive signals in the AsperGenius® system, the T289A PCR assay was positive in 68% (15/22) of the cases. In both approaches no mutations were detected. The in-house TR46 PCR assay, which is not represented in the AsperGenius® PCR kit, showed positive signals in 77% (17/22) of the cases with 73% (16/22) successful DNA-sequencing. No TR46 alterations were detected in BAL specimens. The in-house M220 PCR assay was successful in 68% (15/22) of the cases; all of them were sequenced without the detection of another mutation. This mutation is also not represented in the AsperGenius® PCR kit. Overall 74% (98/132) of our in-house ARAf PCRs showed positive signals accompanied by 67% (88/132) of successful sequencing, whereas 59% (52/88) of the AsperGenius® PCR assays were positive for the detection of *A. fumigatus* DNA from BAL samples. Statistical analysis revealed that the difference between 59 and 67% sensitivity was not significant ($p = 0.252$). One BAL sample revealed the TR34/L98H mutation combination in both systems. This BAL sample was obtained from a neutropenic AML patient characterized first in 2012 (Hamprecht et al., 2012).

Of the biopsies, 69% (62/90) showed positive signals in our ARAf PCR assays, of these 61% (55/90) were sequenced successfully. In contrast, 47% (28/60) of the AsperGenius® PCRs showed positive results, revealing one Y121F and one T289A mutation. Via sequence analysis we were additionally able to detect those two mutations as well as the corresponding TR46 alteration. The difference in sensitivity from biopsies was also not significant ($p = 0.081$). The TR46/Y121F/T289A mutation combination was found in a lung biopsy sample of a patient suffering from AML and described in Rössler et al. (2017). Furthermore, we detected three L98H mutations and one TR34 alteration using our in-house ARAf PCRs with subsequent DNA sequencing. One L98H/TR34 mutation combination was found in a brain biopsy of a patient suffering from ALL and another L98H mutation in a lung biopsy of an AML patient (Spiess et al., 2012, 2014). An additional L98H mutation not yet described was found in a lung biopsy of an osteosarcoma patient. The potentially present corresponding TR34 alteration in this patient was not detectable due to technical reasons. The TR34 ARAf PCR approach provided no positive PCR signal applicable for DNA sequence analysis. None of these mutations were detected by the AsperGenius® system from biopsies.

In CSF specimens, the AsperGenius® system showed better results with 42% (25/60) positive PCRs. The in-house ARAf PCR assays were positive in 39% (34/87) of the cases with only 29% (25/87) successful sequence analysis. The difference in sensitivity between both assays was not significant ($p = 0.104$). No mutations were detected from CSF samples with both methods. Isolates showed 100% positive fungal DNA detection in the in-house ARAf PCRs as well as in the AsperGenius® PCR kit. The known present TR46/Y121F/T289A mutation combinations were found with both methods. With our approach we were able to detect nine mutations directly from clinical samples. Due to its setup the AsperGenius® kit was not able to detect any TR46 alteration. Furthermore, the system did not detect three L98H mutations and one TR34 alteration from biopsy samples.

Detailed information about positivity of the assays in the different clinical specimens and detected *Cyp51A* mutations can

be seen in **Tables 2**, **3**. A comparison of the results for both methods is shown in **Figure 3**.

DISCUSSION

Diagnosis of IA in hematological high risk patients often remains largely unsatisfying, especially since *Aspergillus* culture remains mostly negative in microbiological diagnostics in this group of patients (Ruhnke et al., 2012). In the SEPIA study—a prospective multicenter cohort study in hematological and oncological centers in Germany−179 of 3,067 patients with acute leukemia were diagnosed suffering from IA, among these 96% were classified as probable IA following the EORTC/MSG consensus criteria (De Pauw et al., 2008; Koehler et al., 2017). Only in 14% of these cases *A. fumigatus* was proved in culture (Koehler et al., 2017), reflecting the fact that culture-based IA diagnostics alone is not sufficient in these patients. Studies already indicate that molecular-based detection methods in addition to culture-based diagnostics are beneficial for the outcome of the patients (Rickerts et al., 2007; Guegan et al., 2017); this applies especially to hematological patients showing poorer test-performances with culture-based diagnostic approaches (Guegan et al., 2017).

In this study we compared the commercially available AsperGenius® kit system to our six in-house ARAf PCR assays with subsequent DNA sequence analysis concerning the sensitivity of detection of *Aspergillus* DNA and triazole resistance mutations investigating 22 BAL and 15 CSF samples, 15 biopsies, and three clinical isolates of immunocompromised patients.

We included BAL, CSF and biopsy specimens from the site of infection and explicitly excluded blood samples. At the time of diagnostics for IA most patients at high risk for IPA in Germany are already undergoing antifungal prophylaxis or early pre-emptive antifungal therapy. Springer et al. demonstrated in 2016 that *Aspergillus* PCR had a high false predictive value in patients during antifungal medication (Springer et al., 2016). Furthermore, the diagnostic *Aspergillus* PCR showed a better test performance in BAL than in peripheral blood samples (Boch et al., 2016). Nevertheless, White et al. have examined the diagnostic value of the diagnostic AsperGenius® kit system in serum and plasma samples (White et al., 2015b, 2017). For serum samples a sensitivity of 79% with a specificity of 100% was reached, whereas in plasma samples sensitivity and specificity of 80 and 78% was observed (White et al., 2015b, 2017). In other PCR assays plasma specimens were superior compared to serum specimens (White et al., 2015a). In all studies, patients have been for the most part under antifungal prophylaxis.

For the comparison of the triazole resistance AsperGenius® kit system to our in-house ARAf PCR assays we used the DNA extraction methods described in our previous publications (Skladny et al., 1999; Hummel et al., 2006; Spiess et al., 2012). The extraction method differs from the one depicted in the manual of the AsperGenius® kit system (BioMerieux EasyMag extraction method). This way was chosen due to the fact that DNA of several clinical samples had already been extracted by the time the study started and there was no raw material left over. We have not yet evaluated the impact of the extraction methods on the performance of the AsperGenius® kit system.

The comparative evaluation of the generated data revealed that our in-house ARAf PCR assays are more sensitive for the analysis of BAL and biopsy samples, although the calculated differences were not statistically significant. Nevertheless, carrying out six PCR assays with subsequent DNA sequence analysis is time consuming. Regarding this point the AsperGenius® kit system has an advantage over our in-house ARAf PCR assays. In case of CSF samples both approaches showed no convincing results, with AsperGenius® being lightly more sensitive. Most likely negative results in biopsy samples in the AsperGenius® kit system could be caused by interference with human DNA during the PCR reactions.

Unsuccessful DNA sequencing of the PCR fragments generated by the in-house ARAf PCRs could be due to the low amount of *A. fumigatus* DNA in the clinical samples and therefore to the low amount of DNA generated by the PCR assays. By agarose gel electrophoresis 25 pg of DNA are visible, for the performance of Sanger sequencing, 50 ng of fungal DNA are necessary. Both systems showed a 100% sensitivity when investigating *A. fumigatus* isolates, because in this scenario the amount of fungal DNA and the interference with human DNA are no limiting factors. The reason for the statistically insignificant calculated differences in the determination of the sensitivity of the two test systems may be due to the number of samples investigated; the number of investigated *Aspergillus* DNA positive clinical samples is owing both to the low prevalence

TABLE 2 | Summary of the comparison of positive diagnostic results of ARAf PCR assays and the AsperGenius® system concerning the detection of *A. fumigatus* DNA and *Cyp51A* mutations directly from clinical samples.

		In-house ARAf PCR			AsperGenius®	
		PCR+	Successful sequencing	Mutation+	PCR+	Mutation+
BAL	TR34	82% (18/22)	73% (16/22)	1	64% (14/22)	1
	L98H	91% (20/22)	77% (17/22)	1	31% (7/22)	1
	TR46	77% (17/22)	73% (16/22)	0	–	–
	Y121F	68% (15/22)	59% (13/22)	0	72% (16/22)	0
	T289A	59% (13/22)	50% (11/22)	0	68% (15/22)	0
	M220	68% (15/22)	68% (15/22)	0	–	–
	Total	74% (98/132)	67% (88/132)	2	59% (52/88)	2
BIOPSY	TR34	53% (08/15)	53% (08/15)	1	33% (05/15)	0
	L98H	67% (10/15)	60% (09/15)	3	33% (05/15)	0
	TR46	60% (09/15)	53% (08/15)	1	–	–
	Y121F	80% (12/15)	67% (10/15)	1	60% (09/15)	1
	T289A	80% (12/15)	60% (09/15)	1	60% (09/15)	1
	M220	73% (11/15)	73% (11/15)	0	–	–
	Total	69% (62/90)	61% (55/90)	7	47% (28/60)	2
CSF	TR34	33% (5/15)	27% (4/15)	0	47% (7/15)	0
	L98H	40% (6/15)	40% (6/15)	0	40% (6/15)	0
	TR46	21% (3/14)	21% (3/14)	0	–	–
	Y121F	53% (8/15)	40% (6/15)	0	40% (6/15)	0
	T289A	50% (7/14)	14% (2/14)	0	40% (6/15)	0
	M220	36% (5/14)	29% (4/14)	0	–	–
	Total	39% (34/87)	29% (25/87)	0	42% (25/60)	0

TABLE 3 | Summary of all detected *Cyp51A* mutations from clinical samples and isolates so far.

Clinical samples and isolates	In-house ARAf PCRs plus sequencing						AsperGenius®			
	TR34	L98H	TR46	Y121F	T289A	M220	TR34	L98H	Y121F	T289A
BAL: (AML) (Hamprecht et al., 2012; Spiess et al., 2014)	+	+	–	–	–	–	+	+	–	–
Lung biopsy: (AML) (Spiess et al., 2014)	–	+	–	–	–	–	–*	–*	–*	–*
Brain biopsy: (ALL) (Spiess et al., 2012)	+	+	–	–	–	–	–*	–*	–	–
Lung biopsy: (Osteosarcoma)	–*	+	–*	–	–*	–	–*	–*	–*	–*
Lung biopsy: (AML) (Rössler et al., 2017)	–	–	+	+	+	–	–*	–*	+	+
Isolate of lung biopsy: (AML) (Rössler et al., 2017)	–	–	+	+	+	–	–	–	+	+
Isolate of BAL: (AML) (Hamprecht et al., 2012)	+	+	–	–	–	–	+	+	–	–
Isolate (IMMi 2107): (Steinmann et al., 2015)	–	–	+	+	+	–	–	–	+	+

Both methods found all known present mutations in isolates. The ARAf PCRs plus DNA sequencing detected nine mutations and the AsperGenius® assay four mutations directly from primary clinical samples. AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; PC, positive control.
**DNA not amplified.*

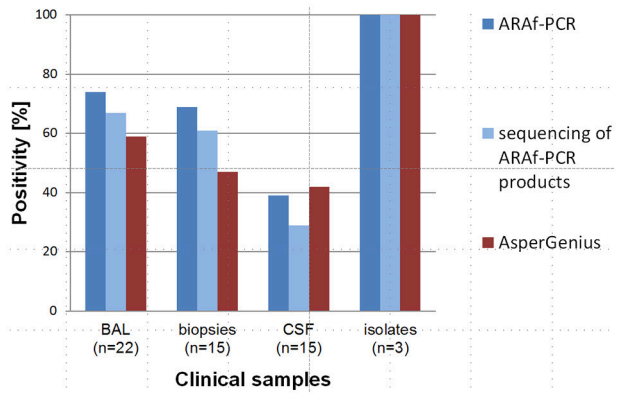

FIGURE 3 | Comparison of diagnostic results of our ARAf PCR assays and the AsperGenius® system concerning the detection of *A. fumigatus* DNA directly from clinical samples as a prerequisite for the characterization of triazole resistance mutations. ARAf PCR results are shown in the dark blue bars, positive ARAf PCR sequencing is shown in light blue bars; the red bars show the AsperGenius® PCR results. In BAL samples the positivity of the ARAf PCRs plus sequencing was slightly higher (8%). In biopsy samples the ARAf PCRs assays plus sequencing showed a 14% higher positivity. Results in CSF (cerebrospinal fluid) samples were nearly identical, whereby after including sequencing results, the sensitivity of the in-house ARAf system decreased to 29%. The amplification of DNA from isolates was 100% for both methods. The detected differences concerning sensitivity of the assays were not statistically significant (BAL, $p = 0.252$; biopsies, $p = 0.081$; CSF, $p = 0.104$).

of IA in hematological high risk patients during antifungal therapy and the low prevalence of *Aspergillus* triazole resistance in Germany.

Meanwhile MycoGENIE®, a new commercial kit has been released. Like AsperGenius® it offers the detection of *Aspergillus* DNA as well as the verification of *Cyp51A* mutations. In contrast to AsperGenius® which detects up to four mutations in the *Cyp51A* gene MycoGENIE® is only able to detect TR34 and L98H mutations (Dannaoui et al., 2017).

Non-culture based molecular detection methods of *A. fumigatus* triazole resistance directly from the site of infection are extremely important in hematological patients at high risk for IA. This can be underlined by the fact that molecular detection methods have the ability to detect triazole-susceptible and triazole-resistant coinfections. Cultural diagnosis may miss these coinfections resulting in insufficient therapy (Schauwvlieghe et al., 2017). To open the possibility to include *Aspergillus* PCR in the revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) definitions for fungal disease, further optimized commercially manufactured assays may be required to provide standardization and accessibility.

Therefore, a prospective study comparing conventional PCR assays from clinical material of the site of infection to commercial kits like AsperGenius® and if possible cultural results in a larger cohort of hematological and oncological patients is ongoing.

AUTHOR CONTRIBUTIONS

PP contributed to the search of scientific literature, data generation and collection, data analysis, and writing of the manuscript. JuS performed the experiments, contributed to data analysis, and writing of the manuscript. TB contributed to reading and editing of the manuscript, and to data analysis. OC, AH, P-MR, JöS, OB, and TM contributed to material collection and editing and writing of the manuscript. AD and NM performed the experiments. W-KH contributed to supervision and editing of the manuscript. DB and BS contributed to search of scientific literature, trial design, data collection, data interpretation, and writing of the manuscript.

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Circulating *Aspergillus fumigatus* DNA Is Quantitatively Correlated to Galactomannan in Serum

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The performance of antigen galactomannan (GM) for diagnosing invasive aspergillosis (IA) is hampered by the occurrence of false-positive results. Quantitative PCR has been proposed to improve the diagnosis of IA. Therefore, we analyzed the value of performing a PCR test to the GM-positive serum sample. Using a quantitative PCR assay specific for *Aspergillus fumigatus* 28S ribosomal DNA, we retrospectively tested 422 GM-positive (Platelia Bio-Rad kit) serum samples collected over 1 year from 147 patients. The cases were classified based on EORTC criteria as "proven," "probable," and "no–IA" before availability of the PCR results. After exclusion of 65 samples for non-reproducibility of GM positivity ($n = 62$) or PCR inhibition ($n = 3$), 75 (21.0%) of the remaining 357 samples were PCR-positive. GM and fungal DNA showed a significantly positive correlation ($p < 0.0001$, $R^2 = 0.27$, slope $= 0.98 \pm 0.19$). At least one PCR-positive result was observed in 63.3% (31/49) of IA patients and in 13.2% (13/98) of non-IA patients ($p < 0.0001$). The PCR positivity was also associated with the presence of other microbiological criteria among the 44 patients with IA and complete mycological workup ($p = 0.014$), as well as a higher mortality rate at six months among the 135 patients with hematological conditions ($p = 0.0198$). Overall, we found a quantitative correlation between serum GM and circulating DNA with an increased likelihood of IA when both were positive. A PCR-positive result also supported a higher fungal load when GM was already positive. We advocate adding a PCR test for every confirmed GM-positive serum sample.

Keywords: *Aspergillus fumigatus*, invasive aspergillosis, galactomannan, quantitative real-time PCR, circulating DNA

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INTRODUCTION

The diagnosis of probable and proven invasive aspergillosis (IA) requires microbiological criteria, which include the galactomannan (GM) antigen (De Pauw et al., 2008). This antigen is produced by several molds including *Aspergillus fumigatus*, the main species responsible for IA (Lortholary et al., 2011). For serum, the test is mainly used as a screening test to initiate a diagnostic workup

or to start antifungal therapy as soon as possible (Marchetti et al., 2012). However, concern has always been raised about the rate of false positivity using the Platelia Aspergillus Ag assay (Bio-Rad Laboratories, Marnes la Coquette, France) (Marchetti et al., 2012). We recently showed the importance of excluding unreproducible positive results by testing GM-positive samples twice (Guigue et al., 2015). However, numerous GM-positive samples cannot be ascribed to IA even after an intensive diagnostic work-up including imaging and mycology with direct examination and culture. At a cut-off value 0.5 ODI and an IA prevalence of 8%, a meta-analysis showed 19% of false positives (Leeflang et al., 2008).

Real-time quantitative PCR assays have been proposed to improve the diagnosis of IA. With the advent of PCR, several technical procedures have been recommended for testing serum (White et al., 2011). With such improvements, PCR should be recommended for diagnosing IA (White et al., 2015). The combination of both tests to improve the clinical utility for the diagnosis of IA has been advocated for many years (Bretagne et al., 1998; Barnes et al., 2013). In a recent meta-analysis, the association of positive results for both tests was highly suggestive of an active infection with a positive predictive value of 88% (Arvanitis et al., 2015). However, a screening strategy utilizing twice weekly GM and PCR testing is questioned with the generalization of anti-mold prophylaxis (Patterson et al., 2016). In decreasing the prevalence of IA, the efficiency of the screening strategy decreases (Leeflang et al., 2008). In these conditions, GM testing is integrated in a diagnostic work-up without the previous serial tests to interpret the results. Thus, a positive GM results should be interpreted on a single result. One possibility is to ask for a second serum sample. However, this can delay the initiation of an appropriate therapy. We wondered whether adding a PCR test for every GM-positive sample without waiting for additional samples could improve the diagnosis of IA.

MATERIALS AND METHODS

Ethics Statement

The present study was a non-interventional retrospective study performed using biological material and clinical data obtained for standard diagnostics without any supplementary sampling and no change in the usual procedures. French Public Health Law (CSP Art L1121-1.1) does not require specific approval from an ethics committee for this study which is exempted from specific informed consent application.

Serum Samples Collection and GM Detection

From January 1st, 2013 to December 31st, 2013, 7628 serum samples were tested using the Platelia Bio-Rad kit mainly as part of the screening of 1374 patients at risk of IA according to previous recommendations (Marchetti et al., 2012). GM detection was performed according to the manufacturer's instructions. The results were inferred from the ratio of the optical density (OD) results from the sample and the controls and are expressed as GM-OD index (GM-ODI). For each positive serum sample (GM-ODI $>$ 0.5), another test on the same serum sample was performed the next day as part of our routine practice (Guigue et al., 2015). Only the samples that tested positive twice were considered as positive, and the mean of the two GM-ODIs was used for further analyses. The serum samples that turned negative were considered as unreproducible results (Guigue et al., 2015). The serum samples were stored at −80°C until further analysis.

DNA Detection

All serum samples tested positive with more than 1 ml available were analyzed. Storage at −80°C before PCR did not exceed 2 years. After thawing, DNA from 1 mL of serum was extracted using the Qiasymphony DSP virus/Pathogen Mini kit (Qiagen) and a Qiasymphony apparatus (Qiagen), eluted in 85 μL, and tested in duplicate using the 28S rDNA PCR assay previously reported (Challier et al., 2004). Primer and probe concentrations were set at 0.3 and 0.1 μM in the 480 probe Master (Roche), respectively, and the PCR assay was performed in a LightCycler 480 instrument (Roche).

The results were expressed in quantification cycles (*C*q), with higher values indicating less targeted DNA in the sample. Positivity was defined by at least one of the two duplicates having *C*q $\leq$ 45 cycles. The mean value of the duplicates was retained for further comparisons when both were positive and the single value when one replicate was positive alone. DNA extraction and amplification yields were assessed using the Simplexa Extraction and Amplification Control Set (Focus Diagnostics, Cypress, CA, United States) as an internal control (IC). The PCR assay was performed blind to interpretation of the GM results (true or false positives) and to the clinical classification.

Mycology Laboratory Result

Respiratory specimens [bronchoalveolar lavage (BAL) fluid, induced sputum, sputum] were split into two parts. One part underwent direct examination using microscopy after staining with calcofluor (BD Biosciences) in KOH (10%). The other part was seeded on Sabouraud dextrose agar with gentamycin and chloramphenicol (Bio-Rad) and incubated at 30 and 37°C. Every positive culture was identified using phenotypic methods. Molecular identification was done based on sequencing three different loci (Internal Transcribed Spacer, beta-tubulin, calmodulin). Sequences were then posted in the Mycobank database[1] and Institut Pasteur FungiBank[2].

Patient Classification

Every four months, a local multidisciplinary medical committee analyses each effective anti-mold therapy recorded in the pharmacy department and classifies the patients as 'proven,' 'probable,' IA, or 'no-IA' according to criteria from the European Organization for Research and Treatment of Cancer and from the Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) (De Pauw et al., 2008). For patients without

[1]http://www.mycobank.org/BioloMICSSequences.aspx?expandparm=f&file=all
[2]http://fungibank.pasteur.fr/

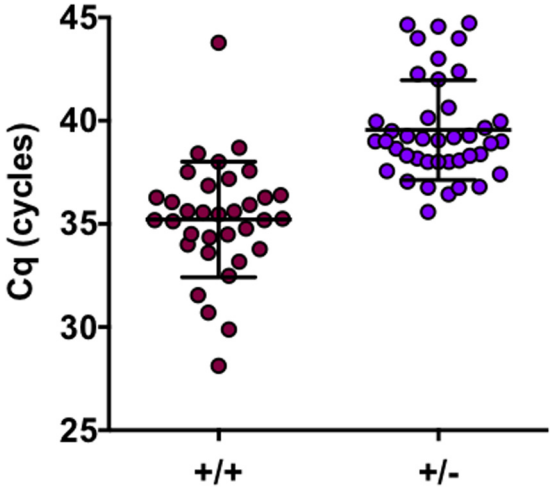

FIGURE 1 | Results of the duplicates performed for each PCR-positive sample. The positive duplicates (+/+) had lower mean *C*q values than the sample (+/–) with only one duplicate positive (*t*-test; $p < 0.001$).

hematological condition or solid organ transplantation not responding to the EORTC/MSG definition, *ad hoc* classification was consensually performed by the local committee. The patient outcome was censored at 6 months (183 days).

Graphs and Statistical Analysis

Chi-2 test and Fisher's exact test were used for contingency tables analyses for calculation of statistical association. The potential relationship between GM and *C*q was studied using linear regression and for comparison of slopes, a *P*-value (two-tailed) testing the null hypothesis that the slopes are all identical (the lines are parallel) was calculated. Survivals were determined by Kaplan–Meier method and compared by the Mantel–Cox test. For comparisons of quantitative data, we performed unpaired two-tailed *t*-test for normally distributed data. *P*-values of <0.05 were considered significant. All analyses and graphs were performed using Prism software v.6.0 (Graphpad).

RESULTS

Of the 422 GM-positive serum samples tested by PCR, three were excluded because of IC amplification failure. Among the 419 remaining samples, 62 were unreproducible GM-positive samples, i.e., the first positive result (median ODI: 0.75, interquartile range: 0.55–1.005) tested negative upon retesting. All were PCR-negative. These 62 samples were from 53 patients (median number 1; interquartile range 1–1 range 1–3). None of these patients developed IA.

The remaining 357 samples were confirmed to be true GM-positive samples after retesting (median difference: ODI 0.11 interquartile range [0.04–0.22], mean ± SD 0.19 ± 0.27). Among these, 75 (21.0%) were PCR-positive with a median of one positive sample per patient [interquartile range 1–2; range 1–13]. For the duplicates, they were more consistently both positive when the *C*q was below 38 (**Figure 1**). When looking at quantitative values of the assays, the GM-ODI and the *C*q showed an inversely significant correlation (slope $= -0.98 \pm 0.19$, $R^2 = 0.27$; $p < 0.0001$). When considering PCR results from samples collected before or during antifungal therapy, the correlation was significantly improved ($p = 0.019$) for the samples before therapy ($n = 28$, slope $= -1.5 \pm 0.29$, $R^2 = 0.50$; $p < 0.0001$) compared to those collected after the initiation of antifungal treatment ($n = 47$, slope $= -0.61 \pm 0.23$, $R^2 = 0.13$; $p = 0.01$) (**Figure 2**).

The 357 GM-positive samples (median 1; range 1–17 samples per patient) were from 147 patients with mainly hematological diseases as an underlying condition (**Table 1**), of whom 49 (33.4%) were classified as having IA (47 with probable IA, 2 with proven IA) and the remaining 98 (66.7%) as not having probable or proven IA (**Table 1**). At least one PCR-positive serum sample was observed in 31 (63.3%) of the 49 patients with IA, including the two proven cases, and in 13 (13.2%) of the 98 patients without IA ($p < 0.0001$) (**Table 1**). Notably, none of the 10 patients with common variable immunodeficiency disease (CIVD) were considered as having IA and all were PCR-negative (**Table 1**).

Among the 18 patients with IA who were PCR-negative, one patient (five samples) had probable IA due to *Emericella quadrilineata*, and one patient (one sample) had probable IA due to *A. flavus*, based on culture results. Both species were not detected by our PCR assay. Among the 98 patients without IA who were GM-positive, one (one sample) had cryptococcosis and one (three samples) had histoplasmosis. Among the remaining 96 patients, who were GM-positive, 18 (18.75%) were given effective anti-mold therapy and 12 (66.7%) of them were PCR-positive, in contrast with only one (1.3%) PCR-positive patient among the 77 GM-positive patients who were not given effective anti-mold therapy ($p < 0.0001$) (**Table 1**).

Among the 49 patients with IA, 44 had other investigations performed (direct microscopic examination, culture, BAL GM testing). Among these 44 patients, the percentage of PCR-positive patients increased with the number of other positive criteria, from 36.8% (7/19) with GM positivity alone to 76.9% (19/25) when GM, culture and/or BAL GM was positive ($p = 0.014$) (**Table 2**).

We also analyzed the survival of the patients for whom a 6-month follow up was available ($n = 135$) and who had underlying hematological diseases or allogeneic hematopoietic stem cell transplantation to avoid confusion with the other underlying diseases (**Figures 3A–C**). Survival was significantly lower ($p = 0.001$) in PCR-positive patients compared to PCR-negative patients (**Figure 3A**). We further analyzed the data according to the presence or not of the EORTC/MSG 2008 criteria (**Figure 3B**). The patients without EORTC/MSG criteria had a better survival ($p < 0.001$) whatever the PCR result than patients who fulfilled the EORTC/MSG criteria who exhibited the worst prognosis when PCR was positive, even if the difference between PCR-negative and PCR-positive patients did not reach significance ($p = 0.073$). When analyzing the quantitative results ($n = 38$ patients), the patients with a *C*q < 36 on the first PCR-positive sample had a shorter survival than patient with a *C*q > 36 ($p = 0.028$) (**Figure 3C**). The *C*q values of the first PCR-positive sample were significantly lower (*t*-test, $p = 0.0066$) in the

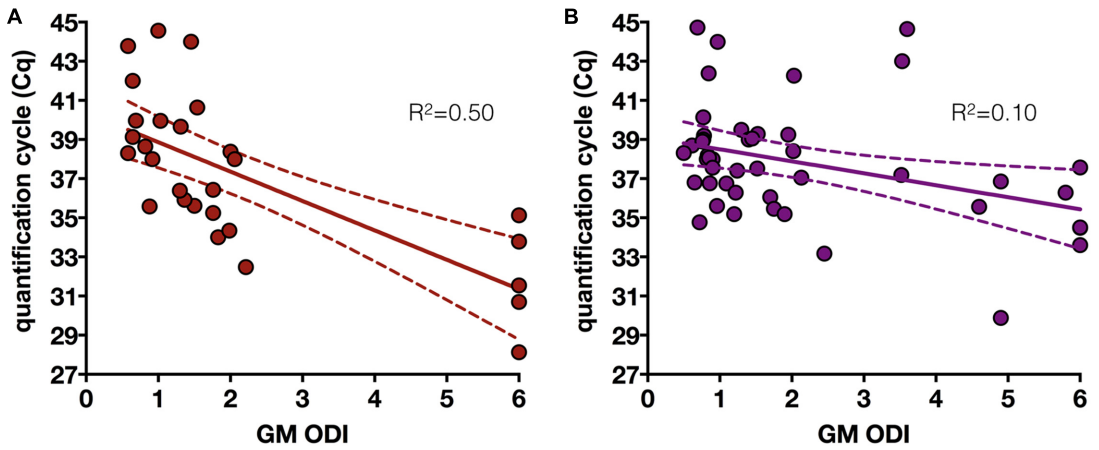

FIGURE 2 | Correlation between galactomannan index (GM-ODI) and quantification cycle (Cq) of *Aspergillus fumigatus* PCR assay in GM-positive serum samples obtained before **(A)** or after **(B)** initiation of antifungal therapy.

TABLE 1 | Underlying disease or risk factors of invasive fungal diseases of the 147 adults patients with the PCR results of the 357 true GM-positive samples tested, and the results according to the final diagnosis of invasive aspergillosis (IA).

Underlying diseases or condition	Number of patients ($n = 147$)	Number of samples ($n = 357$)	Number of PCR samples ($n = 75$)	Number of IA patients ($n = 49$)	Number of PCR-positive IA patients ($n = 31$)	Number of treated patients wo IA diagnosis ($n = 18$)
Myeloid disorders	26 (17.7)	49 [1–6]	14 (28.6)	7 (26.9)	5 (71.4)	5 (28)
Acute myeloid leukemia	22 (15)	39 [1–5]	9 (23.1)	4 (18.2)	4 (100)	3 (17)
Myelodysplasia	4 (2.7)	10 [1–6]	5 (50)	3 (75)	1 (33.3)	2 (11)
Acute lymphoid leukemia	13 (8.8)	26 [1–6]	5 (19.2)	3 (23.1)	2 (66.7)	5 (28)
Chronic lymphoproliferative disorders	39 (26.5)	76 [1–7]	18 (23.7)	22 (56.4)	11 (50)	4 (23)
Lymphoma	26 (17.7)	50 [1–7]	11 (22)	14 (53.8)	7 (50)	3 (17)
Multiple myeloma	11 (7.5)	21 [1–7]	6 (28.6)	6 (54.5)	3 (50)	1 (6)
Chronic lymphoid leukemia	2 (1.4)	5 [1–4]	1 (20)	2 (100)	1 (50)	0 (0)
Allogeneic stem cell transplantation[a]	42 (28.6)	153 [1–17]	29 (19)	11 (26.2)	8 (72.7)	2 (11)
Renal transplantation	6 (4.1)	15 [1–5]	6 (40)	3 (50)	2 (66.7)	1 (6)
CVID[b]	10 (6.8)	17 [1–5]	0 (0)	0 (0)	0 (0)	1 (6)
AIDS	6 (4.1)	8 [1–3]	1 (12.5)	1 (16.7)	1 (100)	0 (0)
Others[c]	5 (3.4)	13 [1–5]	2 (15.4)	2 (40)	2 (100)	0 (0)

Numbers in () represent % and numbers in [] represent range/patient; wo, without.
[a]*Underlying diseases include myeloid disorders (n = 18), acute lymphoid leukemia (n = 7), chronic lymphoproliferative disorders (n = 16), and sickle cell disease (n = 1).*
[b]*Common variable immunodeficiency disease.*
[c]*Includes: pulmonary carcinoma (n = 2); amyloidosis (n = 1); Still disease (n = 1); Intensive care unit (n = 1).*
The last column represents the PCR-positive patients who received effective anti-mold therapy despite the absence of all the EORTC/MSG criteria for diagnosing IA.

deceased patients (mean ± SEM = 34.8 ± 1.2) than in the patients alive at week 2 (mean ± SEM = 38.6 ± 1.3).

DISCUSSION

The aim of the present study was to improve the specificity of the GM test by adding on the same tube the detection of circulating *A. fumigatus* DNA. We found not only an association between PCR positivity and the presence of IA, but also for the first time a quantitative correlation between GM and PCR results in serum. Moreover, all our results suggest that a PCR-positive result once GM is already positive is associated with a poorer prognosis.

Based on the EORTC/MSG classification performed blind to the PCR results, the percentage of patients with at least one PCR-positive result increased from 13.2% in the non-IA group to 63.2% in the IA group ($p < 0.0001$). However, our results clearly show that the EORCT/MSG classification does not always fit with the clinical decision underlining the issue of the GM assay specificity (Leeflang et al., 2008; Marchetti et al., 2012). Indeed, among the GM-positive patients, only 18.75% were prescribed effective anti-mold therapy. Interestingly, 66.7% of the patients who received antifungal therapy were PCR-positive, and this

TABLE 2 | Comparison between PCR-positive results and the presence of other positive microbiological investigation [direct microscopy, culture, and bronchoalveolar lavage (BAL) GM testing] in 44 patients with probable or proven invasive aspergillosis and at least one galactomannan (GM) positive sample.

Results of mycological investigations	PCR-positive patients ($n = 26$) (%)	PCR-negative patients ($n = 18$) (%)	*P*-value
Serum GM positivity alone	7 (36.8)	12 (63.2)	0.014
Serum GM positivity associated with other microbiological criteria	19 (76.0)	6 (24.0)	

result was not known when the clinical decision was made. Therefore, PCR performed when GM is already positive can improve the specificity of the GM result.

We also report for the first time that fungal DNA and GM titers are positively correlated. In parallel, we observed a higher mortality at 6 months in hematology when the patients were PCR-positive. Our hypothesis is that PCR positivity indicates a more advanced stage of the IA, at least when GM is already positive. This is also supported by the significant association of PCR positivity with other microbiological criteria. This finding is consistent with the poorer prognosis reported when several mycological criteria are present (Lortholary et al., 2011). Therefore, if PCR positivity increases the specificity of GM, this positivity is also associated with a higher fungal load, and thus, with a poorer prognosis. This finding is also coherent with the poor prognosis observed when the GM titers remain high (Boutboul et al., 2002; Nouér et al., 2011; Bergeron et al., 2012).

Although PCR could improve the specificity of some GM-positive serum samples, many GM-positive samples remain PCR negative (36.7%), even after the exclusion of unreproducible GM results (Guigue et al., 2015). We have already observed more GM-positive than PCR-positive serum samples in different populations (Bretagne et al., 1998; Millon et al., 2005; Botterel et al., 2008). Similar findings have been recently reported (Aguado et al., 2015; Imbert et al., 2016). For instance, less than 24% of GM-positive patients had at least one PCR-positive serum sample in a randomized trial (Aguado et al., 2015). A few of these GM-positive PCR-negative samples could be related to cross-reactions during infections by other fungi such as cryptococcosis (Dalle et al., 2005) or histoplasmosis (Rivière et al., 2012). They can also be due to *Aspergillus* species not targeted by the present primers specific for *A. fumigatus* (Challier et al., 2004), which represent a very small number of patients given the high predominance of *A. fumigatus* among the species responsible for IA in our patient population (Lortholary et al., 2011). GM contamination of transfused products and specially fungus-derived antibiotics are also always a concern, although to a lesser extend nowadays for piperacillin-tazobactam (Vergidis et al., 2014).

The main explanation for GM-positive PCR-negative results is probably the different kinetics of the two biomarkers. *In vitro* studies have shown that the maximum release of GM and *A. fumigatus* DNA was correlated with increased biomass during culture, with GM being detectable earlier than DNA (Mennink-Kersten et al., 2006; Morton et al., 2010). Several animal models

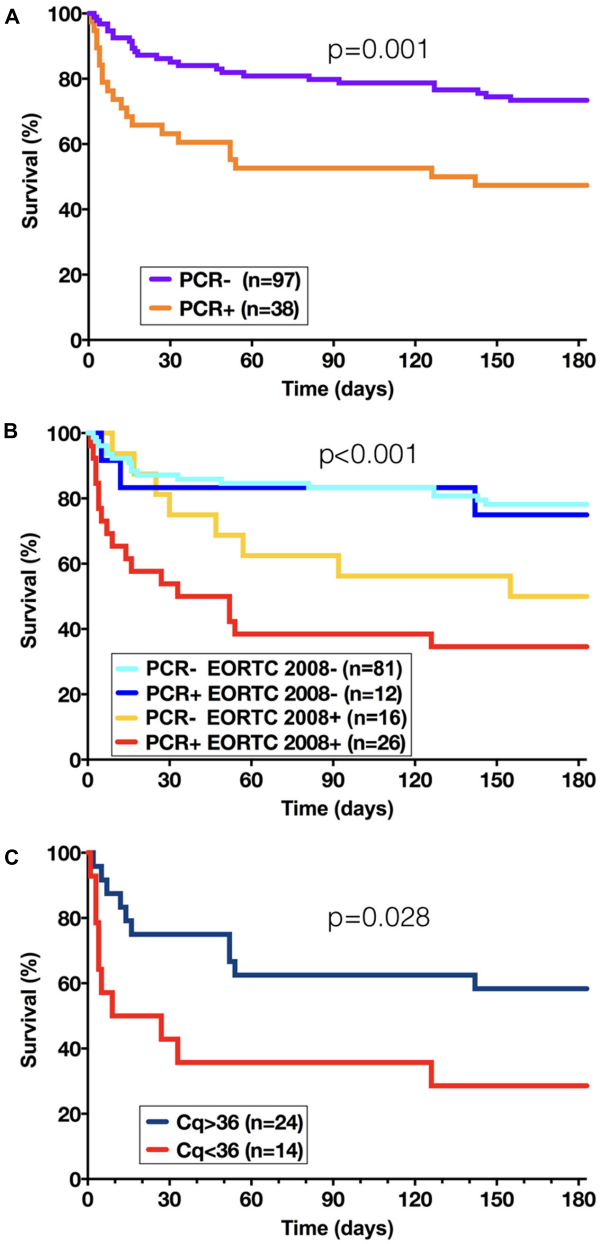

FIGURE 3 | Survival curves according to the presence or not of a PCR-positive result on GM-positive samples **(A)**, to the presence or not of a PCR-positive result associated with EORTC/MSG criteria for IA diagnosis **(B)**, and according to the circulating fungal load as expressed as a *Cq* value **(C)**.

of pulmonary aspergillosis also showed that GM is detected earlier, usually 24–48 h before DNA (Becker et al., 2000; Ahmad et al., 2014; Lin et al., 2014). Using droplet digital PCR, we recently showed that the circulating *A. fumigatus* DNA detected in patients is fragmented, and we hypothesized that such DNA comes from dying hyphae, is extracellular and circulates in the serum (Alanio et al., 2016). Indeed, extracellular DNA has been detected increasingly over time in a biofilm model of *A. fumigatus* and was shown to be produced through autolysis (Rajendran

et al., 2013). Besides the differences of kinetics between the two markers during an active IA, other differences may occur. We observed a lower correlation between GM and DNA when antifungals are given suggesting a different impact of treatment on the release of biomarkers in the bloodstream, which warrants further investigations. This suggests that DNA could be a poorer marker for following the efficiency of therapy compared to serum GM (Boutboul et al., 2002; Nouér et al., 2011; Bergeron et al., 2012). Another reason for discrepancies between GM titers and PCR *C*t may be related to the infecting isolates. Indeed, possible differences in the copy numbers of the rRNA repeats (between 61 and 86 copies/genome) chosen as the target for amplification could affect quantification through PCR according to the infecting isolate (Alanio et al., 2016).

Our study was not designed to address the issue of a PCR-positive signal before the onset of GM positivity. In guinea pigs infected by inhalation, circulating GM antigen was above the threshold of detection at day 3 and steadily rose thereafter whereas the PCR assay detected 10 conidial equivalents as soon as one hour, with a peak of 20 conidial equivalents at 24 h post infection (Vallor et al., 2008). This observation raises the issue of the detection of circulating conidia, which are probably engulfed in macrophages after a pulmonary challenge, either directly or through dendritic cells as reported for *Histoplasma capsulatum* (Lin et al., 2005) and *Cryptococcus neoformans* (Lortholary et al., 1999). Aguado et al. (2015) reported PCR-positive results before GM positivity in 42% of the 30 patients analyzed, while GM before PCR positivity occurred in 38%. We have also reported PCR positivity before GM positivity and concluded that a PCR-positive result accelerates the early detection of IA independently of the other diagnostic information (Schwarzinger et al., 2013). Additional specific studies are needed to determine whether PCR positivity before or after GM positivity could respond to different pathology processes (detection of fungal elements vs. circulating DNA). These studies should nevertheless be difficult to implement given the generalization of anti-mold prophylaxis. Indeed, some authors suggest that the potential of PCR to play a decisive role in the diagnosis and management of IA should be restricted to centers not applying primary antifungal mold prophylaxis (Springer et al., 2016).

We acknowledge some limitations to our study due to the retrospective design. The association between more microbiological criteria and PCR positivity might be a consequence of more investigations in patients with a higher suspicion index. Similarly, the higher mortality observed in PCR patients with hematological conditions does not mean that the patients died of IA in light of the difficulties in assessing the prognosis of IA (Segal et al., 2008). For the PCR protocol, several criticisms can be addressed. We decided to consider PCR-positive samples as having a positive threshold of $Cq \leq 45$, and we considered a sample positive even when the duplicate was not positive. A lower *C*q value is often used for censoring the results when a risk of unspecific positivity is suspected. For instance, Johnson et al. censored their results at 40 cycles when using primers and a probe not specifically designed for *A. fumigatus* (Johnson et al., 2012). If a threshold of 40 had been used in our study, 12 samples from 10 patients would have become PCR negative, restricting the interest of the PCR to very limited patients with a high fungal DNA load. However, there is no consensus on the threshold to be used even if ROC analysis of results of a multicenter study indicated a good diagnostic accuracy of a $Cq \leq 43$ cycles (White et al., 2011). Six patients of the present study had *C*q between $\geq$43 and $\leq$45 cycles. One out these six had a probable IA with *A. fumigatus* positive culture from respiratory specimens. Thus, despite the difficulty to obtain positive duplicates when dealing with low fungal loads (e.g., when $Cq > 38$) because it may be normal to obtain negative results of replicates according to the Poisson's law (Alanio and Bretagne, 2014), we think a $Cq \leq 45$ should be taken into account for diagnosing IA for a given patient. The other possibility is to wait for additional samples and expect a higher fungal load on the subsequent results to confirm the diagnosis but this attitude could be deleterious for the patient. Because no systematic screening of other samples was part of our study restricted to samples known to be GM-positive, we cannot comment the benefit to wait for additional samples to improve the specificity of the PCR results. One can also criticize our choice to focus on *A. fumigatus* in light of the increase of other *Aspergillus* species in IA (Lionakis et al., 2005) or mixed infections (de Fontbrune et al., 2014). However, restriction to a specific species decreases the risk of PCR positivity from environmental non-*fumigatus* DNA, especially if high *C*q are to be considered (Alanio and Bretagne, 2014), although contamination with *A. fumigatus* DNA cannot be completely excluded (Harrison et al., 2010; Millon et al., 2010). Moreover, since all the *Aspergillus* species do not equally produced GM in the same quantity (Xavier et al., 2013), quantitative comparison between GM and circulating DNA would have been biased if a non-*A. fumigatus* specific PCR had been used.

CONCLUSION

The diagnosis of IA is a dynamic process where all the diagnostic elements are not obtained simultaneously. Since prospective screening is questioned with the generalization of antifungal prophylaxis in patients at risk of IA, resulting in a decrease in GM performance (Patterson et al., 2016), GM should become the first test requested by clinicians in case of febrile pneumonia for patients at risk of invasive mold infection. Our results clearly show the added value of PCR tests for every GM-positive serum sample to increase the probability of IA diagnosis, without waiting for additional samples. The two markers provide parallel quantitative information in accordance with the fungal load. A PCR-positive result is associated with a poorer outcome, probably as the witness of a higher fungal load, in accordance with the positivity of the other microbiological investigations.

AUTHOR CONTRIBUTIONS

AA and SB designed the project, performed the analyses, and wrote the manuscript. JM, MG-M, SH, and NG collected and analyzed the laboratory experiments. BD, ER, RPdT, ST, and AB

classified the patients based on medical file analysis. All authors read, commented, and approved the final manuscript.

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frontiers in Microbiology

REVIEW
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Galactomannan, a Surrogate Marker for Outcome in Invasive Aspergillosis: Finally Coming of Age

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Detection of galactomannan has become widely available for diagnosing invasive aspergillosis. The test characteristics, using the Platelia™ enzyme-immunoassay, have been well described. This assay could potentially also be useful for the early evaluation of the efficacy of antifungal therapy and for predicting the outcome in terms of response and survival. In this systematic review, we assessed the available evidence for the use of serum galactomannan at baseline as a prognostic marker, and the predictive value of serum galactomannan kinetics after initiation of antifungal therapy. Overall, serum galactomannan at baseline and galactomannan kinetics appear to be good predictors of therapy response and survival. However, breakpoints for predicting therapy failure and validation in different patient populations are still lacking.

Keywords: galactomannan, kinetics, invasive aspergillosis, prognosis, outcome

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INTRODUCTION

Invasive aspergillosis (IA) is a potentially life-threatening disease, occurring mostly in severely immunocompromised patients such as those with acute myeloid leukemia, those with prolonged neutropenia due to myelotoxic therapy, or following allogeneic hematopoietic cell transplantation or solid organ transplantation, and is estimated to affect around 200,000 patients per year (Brown et al., 2012). Timely initiation of therapy is important for improved survival, but diagnosis remains notoriously difficult, especially when relying on conventional culture or microscopy (Lamoth and Calandra, 2017). Because of this, new biomarkers for early diagnosis of IA have been introduced over the last 2 decades. We have summarized the advantages and disadvantages of these tests in **Table 1**. The diagnostic performance of these biomarkers can be further improved by using them as a combination of tests (Aguado et al., 2015; Neofytos et al., 2015).

Galactomannan (GM) belongs to a group of polysaccharides which consist of a mannose backbone and a variable number of galactofuran side chains. GM makes up a major part of the cell wall of *Aspergillus* spp. (Latgé et al., 1994). These galactofuranose-containing polysaccharides vary in size from 35 to 200 kDa and are secreted *in vivo* by the fungus during invasive growth. In recent years, the detection of galactofuranose-containing antigens, including GM, has been used for diagnosing invasive aspergillosis (IA). To date, the most commonly used method to determine GM in serum and broncho-alveolar lavage (BAL) fluid is a double sandwich enzyme-linked immune assay (Platelia™ Aspergillus antigen, Bio-Rad, Marnes-la-Cocquette, France). This assay is based on the rat-derived EB-A2 monoclonal IgM antibody, which acts as capture and detector antibody, and which selectively binds to four or more β(1→ 5) galactofuranosyl residues of GM

(Mennink-Kersten et al., 2004). This assay is approved by the US Food and Drug Administration, commercially available, and has been incorporated as a microbiological criterion in the European Organization for Research and Treatment of Cancer-Mycosis Study Group consensus definitions of invasive fungal disease (Pauw et al., 2008). Although this assay has been approved for use in serum and BAL fluid only, successful determination of GM in other matrices such as cerebrospinal fluid (Chong et al., 2016), urine (Reischies et al., 2016), plasma (White et al., 2013), and fluid from abscesses (Verweij et al., 2000) has been reported as well. Results are reported as an optical density index (ODI), where the absorbance value of a clinical sample is compared to the mean of two reference samples (the cut-off controls) provided by the manufacturer. However, absorbance levels are only reliable within a given interval, depending on the type of photometer that is used. This represents a major limitation of the assay. At higher optical densities, the relation between the concentration of GM and the absorbance value becomes non-linear (**Figure 1**), resulting in the underestimation of concentrations above the linear range. Since the optical density of the reference standards can vary between assay runs, the cutoff at which the assay turns non-linear can also be variable. According to the manufacturer's instruction, the mean optical density of the cut-off controls has to be $\geq$0.300 and $\leq$0.800. For example, a good quality photometer with a linear range up to an absorbance of 2.5 will therefore be able to accurately report an ODI between 8.33 (for a mean cut-off control of 0.300) and 3.13 (for a mean cut-off control of 0.800). In a lower quality photometer with a linear range up to an absorbance of 1.0, this limit of reliable quantification can be as low as 1.25 (for a mean cut-off control of 0.800). As such, small variations of high ODI's should be interpreted with caution. For an accurate determination of higher values of GM (outside the linear range), the ELISA should be repeated in serially diluted samples, or other, more accurate methods such as mass-spectrometry should be used. Currently, the manufacturer recommends a cut-off of 0.5 in both serum and BAL. However, due to the large number of false positives in BAL at this cutoff, a higher cutoff of 1.0 is proposed in the upcoming revision of the EORTC-MSG criteria.

The test characteristics and limitations of GM detection for diagnosing IA have been well studied and have been the subject of several meta-analyses (Pfeiffer et al., 2006; Zou et al., 2012; Leeflang et al., 2015). Besides providing information on diagnosis, serum GM (sGM) has also been explored for predicting the outcome after initiation of treatment, in particular because the test is easy to perform, widely available, largely *Aspergillus* specific, standardized, and objective. However, the sGM concentration *in vivo* is determined not only by the rate of production and secretion by the growing fungus, but also by the rate of uptake in the bloodstream, as well as the rate of elimination from the circulation.

Due to the relative large size of GM, the antigen cannot freely diffuse from the alveoli through the endothelial lining of the pulmonary capillaries; angio-invasion is required to reach the circulation. This was confirmed in an *in vitro* model of human alveoli, in which GM only appeared in the bloodstream after invasive growth of *Aspergillus* through the alveolar-capillary membrane (Hope et al., 2007). Obviously, as clearly demonstrated in histopathological studies and studies using quantitative polymerase chain reaction (PCR), the degree of angio-invasion (and hence fungal burden) varies with the nature of the underlying condition, with massive invasion and a high fungal burden in neutropenic models and predominantly inflammation with little invasion and a low fungal burden in steroid-induced models (Sheppard et al., 2006). Production of GM is further influenced by therapy; this explains the decreased sensitivity of sGM detection in patients receiving mold-active therapy (Leeflang et al., 2015). This finding was confirmed in animal models, where a concentration-dependent effect on sGM detection was shown for triazoles, polyenes, and investigational drugs such as the orotomides (Petraitiene

TABLE 1 | A summarized overview of diagnostic tests in invasive aspergillosis.

	GM	PCR	LFD	β-D-Glucan
Early detection possible	+	+	+	+
Broad range of pathogens detected	−	±	−	+
Identification to species level	−	+	−	−
Good performance	+	+	+	+
Quantitative results	+	±	−	+
Rapidly available	+	+	++	+
Low cost	± (in house or referral)	−	?	−

GM, galactomannan; PCR, polymerase chain reaction; LFD, lateral flow device.

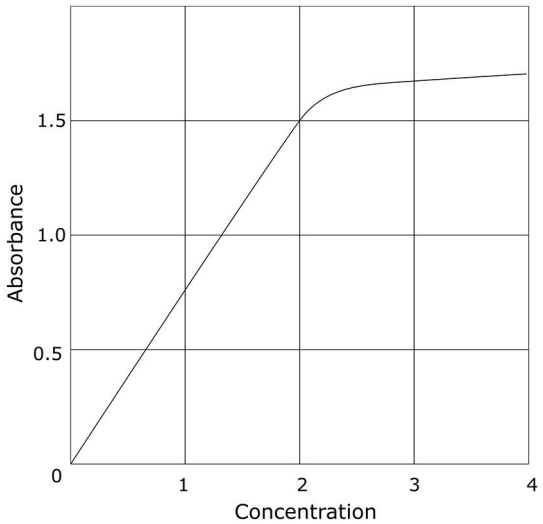

FIGURE 1 | Idealized example of the relation between concentration and optical density in photometry. This is a generalization which holds true for all photometers. The specific numbers will be different depending on the device used.

et al., 2001; Petraitis et al., 2016; Kimura et al., 2017; Negri et al., 2017). One model showed a paradoxical increase in sGM after treatment with caspofungin (Petraitiene et al., 2002), potentially due to interference with fungal cell wall synthesis. However, other models using echinocandins could not replicate this phenomenon (Miceli and Anaissie, 2007). It is more likely that the "paradoxical effect" was caused by ineffective therapy resulting in increased fungal load, rather than an increased release from the cell wall, as echinocandins have been shown to have limited activity against *Aspergillus* spp. in humans (Viscoli et al., 2009). In most comparative animal models, no difference in sGM kinetics was seen between different antifungal drugs when compared at the same level of efficacy.

Elimination of sGM occurs via different routes *in vivo*. Using radioactively labeled *A. fumigatus* GM, a rat and rabbit model of IA showed liver concentration of about one third of the initially injected dose via uptake in Kupffer cells (Bennett et al., 1987). The macrophage mannose receptor plays a central role in this process as hepatic uptake was decreased upon administration of inhibitors of this receptor (Bennett et al., 1987). Another third was excreted renally within 24 h, which is in line with the appearance of GM in urine of patients with IA (Reischies et al., 2016). Renal clearance also depends on the renal function (and the size of the molecule), as is further evidenced by a case report of IA in a patient on hemodialysis who had increasing sGM levels, despite adequate treatment and clinical improvement (Saleeby et al., 2005). Finally, neutrophils are also believed to be involved in the uptake and elimination of circulating GM. This would explain the significantly higher sensitivity of sGM detection in neutropenic patients compared to non-neutropenic ones (Pfeiffer et al., 2006). In addition, a rabbit model confirmed that lower levels of sGM appear in non-neutropenic rabbits, compared to neutropenic rabbits, while no difference in GM could be found in BAL fluid (Petraitiene et al., 2015). Therefore, the interaction between production and secretion during invasive growth, size of the fungal burden, anti-mold therapy, renal and hepatic function, and neutropenic state, results in a complex kinetic profile for sGM.

To determine the current state of the art of the role of GM and its kinetics in the outcome of IA, we searched the MEDLINE database through Pubmed using the following structured query: ("galactomannan"[Supplementary Concept] OR "galactomannan"[All Fields]) AND ("prognosis"[MeSH Terms] OR "prognosis"[All Fields] OR response [All Fields] OR "therapy"[Subheading] OR "therapy"[All Fields] OR "treatment"[All Fields] OR "therapeutics"[MeSH Terms] OR "therapeutics"[All Fields] OR "outcome"[All Fields]). From a total of 911 articles, 56 articles were selected based on title and abstract.

KINETICS IN HUMANS

We failed to identify any data on the kinetics of sGM after its administration to healthy volunteers, which would allow us a detailed exploration of its kinetics and metabolism. However, different sources of false positivity (such as GM-containing electrolyte solutions or beta-lactam antibiotics) allow some insight into its kinetics in the human body. One study looked at sGM after infusion of beta-lactam antibiotics in patients who were previously GM seronegative and who were deemed not to have IA based on clinico- radiological signs and symptoms (Aubry et al., 2006). After infusion, a sudden increase in sGM was seen. Based on the declining sGM levels thereafter, the authors estimated a serum half-life of 2.4 days for eliminating sGM. However, influencing parameters such as creatinine clearance and neutrophil count were not reported. Huurneman et al proposed a pharmacokinetic model for the evolution of sGM during antifungal therapy (**Figure 2**), based on a small number of pediatric patients with IA receiving voriconazole with therapeutic drug monitoring (Huurneman et al., 2016). This model showed a good fit with the actual values, but was limited by the very small number of actual sGM measurements, inclusion of possible cases of IA, and by not taking into account the three different metabolic routes (kidney, liver, and neutrophils).

$$\frac{dx}{dt} = KGM_{prod} \cdot \left[1 - \left(\frac{x}{POP_{max}}\right)\right] \cdot \left(1 - \frac{\frac{D^H}{V}}{EC_{50}{}^H + \frac{D^H}{V}}\right) \cdot x - KGM_{elim} \cdot x$$

FIGURE 2 | A pharmacokinetic model for serum galactomannan in invasive aspergillosis, as proposed by Huurneman et al. (2016). The first part of the equation estimates production of galactomannan, taking into account the effect of antifungal therapy, whereas the second part estimates the elimination from the bloodstream. x, serum galactomannan; KGM_{prod}, maximal rate of galactomannan production; POP_{max}, maximal achievable galactomannan; D, drug concentration in the central compartment; V, volume of the central compartment; H, relationship between drug concentration and reduction in galactomannan production; EC_{50}, drug concentration at which half-maximal reduction in galactomannan production is reached; KGM_{elim}, maximal rate of elimination of galactomannan.

IMPACT OF GM AT BASELINE ON OUTCOME

We identified 16 studies that looked at GM at baseline as a predictor of response and survival (**Table 2**). All included studies used the Platelia™ Aspergillus antigen assay, although at different cut-offs. All studies included adult patients with proven and probable IA, unless stated otherwise in the table. We could not identify conflicting results between the articles: both statistically significant results and non-significant trends pointed in the same direction.

Overall, there was a strong and consistent correlation between the level of sGM and both short-term and long-term survival, from day 42 up to day 180. Indeed, a well performed prospective randomized trial comparing anidulafungin in combination with voriconazole to voriconazole alone found baseline sGM to be only one of three independent predictors of week 6 survival in multivariate analysis (Marr et al., 2015). Stratifying patients by baseline sGM positivity (using a cutoff of 0.5) divided patients in two groups, with sGM positive patients having significantly higher mortality (Fisher et al., 2013; Hoyo et al., 2014; Kim

TABLE 2 | Studies reporting statistics on a relation between baseline serum galactomannan and outcome.

References	Population	*N*	Parameter at baseline	Measured Outcome	*p*-value
Imbert et al., 2016[a]	SOT, hematological, solid tumor, ICU	40	sGM < 2.0	50% of day 90 survivors vs. 25% of day 90 non-survivors had sGM< 2.0 at baseline	0.19
Vehreschild et al., 2017[c]	Majority hematological	40	Mean sGM	0.9 in week 12 survivors vs. 4.3 in week 12 non-survivors	0.047
Jung et al., 2017[a]	SOT, hematological, AIDS, diabetes	102	sGM < 0.5	28% of day 30 survivors vs. 24% of day 30 non-survivors had sGM < 0.5 at baseline	0.81
			sGM < 0.5	51% of day 90 survivors vs. 41% of day 90 non-survivors had sGM < 0.5 at baseline	0.29
Neofytos et al., 2015	SOT, hematological, solid tumor	47	sGM < 0.5	OR 4.5 for good response at week 6	0.05
			sGM < 0.5	OR 7.0 for week 12 survival	0.02
López-Medrano et al., 2016	Kidney transplant recipients	112	Mean sGM	0.5 in week 6 survivors vs. 1.1 in week 6 non-survivors	0.024
			Mean BAL GM	1.0 in week 6 survivors vs. 6.5 in week 6 non-survivors	0.014
Heylen et al., 2015	Kidney transplant recipients	41	sGM	HR 1.371 for week 12 mortality	0.002
			BAL GM	HR 1.742 for week 12 mortality	0.243
Han et al., 2015	Pediatric hematological	45	Median sGM	0.46 in week 12 survivors vs. 1.21 in week 12 non-survivors	0.015
Teering et al., 2014[a,d]	Mixed ICU	44	Mean sGM	Correlated with hospital survival (exact statistic not reported)	NS
Russo et al., 2014	Hematological, solid tumor, COPD (all non-neutropenic)	27	Mean BAL GM	1.9 in week 6 survivors vs. 3.6 in week 6 non-survivors	0.02
Kim et al., 2014[e]	Hematological	391	sGM < 0.5	HR 2.28 for good outcome	0.026
Hoyo et al., 2014	SOT	24	sGM < 0.5	56% of day 30 survivors vs. 18% of day 3 non-survivors had sGM < 0.5 at baseline	0.021
Mikulska et al., 2013[f]	Allogeneic stem cell transplant recipients	57	sGM 0.5–0.99	HR 2.76 for day 42 mortality	NS
			sGM ≥ 2.0	HR 6.98 for day 42 mortality	NS
			sGM 0.5–0.99	HR 1.37 for day 180 mortality	NS
			sGM ≥ 2.0	HR 3.35 for day 180 mortality	NS
Fisher et al., 2013	Allogeneic stem cell transplant recipients	100	sGM ≥ 0.5	Adjusted HR 3.01 for week 6 respiratory mortality	0.038
			sGM ≥ 2.0	Adjusted HR 6.56 for week 6 respiratory mortality	0.003
			sGM ≥ 1.0	Adjusted HR 2.54 for day 180 respiratory mortality	0.024
			sGM ≥ 2.0	Adjusted HR 4.01 for day 180 respiratory mortality	0.003
			sGM < 1.0	Adjusted HR 2.12 for day 180 survival	0.024
			sGM < 2.0	Adjusted HR 4.08 for day 180 survival	0.002
Hadrich et al., 2012	Hematological	58	sGM	HR 1.044 for mortality	NS
Bergeron et al., 2012	Hematological	57	sGM	HR 1.25 for day 60 mortality	<0.05
Koo et al., 2010	Hematological, SOT, solid tumor	93	sGM	Adjusted HR 1.25 for week 6 mortality	0.039
Boutboul et al., 2002[b,g]	Hematological	58	Mean sGM	Correlated with clinical response (exact statistic not reported)	NS

SOT, Solid organ transplantation; sGM, Serum galactomannan; OR, Odds ratio; HR, Hazard ratio; NS, Not significant; [a]Modification of the 2008 EORTC-MSG classification criteria. N; [b]Author's own classification criteria; [c]Only caspofungin treated patients; [d]Also included possible cases; [e]Only possible/probable cases, exclusion of patients with renal or hepatic failure. Outcome was a composite of 5 criteria; [f]Only probable cases; [g]Outcome assessment after at least 7 days (not further specified).

et al., 2014; Neofytos et al., 2015; Jung et al., 2017). Three groups determined a different cutoff of sGM $\geq$ 2.0 based on the Youden index or analysis of the area under the curve (Fisher et al., 2013; Mikulska et al., 2013; Imbert et al., 2016). When stratified by this cutoff, two studies found a trend toward higher 42 and 90 day all-cause mortality (Mikulska et al., 2013; Imbert et al., 2016), with another study showing a statistically significant difference for both 6 week respiratory mortality, 180 day respiratory mortality, as well as 180 day all-cause mortality (Fisher et al., 2013).

This relation demonstrates the interplay between two factors that determine the progression of fungal disease. As shown before, sGM correlates with fungal burden. As such, a higher fungal burden (or higher baseline sGM) can be expected to result in worse outcomes. On the other hand, there is the link between neutrophils and GM, with neutrophils being necessary for clearing both sGM as well as the fungus itself. Indeed, higher sGM at diagnosis have been shown to correlate with lower neutrophil counts (Jung et al., 2017).

One study also reported a significant link between BAL GM and week 6 survival (López-Medrano et al., 2016). However, the relation between BAL GM and outcome should be interpreted with caution as others could not replicate this finding. Of note, BAL GM testing depends on the site of infection, the site of sampling (sampling error), the non-standardized collection of BAL fluid, as well as on the portion of BAL fluid tested (Racil et al., 2011).

IMPACT OF GM KINETICS ON OUTCOME

We identified 21 studies that looked at GM kinetics as predictor of response and survival. Four descriptive studies were excluded due to the lack of a statistical analysis (Kwak et al., 2004; Maertens et al., 2005; Suankratay et al., 2006; Lai et al., 2007). The remainder has been summarized in **Table 3**. All included studies used the Platelia™ Aspergillus antigen assay. All studies included adult patients with proven and probable IA, unless stated otherwise in the table.

As with the baseline sGM, there appears to be a significant correlation between the evolution of sGM after baseline and outcome. Most studies stratified patients by outcome (treatment response or survival), and found significant differences in the mean sGM values at various timepoints (Woods et al., 2007; Maertens et al., 2009; Nouér et al., 2011, 2012; Park S. H. et al., 2011; Park S. Y. et al., 2011; Han et al., 2015; Neofytos et al., 2015; Vehreschild et al., 2017). The studies that took the initial sGM value into account and that evaluated the rate of decline, found this to be a good predictor of outcome as well (Boutboul et al., 2002; Koo et al., 2010; Khanna et al., 2013; Chai et al., 2014; Teering et al., 2014; Neofytos et al., 2015). For example, an increase of sGM values at week 2 of $\geq$1.0 over the baseline value, predicted therapy failure at week 6 with a sensitivity of 66%, a specificity of 87%, and a positive predictive value of 94% (Boutboul et al., 2002). The authors chose the cutoff of 1.0 as they determined this to be the smallest significant variance at higher optical indices. Furthermore, a persistently negative sGM was strongly associated with good outcomes (Neofytos et al., 2015). In another study, a composite of normalized serum 1,3-β-D-glucan (BDG, another biomarker of IA) and sGM (using z-scores) predicted clinical response at week 6 and week 12 (Neofytos et al., 2015). However, this appeared to be entirely due to the sGM kinetics as BDG alone failed to predict either, whereas sGM difference between baseline and week 2 predicted clinical response at week 6 and week 12. No study was able to identify differences in sGM before week 1.

Chai et al. found distinct kinetic profiles depending on the antifungal treatment, with voriconazole treatment showing earlier sGM clearance than amphotericin B treatment (Chai et al., 2014). However, this is in contrast with animal models where no difference in sGM kinetics could be seen between azole and polyene treatment (Petraitiene et al., 2001). Furthermore, another study in 93 patients found no differences in profiles between the antifungal drugs used (Koo et al., 2010).

IMPACT OF OTHER BIOMARKERS ON OUTCOME AND SURVIVAL

Besides GM, other quantitative biomarkers are being used for diagnosing IA such as BDG and *Aspergillus* PCR. These could therefore theoretically offer complementary information on prognosis and response to therapy as they have different sources of production and elimination. Indeed, a declining BDG at week 2 has been shown to correlate with survival at week 6 and week 12 (Neofytos et al., 2015). However, this decline was slower than the decline in sGM, and was less sensitive for predicting therapy response. The rate of decline seems to have an impact on survival however: a decline in BDG levels of 2.51 pg/mL/day had a sensitivity of 73.5% and specificity of 83.5% for predicting survival (Pini et al., 2016). Serum concentrations of bis(methylthio)gliotoxin (bmGT), a secondary metabolite of *Aspergillus* which has been proposed as a complementary biomarker, were shown to be significantly higher in patients who died at day 30 (2.36 ± 4.76 vs. 1.4 ± 7.58 mg/L, $p < 0.01$; Vidal-García et al., 2016).

In another study, a quantitative *Aspergillus* PCR showed good correlation between initial copy number and 90 day mortality, as well as between persistent PCR positivity after 2–3 weeks and 30 and 90 day mortality (Imbert et al., 2016). Similarly, a decline in circulating *Aspergillus* RNA between week 4 and week 6 correlated weakly with week 12 response ($\kappa = 0.621$, $p = 0.026$) but not with week 6 response (Zhao et al., 2016). A relation between sGM and circulating *Aspergillus* RNA could not be found. As such, these non-GM biomarkers appear to be especially useful in sGM negative patients, but are outperformed by sGM in sGM positive patients (which have a worse prognosis from the start), and only allow evaluation of antifungal efficacy during the later stages of treatment.

WHAT'S NEXT?

The data so far indicate a strong correlation between both baseline sGM and outcome, as well as between the kinetics of sGM and outcome. However, these correlations are based

TABLE 3 | Studies reporting statistics on a relation between galactomannan evolution after diagnosis and outcome.

References	Population	*N*	Kinetic parameter	Measured outcome	*p*-value
Vehreschild et al., 2017[d]	Majority hematological	40	Mean sGM at day 7	0.3 in week 12 survivors vs. 1.1 in week 12 non-survivors	0.354
			Mean sGM at day 14	0.3 in week 12 survivors vs. 1.3 in week 12 non-survivors	0.559
			Mean of (day 14 sGM – day 7 sGM)	1.26 in week 12 survivors vs. 0.82 in non-survivors	0.617
Neofytos et al., 2015	SOT, hematological, solid tumor	47	Baseline sGM – Week 2 sGM	Mean difference 0.58 between week 6 responders vs. week 6 non-responders	0.03
			Baseline sGM – Week 6 sGM	Mean difference 0.65 between week 6 responders vs. week 6 non-responders	0.03
			Baseline sGM – Week 2 sGM	Mean difference 0.72 between week 12 responders vs. week 12 non-responders	0.02
			Baseline sGM – Week 6 sGM	Mean difference 0.98 between week 12 responders vs. week 12 non-responders	0.01
			sGM remaining < 0.5	OR 4.1 for week 6 response	0.07
			sGM remaining < 0.5	OR 4.5 for week 12 response	0.05
			sGM remaining < 0.5	OR 4.3 for week 6 survival	0.10
			sGM remaining < 0.5	OR 6.5 for week 12 survival	0.02
Han et al., 2015	Pediatric hematological	45	Week 1 median sGM	0.39 in week 12 survivors vs. 1.64 in week 12 non-survivors	0.015
			Week 2 median sGM	0.38 in week 12 survivors vs. 2.76 in week 12 non-survivors	0.004
			Week 1 sGM < 1.5	Predicts week 12 survival with sensitivity 61.5%, specificity 89.3%, NPV 83.3%, PPV 72.7%	
Teering et al., 2014[a,e]	Mixed ICU	44	Maximum sGM – baseline sGM	0.11 in in-hospital survivors vs. 0.48 in non-survivors	0.017
Chai et al., 2014	Majority hematological	147	Week 1 sGM – baseline sGM	Greater decline in week 12 responders in voriconazole treated patients (effect size not reported)	0.001
			Week 2 sGM – baseline sGM	Greater decline in week 12 responders in voriconazole treated patients (effect size not reported)	0.046
			Week 4 sGM – baseline sGM	Greater decline in week 12 responders in amphotericin B treated patients (effect size not reported)	0.072
Khanna et al., 2013[f]	Adults and children, no pathology specified	57	Increasing sGM	5.4% of day 30 survivors vs. 64.9% of day 30 non-survivors had increasing sGM	0.02
Nouér et al., 2012	Multiple myeloma	98	sGM < 0.5 within 7 days	Adjusted OR 2.9 for favorable week 6 response	0.048
			sGM < 0.5 within 7 days	45.5% of week 6 survivors vs. 22.6% of week 6 non-survivors had sGM < 0.5 within 7 days	0.03
			sGM < 0.5 within 7 days	Adjusted OR 2.9 for week 6 survival	0.048
Hadrich et al., 2012	Hematological	58	7 × (Week 1 sGM – baseline sGM)/days between tests	HR 0.709 for mortality	NS

(Continued)

TABLE 3 | Continued

References	Population	*N*	Kinetic parameter	Measured outcome	*p*-value
Bergeron et al., 2012	Hematological	57	sGM area under the curve	No association found with day 60 survival	
			Rate of sGM decline	No association found with day 60 survival	
Park S. H. et al., 2011	Hematological	58	sGM remaining > 0.5 for more than 2 weeks	Kappa coefficient 0.663 for week 6 clinical failure	<0.05
			sGM remaining > 0.5 for more than 2 weeks	Kappa coefficient 0.819 for week 12 clinical failure	<0.05
Park S. Y. et al., 2011	Hematological, SOT	110	sGM remaining > 0.5 for more than 3 months	HR 7.14 day 90 mortality	<0.001
Nouér et al., 2011	Hematological	115	sGM remaining > 0.5 for more than 2 weeks	Kappa coefficient 0.819 for week 6 clinical failure	<0.001
Koo et al., 2010	Hematological, SOT, solid tumor	93	(Baseline sGM – week 1 sGM)/days between tests	Adjusted HR 0.78 for week 6 survival	0.02
Maertens et al., 2009[g]	Neutropenic hematological	70	sGM remaining > 0.5 for more than 2 weeks	Kappa coefficient 0.588 for week 6 clinical failure	<0.05
			sGM remaining > 0.5 for more than 2 weeks	Kappa coefficient 0.886 for week 12 clinical failure	<0.05
			sGM remaining > 0.5 for more than 2 weeks	Kappa coefficient 0.752 for week 6 EORTC-MSG response failure	<0.05
Woods et al., 2007[b]	Hematological	56	sGM remaining > 0.5 for more than 2 weeks	Kappa coefficient 0.861 for mortality	<0.0001
Boutboul et al., 2002[c]	Hematological	58	Increase of week 1 sGM < 1.0 over baseline	Predicts favorable week 6 response with sensitivity 44%, specificity 87%, PPV 94%	
			Increase of week 2 sGM < 1.0 over baseline	Predicts favorable week 6 response with sensitivity 55%, specificity 92%, PPV 92%	
Salonen et al., 2000[c,h]	Hematological, SOT	18	sGM remaining > 1.0	100% of non-survivors vs. 20% of survivors had sGM remaining > 1.0	0.002

SOT, Solid organ transplantation; sGM, Serum galactomannan; OR, Odds ratio; HR, Hazard ratio; NS, Not significant; PPV, Positive predictive value. N; [a]Modification of the 2008 EORTC-MSG classification criteria; [b]2002 EORTC-MSG classification criteria; [c]Author's own classification criteria; [d]Only caspofungin treated patients; [e]Also included possible cases; [f]Interval between serial sGM assessments not specified; [g]Only pulmonary invasive aspergillosis; [h]No interval specified between tests, or for outcome assessment.

on average sGM values and offer little added value for the management of the individual patient, mainly due to the lack of specific thresholds. Therefore, several authors have proposed clinical decision rules based on their findings. However, validation of these proposed rules is lacking, both in the initial population from which these have been derived, as well as in external validation populations. As such, exact indicators of the accuracy, sensitivity, specificity and other parameters are not available, making these proposed decision rules not suitable yet for use in daily clinical practice. Furthermore, of the studies discussed above which used the Platelia™ Aspergillus ELISA, none addressed the issue of non-linearity of higher levels of sGM. Several studies have applied modifications of the EORTC-MSG consensus definitions, mostly including other host criteria such as AIDS, cirrhosis and chronic obstructive lung disease, and other clinical criteria, making comparison and interpretation of the results more difficult. In addition, many studies suffer from low to very low numbers of sGM samples per patient. This is sometimes circumvented by modeling the average kinetics of sGM in the population, and using this model to predict the expected value on a certain time point based on previous values. The resulting estimate is then used for further analysis. Both approaches are inherently subject to bias as the actual values at the time point of interest are unknown.

Currently, clinical trials evaluating antifungal drugs primarily use survival at week 6 or week 12 as the primary outcome, or the clinical response as defined in the EORTC-MSG criteria (Segal et al., 2008). Surrogate outcomes for earlier assessment of efficacy, which would potentially allow for shorter durations of clinical trials, have been proposed. One such endpoint defines success as

repeatedly negative sGM (<0.5) for at least 2 weeks after the first negative sGM. This showed a good correlation with survival in 56 hematological patients (kappa correlation coefficient 0.861, $p < 0.0001$), which is in line with what would be expected from the kinetic data described above (Woods et al., 2007). This finding was confirmed by three independent studies in hematological patients, all of which found similar kappa correlation coefficients between this surrogate marker and clinical outcome and survival (Maertens et al., 2009; Nouér et al., 2011; Park S. H. et al., 2011). However, this definition does not allow evaluation of efficacy at a predetermined endpoint (e.g., after 1 or 2 weeks of treatment), which could be very useful in guiding decision making. In this setting, a robust and adequately validated early surrogate marker is not yet available.

Although the sensitivity of sGM for the diagnosis of IA is lower in non-neutropenic patients, solid organ transplant recipients, and patients on mold-active antifungal prophylaxis, the prognostic properties of sGM don't appear to be influenced by this. Several studies included non-neutropenic patients or solid organ transplant recipients (Koo et al., 2010; Park S. Y. et al., 2011; Russo et al., 2014; Teering et al., 2014; Neofytos et al., 2015; Imbert et al., 2016; Jung et al., 2017), or looked at these populations exclusively (Hoyo et al., 2014; Heylen et al., 2015; López-Medrano et al., 2016). The findings from these studies were in line with findings from studies in hematological patients. We could not identify any studies that looked at the difference in kinetics between patients on mold-active antifungal prophylaxis. However, several studies included this population in their overall analysis (percentage of study population on mold-active antifungal prophylaxis: range 4.3–85%, median 50%), and found results similar to those in populations not on prophylaxis (Park S. Y. et al., 2011; Hoyo et al., 2014; Kim et al., 2014; López-Medrano et al., 2016; Jung et al., 2017). We can thus conclude that patients with high initial sGM, and patients with an sGM that fails to decrease, are still at increased risk of poor outcome, independent of the underlying condition or prophylaxis. However, the exact kinetics could differ between these different populations, and have not been studied in detail.

CONCLUSION

Baseline sGM and trends in sGM kinetics correlate with outcome (both response and survival) in IA. In addition, sGM appears to have early prognostic potential, especially in hematological patients. However, further studies are urgently needed to determine the precise clinically relevant breakpoints and their test characteristics, followed by validation in both hematological and non-hematological populations. Furthermore, several other biomarkers such as BDG, bmGT, and *Aspergillus* DNA or RNA, appear to offer additional and complementary information, although the amount of evidence for these biomarkers is as of yet sparse.

AUTHOR CONTRIBUTIONS

TM was involved in data collection and drafting the article. TM, EG, KL, and JM were involved in critical revision of the article and final approval of the version to be published.

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MINI REVIEW
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Recent Advances in Diagnosing Chronic Pulmonary Aspergillosis

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Purpose: The diagnosis of chronic pulmonary aspergillosis (CPA) is occasionally complicated due to poor sensitivity of mycological culture and colonization of *Aspergillus* species in the airway. Several diagnostic methods have been developed for the diagnosis of invasive pulmonary aspergillosis; however, their interpretation and significance are different in CPA. This study aimed to review the recent advances in diagnostic methods and their characteristics in the diagnosis of CPA.

Recent findings: Radiological findings of lung, histopathology, and culture are the gold standard of CPA diagnosis. Serodiagnosis methods involving the use of galactomannan and β-D-glucan have low sensitivity and specificity. An *Aspergillus*-specific IgG antibody assay showed good performance and had better sensitivity and reproducibility than conventional precipitant antibody assays. Currently, it is the most reliable method for diagnosing CPA caused by *Aspergillus fumigatus*, but evidence on its effectiveness in diagnosing CPA caused by *non-fumigatus Aspergillus* is lacking. Newly developed lateral flow device Aspergillus and detection of volatile organic compounds in breath have potential, but evidence on its effectiveness in diagnosing CPA is lacking. The increasing prevalence of azole-resistant *A. fumigatus* strains has become a threat to public health. Some of the azole-resistant-related genes can be detected directly from clinical samples using a commercially available kit. However, its clinical efficacy for routine use remains unclear, since resistance-related genes greatly differ among regions and countries.

Conclusion: Several issues surrounding the diagnosis of CPA remain unclear. Hence, further investigations and clinical studies are needed to improve the accuracy and efficiency of CPA diagnosis.

Keywords: aspergillosis, *Aspergillus*, galactomannan, *Aspergillus* IgG antibody, azole resistance

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INTRODUCTION

Aspergillus species are environmental molds that produce airborne spores, and the average human is estimated to inhale hundreds of *Aspergillus* conidia daily (Hospenthal et al., 1998). Host immunity and the underlying pulmonary diseases are critical factors in determining the outcome of this daily exposure. Patients with defects in cell-mediated immunity, including those with neutropenia due to cytotoxic chemotherapy, or T-cell dysfunction due to corticosteroid or other immunosuppressive therapy are at risk of developing invasive pulmonary aspergillosis (IPA) characterized by hyphal invasion of lung tissues and dissemination to other organs (Baddley, 2011; Patterson et al., 2016). However, patients with underlying chronic respiratory disorders,

such as chronic obstructive pulmonary disease, post-pulmonary tuberculosis, non-tuberculosis mycobacteriosis (NTM), cystic fibrosis (CF), bronchiectasis, or allergic bronchopulmonary aspergillosis could develop saprophytic *Aspergillus* colonization and infection, namely, chronic pulmonary aspergillosis (CPA) (Saraceno et al., 1997; Takeda et al., 2016; Lowes et al., 2017). CPA is a slowly progressive pulmonary disease caused by *Aspergillus spp*. (Saraceno et al., 1997) and its prognosis is poor; the 5-year mortality rate of CPA patients is approximately 50–85% (Lowes et al., 2017). CPA is categorized into five disease entities based on the recent guidelines of the European Respiratory Society: *Aspergillus* nodule, simple pulmonary aspergilloma, chronic cavitary pulmonary aspergillosis (CCPA), chronic fibrosing pulmonary aspergillosis (CFPA), and subacute invasive pulmonary aspergillosis (SAIA) (Denning et al., 2016).

The diagnosis of CPA is occasionally complicated, as there are several disease entities in CPA, which are described in the following section, and some patients with underlying pulmonary diseases develop *Aspergillus airway colonization*. Diagnostic methods used for CPA are similar with those of IPA, but their interpretation and significance are different. Clinicians need various clinical information such as patients' background, radiological images, clinical courses, cultural tests, and other supportive diagnostic methods to diagnose CPA. The present review describes the currently available diagnostic methods and discusses new approaches for diagnosing CPA and their future directions.

Radiological and Histopathological Findings

Simple pulmonary aspergilloma is defined as single pulmonary cavity containing a fungal ball in a non-immunocompromised patient with minor or no symptoms and no radiological progression over at least 3 months of observation. Aspergillus nodule is characterized by the presence of one or more nodules without cavitation caused by *Aspergillus spp*. (Denning et al., 2016; Muldoon et al., 2016).

On the contrary, CCPA and SAIA are characterized by one or more cavities with or without fungal ball and its radiological progression such as expanding thick-walled cavities and pericavitary infiltration (Denning et al., 2016). The crucial difference between them is that SAIA involves hyphal invasion into the lung parenchyma (Yousem, 1997; Hope et al., 2005); however, it is not occasionally easy and practical to obtain sufficient histopathological samples to confirm the diagnosis. Therefore, clinical information such as time course of radiological progression (CCPA >3 months; SAIA 1–3 months) and process of cavity formation are indispensable for clinical diagnosis; CCPA usually occurs in pre-existing cavities, whereas in SAIA, cavities can be subsequently formed by the necrotic change of nodules or infiltration lesion due to *Aspergillus* species. infection (Izumikawa et al., 2014). However, it is hard to distinguish them if the serial radiography films are not available. Particularly, the patients with NTM infection are difficult to diagnose due to their similarity in radiological findings such as nodular shadows and cavity formation (Kobashi et al., 2006). CFPA is defined as severe fibrotic destruction of at least two lung lobes complicating CCPA leading to a major loss of lung function and generally the end result of untreated CCPA (Denning et al., 2003, 2016). Thus, these three clinical entities are vague and overlapping in some cases; however, it is essential to distinguish them in order to estimate their prognoses. Although triazole antifungals are recommended in these entities, their efficacy was better in patients with SAIA than in those with CCPA, as reported in a prospective study in France (Cadranel et al., 2012). Recently, "scab-like sign" observed inside the cavitary lesion in CT was proposed as a high-risk sign of hemoptysis in CPA patients, this could be useful when following the CPA patients (Sato et al., 2018).

Mycological Culture

Mycological culture is the basic methods for diagnosing CPA, although it has several limitations. The culture positivity rates of *Aspergillus* species from respiratory specimens in CPA vary widely, ranging from 11.8 to 81.0% depending on reports (Kitasato et al., 2009; Kohno et al., 2010; Nam et al., 2010; Shin et al., 2014). Uffredi et al. reported that 48 (63%) individuals were colonized patients among 76 non-granulocytopenic patients whose respiratory specimens yielded *Aspergillus fumigatus* (Uffredi et al., 2003). In our previous study, only 11 (16.4%) of 67 individuals were colonized patients among those with culture positive for *A. fumigatus*. By contrast, 58 (65.9%) of 88 individuals were colonized patients whose cultures yielded *non-fumigatus Aspergillus* strains (Tashiro et al., 2011). These reports imply that the clinicians need to be careful when interpreting the results of fungal cultures from respiratory specimens, as *Aspergillus* species are ubiquitous organism that is present in the air, and some of them are saprophytic fungus and cannot be the target of treatment. The most important way to distinguish the colonization from infection is to confirm clinical information, such as the transitional change of radiological findings; however, films are not always available. Therefore, we need a biomarker that reflects the invasiveness of *Aspergillus* infection.

Antigen and Antibody Test

It is not always easy to obtain the histopathological specimen, as some patients are not tolerable for invasive diagnostic procedure such as transbronchial lung biopsy due to their general conditions; therefore, serodiagnosis is indispensable for the diagnosis of CPA. Galactomannan (GM) antigen assays in serum and bronchial alveolar lavage (BAL) fluid have high sensitivity and specificity for the diagnosis of IPA, with cutoff values of 0.5 and 1.0, respectively (Maertens et al., 2007, 2009). However, the GM serum assay has lower sensitivity and specificity for CPA, with a cutoff value of 0.5 (Kitasato et al., 2009; Shin et al., 2014), than for IPA. GM antigen in BALF showed relatively higher

Abbreviations: CCPA, chronic cavitary pulmonary aspergillosis; CFPA, chronic fibrosing pulmonary aspergilllosis; CT, Computed tomography; ABPA, Allergic bronchopulmonary aspergillosis; CPA, chronic pulmonary aspergillosis; BAL, bronchial alveolar lavage; BDG, β-D-glucan; GAG, galactosaminogalactan; GM, galactomannan; IPA, invasive pulmonary aspergillosis; LFD, lateral flow device; PCR, polymerase chain reaction; RT-PCR, Reverse transcription-polymerase chain reaction; SAIA, subacute invasive pulmonary aspergillosis; TR, tandem repeats.

sensitivity (77.2%) and specificity (77.0%), with a cutoff value of 0.4, than that in serum (Izumikawa et al., 2012).

Although the β-D-glucan (BDG) assay has high sensitivity for the screening of a wide range of invasive fungal infections such as candidemia, pneumocystis pneumonia, and IPA, its specificity is limited (Karageorgopoulos et al., 2011; Onishi et al., 2012). Furthermore, its sensitivity is very low (about 20%) in CPA patients (Kitasato et al., 2009; Kohno et al., 2010). Urabe et al. recently reported that the combination of GM and BDG assays in BALF had a higher diagnostic accuracy compared with other single or combinations of diagnostic methods including PCR (Urabe et al., 2017).

Detection of the *Aspergillus*-specific antibody plays an important role in the diagnosis of CPA and Allergic bronchopulmonary aspergillosis and this method has been widely used. The precipitating *Aspergillus* IgG antibody has better sensitivity (80–90%) than GM and BDG assays (Kitasato et al., 2009; Kohno et al., 2010) At the moment, commercial *Aspergillus*-specific IgG plate ELISA tests are currently produced by Serion (Germany), IBL (Germany/USA), Dynamiker/Bio-Enoche (China), Bio-Rad (France), Bordier (Switzerland), and Omega/Genesis (UK) (Page et al., 2015). Siemens (Germany) supplies an automated *Aspergillus*-specific IgG ELISA system (Immunolite), while Thermo Fisher Scientific/Phadia (multinational) supplies an automated *Aspergillus*-specific IgG fluoroenzyme immunoassay system (ImmunoCAP), which is an ELISA variant (Page et al., 2015). The Phadia ImmunoCAP IgG assay and Bio-Rad Platelia *Aspergillus* IgG method have been reported to possess better sensitivity and reproducibility compared with the method involving the use of the conventional precipitant antibody (Baxter et al., 2013). These detection kits have excellent performance in the diagnosis of CPA and ABPA (Baxter et al., 2013; Dumollard et al., 2016; Fujiuchi et al., 2016; Page et al., 2016, 2018). However, all these tests use purified antibodies to culture extracts or recombinant antigens of *A. fumigatus*, and were originally designed to detect *A. fumigatus*. As non-fumigatus strains account for 40% (30 of 74) of CPA patients in Japan (Tashiro et al., 2011) and 38% in India (Shahid et al., 2001), these assays might have limitations in diagnosing CPA caused by non-*fumigatus* strains in some areas.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) for the diagnosis of IPA has been used for over 2 decades, though is not included in the European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) definitions of invasive fungal disease (White et al., 2015). *Aspergillus* PCR from blood sample has similar sensitivity and specificity for the diagnosis of IPA (White et al., 2015), but failed to detect *Aspergillus* DNA in patients with SPA and CPA (Imbert et al., 2016), conversely, this implies that PCR could be useful to eliminate disseminated infection from CPA. In BALF sample, PCR showed tolerable sensitivity (66.7–86.7%) and specificity (84.2–94.2%) compared to GM or BDG (Urabe et al., 2017). RT-PCR has advantages, (1) its quantitative aspect offers the possibility to establish precise cutoff values that could distinguish colonization from active infections, (2) since RT-PCR detects RNA, which is an indicator of the living fungal cells.

New Strategies

Aspergillus-specific lateral flow device (LFD) was newly developed. It uses the mouse monoclonal antibody JF5, which binds to a protein epitope present on an extracellular glycoprotein antigen secreted constitutively during the active growth of *A. fumigatus*. This method can detect Aspergillus antigens in human serum within 15 min. An early clinical trial showed that LFD is comparable to GM in serum in terms of diagnosing IPA, with a sensitivity and specificity of 81.8 and 98%, respectively (White et al., 2013). In a single center prospective study, LFD test using BALF specimen also showed tolerable sensitivity (77%) and specificity (92%) for proven/probable IPA (Prattes et al., 2014). However, recently, a single center study reported that LFD showed low sensitivity of 38% for IPA (Castillo et al., 2018). The evidence of LFD's utility in CPA diagnosis is quite limited to date, clinical studies on the diagnosis of CPA are needed to better understand the clinical use of LFD.

Volatile organic compounds (VOCs) are known to be detected from the breath of an infected individual. Initially, 2-pentylfuran was reported as the potential diagnostic VOC in IPA patients (Syhre et al., 2008; Chambers et al., 2009). A recent proof-of-principle study was conducted using electronic noses to detect the characteristic VOC pattern of IPA and showed high sensitivity of 100% and a specificity of 83.3% (de Heer et al., 2013). Other researchers used thermal desorption-gas chromatography/mass spectrometry to detect the specific VOCs pattern of IPA and also showed high sensitivity of 94% and specificity of 93% (Koo et al., 2014). Moreover, Heer et al. applied the same methods to detect *A. fumigatus* colonization in CF patients and showed sensitivity of 78% and specificity of 94% (de Heer et al., 2016). These methods can be useful screening tests, as they are noninvasive diagnostic procedures; however, there might be an issue in distinguishing CPA from *Aspergillus*-colonized patients.

Galactosaminogalactan (GAG) is a newly discovered extracellular polysaccharide of *Aspergillus* species, composed of α-1-4-linked galactose and α-1-4-linked N-acetylgalactosamine. It was observed only in hyphae form (Fontaine et al., 2011). GAG is particularly abundant in *A. fumigatus,* which is the most pathogenic specie among hundreds of *Aspergillus* species (Lee et al., 2015). Furthermore, GAG is required for its virulence (Gravelat et al., 2013). Therefore, this component could be a potential biomarker to estimate the invasiveness of *Aspergillus* infection.

Diagnosis of Infection With Azole-Resistant *A. fumigatus*

In recent years, the global increase of azole-resistant *A. fumigatus* became an emerging concern for public health, despite the fact that the rates of resistant strains vary among regions, countries, or continents, and rates of resistant strains are especially high in European countries (van der Linden et al., 2015; Meis et al., 2016; Rivero-Menendez et al., 2016). Azole antifungals are the mainstay of treatments for pulmonary aspergillosis. The mortality rates in IPA patients infected with azole-resistant strains were higher

TABLE 1 | Diagnostic methods for chronic pulmonary aspergillosis.

Test	Specimen	Sensitivity (%)	Specificity (%)	Note	References
Culture	Respiratory specimens	11.8–81.0	–		Kitasato et al., 2009; Kohno et al., 2010; Nam et al., 2010; Shin et al., 2014
β-D-glucan	Serum	15.4–26.7	95.8		Kitasato et al., 2009; Kohno et al., 2010; Urabe et al., 2017
	BALF	77.8	72.5	Cutoff ≧ 100 (Wako turbidimetric assay)	Urabe et al., 2017
Galactomannan	Serum	22.6–66.7	63.5	Cut off values differ in each study between 0.5 and 1.0	Kitasato et al., 2009; Kohno et al., 2010; Izumikawa et al., 2012; Shin et al., 2014
	BALF	77.2–77.8	77–90	Cut off values differ in each study between 0.4 and 0.5	Izumikawa et al., 2012; Urabe et al., 2017
Aspergillus precipitating antibody	Serum	56–89.3	100		Kitasato et al., 2009; Kohno et al., 2010; Baxter et al., 2013; Page et al., 2016
Aspergillus IgG antibody	Serum	93.2	98.2	Bio-Rad	Page et al., 2018
		83.8–98	84–98	ImmunoCAP	Fujiuchi et al., 2016; Page et al., 2016, 2018
		92.9–96	98–99.3	Immulite	Page et al., 2016, 2018
		84.2–90	91–98	Serion	Page et al., 2016, 2018
		77	97	Dynamiker	Page et al., 2016
		75	99	Genesis	Page et al., 2016
PCR	BALF	66.7–86.7	84.2–94.2	Non-standardized method	Urabe et al., 2017

BALF, Bronchial alveolar lavage fluid.

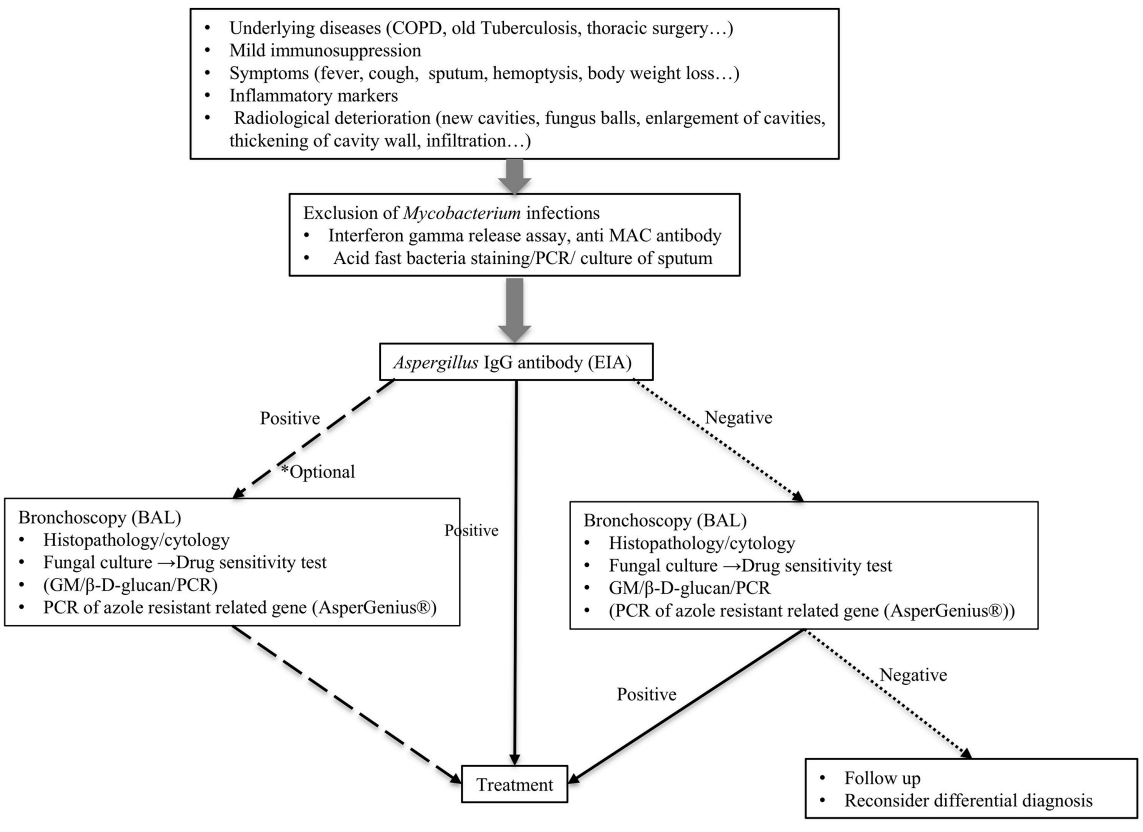

FIGURE 1 | Proposed algorithm for the diagnosis of chronic pulmonary aspergillosis. BAL, bronchoalveolar lavage.

than those infected with azole-sensitive ones (88% vs. 30–50%) (van der Linden et al., 2011). Lowes et al reported that the 10-year survival of CPA patients in United Kingdom with isolates fully susceptible to azoles was 68%, in contrast to 46% in patients with an isolate with reduced susceptibility to azoles, though there was not a significant difference (Lowes et al., 2017). However, it is still unclear how patients acquired azole-resistant strain infection affects the clinical course or mortality in CPA patients, because some azole-resistant strains obtained from aspergillosis patients treated with azoles showed poor condition and attenuated growth activity in *in vitro* condition (Ballard et al., 2018).

CPA patients need at least 6 months of oral azole treatment (Denning et al., 2016); detecting the azole-resistant strain earlier could provide them benefit by changing the treatment regimen. However, it is difficult to diagnose azole-resistant *A. fumigatus* infection in the clinical setting, as *in vitro* antifungal susceptibility testing of *Aspergillus* species is not routinely done in most clinical laboratories due to its cost and technical problems. The screening test with azole containing (itraconazole, 4 mg/L; voriconazole, 1 mg/L; posaconazole, 0.5 mg/L; and no antifungal) 4-well agar plate showed a sensitivity of 99% and a specificity of 99%, to screen the azole-resistant mutants (Arendrup et al., 2017); this could be useful and practical for routine test in clinical laboratories in countries where azole-resistance rate is high.

Azole-resistant *A. fumigatus* strains are mainly categorized into "environmental route" and "patient-acquired route" by means of resistance acquisition. The former was estimated to be generated by the agricultural fungicides used for crop protection and carries the tandem repeats (TR) of 34, 46, and 53 base pairs upstream in the promoter region of CYP51A with a single point mutation of CYP51A gene. By contrast, the latter were generated by the long-term use of medical azoles and carries various single point mutations of CYP51A gene (Meis et al., 2016). The environmentally obtained azole-resistant strains seemed to originate in Europe and have already spread into other regions worldwide (Meis et al., 2016).

The most commonly used method is simple polymerase chain reaction (PCR) amplification of the entire coding and promoter region with sequence analysis of the PCR products; however, this method is not practical for clinical use as it is time consuming. Restriction fragment length polymorphism by AluI is valuable as it can detect TR34 and L89H mutations from DNA samples faster than sequencing (Ahmad et al., 2014). The commercially available AsperGenius® (PathoNostics) can detect L98H, T289A, Y121F, and TR34 mutations as well as *A. fumigatus* gene directly from BALF specimen by multiplex real time PCR. In a multicenter clinical study, it showed good diagnostic performance on BAL and could detect *A. fumigatus* with resistance-associated mutations, including in culture-negative BALF samples, and detection of mutations was associated with azole treatment failure (Chong et al., 2016). However, the efficacy of this detection kit for CPA patients is unclear, as these mutations are relatively rare among patient-acquired azole-resistant strains obtained worldwide (Meis et al., 2016; Chowdhary et al., 2017); on the contrary, 27 (93.1%) of 29 of CPA patients from Europe had an L98H mutation from BALF samples and 16 (55.2%) had a TR34 mutation (Denning et al., 2011).

CONCLUSION

Needless to say, the gold standard of CPA diagnosis is the radiological findings of the lungs, its histopathology, and culture from the focus of infection. The definitive diagnosis by histopathology and culture is not always easy to perform; thereby, other diagnostic tools are also dispensable and biomarkers to reflect the disease status are needed. Diagnostic methods for CPA described in this review are summarized in **Table 1**. Currently, the *Aspergillus*-specific IgG antibody is the most promising tool for diagnosing CPA caused by *A. fumigatus*. We propose the algorithm for the diagnosis and treatment of CPA (**Figure 1**). When the patient is suspected of chronic aspergillus infection, it is important to rule out the mycobacterium infection first. Indication of bronchoscopy examination should be considered depending on the result of Aspergillus IgG antibody test. If it is negative, bronchoscopy examination is strongly recommended, as non-fumigatus Aspergillus infection can be the causative organism. If it is positive, bronchoscopy examination is however, optional, to determine which antifungal agents to be used, or collect more precise epidemiological information.

Since the emergence of azole-resistant *A. fumigatus* strains is a serious concern, convenient detection methods are required to detect these directly from clinical samples; however, further investigation is required. In addition, we need to investigate how these azole mutants are produced inside the lungs and how they affect CPA patients to discover other methods to decrease their prevalence.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Evaluation of Galactomannan Testing, the *Aspergillus*-Specific Lateral-Flow Device Test and Levels of Cytokines in Bronchoalveolar Lavage Fluid for Diagnosis of Chronic Pulmonary Aspergillosis

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Background: Diagnosis of chronic pulmonary aspergillosis (CPA) is challenging. Symptoms are unspecific or missing, radiological findings are variable and proof of mycological evidence is limited by the accuracy of diagnostic tests. The goal of this study was to investigate diagnostic performance of galactomannan (GM), the newly formatted *Aspergillus*-specific lateral-flow-device test (LFD), and a number of cytokines in bronchoalveolar lavage fluid (BALF) samples obtained from patients with CPA, patients with respiratory disorders without CPA and healthy individuals.

Methods: Patients with CPA (n = 27) and controls (n = 27 with underlying respiratory diseases but without CPA, and n = 27 healthy volunteers) were recruited at the Medical University of Graz, Austria and the Research Center Borstel, Germany between 2010 and 2018. GM, LFD and cytokine testing was performed retrospectively at the Research Center Borstel.

Results: Sensitivity and specificity of GM testing from BALF with a cut off level of ≥0.5 optical density index (ODI) was 41 and 100% and 30 and 100% with a cut off level of ≥1.0 ODI. ROC curve analysis showed an AUC 0.718 (95% CI 0.581–0.855) for GM for differentiating CPA patients to patients with other respiratory diseases without CPA. The LFD resulted positive in only three patients with CPA (7%) and was highly specific. CPA patients did not differ significantly in the BALF cytokine profile compared to patients with

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respiratory disorders without CPA, but showed significant higher values for IFN-γ, IL-1b, IL-6, IL-8, and TNF-α compared to healthy individuals.

Conclusion: Both GM and LFD showed insufficient performance for diagnosing CPA, with sensitivities of BALF GM below 50%, and sensitivity of the LFD below 10%. The high specificities may, however, result in a high positive predictive value and thereby help to identify semi-invasive or invasive disease.

Keywords: chronic pulmonary aspergillosis, bronchoalveolar lavage, galactomannan, lateral-flow device, cytokines

INTRODUCTION

Diagnosis of chronic pulmonary aspergillosis (CPA) is challenging. Symptoms are unspecific, radiological findings are variable and proof of mycological evidence is limited by the paucity of diagnostic tests. Current guidelines recommend establishing diagnosis based on several characteristics including a radiological pattern consistent with CPA, proof of mycological evidence and exclusion of alternative diagnosis (Denning et al., 2016; Patterson et al., 2016). Consequently, patients usually undergo bronchoscopy to achieve both exclusion of alternative diagnosis (e.g., lung cancer or mycobacterial infection) and to obtain bronchoalveolar lavage fluid (BALF) at the primary site of infection to obtain mycological evidence.

Galactomannan (GM) testing from BALF is well established in the diagnosis of pulmonary invasive aspergillosis (IPA) (Ullmann et al., 2018), but evidence about the performance in CPA patients is limited. Only four studies have reported GM test results from BALF in CPA patients so far. Sensitivity and specificity were between 78 and 92 and 76 and 90% with an optical density index (ODI) ≥0.5, respectively (Park et al., 2011; Kono et al., 2013; Urabe et al., 2017) and 77–77% with an ODI ≥0.4 (Izumikawa et al., 2012).

The *Aspergillus*-specific lateral-flow device (LFD) is an immuno-chromatographic assay that detects extracellular glycoprotein antigen circulating in BALF secreted during active growth of the fungus (Thornton, 2008). A first clinical evaluation of the newly formatted and CE marked point-of-care LFD in BALF showed a sensitivity of 71% and a specificity of 100% for patients at risk for IPA (Hoenigl et al., 2018). Data about the performance of the LFD in BALF from patients with CPA are lacking.

Genetic association studies of genes involved in the immune response to *Aspergillus fumigatus* indicated that patients with CPA might differ from other patients in the cytokine profile, however, studies comprehensively investigating cytokines in BALF from CPA patients vs. controls are missing (Sambatakou et al., 2006; Smith et al., 2014).

We aimed to investigate diagnostic performance of GM and LFD testing, as well as various cytokine levels in BALF samples obtained from patients with CPA according to ESCMID/ERS/ECMM definition, patients with respiratory disorders without CPA and healthy individuals.

MATERIALS AND METHODS

Study Population

Patients with CPA were recruited at the Medical University of Graz, Austria (n = 4) and the Research Center Borstel, Germany (n = 23). Healthy volunteers (n = 27) were recruited at the Research Center Borstel, Germany and were asked to undergo bronchoscopy for study purposes, which served as control group (Approval number: AZ 15–194). Patients with respiratory disorders without CPA infection who received bronchoscopy and BALF as part of routine clinical and subsequent microbiologic work-up due to suspicion of pulmonary infection were recruited at the Medical University of Graz, Austria (n = 27). Inclusion of residual BALF samples of patients was approved by the local ethics committee at the University of Lübeck, Germany (Approval numbers: AZ 12–220, AZ 14–225, and AZ 18–105) and the Medical University of Graz, Austria (Approval number: 25–221 ex 12/13).

Case Definition

Criteria for CPA were based on the diagnostic criteria expressed by the ESCMID/ERS/ECMM guideline including a) one or more cavities with or without a fungal ball present or nodules on computed tomography scan, b) direct evidence of *Aspergillus* infection or an immunological response to *Aspergillus* spp. (e.g., *Aspergillus*-specific IgG antibody), and exclusion of alternative diagnosis, all present for at least 3 month or 1–3 month in case of subacute invasive aspergillosis (SAIA), respectively (Denning et al., 2016; Salzer et al., 2017).

Clinical Samples

Bronchoscopies with BALF according to professional recommendations were performed in all patients at the Medical Clinic of the Research Center Borstel, Germany and the Medical University of Graz, Austria (Haussinger et al., 2004). All BALF samples were stored at −70°C and samples from the Medical University of Graz, Austria shipped on dry ice to the Medical Clinic of the Research Center Borstel, Germany, where GM, LFD and cytokine testing was performed in June 2018. BALF samples from the Medical University of Graz were, in part, published before (Prattes et al., 2014).

Aspergillus Galactomannan Antigen Assay

BALF *Aspergillus* GM was determined by the Platelia EIA (Bio-Rad Laboratories, Munich, Germany) in clinical routine at

the Medical Clinic of the Research Center Borstel, Germany according to the manufacturer's instructions. BALF samples were processed in accordance to the manufactures protocol. While our analysis primarily focused on the recommended 1.0 ODI cut-off for BALF, we also evaluated a 0.5 GM ODI cut-off, following previous evidence that the 0.5 ODI cut off is preferable in patients on mold-active antifungals (Eigl et al., 2015, 2017).

Newly Formatted Lateral-Flow Device

The newly formatted CE marked *Aspergillus* LFD (OLM Diagnostics, Newcastle upon Tyne, United Kingdom) was performed in accordance to the manufactures protocol (Hoenigl et al., 2018). Stored BALF samples were thawed, vortexed, and centrifuged for 1 min at 14,000 × *g*. Seventy microliter of untreated BALF sample were applied to the port of the cassette, with results read 15 min later. Two interpreters read LFD test results independently without knowing the CPA status, ensuring an unbiased interpretation of the test results. Appearance of the test and the control line were considered as positive test result.

Quantification of Cytokines

Levels of IL-1β, IL-6, IL-8, IL-10, IL-15, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were analyzed by using the Meso Scale Discovery (MSD) U-Plex Platform (MSD, Rockville, MD, Unites States). The panel was selected based on previous findings of BALF cytokines in CPA and invasive aspergillosis (Sambatakou et al., 2006; Smith et al., 2014; Heldt et al., 2017; Heldt et al., 2018). According to the manufacturer's instructions[1] the U-Plex linker (= binding to the U-Plex plate) was coupled to the specific biotinylated capture antibodies (= conjugated with electrochemiluminescent label), followed by the preparation of the multiplex coating solution. Fifty microliter of the multiplex coating solution were added to each 96-well U-Plex plate for a 1 h incubation. Twenty-five microliter BALF and 25 μL standard were incubated in the 96-well plate. Fifty microliter detection antibody was added for 1 h incubation to complete the sandwich immunoassay followed by adding 150 μL of 2× Reading Buffer. Each incubation was performed for 1 h on a shaker at room temperature, afterward the solutions was washed off. Analyses were performed on the MSD instrument by measuring the intensity of emitted light providing a quantitative measure.

Statistical Analyses

Statistical analysis was performed with R Version 3.5.0. Kruskal–Wallis rank sum and pairwise Wilcoxon rank sum test were used to compare the patient characteristics and biomarker values in the three groups (patients with CPA, patients with respiratory disorders without CPA and healthy individuals). The Tukey Test was used to display the mean value differences. In this context, a power analysis was also conducted. Receiver operating characteristic (ROC) curve analyses were performed and Areas under the Curve (AUC) including 95% confidence interval were displayed to assess the diagnostic discriminatory ability of biomarkers to distinguish between CPA patients and healthy individuals and between CPA patients and patients with respiratory disorders without CPA.

[1]https://www.mesoscale.com

RESULTS

A total of 81 patients were included in this analysis. Twenty-seven patients were classified as CPA and were compared to two control groups consisting of 27 patients with respiratory disorders without evidence of CPA and 27 healthy individuals. CPA patients were classified as chronic cavitary pulmonary aspergillosis (CCPA) ($n = 12$), single/simple aspergilloma ($n = 6$), *Aspergillus* nodules ($n = 4$), and subacute invasive aspergillosis [SAIA ($n = 5$)]. In more than one third of CPA patients (37%; 10/27) the diagnosis was histologically proven with exclusion of SAIA or IPA.

For all patients enough BALF sample volume was available for measurements of GM, LFD and cytokines. Patients' characteristics and mean values for GM and cytokines as well as positive test results for the LFD are displayed in **Table 1**.

Aspergillus Galactomannan Antigen Assay

Depending on the cut-off used, GM antigen assay from BALF was positive in 8/27 CPA patients (30%; cut off 1.0 ODI) and in 11/27 CPA patients (41%; cut off 0.5 ODI), respectively. Neither patients with respiratory disorders without CPA nor healthy individuals had a positive GM test result from BALF with an ODI ≥ 1.0 (specificities 100% for both control groups). Two patients with respiratory disorders without CPA had a positive GM test result from BALF with an ODI ≥ 0.5, but none from the healthy individuals (specificity 93 and 100%, respectively) (**Table 2**). Among 10 patients with histologically proven diagnosis of CPA, eight had a GM test result <0.5 ODI and one patient had a GM test result of 0.7 ODI, while only 1 had a GM result > 1.0 ODI.

The diagnostic performance of the GM test tended to be higher when COPD was present in CPA patients (sensitivity 46% vs. 38%; cut off 0.5 ODI and 46% vs. 19%; cut off 1.0 ODI respectively) (**Table 3**).

The distribution of GM test results for all three cohorts is shown in **Figure 1**. GM values were significantly higher in CPA samples compared to patients with respiratory disorders without CPA ($p = 0.013$) and healthy individuals ($p < 0.001$). No significant differences in GM values were found between healthy individuals and patients with other respiratory diseases without CPA ($p = 0.081$) (**Table 3**). Tukey test showed a significant difference in mean values for GM test between CPA patients compared to patients with respiratory disorders without CPA and to healthy individuals (**Figure 2**).

ROC curve analysis showed an AUC 0.718 (95% CI 0.581–0.855) for differentiating CPA patients from patients with other respiratory diseases without CPA (**Figure 3**) and an AUC of 0.875 (95% CI 0.782–0.968) for differentiating CPA patients from healthy individuals (**Figure 3**).

Newly Formatted Lateral-Flow Device

The *Aspergillus* LFD showed positive test results in two CPA patients (sensitivity 7%) and in one patient with respiratory disorder without CPA, but none in healthy volunteers (specificity

96 and 100%, respectively). Both CPA patients with a positive LFD had a high GM test result of 14.0 and 5.4 ODI, respectively, while the one patient without CPA had a negative GM test result of 0.38 ODI.

Cytokines

Median and standard deviation of CPA patients vs. controls including patients with respiratory disorders without CPA and healthy individuals are depicted **Table 1**. Box plots for IL-1β, IL-6, IL-8, IL-10, IL-15, TNF-α, and IFN-γ are depicted in **Figure 4**. AUC for BALF cytokines for differentiating CPA patients vs. controls including patients with respiratory disorders without CPA and healthy individuals are depicted in **Table 2**.

CPA patients did not differ significantly in the BALF cytokine profile compared to patients with respiratory disorders without CPA, but showed significant higher values for IFN-γ, IL-1b, IL-6, IL-8, and TNF-α compared to healthy individuals (**Table 2**). Patients with respiratory disorders without CPA had also significantly higher values for IFN-γ, IL-1b, IL-6, IL-8, and TNF-α compared to healthy individuals.

Sensitivity of IL-1b, IL-6, and IL-8 tended to be higher in patients without COPD, but at the expense of specificity. The sensitivity of TNF-α tended to be higher when COPD was present in CPA patients (**Table 2**).

DISCUSSION

We evaluated diagnostic performance of GM and LFD testing, which are routinely used for the diagnosis of invasive pulmonary aspergillosis (IPA), in patients with CPA compared to patients with respiratory disorders without CPA and healthy individuals. We also explored the diagnostic potential of cytokine levels in BALF in these patient cohorts.

In the present study the sensitivity of the GM test from BALF for CPA (according to ESCMID/ERS/ECMM definition) was

TABLE 1 | Demographic data, underlying respiratory disorders and mean values with standard deviations of GM, LFD, and cytokine levels.

	All patients	CPA	Respiratory disorders without CPA	Healthy individuals	*P*-value
No. of patients	81	27	27	27	
Sex					
- Male	48 (60.0%)	17 (63.0%)	15 (57.3%)	16 (59.3%)	0.922
- Female	32(40.0%)	10 (37.0%)	11 (42.7%)	11 (40.7%)	
Age (median. range)	59 (20–88)	63 (28–88)	66 (48–86)	29 (20–74)	<0.001*
Underlying Respiratory Disorder					
- Asthma	2 (2.5%)	2	0	0	NA
- Bronchiectasis	4 (5%)	3	1	0	NA
- COPD	32 (40%)	12	20	0	0.442
- Lung fibrosis	2(2.5%)	0	2	0	NA
- NTM	2(2.5%)	2	0	0	NA
- Prior pulmonary TB	7 (8.75%)	6	1	0	NA
- Recurrent aspirations	1(1.25%)	1	0	0	NA
- Sarkoidosis	6 (7.5%)	2	4	0	NA
- Lung cancer	6(7.5%)	2	4	0	NA
- Pulmonary embolism	1(1.25%)	1	0	0	NA
- LTOT	1(1.25%)	0	1	0	NA
- OSAS	2(2.5%)	0	2	0	NA
- Pneumonia	2(2.5%)	0	2	0	NA
Tests					
GM (ODI mean, range)	0.69 ± 1.98	1.66 ± 3.22	0.24 ± 0.17	0.15 ± 0.05	0.006*
Positive LFD	3	2	1	0	0.358
Cytokines (pg/ml, mean, range)[#]					
- IFN-γ	102.11 ± 569.34	33.46 ± 75.10	223.77 ± 893.64	1.07 ± 0.88	0.372
- IL-10	2.410 ± 8.65	0.46 ± 0.83	5.43 ± 12.10	0.00 ± 0	0.066
- IL-15	2.29 ± 4.07	2.33 ± 3.37	3.64 ± 5.70	0.92 ± 0.70	0.059
- IL-1b	121.79 ± 507.97	113.74 ± 342.34	255.84 ± 811.74	0.74 ± 0.66	0.189
- IL-6	47.29 ± 102.45	51.38 ± 91.45	92.19 ± 140.45	0.41 ± 0.69	0.004*
- IL-8	1390.34 ± 2087.14	1643.67 ± 2120.37	2545.85 ± 2369.79	24.29 ± 18.76	<0.001*
-TNF-α	4.97 ± 19.64	2.00 ± 4.38	10.08 ± 29.61	0.00 ± 0	0.213

*COPD, Chronic obstructive pulmonary disease; CPA, Chronic pulmonary aspergillosis; GM, Galactomannan; IFN, Interferon; IL, Interleukins; LFD, Lateral-flow device; LTOT = Long-term oxygen therapy; NTM, Non-tuberculous mycobacteria; ODI, Optical density index; OSAS, Obstructive sleep apnea syndrome; TB, Tuberculosis; TNF, Tumor necrosis factor. [#]For one patient in the group of respiratory disorders without CPA cytokine levels were not determined due to insufficient BALF volume. *P-value <0.05 statistically significant.*

TABLE 2 | Results of ROC analysis for all patients and stratification for patients with and without COPD.

Test	All				COPD				Non-COPD			
	CPA vs. healthy		CPA vs. respiratory disorders without CPA		CPA vs. healthy		CPA vs. respiratory disorders without CPA		CPA vs. healthy		CPA vs. respiratory disorders without CPA	
	AUC	Sens and Spec (%)	AUC	Sens/Spec	AUC	Sens/Spec	AUC	Sens/Spec	AUC	Sens/Spec	AUC	Sens/Spec
GM 0.5 ODI	87.5 (78.2–96.8)	Sens: 40.7 Spec: 100	71.8 (58.1–85.5)	Sens: 40.7 Spec: 92.6	95.8% (90.2%–100%)	Sens: 45.5 Spec: 100	64.3 (40.9–87.7)	Sens: 45.5 Spec: 90	22.2% (67.8–95.8%)	Sens: 37.5 Spec: 100	80.5 (64.6–96.3)	Sens: 37.5 Spec: 100
GM 1.0 ODI	87.5 (78.2–96.8)	Sens: 29.6 Sens: 100	71.8 (58.1–85.5)	Sens: 29.6 Spec: 100	95.8% (90.2%–100%)	Sens: 45.5 Spec: 100	64.3 (40.9–87.7)	Sens: 45.5 Spec: 100	81.8% (67.8–95.8%)	Sens: 18.8 Spec: 100	80.5 (64.6–96.3)	Sens: 18.8 Spec: 100
IFN- γ	87.6 (74.2–100)	Sens: 88.0 Spec: 26.7	54.8 (30.8–78.9)	Sens: 88.0 Spec: 0	77.4 (55.5–99.4)	Sens: 88.9 Spec: 26.7	63.5 (9.0–87.9)	Sens: 88.9 Spec: 0	87.6 (74.2–100)	Sens: 93.3 Spec: 26.7	54.8 (30.6–78.9)	Sens: 93.3 Spec: 0%
IL-10	64.6 (55.3–73.9)	Sens: 63.0 Spec: 100	45.8 (32.4–59.1)	Sens: 63.0 Spec: 63.3	70.0 (54.–84.6%)	Sens: 40 Spec: 100	43.7 (24.1–63)	Sens: 40 Spec: 100	75.0 (61.4–88.6)	Sens: 21.4 Spec: 100	60.7(49.6–71.9)	Sens: 21.4 Spec: 100
IL-15	55.2 (33.5–76.8	Sens: 33.3 Spec: 80	55.8 (35.9–75.7)	Sens: 33.3 Spec: 40	71.6% (38.4%–100%)	Sens: 60 Spec: 80	55.0 (26.6–83.4)	Sens: 80 Spec: 38.9	43.3 (18–68.)	Sens: 14.3 Spec: 100	67.3 (34.9–99.8)	Sens: 85.7 Spec: 42.9
IL-1b	93.8 (86.9–100.0)	Sens: 85.1 Spec:100.0	49.6 (33.7–65.59)	Sens: 85.1 Spec: 22.2	96.1 (89.4%–100%)	Sens: 81.3 Spec: 100	55.0 (32.7–77.3)	Sens: 82.3 Spec: 28.6	92.2 (81.5–100)	Sens: 81.8 Spec: 100	62.1 (38.0–86.1)	Sens: 81.8 Spec: 10.5
IL-6	90.2 (81.9–95.5)	Sens: 70.8 Spec: 96.3	61.9 (46.0–77.7)	Sens: 70.8 Spec: 21.1	88.1 (73.2%–100%)	Sens: 73.3 Spec: 96.3	64.0 (40.7–87.4)	Sens: 73.3 Spec: 28.6	91.5 (82.0–100)	Sens. 66.7 Spec: 96.3	47.6 (22.2–73.1)	Sens: 66.7 Spec: 15.8
IL-8	95.4 (90.1–100.0)	Sens: 92.6 Spec: 70.4	63.7 (48.4–78.9)	Sens: 92.6 Spec: 0	96.5% (89.3%–100%)	Sens: 93.8 Spec: 70.4	6.8 (39.3–82.2)	Sens: 93.8 Spec: 0	94.7 (87.3–100.0)	Sens: 90.9 Spec: 70.4	62.5 (35.2–89.8)	Sens: 90.9 Spec: 0
TNF-α	73.9 (63.5–84.3)	Sens: 47.8 Spec: 100	54.1 (38.6–69.6)	Sens: 47.8 Spec: 42.3	72.2% (5.0–89.4%)	Sens: 50.0 Spec: 100	55.3 (33.7–76.8)	Sens: 50.0 Spec: 28.6	75 (61.4–88.6)	Sens: 44.4 Spec: 100	50.5 (26.4–74.6)	Sens: 44.4 Spec: 47.4

AUC, Area under the curve; COPD, Chronic obstructive pulmonary disease; CPA, Chronic pulmonary aspergillosis; GM, Galactomannan; IFN, Interferon; IL, Interleukins; ODI, Optical density index; Sens, Sensitivity; Spec, Specificity; TNF, Tumor necrosis factor.

TABLE 3 | *P*-values of pairwise Wilcoxon rank sum test for GM and cytokines.

	CPA vs. respiratory disorders without CPA	CPA vs. healthy individuals	Respiratory disorders without CPA vs. healthy individuals
GM	$p = 0.013$*	$p < 0.001$*	$p = 0.081$
IFN- γ	$p = 0.477$	$p = 0.001$*	$p < 0.001$*
IL-10	$p = 0.541$	$p = 0.072$	$p = 0.057$
IL-15	$p = 1.00$	$p = 1.00$	$p = 0.14$
IL-1b	$p = 0.96$	$p < 0.001$*	$p < 0.001$*
IL-6	$p = 0.15$	$p < 0.001$*	$p < 0.001$*
IL-8	$p = 0.089$	$p < 0.001$*	$p < 0.001$*
TNF-α	$p = 0.609$	$p = 0.008$*	$p = 0.003$*

*CPA, Chronic pulmonary aspergillosis; GM, Galactomannan; IFN, Interferon; TNF, Tumor necrosis factor. *P-value <0.05 statistically significant.*

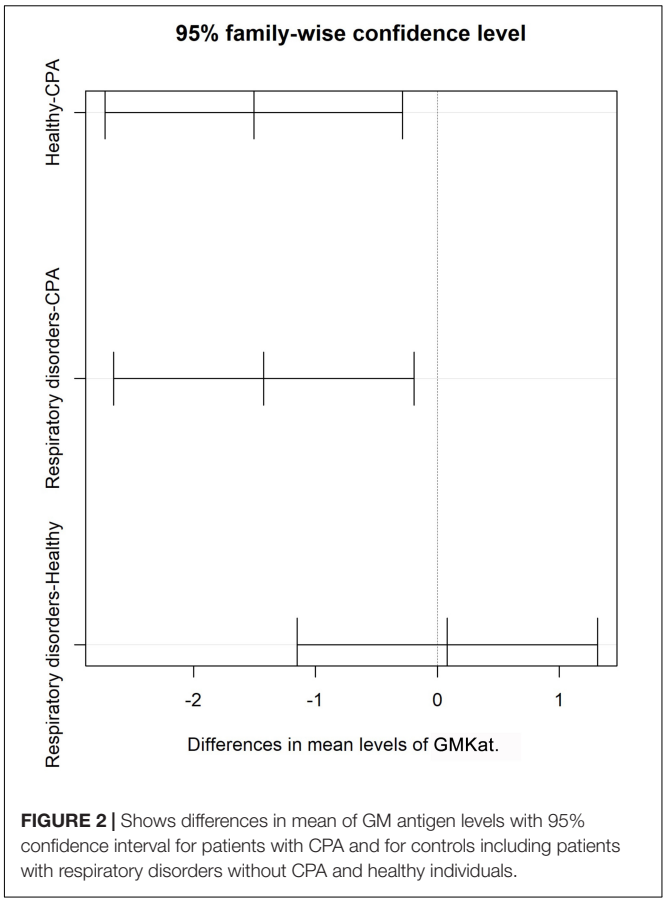

FIGURE 2 | Shows differences in mean of GM antigen levels with 95% confidence interval for patients with CPA and for controls including patients with respiratory disorders without CPA and healthy individuals.

considerably lower than previously reported with sensitivities of 30% (cut off 1.0 ODI) and 41% (cut off 0.5 ODI), respectively. Strikingly, 80% of histologically proven cases of CPA had a negative GM test result when using the 0.5 ODI cut-off (and 90% when using the 1.0 ODI cut off). Previous studies reported higher sensitivities between 77 and 92% for a cut off ≥0.5 ODI or ≥0.4 ODI (Park et al., 2011; Izumikawa et al., 2012; Kono et al., 2013; Urabe et al., 2017). We suggest that one main reason is that studies demonstrating a higher sensitivity from BALF in CPA patients may have included a considerably higher proportion of patients with SAIA (formally chronic necrotizing or semi-IPA), which is in fact an invasive form of the disease and very similar to IPA. This has been demonstrated by the study of Kono et al. (2013) who included a total of 7 patients with pulmonary aspergillosis in their analysis including five patients with SAIA (sensitivity 86%; cut-off ≥0.5 ODI). The most recent study by Urabe et al. (2017) who reported a sensitivity of 78% for BALF GM testing (cut-off ≥0.5 ODI) among 27 CPA patients did not provide information on the SAIA proportion nor on exact GM values hampering the interpretation of the data.

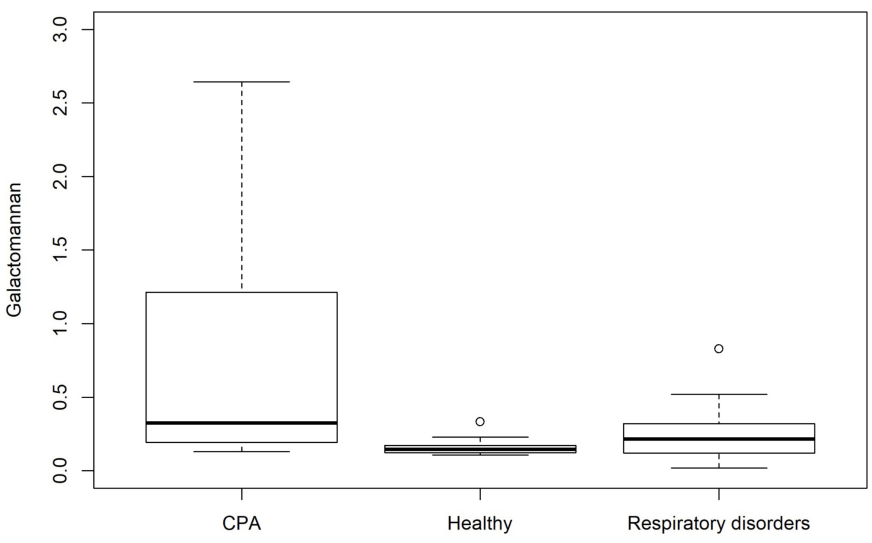

FIGURE 1 | Box plot showing the distribution of GM antigen levels for patients with CPA and controls including patients with respiratory disorders without CPA and healthy individuals.

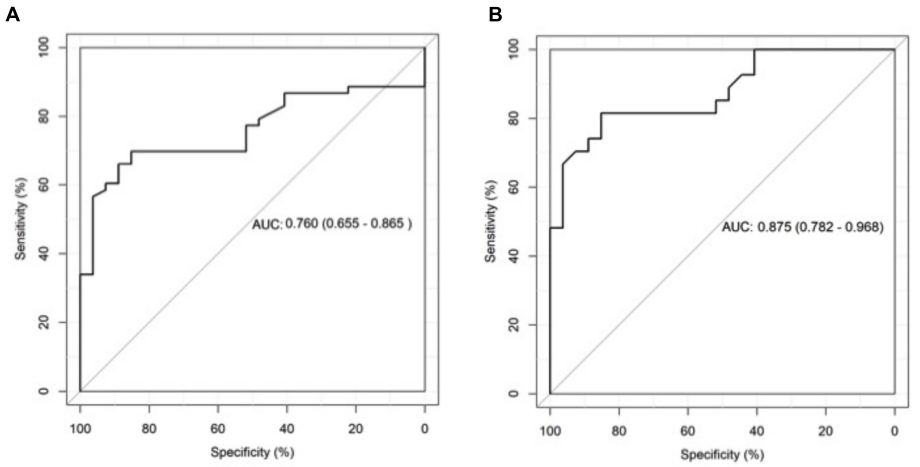

FIGURE 3 | Receiver operating characteristics (ROC) curve analysis of galactomannan test results in bronchoalveolar lavage fluid (BALF) for patients with chronic pulmonary aspergillosis (CPA) and patients with respiratory disorders without CPA **(A)** as well as for patients with CPA and healthy individuals **(B)**.

Another study by Izumikawa et al. (2012) reported a sensitivity of 77% (cut off 0.4 ODI) for the GM test from BALF and included 18 CPA patients. Although they did not classify CPA subtypes, inclusion criteria considered SAIA and we suggest that a considerably proportion might have had SAIA because very high GM test results between 7.3 and 14.1 ODI were observed in 7/18 patients. In contrast, in our study only 3/27 CPA patients had a GM test result >5.0 ODI. We suggest that these three patients might have in fact had SAIA supported by the fact that the two positive LFD test results from BALF in our study were seen in those patients with the highest GM test results.

The highest sensitivity of 92% for GM from BALF (cut off ≥0.5 ODI) was reported in patients with single aspergilloma (Park et al., 2011). Interestingly, 75% of all patients included in that study (n = 48) had hemoptysis, which is usually considered as an expression of angioinvasion by the fungus. Furthermore, 13/34 patients even had a positive GM test in serum (cut off 0.5 ODI), which was also significantly associated with hemoptysis compared to those without hemoptysis (52% vs. 9%; p = 0.02). Therefore it is highly likely that a high proportion of patients in that study might have had a more invasive stage of disease comparable to SAIA or even IPA, which was not the case for our study cohort.

Taken this together it may not be surprising that the sensitivity of GM in BALF, which was initially developed for the diagnosis of IPA, was considerably lower in our study than compared to previous study results. It has to be considered that the vast majority of patients with CPA do not have SAIA. Thus, the diagnostic performance of GM from BALF to establish the diagnosis of CPA is insufficient. However, its high specificity may help to exclude semi-invasive or invasive disease in certain cases. We suggest that patients with a high GM test are at least in a transition stage to semi-invasive or invasive disease, while patients with a non-invasive CPA subtype usually do not respond with a positive GM from BALF. Other factors that may influence the performance of GM in BALF include antifungal treatment, certain antimicrobial drugs (e.g., beta-lactam antibiotics), underlying diseases (e.g., allergic bronchopulmonary aspergillosis), or BALF sampling bias (e.g., volume and site of lavage fluid sampling). However, bronchoscopy is still indispensable and should be integrated in every diagnostic work-up, if possible. Histology (e.g., from transbronchial biopsies) is still the reference standard to differentiate between invasive and non-invasive aspergillosis and to exclude alternative diagnosis as recommended by the current guideline (Denning et al., 2016). BALF also offers the possibility to collect fungal cultures from primary site of infection and when positive, to examine antifungal *in vitro* susceptibility (Alastruey-Izquierdo et al., 2018; Godet et al., 2018).

We did not evaluate GM test in serum, because serum was only availably in a minority of CPA patients included. Furthermore, previous studies clearly demonstrated that GM testing from serum has a very low sensitivity and specificity between 23 and 67% and 64 and 85% with an ODI ≥ 0.7 in CPA patients, which is plausible considering that CPA is a localized chronic disease of the lung without angioinvasion per definition (Izumikawa et al., 2012; Shin et al., 2014; Urabe et al., 2017).

This is to our knowledge the first study investigating cytokine levels in BALF from CPA patients. Generally, CPA patients had cytokine levels in BALF that were comparable to those found in patients with respiratory disorders and suspected infection but without CPA. However, CPA patients had significantly higher cytokine levels when compared to healthy individuals. Previous genetic association studies suggested that patients with CPA might produce lower levels of IL-10 and have ongoing or higher expression of IL-1b and IL-6 leading to a pro-inflammatory response and disease progression (Sambatakou et al., 2006; Smith et al., 2014). In our analysis IL-10 levels did not differ between CPA patients compared to patients with respiratory disorders without CPA (p = 0.541), but tended to be higher compared to healthy individuals (p = 0.072), where IL-10 levels were often below the detection limit. Levels of

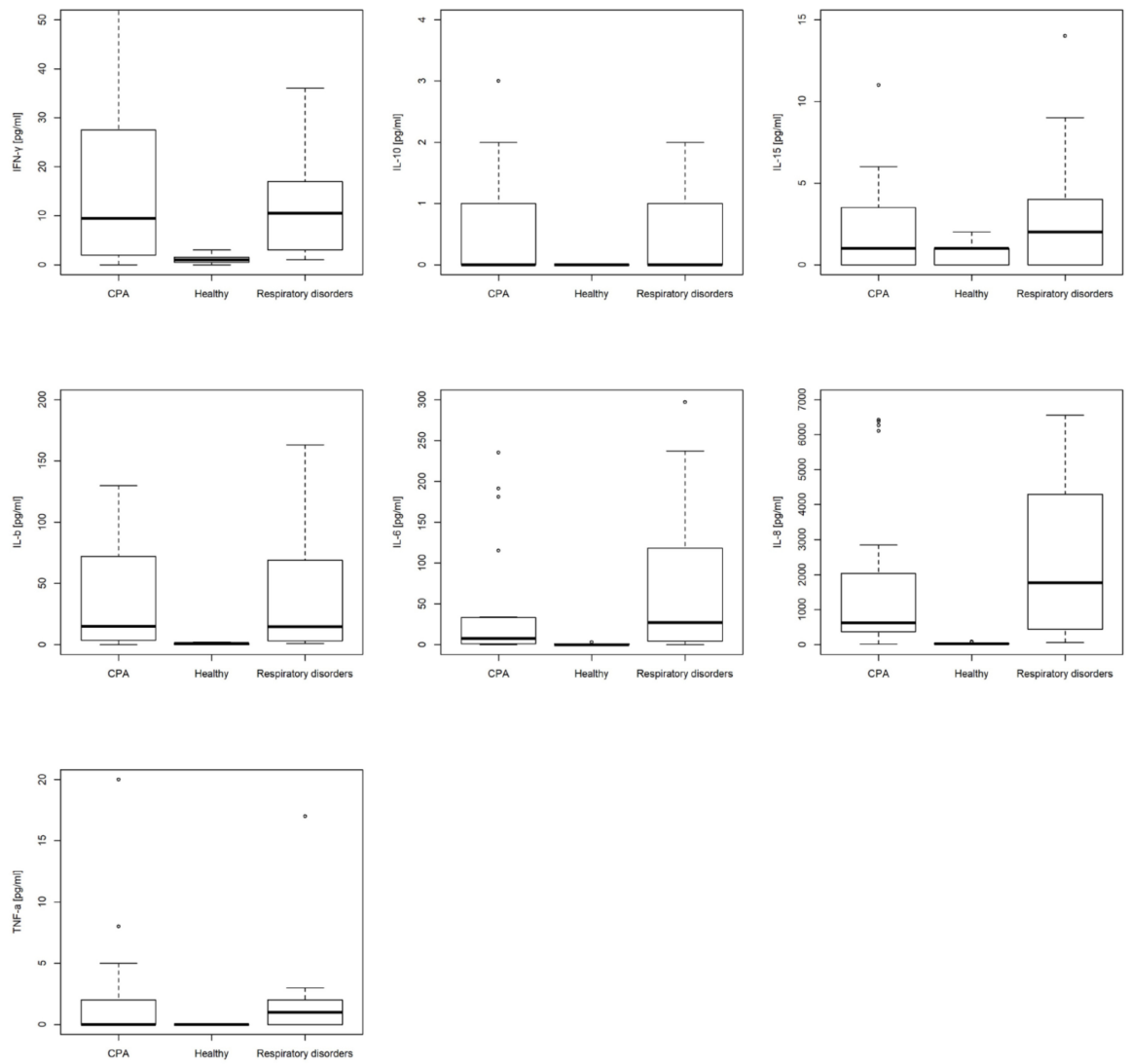

FIGURE 4 | Box plots of bronchoalveolar lavage fluid (BALF) interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-15, interferon-γ, and tumor necrosis factor (TNF)-α in patients with chronic pulmonary aspergillosis (CPA), patients with respiratory disorders without CPA and healthy individuals.

IL-1b, IL-6, IL-8, TNF-α, and IFN-γ were significantly higher in CPA patients compared to healthy individuals ($p < 0.001$ and $p = 0.008$, respectively), but not different than in patients with respiratory disorders without CPA. This stands in contrast with previous suggestions that CPA might be associated with low levels of IFN-γ (Doffinger D, AAA2014 Abstract) and that TNF-α might be linked with aspergillosis and/or chronic cavitary pulmonary asergillosis (Sambatakou et al., 2006).

This analysis has several limitations including the small group size, however, at least in GM a power of over 0.8 was found during the subsequent power analysis. With regard to the LFD, no statement can be made about significant differences or test performance, as only three LFD tests were positive in total. Furthermore, some biomarkers have a very wide range; the mean values are partly outside the third quartile. Box plots showed that, regardless of extreme values and outliers, the values are systematically unequal (or equal) distributed across the individual groups. Furthermore, non-parametric tests were used to minimize distortions due to non-normal distribution and variance heterogeneity. Another limitation is the lack of a reference standard for the diagnosis of patients with CPA (e.g., growth in transbronchial biopsies/histopathology showing fungal elements). Although accepted by ESCMID, ERS, and ECMM the clinical definition of CPA is ambiguous and may be incorrect in a certain number of patients.

CONCLUSION

Both GM and LFD showed insufficient performance for diagnosing CPA, with sensitivities of BALF GM below 50%, and

sensitivity of the LFD below 10%. The high specificities may, however, result in a high positive predictive value and thereby help to identify semi-invasive or invasive disease. This has direct clinical implications, because CPA patients with SAIA have a more rapid disease progression and should be managed like patients with IPA. Any diagnostic assay to proof mycological evidence needs to be interpreted in the clinical and radiological context as recommended by current guidelines.

AUTHOR CONTRIBUTIONS

HS, JP, and MH contributed to the scientific literature search, study design, data collection, data analysis, and drafting the manuscript. HF, JH, BK, SO, KG, CH, and CL were involved in the material collection and data analyses. All authors were involved in revision of the paper and final approval of the version to be published.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Fungi-Induced Upper and Lower Respiratory Tract Allergic Diseases: One Entity

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Introduction: *Aspergillus* can cause different allergic diseases including allergic bronchopulmonary aspergillosis (ABPA) and allergic fungal rhinosinusitis (AFRS). ABPA is allergic pulmonary disease against *Aspergillus* antigens. AFRS is a type of chronic rhinosinusitis (CRS) presented as hypersensitivity reactions to the fungal presence in sinuses. The aim of the present study was to clarify if ABPA and AFRS could be considered as a common disease entity.

Methodology: The prospective cohort study included 75 patients with ABPA. Patients were divided into two groups and compared with each other: (i) patients with CT confirmation of rhinosinusitis and presence of fungi in sinuses (ABPA+AFRS group) and (ii) patients without CT or without mycological evidence of AFRS (ABPA group).

Results: Findings of this study were: (i) AFRS was confirmed in 80% of patients with ABPA; (ii) all ABPA+AFRS patients had allergic mucin while fungal hyphae were present in 60% sinonasal aspirate; (iii) ABPA+AFRS patients had more often complicated CRS with (nasal polyps) NP ($p < 0.001$) and more severe forms of CRS; (iv) culture of sinonasal aspirate revealed fungal presence in 97% patients with ABPA+AFRS; (v) patients with ABPA+AFRS had more common positive skin prick test (SPT) for *A. fumigatus* ($p = 0.037$), while patients without AFRS had more common positive SPT for *Alternaria alternata* and *Penicillium notatum* ($p = 0.04$ and $p = 0.03$, respectively); (vi) 67% of ABPA patients had *Aspergillus* induced AFRS; (vii) larger number of fungi was isolated from the air-samples obtained from homes of patients with ABPA+AFRS than from the homes of patients without AFRS, while the most predominant species were *A. fumigatus* and *A. niger* isolated from almost 50% of the air-samples.

Conclusion: The pathogenesis of ABPA and AFRS is similar, and AFRS can be considered as the upper airway counterpart of ABPA. Fungi-induced upper and lower

respiratory tract allergic diseases present common entity. Next studies should clarify the mechanism by which fungi turn from "normal flora" into trigger of immunological reactions, resulting in ABPA or AFRS as well as to find new approaches for its' diagnosis and treatment.

Keywords: united airway *Aspergillus* disease, allergic bronchopulmonary aspergillosis, allergic *Aspergillus* sinusitis, asthma, chronic rhinosinusitis, respiratory tract

INTRODUCTION

The respiratory tract is continuously exposed to fungal spores present in the environment, and studies showed viable fungus present in the high rates in sinonasal mucus and bronchial sputum cultures, even in healthy subjects (Ponikau et al., 1999; Buzina et al., 2003). Fungal presence is commonly considered as colonization, but it may be an important extrinsic trigger for upper and lower airway allergic diseases especially in patients with asthma, chronic rhinosinusitis (CRS), cystic fibrosis and allergic rhinitis (Kumamoto, 2016). Understanding of fungi-induced allergic airway diseases is complicated by the enormous biodiversity of the fungi, problems with defining major allergens, unclear pathogenesis, and the role of fungi as allergens. Microbial communities, especially fungi, interact with environment, and host inflammatory response could cause or mediate the inflammatory process of upper and lower airway disease (Huffnagle and Noverr, 2013).

One of the most common pathogenic fungi causing upper and lower airway disorders are *Aspergillus* species (Chowdhary et al., 2016; Rick et al., 2016). *Aspergillus* can be found throughout the world. Its spores are ubiquitous and present almost everywhere in the human environment, outdoor and indoor (Woolnough et al., 2015; Chowdhary et al., 2016). In a recent study with patients with CRS, fungal presence in sinuses was identified in 63% patients, while *Aspergillus* was the most predominant fungal genus (Zhao et al., 2017).

Given the ubiquitous nature of fungi, exposure is unavoidable, however, it is not yet known how fungi turn from colonization into triggers of inflammatory reactions, resulting in allergic bronchopulmonary aspergillosis (ABPA) and allergic fungal rhinosinusitis (AFRS) (Lackner et al., 2005). AFRS and ABPA have many similarities: increased levels of serum IgE, and *Aspergillus*-specific IgE and IgG antibodies (Ab), similar immunopathology, and treatment (Chakrabarti et al., 2009; Shin et al., 2015; Barac et al., 2018). Bronchial mucus present in ABPA patients and allergic mucin present in AFRS patients are histologically identical and in some cases contain fungal hyphae, while the culture reveal the presence of different fungal taxa, mostly *Aspergillus* sp. (Chakrabarti et al., 2009; Barac et al., 2015; Kale et al., 2015). Our hypothesis is that fungal infections of upper and lower respiratory tract represent one disease, "united fungal airway disease," as they both depend on the same multiple factors such as exposure levels, anatomy, mucociliary clearance, mucosal health, and host immune factors (Ryan and Clark, 2015).

Literature review identified several review articles on related topic (Ryan and Clark, 2015; Kim, 2016; Rick et al., 2016; Agarwal et al., 2017) but there is a lack of comprehensive prospective study examining paired upper and lower airway mycobiota, clinical and other relevant findings in individuals with AFRS and ABPA. Based on these grounds, we performed a prospective cohort study in patients with upper and lower airway fungi-induced allergic diseases with the aim to further examine the "united airway fungal disease" hypothesis.

METHODOLOGY

Study Population

This prospective cohort study was conducted at the Clinical Centre of Serbia, Faculty of Medicine, University of Belgrade, from 1st February to 1st December 2016. The study was approved by the Ethical Committee of Clinical Centre of Serbia (5030/5) and the Ethical committee of Faculty of Medicine University of Belgrade (29/VI-3). Informed consent has been obtained from all study patients.

Inclusion Criteria

The study included consecutive patients with ABPA presented to ENT department of our hospital. A diagnosis of ABPA was made when at least six of the primary diagnostic Rosenberg-Patterson criteria were fulfilled (Rosenberg et al., 1977; Ishiguro et al., 2016). Diagnoses of ABPA was established by consensus among clinicians, radiologists, and mycologists based on the diagnostic criteria, laboratory findings, or histologic findings obtained via bronchoscopic or thoracoscopic lung biopsy (Ishiguro et al., 2016).

Patients with already confirmed diagnosis of ABPA were included in the study if they fulfilled inclusion criteria: (i) >16 years; (ii) no treatment with systemic corticosteroids over last 7 days and local corticosteroids for 3 days before inclusion, and (iii) absence of invasive fungal infection (screened by serology testing of anti-*Aspergillus* and anti-*Candida* IgM and IgG Ab, as well as the concentration of galactomannan and mannan in patients' sera).

We aimed to reveal how many patients suffer with ABPA and concomitant AFRS. Therefore, once the diagnosis of ABPA was made, we performed further AFRS diagnostic workup including CT imaging and sinonasal aspirate analysis. We defined AFRS as a presence of the hyperattenuating signal density visualized by CT imaging ("double density" sign), presence of allergic mucus and fungi grew from the culture of sinonasal aspirate. Finally, patients were divided into two groups depending on the presence of AFRS: (i) group A (ABPA+AFRS patients)

and (ii) group B (patients with only ABPA but without AFRS).

Data Collection

The following data included: (i) collection of patient's demographics and history data including number of previous endoscopic surgery of sinuses, duration of CRS, duration of AFRS, previous use of local corticosteroids during 3 months, presence of other co-morbidities (asthma, cystic fibrosis, allergic rhinitis, CRS); (ii) examination for fungal allergy: total sera IgE Ab, absolute eosinophil count in blood and skin prick test on fungal allergens (SPT); (iii) anterior rhinoscopy; (iv) CT imaging of paranasal sinuses and thorax; (v) microbiological analysis of induced aspirate; and (vi) cultivation of air samples from patients' homes (bedrooms).

Total Serum IgE, Eosinophil Count and SPT

A concentration of total IgE Ab in serum was measured by enzyme-linked immunosorbent assay (ELISA; Euroimmun AG, Germany). Results were interpreted as follows: (i) negative (<100 kU/L), (ii) low positive (100–500 kU/l) and (iii) high positive (≥500 kU/l). The blood sample was taken before SPT and put into the tube containing ethylene-diamine-tetra-acetic acid (EDTA). Eosinophil counting was performed with Fuchs-Rosenthal counting chamber. Results <350 mm^3 were considered as negative. SPT was done for the most common fungal allergens: *A. fumigatus*, *Alternaria alternata,* and *Penicillium notatum*.

CT Imaging and Anterior Rhinoscopy

All patients underwent sinonasal and chest CT imaging. In addition, all patients were examined by ear, nose, and throat (ENT) specialist to confirm AFRS presence. The presence of nasal symptoms (nasal obstruction, nasal secretion, postnasal discharge, impaired or lost sense of smell, facial sense of pressure) for more than 12 weeks and nasal rhinoscopic/endoscopic finding including the presence of characteristic allergic mucin (thick and viscous, often brown and yellow in color) and unilateral or bilateral nasal polyps and soft-tissue opacification of nasal cavity/paranasal sinuses with the presence of serpiginous areas of high attenuation, suggest the diagnosis of AFRS.

Mycological Analyses

Before sampling induced sinonasal aspirate, nasal cavities were pre-treated with cotton swabs aimed to decrease the contamination possibility. Afterward, each patient did the inhalation by PARI-SINUS nebulizer with 5 ml hypertonic-NaCl solution for 10 min in the quadruped position (PARI, Starnberg, Germany) (**Figure 1**). Quadruped position facilitates drainage of the content from paranasal sinuses (Ford et al., 2011). After the inhalation, mucin was collected from the sinuses by aspiration in a supine position with head tilted backward at a 30° angle.

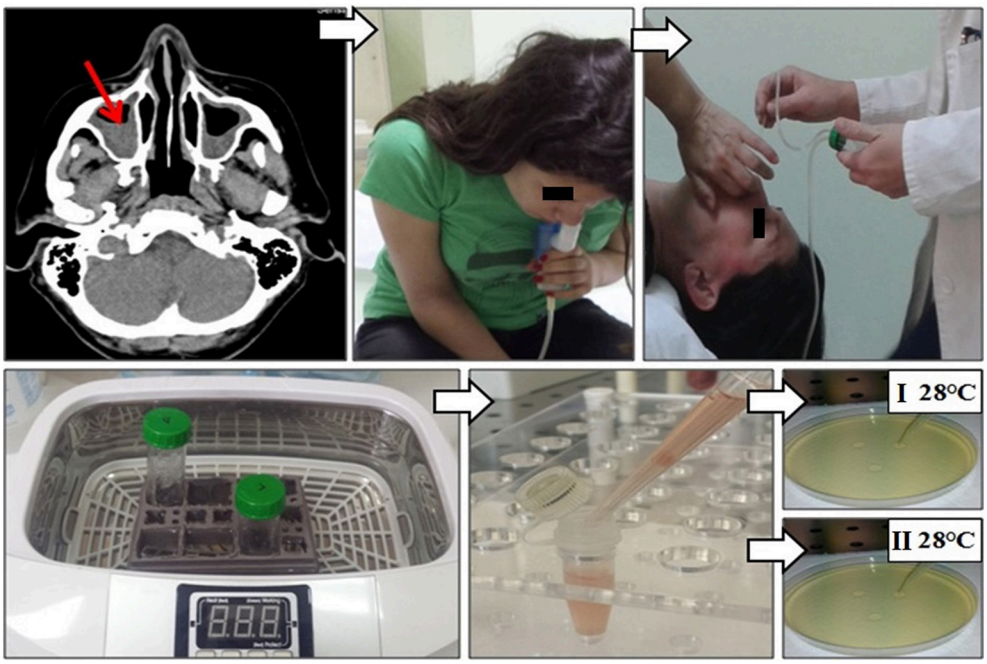

FIGURE 1 | Sinonasal aspirate processing algorithm: (i) CT imaging of paranasal sinuses of patients with ABPA revealed CRS; (ii) each patient did the inhalation by PARI-SINUS nebulizer with hypertonic-NaCl solution for 10 min in the quadruped position; (iii) after the inhalation mucin was collected from the sinuses by aspiration with mucus extractor in a supine position with head tilted backward; (iv) the sinonasal aspirate was additionally processed in ultrasound cleaner aimed to prepare single-cell solution, and (v) afterwards single-cell solution was cultured at Sabouraud dextrose agar at 28°C for 7 days. Fungi were regarded as causative when the same fungi was isolated from sinonasal aspirate repeatedly.

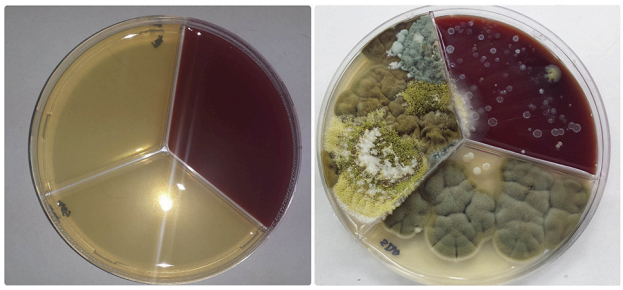

FIGURE 2 | Air-sampling protocol: Petri dish with Potato dextrose agar has been left for 1 h at room temperature in patients bedrooms.

Aspiration was done with mucus extractor (ULTRAMED, Asyut, Egypt), and the obtained sample was additionally processed in ultrasound cleaner (BlueWave Ultrasonic, Davenport, IA, USA). The same sampling procedure has been repeated twice within 7 days. Sabouraud dextrose agar was used for fungal culturing; plates were incubated at 28°C for 7 days. Identification of fungi was based on macroscopic and microscopic characteristics using standard mycological methods (McClenny, 2005). Fungi were regarded as causative if the same fungus was isolated from induced sinonasal aspirate from the first and repeated sinonasal aspirate (Lebowitz et al., 2002; Yeo and Wong, 2002).

Air Sampling

Patients were asked to leave open Petri dishes with potato dextrose agar for 1 h at room temperature in their bedrooms (**Figure 2**). Afterward, Petri dishes that contain air-samples were fixed with parafilm (Sigma-Aldrich, USA) and deposited at the laboratory for further analysis. Plates were incubated at 28°C for 7 days. Identification of fungi was based on macroscopic and microscopic characteristics using standard mycological methods (McClenny, 2005).

Statistical Analysis

Descriptive and inferential statistical analyses were used for evaluation of data using Statistical Package for Social Science (SPSS 17.0, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD) and counts or percentages, where appropriate. The distribution of the continuous variables was checked using the Kolmogorov–Smirnov test which did not show normal distribution. Thus, non-parametric testing was used. Mann–Whitney test was used for nonparametric data, and χ^2 test was used for categorical variables. All differences were considered significant at $p < 0.05$.

RESULTS

Clinical Characteristics and Sociodemographic Data of the Cohort

In total, 75 consecutive patients who signed the written consent and fulfilled the ABPA criteria were enrolled into the study. Mean age of 75 patients was 36 ± 11 (range, 16–50) with an almost equal sex distribution (M:F ratio = 1.1:1). In total, 77% had only asthma, while 23% had asthma and bronchiectasis. Chest CT revealed transient pulmonary infiltrates and central bronchiectasis in 17/75 (23%) patients. All patients have increased total IgE Ab, while 72% had positive SPT for *A. fumigatus*, and 34 and 28% had co-occurrence of positive SPT for *A. fumigatus* and *Alternaria alternata* or *A. fumigatus* and *Penicillium notatum*, respectively. In total, 28% patients were solely sensitized to *Alternaria* or *Penicillium*, and this group was classified as allergic bronchopulmonary mycosis (ABPM).

Clinical Characteristics and Sociodemographic Data of ABPA+AFRS Patients (Group A)

CRS was present in 82.7% (62/75) patients with ABPA. Mean duration of CRS was 12 ± 9 years (range 2–30 years). Complicated CRS with recalcitrant nasal polyposis (NP) and more than one previous endoscopic surgery of sinuses was found in 45.5% patients with ABPA. Rhinological score estimated by sinonasal outcome test (SNOT 22) for scoring of subjective symptoms revealed that 51.3% ABPA patients had severe symptoms CRS while CT imaging score revealed that 34.6% ABPA patients had severe CRS. In 60/75 (80%) ABPA patients CT of paranasal sinuses revealed mucosal thickening with hyperdense lesions, while fungi were isolated from sinonasal aspirate or allergic mucin from the sinuses of these patients, and they were characterized as ABPA+AFRS patients (group A). Mean age of patients from group B was 34 ± 9 (range 18–48) with predominance of male gender (M:F ratio = 1.6:1). Out of all ABPA+AFRS patients, 87% had only asthma, while 13% had asthma and bronchiectasis. Chest CT showed transient pulmonary infiltrates and central bronchiectasis in 9/60 (15%) patients. Sinonasal allergic mucin was seen in all ABPA+AFRS patients and fungal hyphae in 36/60 (60%) (**Figure 3**). All patients from group B had increased total IgE Ab and positive SPT for *A. fumigatus*, 14% had positive SPT for *Alternaria alternata* and 10% for *Penicillium notatum*.

Clinical Characteristics and Sociodemographic Data of Patients With ABPA Without AFRS (Group B)

Patients with ABPA but without AFRS belong to group B. Out of all patients from group B, 48% had only asthma, while 52% had asthma and bronchiectasis. Other relevant sociodemographic and clinical data of this group are presented in **Table 1**.

Relationship of Clinical Characteristics Between Group A and Group B

AFRS was confirmed in a significant proportion (80%). There was a difference in male gender between group A and B (61.7% vs. 20%, respectively; $p = 0.042$). Patients from group B had more common bronchiectasis comparing to group A (53% vs. 15%, respectively; $p = 0.001$). Patients from group A had more

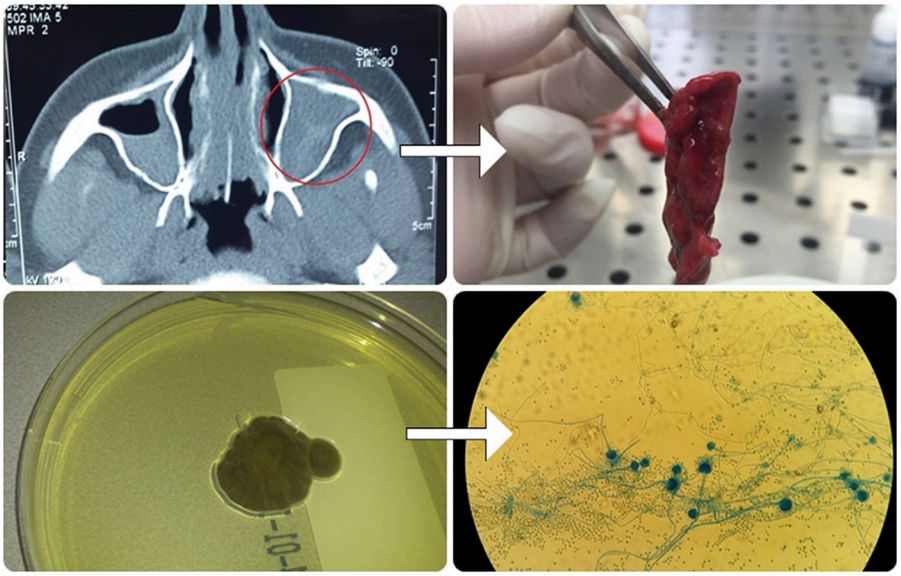

FIGURE 3 | Algorithm for fungal detection in the sinuses: CT imaging of paranasal sinuses of patients with ABPA revealed AFRS; Aspiration of allergic mucin was done, processed and used for mycological examination by microscopy and culturing.

often complicated CRS with NP ($p < 0.001$) and more severe forms of CRS with chronicity more than 10 years ($p < 0.001$), compared to group B. Allergic mucin was seen in all patients from group A, while it was not present in group B. Fungal hyphae in allergic mucin were seen in 60% of patients with AFRS. The culture of sinonasal aspirate revealed fungal presence in 97% patients with ABPA+AFRS, while there was no positive fungal growth in the sinonasal aspirate from the patients in group B.

All patients from both groups had increased total IgE Ab. Patients from group A had more common positive SPT for *A. fumigatus* ($p = 0.037$), while patients from group B had more common positive SPT for *Alternaria alternata* and *Penicillium notatum* ($p = 0.04$ and $p = 0.032$, respectively).

Distribution of Fungi Isolated From Sinonasal Aspirate of ABPA+AFRS Patients

Aspergillus sp. was the most common isolate from sinonasal aspirate found in 80% patients with AFRS, and this group is classified as *Aspergillus* induced AFRS. *A. fumigatus* was isolated in 50% (30/60) cases, followed by *A. flavus* in 17% cases (10/60); *A. niger* in 13% (8/60) cases; *Penicillium* sp. (10%; 6/60); *Cladosporium* sp. (5%; 5/60) and *Alternaria alternata* (5%; 5/60) (**Figure 4**).

Biodiversity of Fungi in Air Samples Obtained From Homes of Patients With ABPA

Out of all fungal isolates ($n = 178$) from the air-samples obtained from the homes of patients with ABPA (one air sample per patient), 114 isolates were from the air-samples of group A, while 64 were isolated from air-samples of group B (**Figure 5**). The most predominant species in group A was *A. fumigatus* and *A. niger* isolated from 47% air-samples, followed by *Penicillium* sp. isolated from 18% air-samples and *Alternaria alternata* isolated from 15% air-samples (**Figure 5**). Macroscopic picture of fungal diversity in air-samples is present (**Figure 6**).

DISCUSSION

Our prospective cohort study revealed that the presence of AFRS in patients with ABPA should not be missed: (i) AFRS was confirmed in 80% of patients with ABPA; (ii) sinonasal allergic mucin was seen in all ABPA+AFRS patients and fungal hyphae in 60%; (iii) ABPA+AFRS patients had more often complicated CRS with NP ($p < 0.001$) and more severe forms of CRS with chronic durations of more than 10 years ($p < 0.001$), comparing to patients without AFRS; (iv) culture of sinonasal aspirate revealed fungal presence in 97% patients with ABPA+AFRS, while there was no positive fungal growth in the sinonasal aspirates of patients without AFRS; (v) patients with ABPA+AFRS had more common positive SPT for *A. fumigatus* ($p = 0.037$), while patients without AFRS had more common positive SPT for *Alternaria alternata* and *Penicillium notatum* ($p = 0.04$ and $p = 0.032$, respectively); (vi) 80% patients with ABPA and AFRS were classified as *Aspergillus* induced AFRS, as *Aspergillus* sp. was isolated from sinonasal aspirates of these patients (67% of all ABPA patients); (vii) larger number of fungi was isolated from the air-samples obtained from the homes of patients with ABPA+AFRS than from the homes of the patients

TABLE 1 | Relationship of sociodemographic data and clinical characteristics between patients with ABPA and AFRS and patients with ABPA only.

Variables	Group n (%)**			*p*
	All patients *n* = 75 (100%)	**Group A** *n* = 60 (80%)	**Group B** *n* = 15 (20%)	
Sex				*0.042
M	40 (53.3%)	37 (61.7%)	3 (20)	
Age (mean ± SD)				0.247
	36.12 ± 11.21	34.54 ± 9.14	37.18 ± 11.81	
Chest CT				*0.005
Pulmonary infiltrates and central bronchiectasis	17 (23.3)	9 (15.1)	8 (53.3)	
Comorbidities				*0.007
Asthma	58 (77.1)	52 (86.7)	7 (48)	
Asthma+bronchiectasis	17 (22.9)	8 (13.3)	8 (52)	
CRS				* < 0.001
Yes	62 (82.7)	60 (100)	2 (13.3)	
Duration of CRS (years)				* < 0.001
<5	18 (29.1)	16 (26.6)	2 (100)	
5–10	11 (17.7)	11 (18.4)	0 (0)	
10–20	9 (14.5)	9 (15)	0 (0)	
>20	24 (38.7)	24 (40)	0 (0)	
Duration of CRS (years) (mean ± SD)				0.427
	12.88 ± 9.5	14.24 ± 10.7	13.78 ± 9.81	
CRS with NP				*0.028
Yes	28 (37.3)	27 (45)	1 (6.7)	
Rhinological index for objective assessment of CRS				* < 0.001
Mild	20 (32.3)	18 (29.5)	1 (50)	
Moderate	10 (16.4)	12 (20.5)	1 (50)	
Severe	32 (51.3)	30 (50)	0 (0)	
CT index for objective assessment of CRS				* < 0.001
Mild	19 (31.5)	20 (33.8)	2 (100)	
Moderate	21 (33.9)	19 (32.8)	0 (0)	
Severe	22 (34.6)	21 (33.4)	0 (0)	
Fungal hyphae				* < 0.001
Yes	36 (48)	36 (60)	0 (0)	
SKIN PRICK TEST				
Aspergillus fumigatus	54 (72.3)	60 (100)	4 (26.7)	*0.004
Alternaria alternata	26 (34.4)	9 (14.2)	7 (46.7)	*0.032
Penicillium notatum	21 (28.1)	6 (10.6)	5 (33.3)	*0.038

*According to Chi-square test, $p < 0.05$; Abbreviations: CRS, chronic rhinosinusitis, CT, computerized tomography; NP, nasal polyposis; ABPA, allergic broncopulmonare aspergillosis.

**Group A (ABPA+AFRS patients); group B (patients with only ABPA but without AFRS).

without AFRS and the most predominant isolated species were *A. fumigatus* and *A. niger* found in almost 50% of the air -samples.

The prevalence of fungal airway's diseases, especially ABPA and AFRS, has been increasing every year last decades (Buzina et al., 2003; Huffnagle and Noverr, 2013). *Aspergillus* has ability to act as an antigen and to invade both, the lower and upper parts of respiratory tract, due to similarities between AFRS and ABPA (Mukherjee et al., 2015).

Although many studies tried to reveal the pathophysiology of ABPA and AFRS, it is still unclear. In patients with ABPA and AFRS, structural abnormalities in the airway epithelium, presence of sinonasal mucin and inappropriate clearance allows fungal growth (Chaudhary and Marr, 2011; Rick et al., 2016; Zhang et al., 2017). The presence of fungi increased neutrophils and eosinophils and IgE Ab (total and *Aspergillus*-specific) in these patients, causing chronic inflammation (Woolnough et al., 2015; Kumamoto, 2016). Formation of NP and bronchiectasis in AFRS and ABPA patients is consequence of chronic inflammation (Agarwal et al., 2017). Although ABPA and AFRS have many histopathological similarities, coexistence of both these clinical entities has not been reported often. Shah et al. revealed concomitant occurrence of ABPA and AFRS in only 7 of 95 patients by postoperative confirmation, while 38 had associated nasal symptoms and additional five asymptomatic patients had radiological evidence of sinusitis (Shah et al., 2001). Unfortunately, only nine patients signed written consent to undergoing surgical procedure for diagnosis. In other studies, ABPA with concurrent AFRS has also been rarely reported (Travis et al., 1991; Schubert and Goetz, 1998). However, in the present study, AFRS was confirmed in 80% ABPA patients, while *Aspergillus* induced AFRS was confirmed in 67% ABPA patients, by radiological and microbiological evidence. One reason for the high co-occurrence of ABPA and AFRS could be that all study patients were referred to an ENT-division due to sinonasal discharge. If all ABPA-patients and the ones without ENT-symptoms were screened, the rate would probably be lower. On the other side, the divergence in results could be explained by differences in the methodology used for fungal detection, isolation and identification. In the present study, we used new methodology for sampling and processing of sinonasal mucin and extraction of fungi from tick allergic mucin (**Figure 1**). In addition, this study was designed as prospective cohort study aimed to reveal the number of AFRS patients within ABPA group and relationship between clinical findings of these groups, in contrast to other studies that mostly were retrospective or review studies. In addition, in studies such as the one published by Shah et al. AFRS could not be ruled out in patients with sinusitis because some refused to undergo surgery, that is required for establishing diagnosis (Shah et al., 2001). It is supposed that the frequency of AFRS among patients with ABPA could be higher.

Tenacious secretions in bronchi of patients with asthma provide favorable environment for the fungal growth and subsequent release of antigenic material, which explain occurrence of ABPA in asthmatic patients (Woolnough et al., 2015; Rick et al., 2016). Similar chain of events may be responsible for AFRS onset (Woolnough et al., 2015). On the other side, the scenario could be reversed; the changes in the bronchial or sinus mucous and the secretory immune system exist in patients who develop ABPA and AFRS, favoring growth of fungi (Woolnough et al., 2015; Agarwal et al., 2017). In ABPA fungal diversity is increased, patients usually have poorly controlled asthma, recurrent pulmonary infection and bronchiectasis (Wieringa et al., 2001; Chishimba et al., 2015; Ryan and Clark,

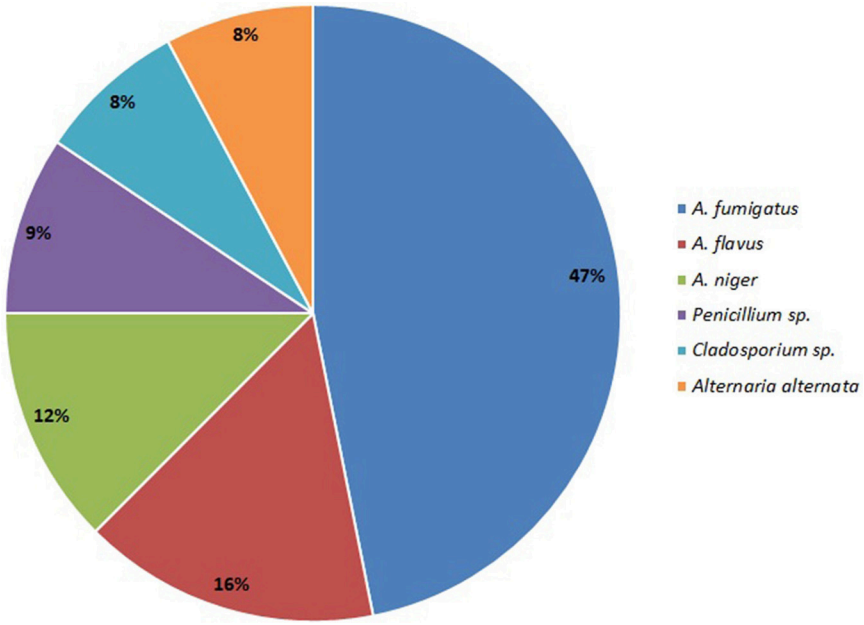

FIGURE 4 | Distribution of fungal species in sinonasal aspirate of patients from patients AFRS+AFRS.

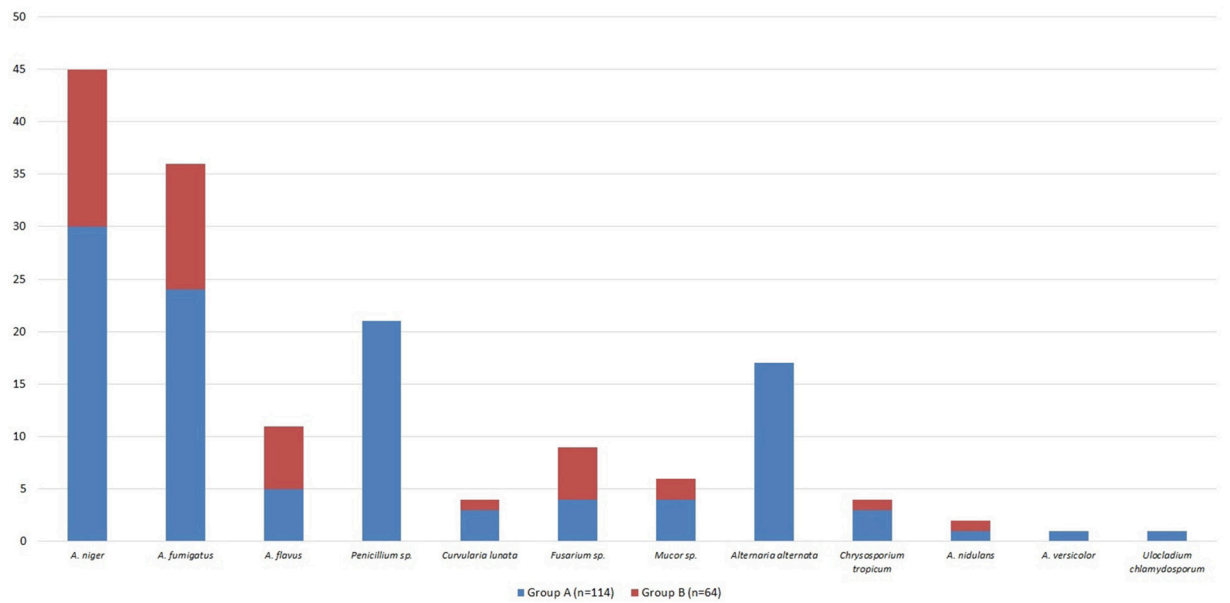

FIGURE 5 | Fungal biodiversity in environment of patients from group A and B.

2015). In the study of Chishimba et al. the relative abundance of *Aspergillus* increased approximately 15-fold in severe asthmatics compared to mild asthmatics (Chishimba et al., 2015). In our study, 77% patients with ABPA had only asthma, while 23% had asthma and bronchiectasis. Chest CT showed transient pulmonary infiltrates and central bronchiectasis in 23% patients. All patients have raised IgE Ab, while 72% had positive SPT for *A. fumigatus*. In the present study, 83% patients with ABPA had CRS, almost 50% had complicated forms with recurrent NP and severe subjective rhinological symptoms. In all our patients with ABPA and AFRS the chest symptoms preceded the nasal symptoms, but nasal symptoms were dominant, while in the patients without AFRS, presence of bronchiectasis was more frequent compared to patients with AFRS. Rhinological score for estimation of subjective symptoms revealed that 51.3% ABPA patients had severe nasal symptoms. Presence

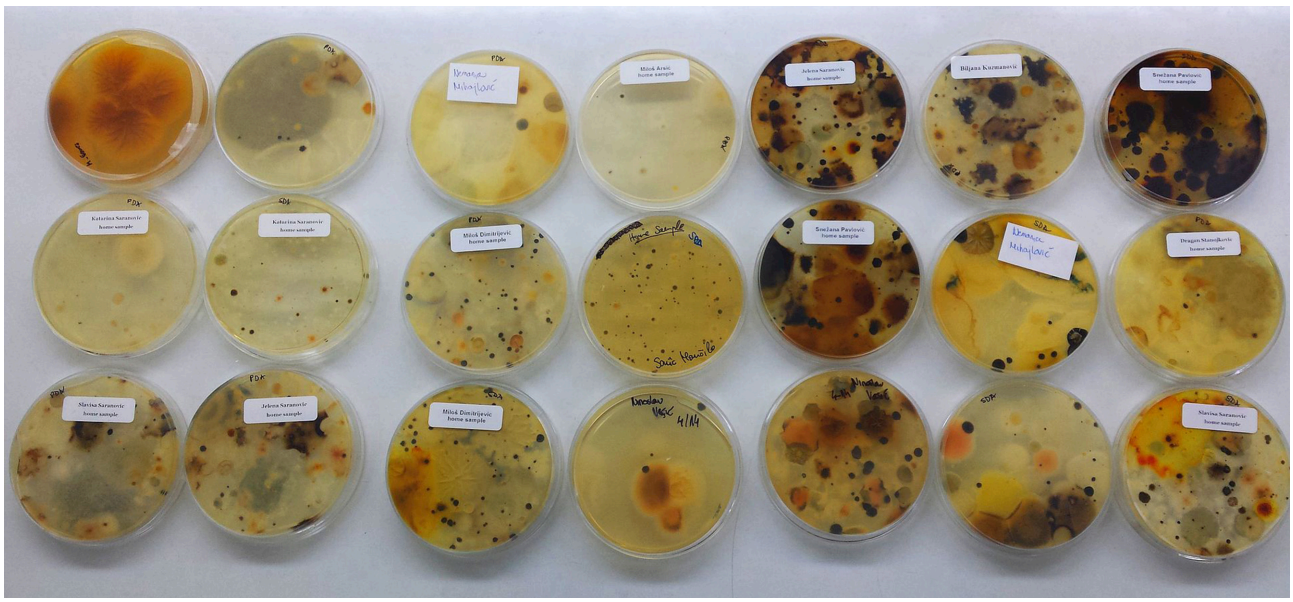

FIGURE 6 | Variety of fungi isolated from air-samples sampled from bedrooms of patients with ABPA+AFRS.

of sinonasal and sputum plugs is increased in patients with ABPA and AFRS (McCarthy and Pepys, 1971). McCarthy and Pepys, in their review of 111 patients with ABPA, reported the passage of sputum plugs in 54% and nasal plugs in 10% cases, but further investigation to confirm the diagnosis of AFRS has not been done (McCarthy and Pepys, 1971). Schubert and Goetz also reported passage of nasal and chest casts in 75% of their 67 patients with AFRS (Schubert and Goetz, 1998). Patient's observation that presence of both sinonasal and sputum secretion is increased should alert the physician to the possibility of coexistent AFRS and ABPA (McCarthy and Pepys, 1971; Schubert and Goetz, 1998). Future studies examining paired upper and lower airway mycobiota in individuals with CRS and asthma are desirable.

Although *Aspergillus* is the most common fungi isolated from respiratory tract of patients with ABPA or AFRS, some previous studies reported that patients with concomitant ABPA and AFRS were attributed to *Curvularia* spp. and *Bipolaris* spp. (Travis et al., 1991; Schubert and Goetz, 1998). These discrepancies could be explained by the cross-reactivity of allergens derived from common airborne fungi that could also play an important role in ABPA and AFRS patients (Kale et al., 2015; Chaaban et al., 2016). *Aspergillus* was the most common isolate from sinonasal aspirate in the present study, isolated from sinonasal aspirate of 80% patients with AFRS, and this group was classified as *Aspergillus* induced AFRS. *A. fumigatus* was isolated in 50% cases, followed by *A. flavus* (17%), *A. niger* (13%), and *Penicillium* sp. (10%). Air-samples from the houses of patients with AFRS and ABPA revealed that *Aspergillus* is the most common isolate, so this could be related to the frequent presence of *Aspergillus* in sinonasal aspirate. Considering various results of different studies, geographical differences in environmental and clinical fungal agents exist (Travis et al., 1991; Schubert and Goetz, 1998; Barac et al., 2015). Avoidance of places with fungal overload in air may help patients with ABPA or AFRS in the prevention of possible sharing of airborne fungal epitopes.

The drawback of the present study is that the molecular diagnosis of fungal isolates was not done, and consequently genotyping results were not available that should confirm if the same fungal strains were isolated form the patient's upper respiratory tract and air-samples. Air-sample fungal isolates were reported only as percentages, because the present study was focused on the clinical evaluation of diseases. The mycological study is started as continuation of the present clinical study, which is focused on a comparison of the strains isolated from sinonasal aspirates of the present cohort and air samples.

In conclusion, this first prospective study suggests that the co-occurrence of ABPA and AFRS is more common than previously expected. The pathogenesis of ABPA and AFRS share several common features, and AFRS can be considered as the upper airway counterpart of ABPA. Although these diseases are often treated by two different specialities, it seems that both are very much connected. This study confirms that the most common cause of these airway diseases is *Aspergillus*, and there are sufficient arguments to categorize this as united airway *Aspergillus* disease. Because *Aspergillus*-related upper and lower respiratory tract allergic diseases are inseparably connected, treating allergic *Aspergillus* lung diseases means also treating the nose, while treating patients with allergic *Aspergillus* sinonasal diseases has to be associated with a proper lung function evaluation, since the sinonasal and broncho-pulmonary systems should always be considered as a unique entity. Future studies should clarify the mechanisms

by which *Aspergillus* turn from "normal flora" into the trigger of immunological reactions, resulting in ABPA or AFRS as well as to find new approaches for its' diagnosis and treatment.

AUTHOR CONTRIBUTIONS

AB, SR and VT made substantial contributions to conception and design, and acquisition of data. PS and DO did a collection of data and statistical analyses. AP and LJ did an interpretation of data and final review of the paper. All authors contributed in writing the paper.

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Evaluation of Bronchoalveolar Lavage Fluid Cytokines as Biomarkers for Invasive Pulmonary Aspergillosis in At-Risk Patients

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Background: Invasive pulmonary aspergillosis (IPA) is an infection that primarily affects immunocompromised hosts, including hematological patients and stem-cell transplant recipients. The diagnosis of IPA remains challenging, making desirable the availability of new specific biomarkers. High-throughput methods now allow us to interrogate the immune system for multiple markers of inflammation with enhanced resolution.

Methods: To determine whether a signature of alveolar cytokines could be associated with the development of IPA and used as a diagnostic biomarker, we performed a nested case-control study involving 113 patients at-risk.

Results: Among the 32 analytes tested, IL-1β, IL-6, IL-8, IL-17A, IL-23, and TNFα were significantly increased among patients with IPA, defining two clusters able to accurately differentiate cases of infection from controls. Genetic variants previously reported to confer increased risk of IPA compromised the production of specific cytokines and impaired their discriminatory potential toward infection. Collectively, our data indicated that IL-8 was the best performing cytokine, with alveolar levels ≥904 pg/mL predicting IPA with elevated sensitivity (90%), specificity (73%), and negative predictive value (88%).

Conclusions: These findings highlight the existence of a specific profile of alveolar cytokines, with IL-8 being the dominant discriminator, which might be useful in supporting current diagnostic approaches for IPA.

Keywords: invasive pulmonary aspergillosis, biomarkers, cytokines/chemokines, bronchoalveolar lavage, fungal diagnostics

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is a life-threatening infection caused predominantly by the opportunistic fungus *Aspergillus fumigatus* (Segal, 2009). It is commonly diagnosed among patients with immunological deficits, namely those with hematologic malignancies during chemotherapy or undergoing solid organ or allogeneic hematopoietic cell transplantation (Kontoyiannis et al., 2010; Pagano et al., 2010; Pappas et al., 2010). There are currently no licensed vaccines, and despite

improved diagnosis and therapy, management of IPA remains challenging, with mortality rates of infected patients exceeding 30% (Bitar et al., 2014; Maertens et al., 2016). Given the variable risk of infection and its clinical outcome among patients with comparable predisposing clinical and microbiological factors, susceptibility to IPA is thought to rely largely on genetic predisposition (Cunha et al., 2013; Camargo and Husain, 2014).

Early diagnosis of IPA is critically required to decrease morbidity and mortality, particularly in vulnerable populations of immunocompromised patients, since the delayed initiation of antifungal therapy may contribute to fatal outcomes (Arvanitis et al., 2015). However, definitive diagnoses are challenging, typically because traditional diagnostic tools, such as histology and culture, are often difficult to obtain, with relatively low sensitivities, particularly in patients under antifungal prophylaxis. The incorporation of fungal surrogate markers, such as galactomannan, into clinical algorithms has facilitated diagnostic-driven strategies in at-risk patients (Morrissey et al., 2013). Notwithstanding, treatment of IPA remains mostly empirical based on a high index of suspicion, combined with non-specific clinical signs and symptoms, and radiological findings (Kozel and Wickes, 2014). This results in an excessive prescription of antifungal drugs, ultimately associated with a remarkable economic burden to the healthcare systems, highlighting therefore a pressing demand for new and improved diagnostic methods for IPA (Oliveira-Coelho et al., 2015).

Our current view of the pathogenesis of IPA identifies the concerted action of the ciliated epithelium and cells of the innate immune system, including resident alveolar macrophages and dendritic cells, and recruited inflammatory cells, as the first line of defense against inhaled fungal spores (Espinosa and Rivera, 2016). These cells express a large repertoire of immune receptors that sense pathogen motifs and drive the secretion of cytokines and chemokines that control innate and adaptive immune responses (Carvalho et al., 2012b). Because of their production in response to infection, specific cytokines and chemokines, such as interleukin (IL)-6, IL-8, and IL-10, have been reported at higher concentrations in the serum of patients suffering from IPA compared to controls (Chai et al., 2010a,b; Reikvam et al., 2012; Ceesay et al., 2016; Shen et al., 2016). Remarkably, IL-6 was also reported to discriminate cases of IPA from *Pneumocystis* pneumonia in pediatric oncology patients (Shen et al., 2016), thereby raising the appealing possibility for cytokine detection in differential fungal diagnostics. Besides their diagnostic value, certain cytokines have been endowed with prognostic significance. For example, persistently elevated levels of circulating IL-6 and IL-8 were identified as important early predictors of adverse outcomes in IPA (Chai et al., 2010a).

The genetic profile of the patient is regarded as a critical factor contributing to the risk of IPA (Cunha et al., 2013). Accordingly, variants in cytokine and chemokine genes impairing their expression or functional activity have been disclosed as major determinants of susceptibility to infection (Mezger et al., 2008; Wojtowicz et al., 2014; Cunha et al., 2017). Likewise, genetic variants affecting the β-glucan receptor dectin-1, and known to predispose to IPA, triggered a significant defect in cytokine production following experimental fungal infection (Cunha et al., 2010). Although the list of circulating cytokines and chemokines that correlate with the risk and clinical outcome of IPA continues to grow, a systematic profiling of alveolar cytokines during infection and the evaluation of their diagnostic significance has never been performed.

To determine whether alveolar cytokines were endowed with diagnostic potential in IPA, we measured 32 analytes in bronchoalveolar lavage (BAL) samples from a nested case-control study involving 113 patients at-risk of infection. We found that a subset of alveolar cytokines could significantly discriminate cases of infection from controls. In addition, we identified two distinct clusters of highly correlated cytokines that were differentially expressed between cases of IPA and controls. The diagnostic performance of individual or clustered cytokines was found to depend on the genetic background of the patient. Taken together, the results presented herein provide crucial insights into the pulmonary immune profile of patients with IPA and disclose individual and clustered cytokines that may serve as important diagnostic adjuvants in combination with classical diagnostic methods.

MATERIALS AND METHODS

Patients and Sample Collection

BAL and serum samples were collected from hospitalized adult patients (≥18 years of age) during routine diagnostic workup following suspicion of infection at the Leuven University Hospitals, Leuven, Belgium. The demographic and clinical characteristics of the patients enrolled are summarized in **Table 1**. Fifty-seven cases of "probable" or "proven" IPA were identified according to the revised standard criteria from the European Organization for Research and Treatment of Cancer/Mycology Study Group (EORTC/MSG) (De Pauw et al., 2008). The control group included patients with no evidence for the presence of *Aspergillus* spp. in the BAL (negative culture and galactomannan testing). Patients with "possible" disease were excluded from the study and no mold-active drugs were administered by the treating physician(s) before sample collection. This study was approved and carried out in accordance with recommendations of the Ethics Subcommittee for Life and Health Sciences of the University of Minho, Portugal, and the Ethics Committee of the University Hospitals of Leuven, Belgium. Written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

BAL Fluid Collection

BAL specimens were collected using a flexible fiberoptic bronchoscope following local anesthesia with 2% lidocaine (Xylocaine), when infection was clinically suspected. Samples were obtained by instillation of a pre-warmed 0.9% sterile saline solution (20 mL twice). The sampling area was determined based on the localization of lesion on chest imaging (X-ray or computed tomography scan). BAL specimens with comparable recovery rates were used. All samples were stored at −80°C until use.

TABLE 1 | Baseline characteristics of patients enrolled in the study.

Variables	IPA ($n = 57$)	No IPA ($n = 56$)	*P*-value
Age, no (%)			
≤50 years	9 (15.8)	14 (25.0)	0.25
>50 years	48 (84.2)	42 (75.0)	
Gender, no (%)			
Female	26 (45.6)	24 (42.9)	0.85
Male	31 (54.4)	32 (57.1)	
Underlying disease, no. (%)			
SOT[†]	12 (21.1)	20 (35.7)	0.13
Allogeneic SCT	10 (17.5)	12 (21.4)	
Acute leukemia	10 (17.5)	10 (17.9)	
Chronic lymphoproliferative diseases	10 (17.5)	7 (12.5)	
Influenza A (H1N1)	6 (10.5)	0 (0.0)	
Chronic lung diseases	4 (7.0)	1 (1.8)	
Solid tumors	2 (3.5)	1 (1.8)	
Other	3 (5.3)	5 (8.9)	
Neutrophil counts, $\times 10^3$ cells/μL (range)	5.6 (0.0–24.8)	5.2 (1.9–17.3)	0.40
GMI, mean (range)	5 (1.0–6.9)	0.2 (0.1–0.4)	<0.001
Other pathogens detected in BAL fluid			
Bacteria	8 (14.0)	8 (14.3)	0.42
Viruses	22 (38.6)	10 (17.9)	
Fungi[‡]	6 (10.5)	3 (5.4)	

SOT, solid organ transplantation; SCT, stem-cell transplantation; GMI, galactomannan index; BAL, bronchoalveolar lavage; P-values were calculated by Fisher's exact probability t-test or Student's t-test for continuous variables. [†]The study included 32 patients who received an SOT from lung (n = 27), kidney (n = 3), and liver (n = 2). Among those, 12 were diagnosed with IPA (lung, n = 8; kidney, n = 2; and liver, n = 2). [‡]Among fungi, Pneumocystis spp. was detected in five patients with IPA and three controls.

Galactomannan Testing

The Platelia *Aspergillus* EIA (Bio-Rad, Marnes-la-Coquette, France) was used during routine microbiological workup to detect the presence of galactomannan on uncentrifuged BAL specimens, as described (D'Haese et al., 2012). The enzyme immunoassay data was expressed as galactomannan index (GMI).

ELISA

Cytokines were quantified in BAL and serum samples using customized Human Premixed Multi-Analyte Kits (R&D Systems, MN, USA). All cytokine determinations were performed in duplicates, and concentrations were reported in pg/mL.

Single Nucleotide Polymorphism (SNP) Selection and Genotyping

SNPs were selected based on previously reported associations with increased risk of developing IPA (Cunha et al., 2010, 2014) that were independently validated (Chai et al., 2011; Fisher et al., 2017). Genomic DNA was isolated from whole blood using the QIAcube automated system (Qiagen, Hilden, Germany). Genotyping of rs2305619 in *PTX3* and rs16910526 in *CLEC7A* (dectin-1) was performed using KASPar assays (LGC Genomics, Hertfordshire, UK) in an Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, MA, USA), according to the manufacturer's instructions. Mean call rate for the SNPs was >98%. Quality control for the genotyping results was achieved with negative controls and randomly selected samples with known genotypes.

Statistical Analysis

Statistical analyses were performed using the Prism Version 7.0 (GraphPad Software) or R version 3.4.1. *P*-values < 0.05 were considered statistically significant, unless indicated otherwise. The concentrations for each cytokine in BAL and serum specimens were compared between cases of IPA and controls using either the Student's *t*-test or the non-parametric Mann-Whitney *U*-test, following the Shapiro-Wilk normality test. Cytokine levels were expressed as $\log_{10}$ pg/mL, and data were represented as mean ± SEM. Categorical variables were compared using the Fisher's exact test.

Random Forest Analysis (RFA) was used to rank cytokine levels in importance toward phenotype prediction. Calculations were carried out tree-by-tree as the random forest was constructed and were run >100 times to assess the robustness of ordering using the *randomForest* package for R. To identify sets of cytokines whose expression levels were correlated among patients, an unsupervised hierarchical clustering was applied using $\log_2$ transformed cytokine values. A heatmap was produced using the Morpheus platform (Broad Institute, MA, USA) using average linkage on a similarity matrix derived by Pearson moment correlations between patients (vertical clusters) or cytokines (horizontal clusters). Cytokine levels in clusters 1 (C1) and 2 (C2) for a given patient were combined by adding contributions based on the $\log_2$ transformed levels of the individual cytokines, as described (Yan et al., 2011). The median level for each cluster for all the patients was determined and patients were evaluated as to whether they were high ("Hi") or low ("Lo") for that cytokine cluster based on their individual value relative to the median.

The discriminatory ability of each cytokine was measured as the area under the receiving operating characteristic curve (AUC^{ROC}). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Youden index values were computed to evaluate the cytokine thresholds with the highest discriminatory power. The net reclassification index (NRI) was used to compare the performance of the identified cut-off levels of specific cytokines in BAL with other variables, as indicated.

RESULTS

Alveolar Immune Profiling Reveals a Subset of Cytokines Differentially Expressed in IPA

To gain insight into the alveolar cytokine profile associated with the development of IPA, we compared the levels of single cytokines in the BAL from patients diagnosed with IPA and

matched controls. BAL samples from 48 patients with IA and 48 matched controls were available and analyzed for cytokine levels. From the initial set of 32 analytes tested, we found that patients with IPA displayed significantly higher levels of IL-1β, IL-6, IL-8, IL-17A, IL-23, and TNFα after adjusting for multiple comparisons (**Figure 1A**; Table S1). Among the differentially expressed BAL cytokines, random forest analysis (RFA) revealed that IL-8, IL-6, and IL-23 best differentiated between cases of IPA and controls, whereas the remaining cytokines displayed an inconsistent contribution to discrimination (**Figure 1B**). Levels of IL-8 were the dominant discriminator, although the full set distinguished cases from controls. No differences were observed in the BAL cytokine levels according to the neutropenic status of the patients with IPA (Figure S1). Consistent with the BAL data, and despite the globally lower absolute cytokine concentrations detected in the serum, circulating IL-6, IL-8, IL-17A, and IL-23 were also significantly increased among patients with IPA (only IL-17A remained significant after adjustment for multiple comparisons) (**Figure 1C**; Table S2).

Two-Dimensional Cluster Analysis Defines Two BAL Cytokine Sets Related with IPA

Previous studies have shown that cytokines can be released in clusters, and this coordinated release may reflect common regulatory mechanisms (Ter Horst et al., 2016). Unsupervised hierarchal clustering analysis revealed that patients with IPA clustered separately from controls based on the levels of individual BAL cytokines (**Figure 2A**). In addition, two cytokine sets able to differentiate cases of IPA from controls were defined: cluster 1 (C1), including IL-6, IL-17A, IL-23, and TNFα, and cluster 2 (C2), which included IL-1β and IL-8. Further supporting these results, higher median levels of C1 and C2 cytokines in the BAL were more frequently observed in patients with IPA (37 $C1^{Hi}$ and 28 $C2^{Hi}$ patients out of 48) than controls (13 $C1^{Hi}$ and 9 $C2^{Hi}$ patients out of 48) (**Figure 2B**). The combined analysis of C1 and C2 clusters also revealed that higher levels of cytokines from both clusters were more common among patients with IPA compared to controls (24 $C1^{Hi}/C2^{Hi}$ patients out of 48) (**Figure 2C**).

Positivity for Galactomannan Influences the Levels of BAL Cytokines

The detection of galactomannan in BAL specimens has been advocated as a sensitive test for diagnosing IPA, particularly when interpreted in combination with clinical and radiological findings (D'Haese et al., 2012). To understand whether galactomannan in the BAL could influence the ability of cytokines to discriminate cases of IPA from controls, we analyzed individual and clustered cytokine data according to GMI: <0.5 (identifying controls), 0.5–2.0 (representing the most commonly used cut-off values for positivity), and >2.0. We found that patients with high, but not intermediate, GMI displayed significantly elevated concentrations of BAL cytokines compared to controls (**Figure 3A**). Importantly, only IL-6 and IL-8 retained their discriminatory potential for IPA in patients with intermediate

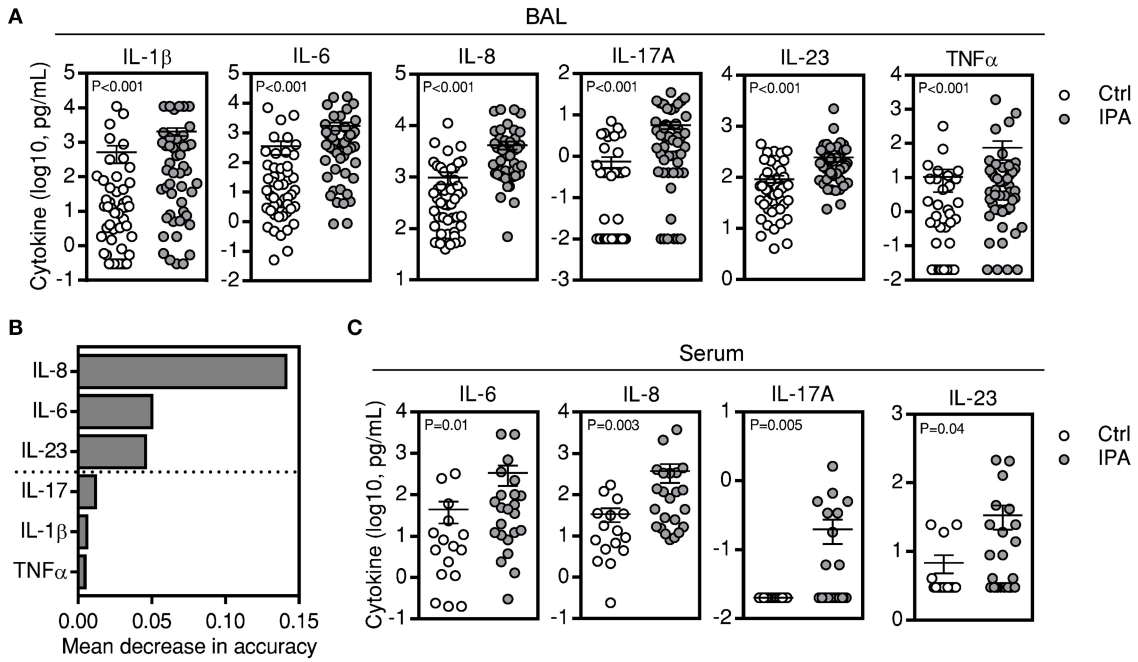

FIGURE 1 | A subset of BAL cytokines is differentially expressed in IPA. **(A)** Levels of cytokines present in the BAL of patients with IPA compared with controls (Ctrl). Data are presented as mean ± SEM values. **(B)** Ranking of cytokines by their relative importance in discriminating cases of IPA from controls using RFA. The horizontal axis represents the average decrease in classification accuracy, and bars indicate the relative importance of each individual cytokine to discrimination. The dashed line divides cytokines at the mean value of decrease in accuracy and defines the number of cytokines required for maximum classification accuracy. **(C)** Levels of cytokines present in the sera of patients with IPA compared with controls (Ctrl). Data are presented as mean ± SEM values.

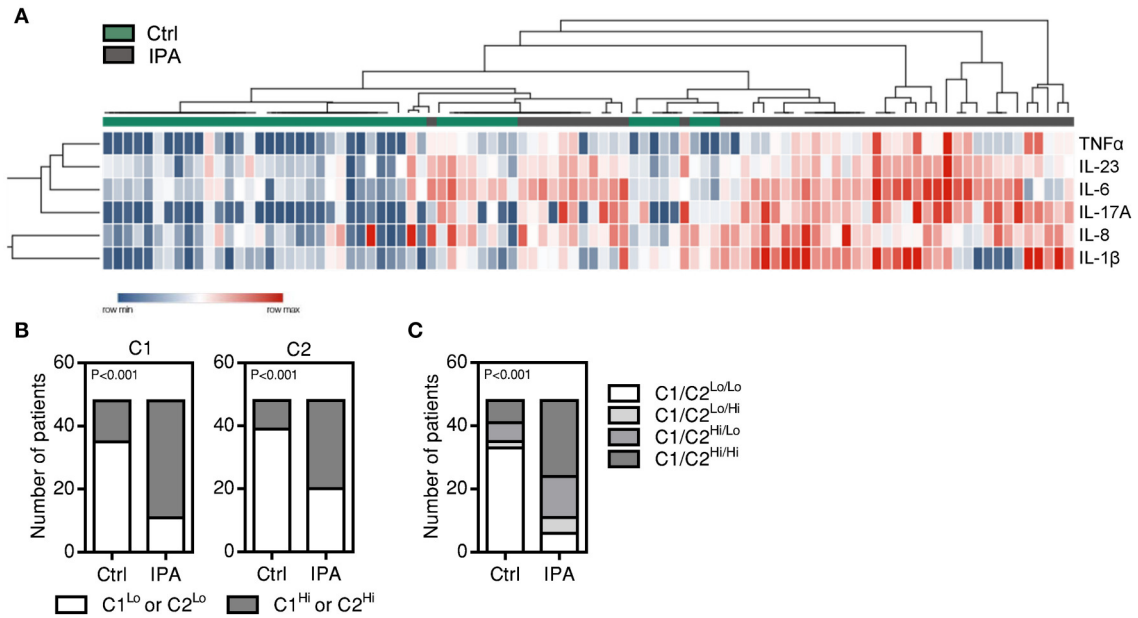

FIGURE 2 | Cluster analysis reveals two groups of highly expressed BAL cytokines in IPA. **(A)** Unsupervised hierarchical clustering for IL-1β, IL-6, IL-8, IL-17A, IL-23, and TNFα. Expression levels of individual cytokines are represented by shades of blue to red in the heatmap, with highest values in dark red and lowest values in dark blue. The top dendrogram illustrates the separate clustering of cases of IPA (indicated by gray boxes) from controls (Ctrl) (indicated by green boxes). The left dendrogram illustrates the identification of two cytokine clusters: C1 (TNFα, IL-23, IL-6, and IL-17A) and C2 (IL-8 and IL-1β). **(B)** Number of patients presenting low and high values of C1 ($C1^{Lo}$ and $C1^{Hi}$, respectively) and C2 cytokines ($C2^{Lo}$ and $C2^{Hi}$, respectively) among controls (Ctrl) and cases of IPA. **(C)** Number of patients with combined information on the levels of C1 and C2 cytokines among controls (Ctrl) and cases of IPA. Four categories are indicated: $C1^{Lo}/C2^{Lo}$, $C1^{Lo}/C2^{Hi}$, $C1^{Hi}/C2^{Lo}$, $C1^{Hi}/C2^{Hi}$.

GMI. In addition, by comparing the expression of cytokine clusters, we found that the C1 profile was not influenced by the GMI (**Figure 3B**). Instead, $C2^{Hi}$ patients displayed significantly higher mean values of GMI than $C2^{Lo}$ patients. Consistent with this, patients with a combined $C1^{Hi}/C2^{Hi}$ cytokine profile presented higher mean values of GMI than patients belonging to the $C1^{Lo}/C2^{Lo}$ category (5.7 vs. 4.0) (**Figure 3C**).

Genetic Variants Conferring Risk of IPA Impair the Discriminatory Ability of BAL Cytokines

A number of studies has disclosed an important contribution of host genetics in defining susceptibility to IPA (Cunha et al., 2013). However, with a few exceptions, their consequences to cytokine production remain elusive. We analyzed cytokine data according to the genotypes of two of the most robust genetic markers for IPA identified to date, rs2305619 in *PTX3* (Cunha et al., 2014, 2015; Wojtowicz et al., 2015) and rs16910526 in *CLEC7A* (Cunha et al., 2010; Chai et al., 2011). We found that patients harboring AA or AG (referred to as A+) genotypes at rs2305619 retained increased levels of BAL cytokines when compared to controls (**Figure 4A**). However, the production of IL-6 and IL-8 was significantly impaired by the presence of the high-risk GG genotype. Accordingly, cluster analysis revealed differences in the expression of cytokines between cases of IPA and controls with the A+, but not GG, genotypes (**Figure 4B**). Similar findings were observed for the rs16910526 variant in *CLEC7A*, with increased levels of BAL cytokines detected in patients carrying the TT genotype compared to controls (**Figure 4C**). Production of IL-1β and IL-23 was instead impaired among patients with IPA carrying the high-risk TG genotype. As expected, expression of clustered cytokines was different between cases and controls with the TT, but not TG, genotypes (**Figure 4D**). Similar results were obtained for combined cluster analysis (Figure S2). No differences were noted in cytokine levels among control subjects according to the *PTX3* or *CLEC7A* genotypes (data not shown).

BAL Cytokines Represent Potentially Useful Diagnostic Biomarkers for IPA

To corroborate the utility of BAL cytokines for the diagnosis of IPA, we analyzed the AUC^{ROC} for each cytokine (**Figure 5**; **Table 2**). As expected, all cytokines tested, except for TNFα, demonstrated reasonable sensitivity and specificity. IL-8 was the best performing analyte (AUC^{ROC} = 0.84; 95% CI, 0.75–0.91; P<0.001), with a cut-off level of IL-8 ≥904 pg/mL associated with 90% sensitivity, 73% specificity, 78% PPV, and 88% NPV. Total accuracy conferred by alveolar IL-8 was 82%: five patients with IPA presented values below the cut-off, whereas 13 control subjects displayed instead values above the cut-off.

Consistent with the results pointing to a detrimental role of specific risk genotypes for IPA regarding cytokine production, we observed that the high-risk GG genotype at rs2305619 in *PTX3* led to a decrease of 23% sensitivity for IL-8 and 29% specificity for

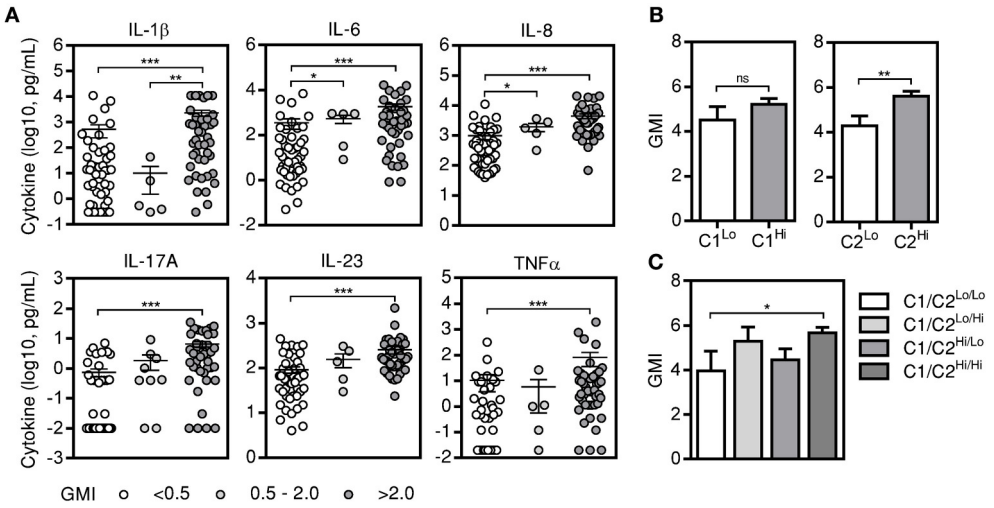

FIGURE 3 | BAL cytokine levels depend on galactomannan positivity. **(A)** Levels of alveolar cytokines in patients with negative (<0.5) and different ranges of GMI positivity (0.5–2.0 and >2.0). Data are presented as mean ± SEM values; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$. **(B)** GMI of patients displaying low and high values of C1 ($C1^{Lo}$ and $C1^{Hi}$, respectively) and C2 cytokines ($C2^{Lo}$ and $C2^{Hi}$, respectively). Data are presented as mean ± SEM; $^{**}p < 0.01$. **(C)** GMI of patients with combined information on the levels of C1 and C2 cytokines. Four categories are indicated: $C1^{Lo}/C2^{Lo}$, $C1^{Lo}/C2^{Hi}$, $C1^{Hi}/C2^{Lo}$, $C1^{Hi}/C2^{Hi}$. Data are presented as mean ± SEM; $^{*}p < 0.05$.

IL-6, hindering their performance in identifying cases (AUC^{ROC} = 0.67; 95% CI, 0.33–0.97; $P = 0.70$) and controls (AUC^{ROC} = 0.58; 95% CI, 0.22–0.89; $P = 0.80$), respectively (Table S3). Likewise, the high-risk TG genotype at rs2305619 in *CLEC7A* precluded the ability of IL-1β ($AUC^{ROC} = 0.63$; 95% CI, 0.37–0.89; $P = 0.51$) and IL-17A ($AUC^{ROC} = 0.69$; 95% CI, 0.41–0.94; $P = 0.21$) to identify controls by impairing specificity by 12 and 14%, respectively. Collectively, these results highlight IL-8 as the most relevant discriminator between cases of IPA and controls and highlight the genetic background of the patients as a critical factor to consider when evaluating the diagnostic performance of host-derived biomarkers.

DISCUSSION

The initiation of an efficient antifungal immune response depends on a complex set of signals circulating within the microenvironment, including cytokines and chemokines (Hohl, 2017). Understanding how each of these pathways is regulated is essential to uncover the molecular and cellular processes underlying the pathogenesis of IPA, and may also offer crucial insights toward the identification of molecules that correlate strongly with infection and the network of signals that could be therapeutically targeted.

In our exploratory study, we quantified the alveolar levels of 32 analytes in patients with IPA and matched controls, and analyzed these findings based on individual or analytically clustered sets of mediators. Our data provide strong evidence for an alveolar cytokine profile that is differentially expressed in patients with IPA. The observed inflammatory phenotype is largely attributable to IPA rather than to a general response to infection since events of viral, bacterial and even *Pneumocystis* pneumonia were diagnosed within the control group (**Table 1**). This is consistent with the recent finding that cytokine production is organized around the physiological response toward specific pathogens rather than through specific immune pathways (Li et al., 2016).

Prior studies of biomarker evaluation have mostly addressed candidate cytokines, reporting altered circulating levels to be correlated with the development of IPA (Camargo and Husain, 2014). In addition, the generation of specific cytokine-producing T-cells in response to *A. fumigatus* antigens have also been exploited as a potential immunodiagnostic approach (Potenza et al., 2013). Our study is among the first demonstrations that alveolar cytokines may also be relevant biomarkers for the diagnosis of IPA, regardless of the neutrophil counts at the time of diagnosis. In fact, we found that BAL cytokines performed better than the circulating counterpart, likely because they mirror more accurately the pathophysiology of IPA, with cytokines being produced mostly by immune cells at the site of infection rather than in the periphery (Kontoyiannis, 2011). Of note, and although galactomannan has been suggested to suppress cytokine responses (Chai et al., 2009), patients with high GMI displayed increased levels of alveolar cytokines than controls, demonstrating that the combination of relevant cytokines with fungal surrogate markers may improve our capacity to predict disease outcome.

Within the differential alveolar profile, we identified several inflammatory cytokines with well-known roles in antifungal immune responses, such as IL-1β, IL-6, IL-8, and TNFα (Becker et al., 2015). Among these, IL-8 was the best performing analyte with a total accuracy above 80%. These findings are supported by a recent report on a small patient cohort that also disclosed alveolar IL-8 as a potentially useful biomarker

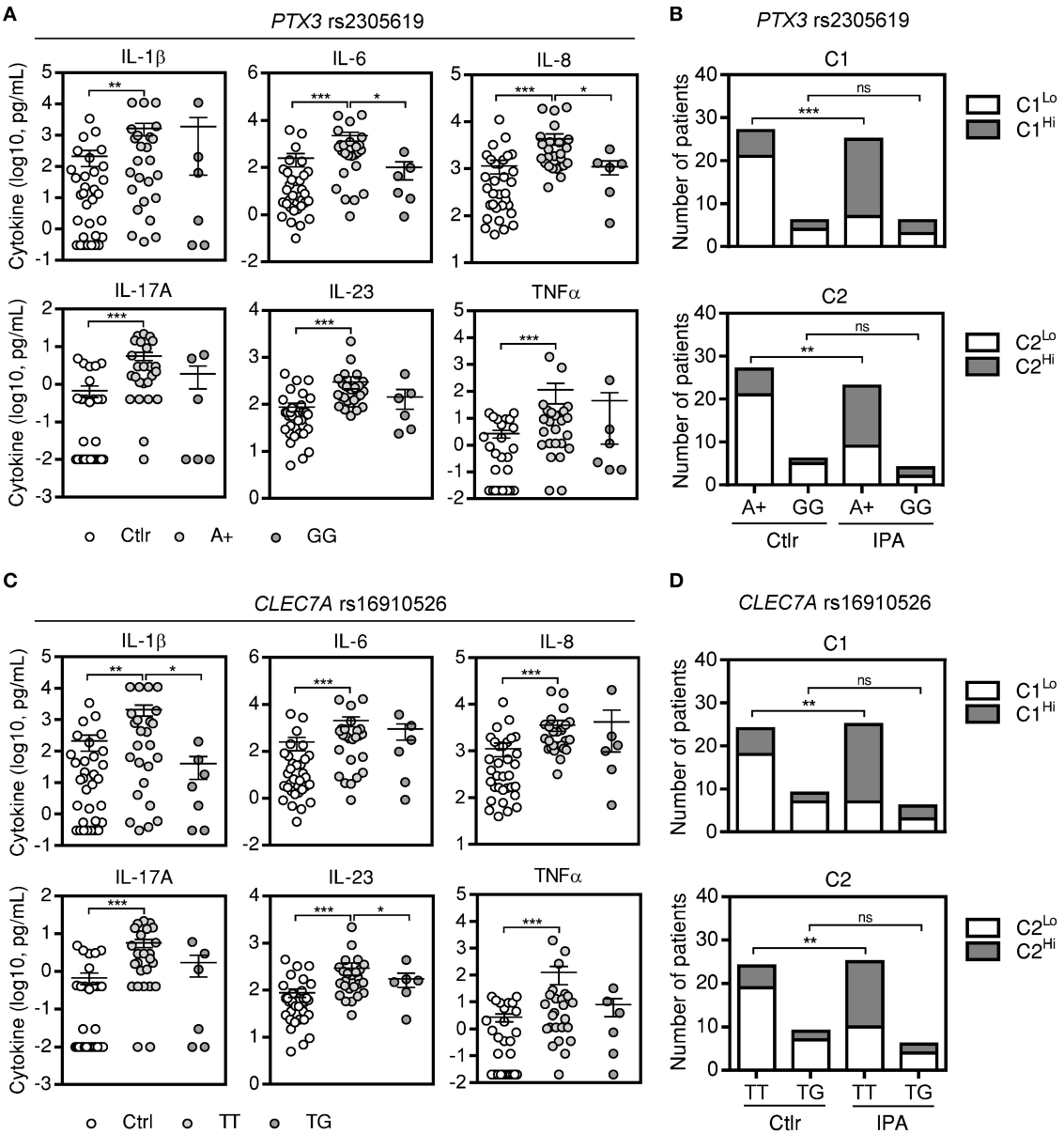

FIGURE 4 | Genetic variants conferring risk to IPA influence the levels of BAL cytokines. **(A)** Levels of alveolar cytokines in patients with IPA carrying different genotypes at rs2305619 in *PTX3*. A+ indicates combined AA and AG genotypes. Data are presented as mean ± SEM values; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. **(B)** Distribution of low and high values of C1 and C2 in IPA and controls (Ctrl) in the presence of different genotypes at rs2305619 in *PTX3*. Vertical axis represents the number of patients with low or high values of C1 and C2 while horizontal axis represents the different genotypes; $^{***}p < 0.001$, $^{**}p < 0.01$. **(C)** Levels of alveolar cytokines in patients with IPA carrying different genotypes at rs16910526 in *CLEC7A*. Data are presented as mean ± SEM values; $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. **(D)** Distribution of low and high values of C1 and C2 in IPA and controls (Ctrl) in the presence of different genotypes at rs16910526 in *CLEC7A*. Vertical axis represents the number of patients with low or high values of C1 and C2 while horizontal axis represents the different genotypes; $^{**}p < 0.01$.

for IPA (Heldt et al., 2017). This is in accordance with its role as a central regulatory cytokine produced mainly by alveolar macrophages and epithelial cells early after infection to coordinate the recruitment of inflammatory cells (Balloy et al., 2008). Interestingly, and despite previous reports pointed to a minor contribution of T helper (Th)17 responses to *A. fumigatus* in cellular models of infection (Chai et al., 2010b), IL-17A and IL-23 in the BAL were also upregulated, supporting a role for the Th17 pathway during IPA (Zelante et al., 2007).

The risk of IPA and its clinical outcome vary significantly even among patients with similar predisposing clinical conditions and microbiological exposure (Cunha et al., 2013). Although a number of genetic variants in cytokine genes have also been associated with the development of IPA (Loeffler et al., 2010), rs2305619 in *PTX3* and rs16910526 in *CLEC7A* have been

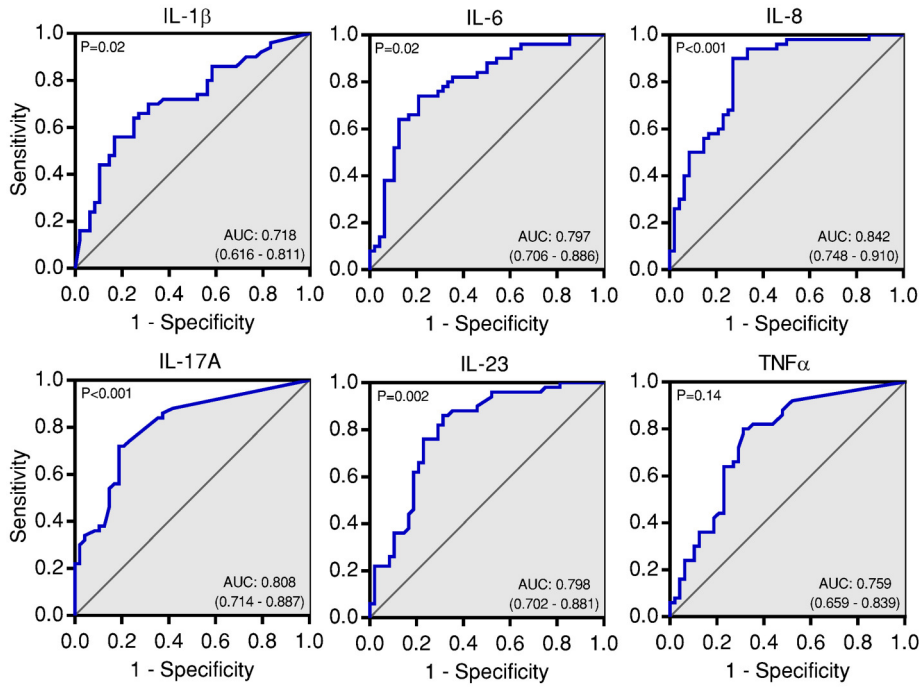

FIGURE 5 | BAL cytokines accurately predict the development of IPA. Area under the receiver operating characteristic curve (AUC^{ROC}) analysis for each BAL cytokine demonstrating sensitivity as a function of one-specificity for the prediction of IPA.

TABLE 2 | Performance of BAL cytokines as diagnostic biomarkers for IPA.

Cytokine	Cut-off†	Sensitivity	Specificity	PPV	NPV	NRI
		(95% CI)				
IL-1β	27.1	70 (55–83)	68 (55–81)	70 (60–79)	69 (58–78)	0.34
IL-6	89.8	74 (63–85)	79 (68–89)	78 (67–87)	73 (63–81)	0.51
IL-8	904	90 (81–98)	73 (60–85)	78 (68–85)	88 (75–94)	0.63
IL-17A	0.66	72 (58–84)	81 (70–90)	80 (68–88)	74 (64–82)	0.53
IL-23	103	76 (66–90)	77 (67–90)	78 (67–86)	76 (65–84)	0.53
TNF-α	0.94	80 (70–90)	69 (55–81)	73 (63–81)	77 (65–86)	0.49

†*Cut-off values of cytokines are expressed as pg/mL. Statistically-derived optimal cut-off was determined by Youden's index (maximum sensitivity and specificity given by the inflection point of the* AUC^{ROC}*). The net reclassification index (NRI) was used to compare the performance of each cytokine cut-off with the known diagnosis of IPA. IL, interleukin; TNF, tumor necrosis factor; PPV, positive predictive value; NPV, negative predictive value.*

recently proposed as the most robust genetic markers identified to date (Fisher et al., 2017). PTX3 is an important fluid-phase pattern recognition molecule with ancestral antibody-like properties that recognizes and interacts with *A. fumigatus* to exert critical roles in antifungal innate immunity (Garlanda et al., 2002). Remarkably, IL-6 and IL-8 were influenced by the presence of the risk genotype at rs2305619, known to impair the expression of PTX3 in the lung (Cunha et al., 2014). This may suggest that, similar to other opsonins such as L-ficolin (Bidula et al., 2015), PTX3-mediated conidia opsonization is critically required to potentiate IL-8 secretion in the lung. Whatever the mechanism(s), the functional crosstalk between molecules with opsonic activity and other inflammatory mediators in antifungal immunity remains to be thoroughly explored. In any case, it is not surprising that alveolar, but not serum, PTX3 has been disclosed as a valuable early marker for microbiologically-confirmed pneumonia (Mauri et al., 2014).

The levels of IL-1β and IL-17A were instead preferentially impacted by the genetic deficiency of dectin-1 (Cunha et al., 2010), highlighting likely different mechanisms through which these variants confer risk to IPA. The production of IL-1β, a critical regulator of early Th17 differentiation (Chung et al., 2009), has been shown to depend on dectin-1 activation (Karki et al., 2015). In turn, dectin-1-mediated signals were reported to affect adaptive immunity to *A. fumigatus* by restraining Th1 responses and enabling Th17 differentiation (Rivera et al., 2011). Taken together, these results confirm the importance of Th17 responses during IPA and highlight the detrimental consequences of genetic deficiency of dectin-1 to their activation.

Despite its exploratory and unbiased nature, our nested case-control study presents however certain limitations. The most relevant regard the inability to conclude about the added benefit of cytokine measurements compared to galactomannan testing or fungal PCR, the definition of cytokine levels as the cause or consequence of the infectious process, and the heterogeneity of the study population regarding the underlying conditions. Finally, the number of patients carrying risk-associated genetic variants do not allow to definitively estimate their effect size on cytokine levels. However, it also raises interesting questions that warrant further investigation. For example, functional studies are required to understand the interrelationships

among the identified cytokines and the protective vs. pathophysiological mechanisms underlying IPA, and to identify therapeutic targets amenable to immunomodulation (Carvalho et al., 2012a). In conclusion, our results provide support to additional, well-controlled studies in larger cohorts evaluating whether the diagnostic potential of cytokines, particularly in combination with fungal surrogate markers and integrating the genetic risk profile of the patient, holds clinical value.

AUTHOR CONTRIBUTIONS

SG, LA-F, CC, and AC designed the study; KL and JM oversaw patient recruitment and collection of clinical specimens and data; SG, CR, CFC, and LB-M performed the laboratory assays; SG, FR, RS, CC, and AC performed the statistical analysis and/or interpreted the data; all authors critically revised and approved the manuscript and are accountable for the accuracy and integrity of the work.

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Production of the Invasive Aspergillosis Biomarker Bis(methylthio)gliotoxin Within the Genus *Aspergillus: In Vitro and in Vivo* Metabolite Quantification and Genomic Analysis

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Gliotoxin (GT) is a fungal secondary metabolite that has attracted great interest due to its high biological activity since it was discovered by the 1930s. An inactive derivative of this molecule, bis(methylthio)gliotoxin (bmGT), has been proposed as an invasive aspergillosis (IA) biomarker. Nevertheless, studies regarding bmGT production among common opportunistic fungi, including the *Aspergillus* genus, are scarce and sometimes discordant. As previously reported, bmGT is produced from GT by a methyl-transferase, named as GtmA, as a negative feedback regulatory system of GT production. In order to analyze the potential of bmGT detection to enable identification of infections caused by different members of the *Aspergillus* genus we have assessed bmGT production within the genus *Aspergillus*, including *A, fumigatus*, *A. niger*, *A. nidulans*, and *A. flavus*, and its correlation with *gtmA* presence. In order to validate the relevance of our *in vitro* findings, we compared bmGT during *in vitro* culture with the presence of bmGT in sera of patients from whom the *Aspergillus* spp. were isolated. Our results indicate that most *A. fumigatus* isolates produce GT and bmGT both *in vitro* and *in vivo*. In contrast, *A. niger* and *A. nidulans* were not able to produce GT or bmGT, although *A. niger* produced bmGT from a exogenous GT source. The frequency and amount of bmGT production in *A. terreus* and *A. flavus* isolates *in vitro* was lower than in *A. fumigatus*. Our results suggest that this defect could be related to the *in vitro* culture conditions, since isolates that did not produce bmGT *in vitro* were able to synthetize it *in vivo*. In summary, our study indicates that bmGT could be very useful to specifically detect the presence of *A. fumigatus*, the most prevalent agent causing IA. Concerning *A. terreus* and *A. flavus* a higher number of analyses from sera from infected patients will be required to reach a useful conclusion.

Keywords: bis(methylthio)gliotoxin, *Aspergillus* spp., *gtmA*, invasive aspergillosis, biomarker

INTRODUCTION

More than 20 years ago the first invasive aspergillosis (IA) biomarker, galactomannan (GM), was developed based on an enzyme linked immunosorbent assay (Stynen et al., 1995). It stirred up the diagnosis of this lethal infectious disease, as it allowed to detect the infection when combined with clinical signs and symptoms (Maertens et al., 1999, 2002). During the last few years, the biomarker weaponry has arisen with the development of a system to detect β-D-glucan, *Aspergillus* PCR and lateral flow device to detect an *Aspergillus*-derived protein among others (Odabasi et al., 2004; Thornton, 2008; White et al., 2015). New diagnostic approaches were developed based on the increased accuracy of these tests, such as pre-emptive therapy (Wingard, 2007; Riwes and Wingard, 2012). Despite of these advances, IA management continues to be challenging due to the heterogeneous population at risk, the diversity of clinical and radiological presentations and the lack of a gold standard (Lamoth and Calandra, 2017). Thus, at present, it is required to understand the limitations of each biomarker and the corresponding diagnosis test in order to accurately diagnose these challenging infections (Maertens et al., 2016). In this line, the future directions in IA diagnosis research need to focus on the development of new biomarkers, including a clear understanding of their strengths and limitations, along with the assessment of their utility in well-designed clinical trials (Arvanitis and Mylonakis, 2015; Mercier and Maertens, 2017).

In recent years, bis(methylthio)gliotoxin (bmGT) has generated great interest as an IA biomarker (Maertens et al., 2016; Mercier and Maertens, 2017). Its detection in serum by High Performance Thin Layer Chromatography (HPTLC) was shown to be reliable (Domingo et al., 2012). Moreover, it has been clinically validated in a small prospective study in comparison with GM quantification (Vidal-García et al., 2016). Data suggest a good diagnostic performance (61.5% sensitivity and 93% specificity) and importantly, high positive and negative predictive values when used in combination with GM detection (100% and 97.5%, respectively), which suggest a potential utility in pre-emptive approaches. Pending further validation, unlike GM, bmGT detection could be useful in non-immunocompromised populations as it was previously found to be positive in a non-compromised patient suffering from IA that presented negative GM values (Vidal-García et al., 2017). Nevertheless, data regarding the frequency and distribution of bmGT production by different opportunistic molds are scarce and in most cases based on bioinformatics analysis (Bergmann et al., 2007; Andersen et al., 2013; Dolan et al., 2014). These data would be very important for understanding the specificity and the clinical sensitivity of this biomarker to differentiate between species within the *Aspergillus* genus and, thus, treat this infection more effectively.

Bis(methylthio)gliotoxin is an inactive derivative of gliotoxin (GT). *A. fumigatus* is, to date, the most important opportunistic fungi producing bmGT (Li et al., 2006; Guimarães et al., 2010; Domingo et al., 2012; Sun et al., 2012; Liang et al., 2014). BmGT serves as a negative regulator of the GT biosynthesis, and it is produced by methylation of GT by an S-adenosylmethionine-dependent bis-thiomethyltransferase (Dolan et al., 2014, 2015, 2017), eliminating the ability of GT to produce toxic reactive oxygen species (ROS) (Dolan et al., 2015). BmGT formation from an exogenous source of GT has been described in *A. niger* and *A. nidulans* (Scharf et al., 2014; Manzanares-Miralles et al., 2016). The enzyme responsible for bmGT biosynthesis, which is an *S*-adenosylmethionine (SAM)-dependent methyltransferase called GtmA, has been characterized in *A. fumigatus*; and other orthologs have been found on species like *A. niger* or *A. terreus* (Dolan et al., 2014; Scharf et al., 2014; Manzanares-Miralles et al., 2016). The best characterized enzyme is GtmA, which is known to be encoded by the *gtmA* gene, located in the chromosome 2 (Dolan et al., 2014). Bioinformatics analysis of the *Ascomycota* phylum showed 124 orthologs of GtmA. However, it is known that toxin production is discontinuous among different species and it is not clear which species within *Aspergillus* genus are able to produce GT and, subsequently, the inactive derivative bmGT (Gardiner and Howlett, 2005; Patron et al., 2007). The aim of the present study was to assess the frequency and species distribution of bmGT within the *Aspergillus* genus in cultures *in vitro* as well as *in vivo* in sera of patients from whom fungi were isolated. We also characterized the ability of different clinical isolates from *Aspergillus* genus to methylate GT in cultures *in vitro*, to confirm the presence and activity of methyltransferases in *Aspergillus* species isolated from probable and proven cases of IA. Our findings indicate that bmGT could be considered as an specific biomarker to detect infections by *A. fumigatus*, the most common agent causing IA, excluding the presence of *A. nidulans* and *A. niger*.

MATERIALS AND METHODS

Gliotoxin and Bis(methylthio)gliotoxin Production

We analyzed GT and bmGT production within 252 *Aspergillus* spp. isolates. Most *A. fumigatus* complex (n = 119) were clinical isolates from Canisius-Wilhelmina Hospital, Nijmegen (Netherlands). Eighteen of those isolates were cryptic species from Section *Fumigati* from Gregorio Marañón University Hospital, Madrid (Spain). Other *Aspergillus* species were clinical isolates from Miguel Servet University Hospital, Zaragoza (Spain) and corresponded to 36 *A. flavus* complex, 35 *A. terreus* complex, 40 *A. niger* complex, and 22 *A. nidulans* complex. One milliliter of 12 McFarland conidial suspension (approximately 3–5 × 10^7 conidia/mL) was added to 9 mL of liquid medium (Roswell Park Memorial Institute [RPMI] 1640 + glucose 20 g/L + glutamine 2 mM + HEPES 25 mM) in 50 mL culture flasks and incubated at 37°C for 96 h. A 2 mL sample of supernatant was obtained and frozen at −20°C and subsequently used for GT and bmGT detection and quantification by HPTLC as described below. In those cases where GT and/or bmGT was not detected, fungal isolates were cultured employing Czapek Dox Broth (+ glutamine + HEPES) to confirm that this defect was not specific for RPMI medium. Czapek Dox Broth is a medium of a different composition to RPMI1640, and similarly to

the last one, it is commonly used in *Aspergillus* cultures *in vitro*. Thus, we decided to compare both in order to discard effects relative to specific culture media conditions *in vitro*.

Bis(methylthio)gliotoxin Production From Exogenous Gliotoxin

Bis(methylthio)gliotoxin production from an exogenous source of GT was assessed in a total of 35 isolates of the species complexes *A. flavus* (n = 12), *A. terreus* (n = 9), *A. niger* (n = 8), and *A. nidulans* (n = 6). Conidial inoculum was prepared as described above and added to 50 mL culture flasks with Czapek Dox Broth (+ glutamine + HEPES). These cultures were incubated at 37°C for 45 h. At 45 h, GT was added to a final concentration of 2.5 mg/L and methanol was added as solvent control. At 0, 3, and 6 h, 2 mL aliquots of supernatant were taken and frozen until GT and bmGT analysis.

Detection of Bis(methylthio)gliotoxin in Sera From IA Patients

We assessed sera from patients hospitalized in the Miguel Servet University Hospital with probable/proven IA according to the EORTC/MSG definitions (De Pauw et al., 2008). We included in the study those cases with *Aspergillus* spp. growth in clinical samples. Serum were prospectively collected and frozen at −20°C until GT and bmGT detection. All protocols were supervised and approved by the Ethics Committee of Clinical Research from Aragón (CEICA), number PI15/0203.

Metabolite Identification and Quantification by High Performance Thin Layer Chromatography (HPTLC)

Gliotoxin and bis(methylthio)gliotoxin detection and quantification were performed both in serum and supernatant samples by HPTLC as described by Domingo et al. (2012). Briefly, GT and bmGT were extracted together using dichloromethane. After agitation and two phase's separation, non-aqueous phase was added onto silica gel plates. Then, they were developed using a horizontal development chamber (Camag). The mobile phase was a mixture of tetrahydrofuran/*n*-heptane/acetonitrile (40:58:2 [v/v/v]). After 25 min development, plates were scanned with an ultraviolet scanning densitometry (TLC Scanner 3, Camag; λ = 280 and 367 nm; linear scanning). GT and bmGT identification was performed by retention time and spectral analysis and quantification was performed by peak area under curve analysis using Camag's personal computer software.

Genetic Detection of *gtmA* Gene

Chromosomal DNA of Aspergillus spp. isolates was extracted using cetyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO, United States). The specie-specific primers used for *gtmA* detection are summarized in **Table 1**, along with expected amplicon length. PCR was performed using HotTaq Master Mix, (IBIAN Technologies, Zaragoza, Aragón, Spain). An initial denaturation of 2 min at 94°C was followed by 35 cycles at 94°C for 30 s, 56°C for 15 s, and 72°C for 1 min. DNA amplification products were visualized after electrophoresis on 2% agarose gels.

TABLE 1 | Primers.

Name	Sequence (5′–3′)	Amplification length (bp)
A. terreus Fw	TCG GAG GCC CTA AAC CG	291
A. terreus Rv	GGA TTC GGA AGT CCA ACA AGG	
A. flavus Fw	TCA AGC GTC CTT CAT CAT AC	289
A. flavus Rv	TCG TCA GGG AAG AGA TTA AAA GC	
A. niger Fw	TCT AGT GCC CTT CAT CGT GC	223
A. niger Rv	TCG TCA GGG AAG AGG TTG AAC	
A. fumigatus Fw	TCC AGC GTA CTC AAC CAC AC	293
A. fumigatus Rv	CGT CTG GAA AGA TCT GGA AG	
ITS 1 Fw	TCC GTA GGT GAA CCT GCG G	565 to 613
ITS 4 Rv	TCC TCC GCT TAT TGA TAT G	

UView 6x loading dye (Bio-Rad, Hercules, CA, United States) was used for nucleic acid staining. As a length standard 0.1–1 kbp molecular mass marker was used.

Statistical Analysis

Statistical analyses were performed using Prism 6, Graphpad (San Diego, CA, United States). GT and bmGT concentration in culture filtrates are given as mean ± standard error of mean (SEM). A significance level of 0.05 was considered statistically relevant for multiple comparison test and chi-square test, as appropriate.

RESULTS

Gliotoxin and Bis(methylthio)gliotoxin Production by *Aspergillus* spp. Isolates

The production of GT and bmGT was tested in culture supernatants of 252 *Aspergillus* isolates from the species complexes *A. fumigatus, A. flavus, A. terreus, A. niger,* and *A. nidulans* after 4 days of incubation. Non-cryptic *A. fumigatus* isolates (n = 101) produced GT and bmGT at highest frequencies, 77.23% and 84.16%, respectively. Among the five cryptic species analyzed: *A. calidoustus* (n = 1), *A. fumigatiaffinis* (n = 3), *A. lentulus* (n = 11), *N. udagawae* (n = 2), and *A. novofumigatus* (n = 1), all but the last produced GT and/or bmGT. *A. fumigatiaffinis* seemed to be the most frequent GT and bmGT producing species, as the three tested isolates (100%) produced bmGT. In contrast, just two of the eleven isolates (18%) of *A. lentulus* produced bmGT. BmGT was also more frequently detectable than GT in culture supernatants of *A. flavus*, 11.11% vs. 8.33%, respectively. In contrast, *A. terreus* produced GT more frequently than bmGT (22.86% vs. 2.86% respectively) (**Figure 1**). Notably, none of the *A. niger* and *A. nidulans* isolates tested produced GT and/or bmGT. All cultures were analyzed by HPTLC, a method to detect GT and bmGT previously optimized and validated versus LC-MS (Domingo et al., 2012), and some of the results in selected culture isolates of *A. fumigatus, A. niger, A. nidulans,* and *A. flavus*, were confirmed by LC-MS (data not shown). The absence of GT and/or bmGT production was not specific for the culture conditions *in vitro* since culture

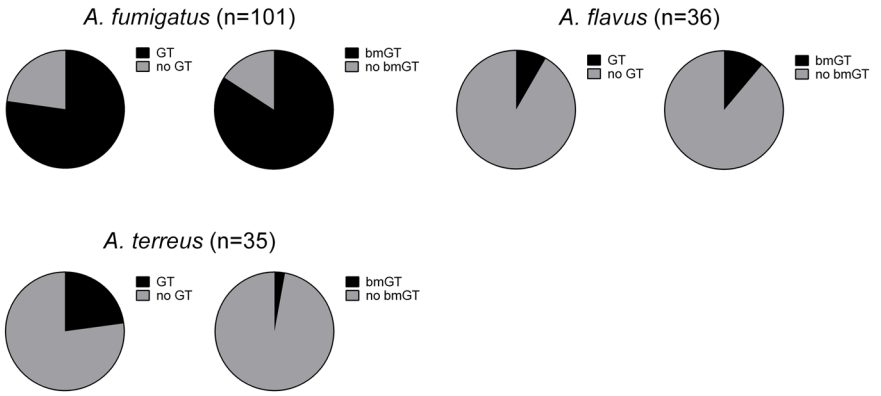

FIGURE 1 | Percentage of gliotoxin and bis(methylthio)gliotoxin producing isolates among different *Aspergillus* species.

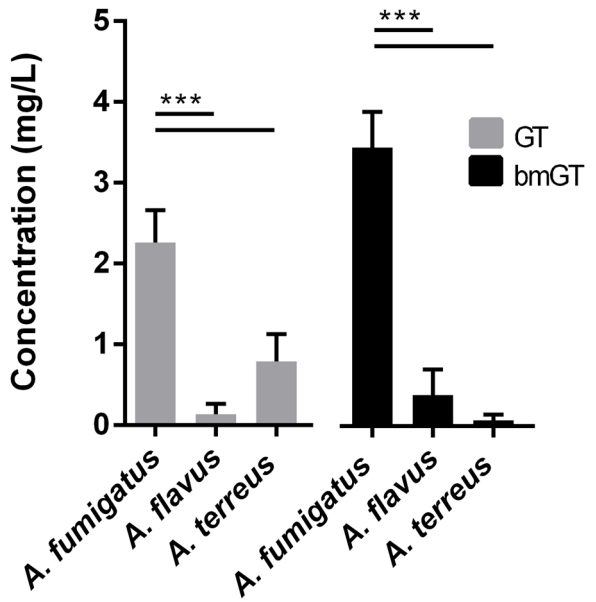

FIGURE 2 | Mean concentrations of gliotoxin and bis(methylthio)gliotoxin detected in culture supernatans of different *Aspergillus* species. ***Statistically significant differences ($p < 0.001$).

of GT/bmGT negative fungal isolates employing Czapek Dox Broth yielded similar results (data not shown). In addition, the differences were not due to different fungal growth since cell cultures showed a similar behavior and growth as analyzed by XTT reduction assay. Optimal culture conditions, as well as analytical specificity of the method, were confirmed by employing cell cultures from an *A. fumigatus gliP* deletion mutant, which is unable to produce GT and bmGT (Sugui et al., 2007).

A. fumigatus isolates also produced GT and bmGT in higher concentration than other *Aspergillus* spp. did. These differences were statistically significant ($p < 0.05$) (**Figure 2**). The mean concentration of GT was 2.26 ± 0.40 mg/L and of bmGT was 3.45 ± 0.44 mg/L for *A. fumigatus*. *A. flavus* isolates yielded a mean concentration of 0.14 ± 0.13 mg/L of GT and 0.39 ± 0.31 mg/L of bmGT. The mean concentration of GT and bmGT were 0.79 ± 0.34 mg/L and 0.07 ± 0.07 for *A. terreus*.

Bis(methylthio)gliotoxin Production From Exogenous Gliotoxin

In order to confirm the results obtained in cell cultures concerning bmGT production, we analyzed the ability of some fungal isolates to generate bmGT from an external GT source as well as the presence of methyltransferase genes. This is of special utility to find out whether the *Aspergillus* spp. that did not produce GT and bmGT (*A. niger* and *A. nidulans*) are also unable to methylate exogenous GT. This finding would mean that these isolates do not express methyl-transferase activity and, thus, confirm that they are unable to generate bmGT. Moreover, this would indirectly suggest that they are also unable to generate GT, since GT methylation has been proposed as a negative feedback regulatory system, inherent to all GT-producing species.

First, we analyzed if fungal isolates presented GT methyl-transferase activity by adding pure GT and monitoring the generation of bmGT. The ability to produce bmGT from an exogenous source of GT was assessed in the isolates of *A. flavus, A. terreus, A. niger,* and A. *nidulans* that did not produce GT or bmGT in the previous experiment. All isolates from the *A. flavus* ($n = 12$) and *A. terreus* species ($n = 9$) were able to methylate exogenous GT in order to produce bmGT. Among *A. niger* isolates, this ability was less consistent, nevertheless, 5/8 isolates (62.5%) showed such ability. Finally, none of the *A. nidulans* isolates ($n = 6$) methylated GT to generate bmGT (**Figure 3**). This result confirms that *A. nidulans* does not express methyl-transferase activity and, thus, it is unable to generate endogenously bmGT, and, likely, GT, in line with the results of **Figure 1**. Concerning *A. niger*, some isolates seem to express methyl-transferase activity against exogenous GT. Indeed, it has been previously shown that the methyl-transferase MT-ii is expressed in *A. niger* and methylates exogenous GT (Dolan et al., 2017). However, since they do not produce GT (**Figure 1** and Manzanares-Miralles et al., 2016), this would explain that they are unable to endogenously produce bmGT.

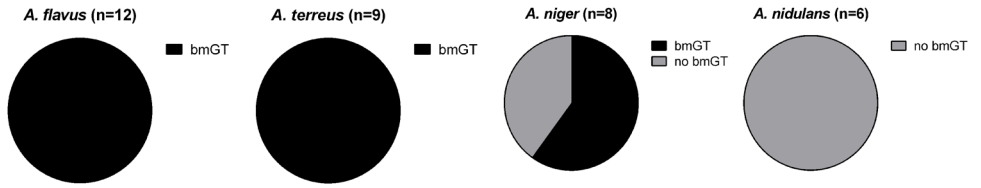

FIGURE 3 | Percentage of GT-methylating isolates of *Aspergillus* species.

Among bmGT producing isolates, immediately after GT addition (t = 0 h), this was recovered in a mean concentration of 0.86 ± 0.04 mg/L. None of the isolates produced bmGT at this time (**Figure 4**). At 3 h after GT addition, GT concentration decreased and bmGT concentration increased. This observation continued at 6 h, when the maximum bmGT and the minimum GT concentrations were detected. There were no differences between mean concentration of GT and bmGT among species ($p > 0.05$) indicating a similar methylating activity.

Comparison of Serum bmGT Detection *in Vivo* With bmGT Production in *in Vitro* Cultures

Our results confirm that most *A. fumigatus* isolates and some *A. terreus* and *A. flavus* isolates were able to endogenously and exogenously produce bmGT. However, the frequency of bmGT production within *A. terreus* and *A. flavus* isolates was much less than in *A. fumigatus* isolates. Since the analyses of the genes involved in GT synthesis is difficult due to the complexity of the pathways involved, we decided to analyze if the isolates that did not produce endogenously GT and bmGT *in vitro*, were able to synthesis bmGT in humans *in vivo*. To this aim, we included six cases of probable/proven IA with mycological growth from whom *in vitro* cultures had been established and analyzed. Serum bmGT concentration for these patients, fungal isolation, sample type and fungal ability to produce bmGT *in vitro* (*de novo* and from exogenous GT) as well as detection of *gtmA* gene and the MT-ii homolog are summarized in **Table 2**.

All, but one serum, were positive for bmGT. This serum belonged to a patient with probable IA diagnosed by *A. fumigatus* growth in bronchial aspirate. The other three patients with *A. fumigatus* isolation had positive bmGT (range 0.19–13.68 mg/L). There was a case of IA by *A. flavus* and a case of IA by *A. terreus*. Both had detectable bmGT in serum. Notably, these isolates corresponded to those ones in which we were not able to detect either endogenous GT or bmGT during *in vitro*

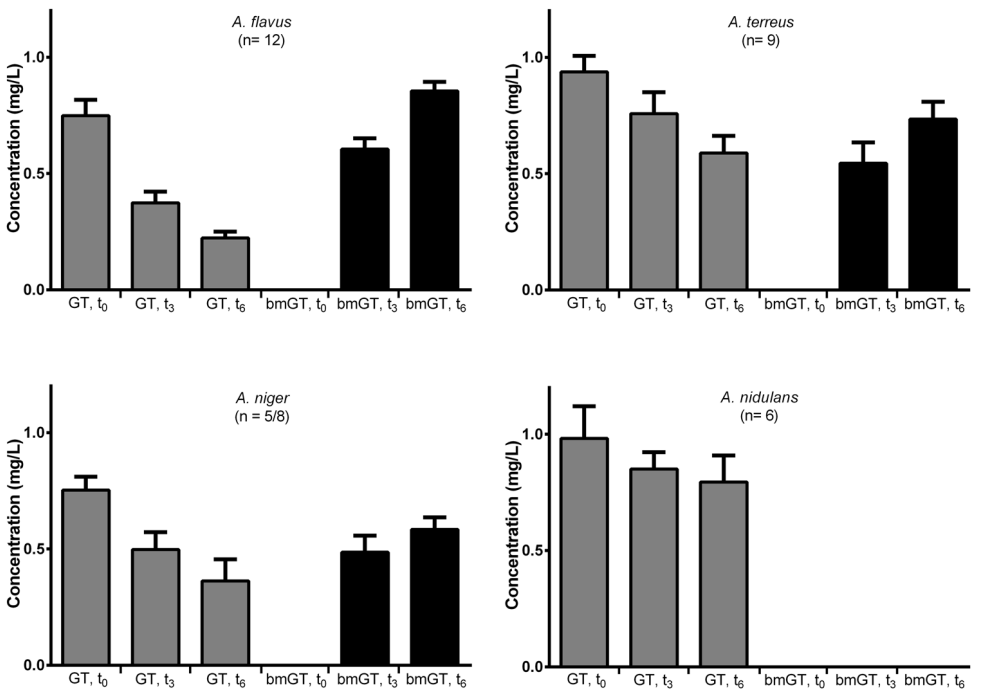

FIGURE 4 | Bis(methylthio)gliotoxin production from exogenous gliotoxin among different *Aspergillus* species. The bars represent mean concentrations with standard error of means.

TABLE 2 | bmGT production, methylation of exogenous GT and carriage of *gtmA or MT-ii* genes for *Aspergillus* spp. isolates from probable and proven invasive aspergillosis cases.

Case	IA type	Culture (sample)	Serum bmGT (mg/L)	Specie	Supernatant bmGT (mg/L)	GT methylation ($[bmGT]_{t=6}$)	*gtmA* gene
1	Proven	Sinus biopsy	1,66	*A. flavus*	Not detected	0.93	+ (mt-ii)
2	Proven	Vitreous, thrombus	0,19	*A. fumigatus*	0.26 ± 0.05	0.90	+
3	Probable (proven IFI)	Bronchial aspirate	6,84	*A. fumigatus*	0.52 ± 0.05	0.55	+
4	Probable	Sputum	13,68	*A. terreus*	Not detected	0.16	+ (mt-ii)
5	Probable	Bronchial aspirate	–	*A. fumigatus*	0.48 ± 0.14	0.98	+
6	Probable	Sputum	2,6	*A. fumigatus*	0.18 ± 0.02	0.48	+

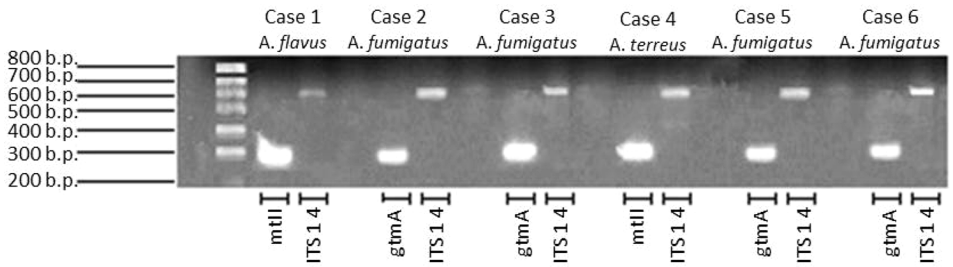

FIGURE 5 | Detection of *gtmA* and mt-ii genes in clinical isolates from probable/proven invasive aspergillosis cases.

culture. Nevertheless, all of them methylated the exogenous GT and carried the mt-ii methyl-transferase gene, as seen in **Figure 5**. Of note, those isolates which produced bmGT in higher amounts *in vitro,* did not correlate with the highest bmGT production *in vivo*. All the clinical isolates from *A. fumigatus* showed the ability to methylate exogenous GT and carry the *gtmA* gene.

Finally, in order to confirm that *A. nidulans* and *A. niger* did not produce GT and, thus, are unable to endogenously synthesize bmGT, we analyze the presence of *gliP* gene (a critical gene within the gli cluster responsible for GT synthesis) by PCR. None of the isolates from *A. niger* and *A. nidulans* carry the *gliP* gene (data not shown) confirming that they are unable to produce GT and bmGT, as found in the cell culture analysis (**Figure 1**). Moreover, a bioinformatic analyses searching for the presence of gli cluster homology sequences in the genome of sequenced *A. niger* and *A. nidulans* strains, yielded negative results, confirming our experimental data and in line with previous findings (De Pauw et al., 2008; Manzanares-Miralles et al., 2016). In contrast, sequences with some homology to gli cluster were found in both *A. terreus* and *A. flavus* genomes (data not shown) as previously indicated (Patron et al., 2007).

DISCUSSION

Despite recent advances, the lack of a single gold standard technique and the limitations of the available ones, make diagnosis of IA still challenging (Maertens et al., 2016; Mercier and Maertens, 2017). In recent years, new metabolite based diagnostic tools have been under research, such as GT or volatile organic compounds (Lewis et al., 2005a; Chambers et al., 2009).

Regarding GT, its high biological reactivity and its potential ability to interact with cells and tissues (Domingo et al., 2012) make it hard to detect in body fluids (Scharf et al., 2012). This limitation is overcame by bmGT, which is more stable and reliably detected in serum (Domingo et al., 2012). It is known that *A. fumigatus* produces GT in the highest concentrations and more frequently than other *Aspergillus* species (Lewis et al., 2005b; Kupfahl et al., 2008). This conclusion has been supported by our results, in which 77% of the *A. fumigatus* isolates were GT-producers in significantly higher concentrations and frequencies than those obtained for *A. flavus* and *A. terreus* species complexes, the other *Aspergillus*-producing GT species. With reference to bmGT, *A. fumigatus* was also the most common bmGT producing complex. Notably, and in line with previous findings (De Pauw et al., 2008; Manzanares-Miralles et al., 2016), neither *A. niger* or *A. nidulans* strains were able to endogenously produce GT and/or bmGT, although methyl-transferase activity was found in *A. niger* isolates when using exogenous GT. These results are supported by both PCR analyses and bioinformatic studies confirming the absence of the gli cluster in these species. These findings contrast to previous studies in which a high proportion of *A. niger* isolates were shown to produce GT (Kupfahl et al., 2008). We have no explanation for these contradictory findings although, in line with our findings, Kupfahl et al. (2008) did not detect *gliP* gene in *A. niger*, which has been shown to be critical for GT synthesis, at least in *A. fumigatus* isolates (Sugui et al., 2007).

Some authors have been interested in the secondary metabolite profiles of cryptic species of the Fumigati section. Unlike other authors, we found out that *A. lentulus* and *A. fumigatiaffinis* were able to produce GT and bmGT (Larsen et al., 2007; Sugui et al., 2010; Tamiya et al., 2015). Regrettably, we

just analyzed one isolate of *A. calidoustus* and *A. novofumigatus*. The *A. calidoustus* isolate was GT and bmGT producer, but *A. novofumigatus* was not. This result does not rule out the ability to produce GT and bmGT by *A. novofumigatus* since culture conditions (medium, aeration, temperature, sampling time. . .) affect to secondary metabolite synthesis (Belkacemi et al., 1999; Watanabe et al., 2004). This could explain the low GT and bmGT detection among non-*A. fumigatus* species since all experiments were performed in the same conditions and confirmed employing other culture protocols.

Aiming to avoid such a limitation, we analyzed the ability to produce bmGT from an exogenous source of GT among non-toxigenic isolates. We detected bmGT in culture filtrates of all the *A. flavus* and *A. terreus* analyzed, thus suggesting a consistent ability to produce bmGT. To our knowledge no specific methyltransferases had been described to date for these species. Nevertheless, it has been described an ortholog and a homolog of GtmA for *A. terreus* and *A. flavus* by bioinformatics analysis but it is the first time in which its expression has been described. In our study, *A. niger* also showed the ability to methylate GT, but less frequently. Curiously, none of the *A. nidulans* isolates analyzed produced bmGT even when it has been described that a specific methyltransferase (and the encoding gene) able to produce bmGT from GT for this species (Manzanares-Miralles et al., 2016). This discrepancy could be due to the known fact that culture conditions do not reflect the genetic potential and that not all the strains of the same species have the same metabolic profile (Bergmann et al., 2007). Indeed, secondary metabolism confers a survival benefit to the producing isolate and the *in vitro* culture conditions are not optimal to activate this survival pathway, depending on the *Aspergillus* spp. and/or isolate.

In order to overcome the limitations of the *in vitro* culture to analyze secondary metabolism, we have employed some clinical isolates from patients with probable/proven IA and compared bmGT production in *in vitro* culture with bmGT in serum from those patients. In this scenario, where fungi has to colonize the host and adapt itself to the new environmental conditions, the fungi would activate secondary metabolism and display all potential virulence factors such a GT (Cramer et al., 2006; Sugui et al., 2007) In these conditions, all but one of the seven probable/proven patients had bmGT detectable in serum, even those that did not produce GT and bmGT *in vitro*. Importantly, all of them were able to produce bmGT from exogenous GT and carried an ortholog of GtmA, MT-ii.

In summary, our findings indicate that bmGT production is useful to diagnose IA caused by *A. fumigatus* and, at some extent, by *A. terreus* and *A. flavus,* although at a much lower frequency, since they present the ability to methylate GT and endogenously produce bmGT *in vitro* and *in vivo*. Moreover, and pending of validation with a higher number of samples, our novel findings indicate that conclusions about the expression of molecules that could be used as potential diagnostic biomarkers based on *in vitro* fungal cultures cannot be reached unless they are confirmed in proper *in vivo* studies employing animal models or patients suffering from IA.

AUTHOR CONTRIBUTIONS

MV-G and SR carried out the experiments. PM helped with genomic analysis and CC helped with *in vitro* experiments. MD performed the HPTLC analysis. MV-G wrote the manuscript with support from AR, JP, and EG. JM provided and characterized different isolates of *Aspergillus* spp. JP and EG conceived the original idea and supervised the project with the support of AR.

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REVIEW
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Molecular Imaging of Invasive Pulmonary Aspergillosis Using ImmunoPET/MRI: The Future Looks Bright

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Invasive pulmonary aspergillosis (IPA) is a life-threatening lung disease of immuno-compromised humans caused by the ubiquitous environmental mold *Aspergillus*. Biomarker tests for the disease lack sensitivity and specificity, and culture of the fungus from invasive lung biopsy is slow, insensitive, and undesirable in critically ill patients. A computed tomogram (CT) of the chest offers a simple non-intrusive diagnostic procedure for rapid decision making, and so is used in many hematology units to drive antifungal treatment. However, radiological indicators that raise the suspicion of IPA are either transient signs in the early stages of the disease or not specific for *Aspergillus* infection, with other angio-invasive molds or bacterial pathogens producing comparable radiological manifestations in a chest CT. Improvements to the specificity of radiographic imaging of IPA have been attempted by coupling CT and positron emission tomography (PET) with [^{18}F]fluorodeoxyglucose ([^{18}F]FDG), a marker of metabolic activity well suited to cancer imaging, but with limited use in invasive fungal disease diagnostics due to its inability to differentiate between infectious etiologies, cancer, and inflammation. Bioluminescence imaging using single genetically modified strains of *Aspergillus fumigatus* has enabled *in vivo* monitoring of IPA in animal models of disease. For *in vivo* detection of *Aspergillus* lung infections in humans, radiolabeled *Aspergillus*-specific monoclonal antibodies, and iron siderophores, hold enormous potential for clinical diagnosis. This review examines the different experimental technologies used to image IPA, and recent advances in state-of-the-art molecular imaging of IPA using antibody-guided PET/magnetic resonance imaging (immunoPET/MRI).

Keywords: monoclonal antibody (mAb), invasive pulmonary aspergillosis, positron emission tomography, magnetic resonance imaging, immunoPET-MRI

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INTRODUCTION

Invasive pulmonary aspergillosis is a frequently fatal lung disease of immuno-compromised individuals caused by inhalation of spores of the air-borne fungus *Aspergillus*. While a number of species of *Aspergillus* can cause IPA, the principal agent of the disease is *Aspergillus fumigatus*, responsible for >80% of recorded cases. As an opportunistic pathogen, it causes more than 200,000

Abbreviations: CT, computed tomography; IFD, invasive fungal disease; IPA, invasive pulmonary aspergillosis; MRI, magnetic resonance imaging, PET, positron emission tomography.

life-threatening infections in humans every year, mainly in high-risk patient groups such as those with hematological malignancies, and in hematopoietic stem cell (HSC) and solid organ transplant recipients, with mortality rates of between 30 and 90% (Segal, 2009). The disease is typically seen during periods of prolonged neutropenia, but non-neutropenic patients with underlying lung diseases such as steroid-treated chronic obstructive pulmonary disease (COPD), asthma, lung cancer, or autoimmune diseases with pulmonary involvement can develop IPA (Prattes et al., 2014). Furthermore, other *Aspergillus* lung diseases such as chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA) are thought to affect >5 million people globally each year (Brown et al., 2012). Taken together, *Aspergillus* diseases represent a significant burden to human health, contributing to patient morbidities and prolonging hospitalization. Much of this burden is caused by the lack of diagnostic tests with sufficient accuracy to allow early identification and timely intervention with effective antifungal drugs.

Early detection of IPA and treatment with mold-active drugs is vital for patient survival. However, clinical symptoms of the disease (fevers and chills, hemoptysis, shortness of breath, chest pains, and headaches) are not specific for *Aspergillus* infections. The gold standard test for IPA is culture of *Aspergillus* from a sterile biopsy, but this is limited by poor sensitivity, lengthy turnaround time, and requires invasive recovery of lung tissue. Assays that detect circulating biomarkers of infection such as the Platelia galactomannan enzyme-linked immunosorbent assay (ELISA) and "pan-fungal" β-D-glucan tests lack either sensitivity or specificity (Prattes et al., 2014). The *Aspergillus* lateral-flow assay (LFA; Thornton, 2008) will be available commercially as a CE-marked *in vitro* device (IVD) for IPA diagnosis in March 2018. When used with BAL samples, it has the ability to be used as a point-of-care test, and so has the potential to improve the speed and accuracy of disease detection (Hoenigl et al., 2017). Despite this, the current inadequacies of IPA diagnostics have led to the empiric or "fever-driven" use of antifungals. This contributes to the erroneous treatment of already sick patients with costly and noxious drugs and promotes the emergence in *Aspergillus* of resistance to mold-active triazoles and to breakthrough infections. Empiric antifungal treatments also impact the sensitivities of fungal culture and biomarker-assisted tests, which are needed for diagnosis, for establishing drug sensitivities, and for monitoring responsiveness to treatments.

Diagnostic-driven approaches to antifungal treatment have been shown to be more effective than empiric treatment with respect to both cost and patient outcome (Barnes, 2013). Diagnostic-driven approaches to IPA treatment habitually rely on radiographic imaging, coupled with frequent testing for fungal biomarkers. Radiographic imaging is an attractive means of detecting *Aspergillus* lung infections *in vivo* because it is a non-invasive procedure, but basic radiographic findings in IPA are largely non-specific (Panse et al., 2016). A computed tomogram (CT) of the chest provides a quick non-intrusive clue for rapid decision making (Prasad et al., 2016), with the earliest sign suggestive of the disease being a nodule. The "halo sign," a transient CT finding, is also suggestive of probable disease, and initiation of antifungal treatment in patients with this indicator at baseline has been associated with improved patient outcomes for early stages compared to later stages of disease (Greene, 2005; Greene et al., 2007). However, other mold pathogens such as mucormycetes species, and angio-invasive bacterial pathogens such as *Pseudomonas aeruginosa*, can give similar appearances (Segal, 2009; Stanzani et al., 2015). The "halo sign" therefore provides only limited accuracy for the diagnosis of IPA.

Over recent years, alternative techniques for non-invasive imaging of IPA have been developed and tested in pre-clinical animal models of disease (Velde and Wiehr, 2017). One such method is bioluminescence, that has been used to track *Candida albicans* and *A. fumigatus* infections and to monitor their responsiveness to antifungal treatments (Doyle et al., 2006; d'Enfert et al., 2010; Brock, 2012; Jacobsen et al., 2014). Bioluminescent strains of *A. fumigatus* have been generated through constitutive expression of the firefly luciferase gene under the fungal promoter *gpdA* (Brock et al., 2008). Transformed strains of the pathogen have been used to monitor antifungal drug efficacies *in vitro* and *in vivo* (Brock et al., 2008; Galiger et al., 2013) and to investigate the roles of resident and recruited immune effector cells in defense against invasive *A. fumigatus* infections (Ibrahim-Granet et al., 2010). The limitation of this technique is the requirement for genetically modified strains, which restricts studies to single mutants of the pathogen expressing luciferase. Different approaches for imaging IPA have therefore been explored using, for example, small molecules such as peptides (Yang et al., 2009), and the antifungal drug fluconazole coupled to ^{18}F or ^{99m}Tc (Lupetti et al., 2002), for scintigraphic imaging of infections. For instance, using a ^{111}In-labeled peptide c(CGGRLGPFC)-NH_2 selected from a bacteriophage phage library, γ-imaging is able to delineate experimental IPA in mice (Yang et al., 2009). However, because the peptide corresponds to extracellular matrix proteins of the lung parenchyma, it is probable that the peptide binds to other fungi that are able to interact with extracellular matrix components of the lungs. Further specificity tests would therefore need to be conducted *in vivo* to determine the spectrum of IFDs detectable with this probe. While ^{99m}Tc-fluconazole proved to be superior to ^{18}F-fluconazole for imaging of *C. albicans* infections in mice, it was found to be unsuitable for imaging of *A. fumigatus* infections (Lupetti et al., 2002).

The limitations of bioluminescence and small molecule imaging have led to efforts to improve the specificity of radiographic imaging of IPA by combining well-established hospital imaging technologies [high-resolution computed tomography (HRCT), MRI, and PET] with specific markers of infection. The aim of this mini-review is to examine recent advances in molecular imaging of IPA using radiolabeled *Aspergillus*-specific monoclonal antibodies (mAbs), and iron siderophores, and their potential for translation to the clinical setting.

ASPERGILLOSIS IMAGING WITH COMPUTED TOMOGRAPHY AND MAGNETIC RESONANCE IMAGING

According to current EORTC/MSG guidelines (De Pauw et al., 2008), a CT examination of the chest that reveals "dense, well-delineated, nodular infiltrates in the lung with or without ground glass attenuation ('halo sign') in a patient with an ongoing or recent history of prolonged neutropenia, or hematopoietic stem cell transplant (HSCT)," is defined as having a "possible" mold infection. Importantly, while 88–96% of neutropenic patients exhibit this sign in the first day of IPA, it disappears in one-third of patients within 72 h and in the remaining two-thirds of patients within 2 weeks (Caillot et al., 1997; Brodoefel et al., 2006). Furthermore, this diagnosis is not specific for IPA, as other infections, and neoplastic and inflammatory processes, can produce similar opacities with ground glass attenuation (Georgiadou et al., 2011), and the disease can also manifest as atypical presentations in liver transplant patients (Mucha et al., 2013) and during invasive bronchial-pulmonary aspergillosis in critically ill patients with COPD (Huang et al., 2016). Radiological indications are rare in the initial stages of IPA in non-neutropenic patients (Prattes et al., 2014), and differ between children and adults with IFDs (Ankrah et al., 2016).

Notwithstanding these limitations, CT acts as a prompt for instigating antifungal treatment in numerous hematology units, with HRCT providing opportunities for improved antifungal stewardship (Stanzani et al., 2016). Reductions in the unnecessary use of antifungal drugs have been reported in centers using HRCT to drive treatment strategies in allogeneic transplant patients who have persistent febrile antibiotic-resistant neutropenia (Dignan et al., 2009). Therefore, in certain settings, refractory fever/HRCT-based approaches to diagnosis may result in significant reductions in parenteral antifungal drug usage in patients who would otherwise have received empirical treatment. However, due to the limited specificity of HRCT, such an approach cannot be used for IPA specifically, but rather IFDs as a whole (Dignan et al., 2009). Improvements to the specificity of HRCT for the detection of fungal lung infections have been attempted by combining it with pulmonary angiography (CTPA), but the performance of CTPA relative to other signs (halo sign, hypo-dense sign, pleural effusion, and reversed halo sign) is not known. Nevertheless, vessel occlusion detected by CTPA may be a more sensitive and possibly more specific radiographic sign in patients with hematological malignancies (Stanzani et al., 2015).

Pre-clinical studies using MORF oligomers that target fungal ribosomal RNA have shown that CT specificity can be dramatically improved when ^{99m}Tc-labeled *Aspergillus*-specific probes are combined with SPECT (Wang et al., 2013). Two probes, AGEN and AFUM, have been investigated that are genus-specific and *A. fumigatus*-specific, respectively. Single photon emission tomography (SPECT)/CT imaging of mice with experimental IPA showed a twofold increased accumulation of both ^{99m}Tc-labeled probes in *A. fumigatus* infected lungs compared to uninfected controls. While the AGEN oligomer was found to cross-react with *C. albicans*, and the AFUM oligomer would preclude detection of infectious *Aspergillus* species other than *A. fumigatus*, the work nevertheless elegantly demonstrates that CT imaging can be rendered disease-specific by using pathogen-specific probes.

Magnetic resonance imaging is now the non-invasive imaging tool of choice, with high spatial resolution and the highest soft tissue contrast. However, unlike HRCT, MRI has received far less attention as a diagnostic imaging modality for *Aspergillus* lung infections, but has been studied extensively as a detection aid for *Aspergillus* cerebral and central nervous system infections (Starkey et al., 2014; Marzolf et al., 2016). While CT is highly suitable for lung applications because it produces high-resolution 3D images with an excellent air–tissue contrast, MRI of the lung is challenging owing to the lack of detectable protons in air-filled spaces and potential artifacts between air–tissue interfaces. Despite these shortcomings, a longitudinal *in vivo* study in mice showed that *A. fumigatus* lung lesions could be visualized and quantified using MRI (Poelmans et al., 2016). By using an advanced MR pulse sequence with ultra-short echo times, pathological changes within the infected lung were visually and quantitatively detectable and with a high degree of sensitivity. In humans, dynamic contrast-enhanced MRI (DCE-MRI) might also be useful for imaging IPA in immunosuppressed acute myeloid leukemia patients (Araz et al., 2014).

ASPERGILLOSIS IMAGING WITH [^{18}F]FDG POSITRON EMISSION TOMOGRAPHY

In contrast to HRCT and MRI, PET can give information about the physiological status of the particular target organ. It has emerged as an immensely powerful imaging technique in the field of oncology, but its use in infectious disease imaging is very much in its infancy (Glaudemans et al., 2012, 2015; Signore et al., 2015). Compared to SPECT of IPA using ^{67}Ga scintigraphy (Tzen et al., 1999; Goméz et al., 2000), PET provides increased sensitivity and resolution through coincidence detection of photons emitted from radionuclei resulting from positron annihilation, with its success in oncology resulting from the use of [^{18}F]fluorodeoxyglucose ([^{18}F]FDG), a diagnostic tracer that specifically accumulates in metabolically active inflammatory cells (neutrophils, macrophages, and lymphocytes), cancer cells, and during infectious processes (Imperiale et al., 2010; Bassetti et al., 2017).

Several studies have indicated that [^{18}F]FDG might be useful for imaging fungal infections (Sharma et al., 2014), for differentiating between non-invasive and invasive aspergillosis (Kim et al., 2013), for identification of extra-pulmonary sites of infection (Chamilos et al., 2008), and for therapy monitoring (Ho et al., 1998; Ozsahin et al., 1998; Franzius et al., 2001; Stanzani et al., 2016). However, a recent study by Rolle et al. (2016), which employed PET and MRI (PET/MRI) to detect lung infections by *A. fumigatus in vivo*, showed that the increase in [^{18}F]FDG uptake during *Aspergillus* lung infection could not be distinguished from that seen during sterile inflammation or during bacterial lung infections caused by *Streptococcus pneumonia* or *Yersinia enterocolitica*, further demonstrating the lack of specificity of this

tracer which has been reported elsewhere (Petrik et al., 2014). While these studies are pre-clinical investigations using mouse models of IPA, numerous clinical studies have also reported the lack of specificity of FDG-PET for diagnosing the disease in humans, with *Aspergillus* lung diseases mimicking lung cancer (Wilkinson et al., 2003; Baxter et al., 2011; Garcia-Olivé et al., 2011), metastatic thyroid cancer (Ahn et al., 2011), lymphoma (Sonet et al., 2007), and tuberculoma (Nishikawa et al., 2011). Pulmonary IFIs caused by fungi other than *Aspergillus* (e.g., *Candida*, *Blastomyces*, *Cryptococcus*, *Coccidioides*, *Histoplasma*, mucormycetes, and *Pneumocystis*) are also detected by FDG-PET in humans (Ankrah et al., 2016). Consequently, while FDG-PET may confirm IPA lesions observed using HRCT and other imaging modalities (Chamilos et al., 2008; Hot et al., 2011), and to guide needle aspirations of lung tissues for fungal culture (Casal et al., 2009), it cannot be used for definitive *in vivo* diagnosis of IPA, or for its differentiation from ABPA (Nakajima et al., 2009) or aspergillomas (Franzius et al., 2001; Ahn et al., 2011).

ASPERGILLOSIS IMAGING WITH ^{68}Ga-SIDEROPHORES

Other than the *Aspergillus*-reactive ^{99m}Tc-labeled MORF probes (Wang et al., 2013), few imaging tracers have been developed that specifically target *Aspergillus* infections, but substantial success has been achieved by combining microPET/CT with iron-scavenging siderophores labeled with ^{68}Ga or ^{89}Zr. Iron is essential for fungal growth and, in iron-poor environments such as serum, bacteria and fungi produce low molecular weight ferric iron-specific chelators to scavenge iron from the host (Haas, 2003). *A. fumigatus* lacks specific uptake systems for host iron sequestered in heme, ferritin or transferrin, and instead uses two high-affinity iron uptake mechanisms, reductive iron assimilation and siderophore-assisted iron mobilization, both of which are induced under conditions of iron starvation. The pathogen produces three hydroxamate-type siderophores, extracellular fusarinine C (FSC) and triacetylfusarinine C (TAFC), and intracellular ferricrocin (FC; Schrettl et al., 2004; Haas et al., 2015). TAFC is secreted soon after spore germination in iron-limited media (Hissen et al., 2004), and is detectable in serum from patients with proven/probable IPA (Carroll et al., 2016). Its biosynthesis is an essential requirement for spore germination, and for virulence in a mouse model of disease (Schrettl et al., 2004; Hissen et al., 2005). Using ^{68}Ga, a positron emitter with complexing properties similar to those of Fe(III), Petrik et al. (2010) evaluated the potential of [^{68}Ga]TAFC and [^{68}Ga]FC as radiopharmaceuticals for imaging of IPA. They showed that uptake by *A. fumigatus* was highly dependent on iron load, but that [^{68}Ga]TAFC displayed excellent *in vitro* stability, and highly selective accumulation in iron-starved cells. Uptake of [^{68}Ga]TAFC in the lungs of immunosuppressed rats correlated with severity of *Aspergillus* infection, while the bio-distribution of [^{68}Ga]FC was inferior to [^{68}Ga]TAFC, and showed poor stability both *in vitro* and *in vivo*. [^{68}Ga]TAFC was again shown in a subsequent pre-clinical study to selectively accumulate in infected lung tissues in a rat infection model, and that another siderophore ferrioxamine (FOXE), when coupled to ^{68}Ga ([^{68}Ga]FOXE), also displayed excellent pharmacokinetics, highly selective accumulation in *Aspergillus* infected lung tissues, and similarly good correlation with disease severity (Petrik et al., 2012a,b). A downside of using the radionuclide ^{68}Ga in PET imaging, compared to longer-lived positron emitters such as ^{64}Cu ($t1/2$ = 12.7 h), ^{124}I, ^{86}Y, ^{90}Nb, or ^{89}Zr ($t1/2$ = 78.41 h), is its relatively short half-life ($t1/2$ = 67.7 min), which limits its use in longitudinal studies. For this reason, alternative siderophores have been investigated for radiolabeling with ^{89}Zr and for use as imaging agent for *Aspergillus* infections (Petrik et al., 2016). Small animal imaging studies of all ^{68}Ga- and ^{89}Zr-labeled siderophores injected in mice displayed similar pharmacokinetics and minimal accumulation of radioactivity in blood and other organs and tissues, with the exception of [^{89}Zr]FOXE which caused significant retention in the gastrointestinal tract. [^{89}Zr]TAFC showed favorable properties for potential longitudinal *Aspergillus* infection imaging.

Using the radiolabeled siderophores as diagnostic tracers should allow for highly specific detection of IPA since TAFC and FC have no function in human physiology and uptake is not detectable in human lung cancer cells (Petrik et al., 2014). However, while the energy-dependent siderophore transporter system might appear advantageous compared to cell wall-labeling approaches using, for example, *Aspergillus*-specific mAbs, the use of radiolabeled siderophores does have its limitations. The first is specificity. While pathogenic bacteria have been shown not to use TAFC- or FOXE-mediated uptake of iron (Petrik et al., 2014), Fe-TAFC uptake under iron depletion has been demonstrated in the human pathogenic fungi *C. albicans* (Lesuisse et al., 2002) and *Fusarium oxysporum* (Leal et al., 2013), and [^{68}Ga]TAFC and [^{68}Ga]FOXE uptake under iron deficiency has been shown in the human pathogens *Fusarium solani* and *Rhizopus oryzae* (Petrik et al., 2014). Consequently, further work is needed to determine whether [^{68}Ga]TAFC (or [^{68}Ga]FOXE) uptake is able to discriminate between *Aspergillus* infections *in vivo* and commensal *C. albicans* colonization of the gastrointestinal tract, invasive candidiasis, mucormycosis, and disseminated *Fusarium* infections (fusariosis). Furthermore, while *A. fumigatus* is the principal agent of IPA, other *Aspergillus* species such as *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger*, and *Aspergillus terreus* are able to cause the disease (Willinger et al., 2014). While uptake of Fe-TAFC has been shown in *A. nidulans* (Oberegger et al., 2001) and [^{68}Ga]TAFC uptake has been shown in *A. flavus* and *A. terreus* (albeit at significantly lower levels than *A. fumigatus*), it is unclear whether all infectious *Aspergillus* species are detectable *in vivo* using this system. The second consideration is the important role of iron overload in the development of invasive fungal diseases. Many patients at high risk for developing IPA (heavily transfused AML patients, neutropenic patients, liver and allogeneic HSCT recipients, and those receiving chemotherapy) frequently have iron overload, which has been shown to contribute to the increased susceptibility of these groups to invasive fungal infections (Alexander et al., 2006; Bullen et al., 2006; Pagano et al., 2011). In these patients, freely available

iron could arguably lead to decreased imaging sensitivity using [^{68}Ga]TAFC (or [^{68}Ga]FOXE), given the strong correlation between iron availability and uptake of radiolabeled siderophores by *A. fumigatus*. Despite these potential drawbacks, the possibility of accurately diagnosing IPA in humans using [^{68}Ga]TAFC or [^{68}Ga]FOXE imaging merits clinical evaluation.

ASPERGILLOSIS IMAGING WITH MONOCLONAL ANTIBODY JF5

Despite the abilities of mAbs to differentiate different genera and species of human pathogenic fungi, and their capacity to discriminate between inactive spores and invasive hyphae (Thornton and Wills, 2015), their use in molecular imaging of IFDs has yet to be fully realized. Imaging with antibodies has, until very recently, been the domain of cancer detection (Mestel, 2017), with limited application in infectious disease diagnostics (Rolle and Wiehr, 2017). However, recent studies have demonstrated the enormous potential of antibody-guided PET/MRI (immunoPET/MRI) technologies to dramatically improve the molecular imaging of viral (Santangelo et al., 2014), bacterial (Wiehr et al., 2016), and fungal (Rolle et al., 2016; Davies et al., 2017) infections *in vivo*, with the real possibility of precision medicine for infectious diseases in the near future (Jain, 2017). ImmunoPET/MRI marries functionality of PET with the specificity of mAbs and anatomical depiction of MRI. Any infectious disease can potentially be detected with this technology provided that high-integrity antibodies are available that have sufficient specificity for the target organism and which detect signature molecules of active infection. In the case of IPA, these diagnostic requirements have been met through the *Aspergillus*-specific mouse mAb mJF5 (Thornton, 2008) and its humanized derivative hJF5 (Davies et al., 2017). mAb mJF5, which forms the basis of the *Aspergillus* LFA (Thornton, 2008; Prattes et al., 2014; Hoenigl et al., 2017), binds to extracellular (galacto)mannoprotein antigens produced by all clinically relevant *Aspergillus* species (Thornton, 2014; Davies et al., 2017), and is able to detect IPA in humans caused by *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus*, either as single or as mixed species infections (Willinger et al., 2014). Its high-level specificity for *Aspergillus* species means that it is able to discriminate between IPA and lungs infections caused by other mold pathogens including *F. solani* (Willinger et al., 2014). Furthermore, the JF5 antigen is produced during active growth only, which means that it is able to differentiate between inactive spores present in inhaled air and invasive hyphae that infect or colonize the lung (Thornton, 2008, 2014).

ASPERGILLOSIS IMAGING WITH ANTIBODY-GUIDED POSITRON EMISSION TOMOGRAPHY/MAGNETIC RESONANCE IMAGING

Novel probes for the non-invasive detection of *A. fumigatus* lung infection based on antibody-guided PET/MR imaging (immunoPET/MRI) have recently been reported (Rolle et al., 2016; Davies et al., 2017). Administration of [^{64}Cu]DOTA-labeled mAb mJF5 to neutrophil-depleted *A. fumigatus*-infected mice allowed specific localization of lung infections when combined with PET, while optical imaging with a fluorophore (DyLight650)-labeled version of the mAb showed co-localization with invasive hyphae (Rolle et al., 2016). The [^{64}Cu]DOTA-mJF5 tracer distinguished *Aspergillus* from bacterial lung infections and, unlike [^{18}F]FDG-PET, differentiated *Aspergillus* infection from lung inflammation caused by a sterile inflammatory stimulus. The long *in vivo* half-life of [^{64}Cu]DOTA-mJF5 allows repeat imaging of infections following a single injection of the radioactive tracer and, because it is hyphal-specific, may prove useful in monitoring infection in response to antifungal treatment.

Despite specific uptake of the [^{64}Cu]DOTA-mJF5 tracer in the lungs of *A. fumigatus*-infected animals compared to uninfected controls, high liver uptake of the tracer was also evident. It was hypothesized that this liver uptake might be due to hepatic removal of the radiolabeled antibody circulating in the bloodstream, specific binding to antigen in liver tissues following shedding of soluble antigen from hyphae in the lungs, and transchelation of ^{64}Cu to liver proteins due to insufficiently strong binding to the chelator DOTA. Transchelation of ^{64}Cu to serum protein was shown not to occur, although it has been shown elsewhere that DOTA has a poor *in vivo* stability, which results in loss of the radio-metal and its non-specific accumulation in off-target tissues. In a subsequent study (Davies et al., 2017), the chelator DOTA was exchanged with the alternative ^{64}Cu chelators DOTAGA and NODAGA, which have increased *in vivo* stability. This decreased the uptake of the radiolabeled immuno-conjugates in the liver, while preserving specific accumulation in the *A. fumigatus*-infected lung. In particular, a NODAGA conjugated tracer ([^{64}Cu]NODAGA-mJF5) provided the lowest liver uptake, while enabling the greatest uptake in infected lungs.

The immunoPET/MR imaging technology based on mAb mJF5 is fundamentally translatable to human disease detection since the antibody tracks a biomarker of *Aspergillus* infection that has been clinically validated for human IPA diagnosis using LFA tests of serum and BAL fluids (Thornton, 2008; Held et al., 2013; White et al., 2013; Prattes et al., 2014; Willinger et al., 2014; Hoenigl et al., 2017). To allow translation of the antibody tracer to the clinical setting, a humanized version of the antibody (hJF5) has been generated by grafting of the mJF5 CDRs into a human IgG1 framework (Davies et al., 2017). Pre-clinical imaging with a [^{64}Cu]NODAGA-hJF5 tracer in a neutropenic mouse model of IPA (**Figure 1**) has demonstrated improved PET/MR image resolution of *A. fumigatus* lung infections compared to its murine counterpart [^{64}Cu]NODAGA-mJF5 (Davies et al., 2017), with the lowest liver uptake but highest uptake in infected lungs (**Figure 2**).

Using targeted deletion of the gene encoding UDP-galactopyranose mutase, an enzyme involved in the production of galactofuranose-containing glyco-conjugates in *Aspergillus* species, mAb JF5 has been shown to bind to

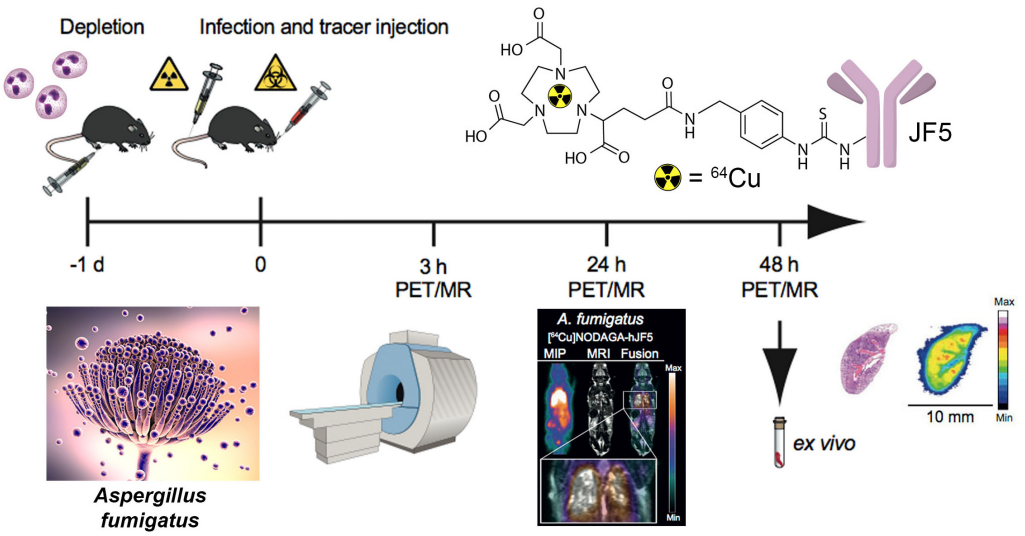

FIGURE 1 | Schematic representation of antibody-guided immunoPET/MR imaging of IPA in a neutropenic mouse model of disease. Twenty-four hours (–1 day) prior to infection with *A. fumigatus*, and administration of the PET tracer, neutropenia is induced in mice by the injection of 100 μg of the anti-Ly-6G/anti-Ly6C antibody RB6-8C5. At time 0, mice are infected by intratracheal injection with an *A. fumigatus* spore suspension and with simultaneous tail vein injection with a JF5-based PET tracer. The tracer shown is mAb JF5 conjugated to the positron emitter ^{64}Cu using the chelator NODAGA. Simultaneous PET/MR imaging of animals is then performed 3, 24, and 48 h after infection/tracer injection. *Ex vivo* bio-distribution and autoradiography are conducted after the last PET scan at 48 h. Adapted from Davies et al. (2017).

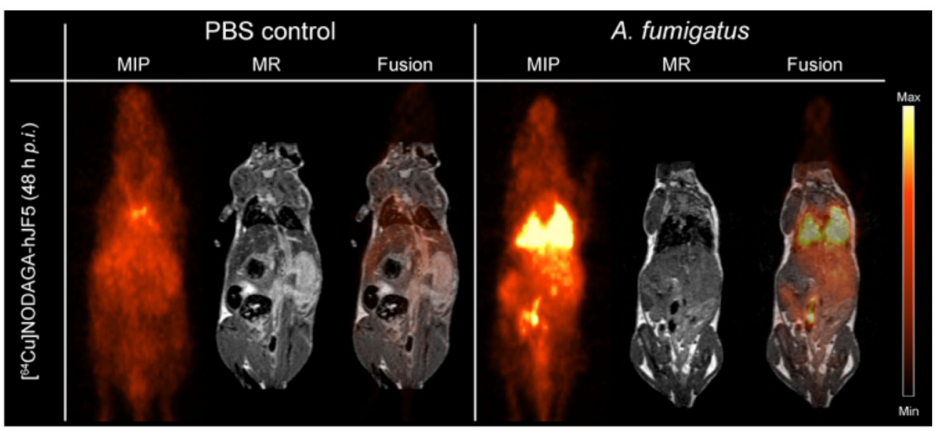

FIGURE 2 | ImmunoPET/MR imaging of IPA using the humanized *Aspergillus*-specific tracer [^{64}Cu]NODAGA-hJF5. The images show coronal maximum intensity projections (MIP), magnetic resonance (MR) imaging, and fused positron emission tomography (PET)/MR images of uninfected mice (PBS control) and mice infected with *A. fumigatus*. In order to render animals neutropenic, they received an intraperitoneal injection of the antibody RB6-8C5 and, 24 h later, were injected with the pathogen and [^{64}Cu]NODAGA-hJF5 tracer, according to the experimental procedure shown in **Figure 1**. Forty-eight hours after injection with the pathogen and humanized tracer, PET/MR imaging shows specific accumulation of the tracer in *A. fumigatus*-infected lung tissues. Image courtesy of MATHIAS Consortium.

β1,5-galactofuranose (Gal*f*), an immuno-dominant epitope present in its (galacto)mannoprotein target (Davies et al., 2017). The absence of the epitope Gal*f* in mammalian carbohydrates (Tefsen et al., 2012), in addition to the enhanced imaging capabilities of the hJF5 antibody, reduces the likelihood of the [^{64}Cu]NODAGA-hJF5 tracer binding to human structures non-specifically, while providing a highly sensitive, non-invasive, procedure for visualizing real-time *Aspergillus* infections of the human lung.

While the pre-clinical imaging studies in the mouse model of disease have shown the enormous potential of the humanized antibody tracer to detect IPA in the context of neutropenia, a number of issues have yet to be resolved. The ability of the tracer to detect chronic semi-invasive aspergillosis syndromes in the setting of underlying lung diseases such as COPD and cystic fibrosis has yet to be established, as has its ability to detect IPA in immunocompetent patients presenting with diffuse bilateral chest infiltrates, with or without cavity (Pathak et al., 2011).

In addition, while pre-clinical studies have demonstrated excellent performance of the JF5 tracer in detecting *Aspergillus* lung infections under high inoculum load, its capability in detecting extra-pulmonary infections involving, for example, the brain, spleen, and bone (Davoudi et al., 2014), and its ability to penetrate necrotic tissues, also needs to be determined. However, given the very low background uptake of the tracer in these organs (Rolle et al., 2016; Davies et al., 2017; **Figure 2**), it is likely that the [^{64}Cu]NODAGA-hJF5 tracer will be able to detect deep-seated infections in tissues other than the lung.

Despite these caveats, the humanized JF5 antibody represents an ideal candidate for molecular imaging of IPA in humans and translation of the antibody-guided imaging technology to the clinical setting. To this end, the NODAGA-labeled hJF5 antibody has entered mammalian toxicity testing and will enter first-in-human clinical trials in 2018.

CONCLUSION

Clinical diagnosis of IPA remains extremely challenging, with non-specific patient symptoms, and insufficient specificity and sensitivity of diagnostic biomarker tests. Radiological imaging of the lung is an attractive means of diagnosing invasive fungal infections since it is a non-invasive procedure, but abnormalities seen in a chest CT which are suggestive of IPA are not sufficiently specific for definitive diagnosis of the disease. Attempts have been to improve radiological detection of IPA using [^{18}F]FDG-PET, but uptake of the tracer during IPA is indistinguishable from that seen during cancer, inflammatory reactions, or during bacterial infections. The specificity of PET has been dramatically improved through the use *Aspergillus* siderophores, and mAbs conjugated to radionuclides, but all studies to date have been conducted in animal models of IPA.

For translation to the clinical setting, the *Aspergillus*-specific mAb JF5 has been humanized using CDR grafting of the mouse IgG3 heavy and light chain variable fragments into a human IgG1 framework. The humanized antibody (hJF5) is currently undergoing toxicity testing prior to clinical trials to allow immunoPET/MR imaging of IPA in humans with the *Aspergillus*-specific PET tracer [^{64}Cu]NODAGA-hJF5. Once the accuracy of the tracer has been established in human clinical trials, its cost effectiveness as a hospital diagnostic procedure for IPA, and its usefulness in monitoring disease in humans in response to antifungal treatment, will then need to be established. These are lengthy and costly investigations but, if successful, may herald a new age in molecular imaging of IPA, and could act as a paradigm for antibody-guided imaging of other invasive fungal diseases of humans. As a platform technology, immuno-PET/MR can be used to image any invasive fungal disease providing that well-characterized disease-specific antibodies are available. Highly specific mAbs have been reported for a number of the most serious mold pathogens of humans such as *Fusarium*, *Scedosporium*, and *Lomentospora* (Thornton, 2009, Thornton et al., 2015; Al-Maqtoofi and Thornton, 2016), enabling molecular imaging of invasive diseases (fusariosis and scedosporiosis) caused by these pathogens. However, it is important to note that despite the unparalleled sensitivity and specificity of immuno-PET/MRI, the high cost of its development, clinical evaluation, and implementation in healthcare systems means that it will likely only be accessible in specialist centers, and will not replace but rather complement less sophisticated diagnostic tests such as ELISA, PCR, and LFA.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Early and Non-invasive Diagnosis of Aspergillosis Revealed by Infection Kinetics Monitored in a Rat Model

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Background: *Aspergillus fumigatus* is a ubiquitous saprophytic airborne fungus responsible for more than one million deaths every year. The siderophores of *A. fumigatus* represent important virulence factors that contribute to the microbiome-metabolome dialog in a host. From a diagnostic point of view, the monitoring of *Aspergillus* secondary metabolites in urine of a host is promising due to the non-invasiveness, rapidity, sensitivity, and potential for standardization.

Methods: Using a model of experimental aspergillosis in immunocompromised Lewis rats, the fungal siderophores ferricrocin (FC) and triacetylfusarinine C (TAFC) were monitored in rat urine before and after lung inoculation with *A. fumigatus* conidia. Molecular biomarkers in high-dose (HD) and low-dose (LD) infection models were separated using high performance liquid chromatography (HPLC) and were detected by mass spectrometry (MS). In the current work, we corroborated the *in vivo* MS infection kinetics data with micro-positron emission tomography/computed tomography (μPET/CT) kinetics utilizing ^{68}Ga-labeled TAFC.

Results: In the HD model, the initial FC signal reflecting aspergillosis appeared as early as 4 h post-infection. The results from seven biological replicates showed exponentially increasing metabolite profiles over time. In *A. fumigatus*, TAFC was found to be a less produced biomarker that exhibited a kinetic profile identical to that of FC. The amount of siderophores contributed by the inoculating conidia was negligible and undetectable in the HD and LD models, respectively. In the μPET/CT scans, the first detectable signal in HD model was recorded 48 h post-infection. Regarding the MS assay, among nine biological replicates in the LD model, three animals did not develop any infection, while one animal experienced an exponential increase of metabolites and died on day 6 post-infection. All remaining animals had constant or random FC levels and exhibited few or no symptoms to the experiment termination. In the LD model, the TAFC concentration was not statistically significant, while the μPET/CT scan was positive as early as 6 days post-infection.

Conclusion: Siderophore detection in rat urine by MS represents an early and non-invasive tool for diagnosing aspergillosis caused by *A. fumigatus*. μPET/CT imaging further determines the infection location *in vivo* and allows the visualization of the infection progression over time.

Keywords: liquid chromatography, mass spectrometry, PET, *Aspergillus fumigatus*, siderophores

INTRODUCTION

Aspergillus fumigatus is a ubiquitous saprophytic airborne fungus that causes life-threatening pulmonary infections in immunocompromised hosts. *A. fumigatus* has also recently emerged as an important pathogen in immunocompetent patients in intensive care units. In 2018, David W. Denning reported that estimated 14,700,000 cases of aspergillosis occur resulting in 1,010,000 deaths every year[1]. On an annual basis, mainly invasive pulmonary aspergillosis (IPA; 200,000–400,000), chronic pulmonary aspergillosis (CPA; 1.5–3 M) and allergic bronchopulmonary aspergillosis (ABPA; 6–20 M) account for these aspergillosis cases, with mortality rates of 30–85%, 45% within 5 years, and <1%, respectively, for the treated cases of these three prevailing aspergillosis forms. These rapidly growing incidence numbers together with emerging pan-azole-resistant *Aspergillus* strains define aspergillosis as an important infectious disease for which no vaccine is yet available (Moore et al., 2017).

Methods routinely used in clinical practice to detect IPA, such as culture, serology, molecular, and radiology techniques, are often slow, invasive, and lack specificity or sensitivity (Luptakova et al., 2017). The identification of patients at high risk, appropriate prophylaxis, diagnostic surveillance, and early and reliable diagnostic tests remain important for improving patient management and underline the need for specific and sensitive diagnostic tools for the identification of IPA (Zhao et al., 2018). There are multiple as yet unvalidated molecular diagnostic tools, e.g., the detection of volatile compounds in the breath by gas chromatography and mass spectrometry (MS) (Koo et al., 2014) or proton nuclear magnetic resonance spectroscopy (Pappalardo et al., 2014). Newer molecular imaging and serology approaches have been identified, including the use of radiolabeled siderophores and antibodies, for positron emission tomography (Haas et al., 2015; Davies et al., 2017) and cytokine IL-8 detection (Goncalves et al., 2017b), respectively. All these techniques require rigorous examination as in our previous work dedicated to siderophore monitoring during *Aspergillus* infection (Pluhacek et al., 2016b). The validation of newly developed tools in a clinical setting represents a prerequisite for future inclusion in the *European Society of Clinical Microbiology and Infectious Diseases* guidelines (Ullmann et al., 2018) or the *European Organization for Research and Treatment of Cancer* criteria[2].

[1]https://www.aspergillus.org.uk/slides/introduction-and-summary-global-disease-burden (access date April 21, 2018). Introductory opening talk by David W. Denning at 8th Advances against Aspergillosis international conference (February 1–3, 2018, Lisbon, Portugal).

[2]http://www.eortc.org/guidelines/ (access date April 21, 2018).

Siderophores are low-molecular-weight iron-chelating molecules secreted by a diverse set of bacteria and fungi. Recent research has revealed that siderophores represent important microbial virulence factors that contribute to the microbiome-metabolome dialog in a host (Goncalves et al., 2017a), and the release of these factors is associated with nutrient procurement for microbial growth. After chelating iron, the uptake of ferri-siderophores is mediated by specific transporters, termed siderophore iron transporters (SITs) (Schrettl and Haas, 2011). Genomic investigation has revealed that SITs are encoded by all fungal species with genome sequences available. Consistently, non-siderophore-producing species have been shown to use SITs for the uptake of siderophores produced by other microorganisms. It is important to note that siderophore usage is confined to the fungal and bacterial kingdoms and that bacteria and mammals do not possess SIT-type transporters (Haas et al., 2008). Bacteria employ structurally different transporter types, e.g., ABC-transporters, for siderophore uptake (Schalk and Guillon, 2013). These differences among fungi, bacteria, and mammals significantly contribute to the specificity of an *in vivo* diagnostic strategy exploiting the siderophore system to improve disease diagnosis.

Further, siderophore detection in host specimens poses multiple advantages over other methods of aspergillosis detection (Savelieff and Pappalardo, 2017), including non-invasiveness, rapidity, sensitivity, and the potential for standardization upon careful quantitation. Conversely, metabolite-based methods do not allow the determination of infection location and require sample preparation and extraction, and the high performance liquid chromatography (HPLC)-MS/MS equipment is costly at the research stage of development. Although, the use of matrix-assisted laser desorption time-of-flight MS for the ribosomal typing of microbes involved in childhood diseases had to overcome the same obstacles a decade ago, now it is a common MS tool in many hospital microbiology departments (Havlicek et al., 2013).

There are several issues deserving further research in siderophore applications, such as specificity and the infection kinetics of biomarkers. TAFC and FC are considered panfungal markers because the production of these molecules by other microbial genera has been described (Pluhacek et al., 2016a; Ramirez-Garcia et al., 2018). This work is aimed to address the precise relationship between the *Aspergillus* siderophore level over time and the severity of infection in a rat model of pulmonary aspergillosis (Petrik et al., 2010).

MATERIALS AND METHODS

Rat Pulmonary Infection Model

Aspergillus fumigatus 1059 CCF was obtained from the Culture Collection of Fungi, Faculty of Science, Charles University in Prague and was maintained on yeast medium slants (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, and 0.5% glucose) at 4°C. To maintain a low siderophore content in the inoculation material, fungal conidia were harvested with Tween 80 in phosphate buffer, and the fungal hyphae were partly removed by filtration through 20 μm porosity Millipore paper (Merck Millipore, Prague, Czechia). The filtrate containing spores was then centrifuged, and wet conidia were resuspended in 0.05 M phosphate buffer with 0.1% Tween 80.

The progression of *A. fumigatus* infection was studied in a rat pulmonary infection model as described previously (Luptakova et al., 2017). Briefly, to induce neutropenia, the rats received repeated (5 days and 1 day before inoculation; 75 mg/kg) intraperitoneal injections of DNA-alkylating agent cyclophosphamide (Endoxan, Baxter, Prague, Czechia). To prevent possible bacterial superinfection, the animals were given antibiotics throughout the experiment. To minimize animal suffering, the introduction of *Aspergillus* spores into animals, ^{68}Ga-tracer injection and small animal imaging were performed under 2% isoflurane anesthesia (Forane, Abbott Laboratories, Abbott Park, IL, United States). *A. fumigatus* infection was established by the intratracheal administration of *Aspergillus* spores at two different concentrations (1×10^4 or 1×10^8 CFU/mL). A 100 μL dose of *A. fumigatus* spores was administered using the TELE PACK VET X LED system equipped with a flexible endoscope (Karl Storz GmbH & Co., KG, Tuttlingen, Germany) under inhalation anesthesia. This homogenized extract (100 μL) was examined by HPLC-MS and served as the false-negative control for the siderophore signal derived solely from the inoculation. The three control animals (CTRL1, CTRL2, and CTRL3) underwent the same treatment as the infected animals, except for the administration of the *Aspergillus* spores.

For MS detection, the urine from experimental animals was collected twice a day starting 1 day before the inoculation until the end of the experiment. The animals treated with the high dose (HD) (1×10^8 CFU/mL) of *Aspergillus* spores were sacrificed by exsanguination 2–3 days (depending on the infection progression) after the *Aspergillus* administration. The rats infected with the low dose (LD) of *Aspergillus* (1×10^4 CFU/mL) were sacrificed by exsanguination 10 days after the inoculation. At the end of the experiment, serum samples and whole lungs were also collected.

All animal experiments were conducted in accordance with the regulations and guidelines of the Czech Animal Protection Act (No. 246/1992) and with the approval of the Ministry of Education, Youth and Sports of the Czech Republic (MSMT-21275/2016-2) and the institutional Animal Welfare Committee of the Faculty of Medicine and Dentistry of Palacký University in Olomouc. The care of research staff conformed to the general guidelines for the protection of the European Community (86/609/EEC, 200/54/EC 16) and included the use of respiratory protective equipment with the standard FFP2 equivalent to an N95 HEPA filter. The animal studies were performed using female 2- to 3-month-old Lewis rats (Envigo, Horst, Netherlands). For PET/CT, the number of animals was reduced to 3 for both (HD and LD) *in vivo* experiments. For MS metabolomics, we used 7 and 9 infected animals in the HD and LD models, respectively.

Animal PET/CT Imaging

PET/CT images were acquired with an Albira PET/SPECT/CT small animal imaging system (Bruker Biospin Corporation, Woodbridge, CT, United States). Rats were intravenously (i.v.) injected with a ^{68}Ga-radiolabeled tracer, prepared as described elsewhere (Petrik et al., 2012), at a dose of 5–10 MBq corresponding to 1–2 μg of TAFC per animal. Anesthetized animals were placed in a prone position in the Albira system before the start of imaging. Static PET/CT images were acquired over 40 min starting 45 min after the ^{68}Ga-TAFC injection. A 10 min PET scan (axial field of view 148 mm) was performed, followed by a triple CT scan (axial field of view 65 mm, 45 kVp, and 400 μA with 400 projections). Scans were reconstructed with the Albira software (Bruker Biospin Corporation, Woodbridge, CT, United States) using the maximum likelihood expectation maximization (MLEM) and filtered backprojection (FBP) algorithms. After reconstruction, the acquired data were viewed and analyzed using the PMOD software (PMOD Technologies Ltd., Zürich, Switzerland). Three-dimensional volume-rendered images were obtained using the VolView software (Kitware, Clifton Park, NY, United States).

To follow the progression of *Aspergillus* infection over time, the animals were imaged at selected time points after the inoculation. The animals receiving a low *Aspergillus* dose (1×10^4 CFU/mL) were scanned 3 h after the inoculation and then on days 2, 6, and 10 post-infection. The rats infected with the high (1×10^8 CFU/mL) dose of *Aspergillus* spores were imaged at 3 h and on days 1, 2, and 3 post-infection. PET/CT images of the control animals were acquired at the beginning and at the end of the experiment (day 10).

Chromatography and Mass Spectrometry

Mass spectrometry experiments were performed using 7 high dose (HD1–HD7), 9 low dose (LD1–LD9) and 3 control (CTRL1–CTRL3) animals (biological replicates) in the HD and LD models, respectively. The siderophore extractions from urine were performed according to a protocol adapted for metabolite profiling (Luptakova et al., 2017). Briefly, the urine samples (20 μL) were mixed with the FOX-MIX standard (EMC Microcollections GmbH, Tübingen, Germany; 10 μL; 1 μg/mL in 5% ACN, ferrioxamines B, D, G, and E), which served as an internal standard, and were diluted with methanol (110 μL) to provide a final methanolic solution of approximately 80%. The samples were gently shaken and incubated overnight at −80°C. The samples were then centrifuged at 14,000 × *g* for

10 min (4–8°C), and the supernatant was transferred to a new 1.5 mL microcentrifuge tube, lyophilized to dryness and stored at −80°C.

Prior to HPLC/MS analysis in triplicate, all the samples were re-dissolved in 5% acetonitrile (60 μL). Each sample (5 μL) was loaded (20 μL loop) onto a BEH C18/1.7 μm, 2.1 mm × 5 mm VanGuard precolumn (Waters, Prague, Czechia) at a 50 μL/min flow rate. The siderophores were then separated on an analogous analytical column (1 × 100 mm) at 60°C with the following gradient: 0–1 min (2% B), 25 min (60% B), 28–33 min (95% B), and 34–45 min (2% B); where A and B represent 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively.

The positive-ion ESI mass spectra were collected using a 12 T SolariX FTICR mass spectrometer (Bruker Daltonics, Billerica, MA, United States). The ion source was operated at 4,800 V with drying gas (N_2) at a flow rate of 2.5 L/min. The collision voltage and time-of-flight values were −8.5 V and 1.2 ms, respectively. The mass spectra were collected in the CASI mode (700–1,000 *m/z* window) at an approximate 0.3 Hz frequency with a mass accuracy better than 3 ppm. The extracted ion chromatograms with 0.005 Da spectral width of Fe-ferricrocin (Fe-FC) and Fe-triacetylfusarinine C (Fe-TAFC) were used for the integration and adjusted to the response of Fe-ferrioxamine E (Fe-FOXE).

Serology

Tests for 1,3-β-D-glucan (BDG) were performed using a Fungitell kit (Fungitell, Associates of Cape Cod, Falmouth, United States) with the rat serum and urine samples. The sample was considered positive if the cut-off value was >80 pg/mL.

Statistical Analysis

The variability in the concentration of Fe-FC and Fe-TAFC within the technical and biological replicates was assessed by the coefficient of variation (**Supplementary Tables S3**, **S4**). The concentrations of Fe-FC and Fe-TAFC are presented as the mean ± standard error of mean (**Supplementary Table S4**). The diagnostic performance of LC-CASI-FTICR methodology was expressed as sensitivity and specificity for Fe-FC, and Fe-TAFC using the procedure described in literature (Trevethan, 2017; **Supplementary Table S1**). The statistical analyses were performed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, United States). Standard uptake values (SUVs) in the lungs of the imaged animals were calculated by PMOD software after the selection of corresponding region of interest (ROI) – the lungs.

RESULTS

The siderophores were quantified in the urine from infected rats using external matrix-matched calibration standards (EMC Microcollections GmbH, Germany). The calibration range for Fe-FC ranged from 0.05 to 10,000 ng/mL, which fully covered the concentration range in the actual samples (**Supplementary Figure S1**). The Fe-TAFC concentration in the urine from the infected animals was lower, and the calibration range was reduced accordingly (0.05–500 ng/mL).

High-Dose (HD) Infection Model

In the HD model, siderophore signals were clearly detected in six (HD1, HD2, HD3, HD4, HD6, and HD7) out of seven animals at 4 h post-infection (**Figure 1**). Analysis of the *A. fumigatus* inoculum revealed that negligible siderophore signal could potentially come from intra-cellular FC or extra-cellular TAFC, representing a potential background signal in this experiment. The maximum Fe-FC and Fe-TAFC false-positive signals were quantified as 322.5 and 34.2 pg, respectively, assuming that all siderophores in the inoculum (100 μL) were quantitatively secreted into the urine (0.217 μL) of an average animal.

An exponential increase in the siderophore signal was recorded 1 day post-infection, which indicated a severe *A. fumigatus* infection leading to prompt animal death. Out of seven animals, two (HD2, HD3) died during the experiment, one (HD1) or four (HD4–HD7) were sacrificed 48 or 54 h post-infection, respectively. A single immunocompromised animal resisted the infection with *Aspergillus*, revealing few aspergillosis symptoms and maintaining a constant urine Fe-FC concentration (HD1 animal in red, **Figure 1**). Interestingly, Fe-TAFC was found to be a biomarker produced less by the *A. fumigatus* CCF 1059 strain that exhibited an exponential profile identical to that of Fe-FC (**Supplementary Figure S2**). Moreover, we observed that urine samples from humans infected with *A. fumigatus* (data not reported here) have a siderophore ratio opposite to that of other *fumigati* strains. To evaluate the diagnostic strength of our highly selective LC-CASI-FTICR methodology, the sensitivity and specificity of the Fe-FC and Fe-TAFC screening were calculated for each data point of the HD kinetic experiments (**Supplementary Table S1**). The proposed LC-CASI-FTICR approach consistently provided 100% specificity and sensitivity for Fe-FC. In contrast, the sensitivity for Fe-TAFC gradually increased from 0 to 100% throughout the kinetics experiment, while the specificity remained unchanged at 100%.

In the μPET/CT scans, the first detectable radiodiagnostic signal was recorded 48 h post-infection (**Figure 2**). The presence of gray halo signs in the CT scans was apparent 72 h post-infection. SUVs in the lungs of the imaged HD animals were 0.21 ± 0.03 and 0.73 ± 0.10 percent of the injected dose per cubic centimeter (%ID/g) at the beginning and at the end of the experiment, respectively. No uptake in the lung region over time was detected in the non-infected rats in which the only visible organs were the following excretory organs: the kidneys, portions of the gastrointestinal tract and the bladder (**Supplementary Figure S3**). The SUVs in the lungs of the control animals were 0.21 ± 0.03 and 0.20 ± 0.03% ID/g at the beginning and at the end of the experiment, respectively.

Regarding the 1,3-β-D-glucan (BDG) testing, almost all the infected rat sera (HD2, HD4, HD5, HD6, and HD7) had BDG concentrations above the upper cut-off value (>617 pg/mL), indicating a severe panfungal infection (**Supplementary Figure S4**). Note that all the serum samples were collected at the end of the experiment. One-third of the control samples (CTRL1) yielded false-positive results. A better dynamic range for the

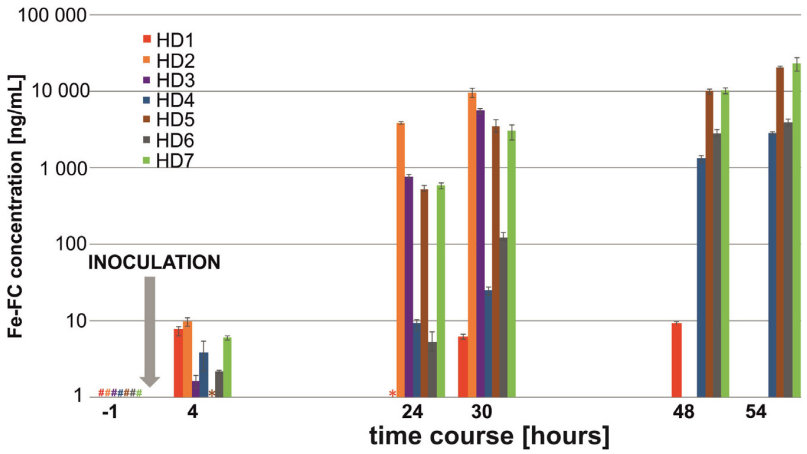

FIGURE 1 | Infection diagram indicating the progression of aspergillosis in the HD rat model with seven biological replicates. Fe-FC concentration detected in rat urine is plotted on a logarithmic scale. A hash (#) denotes the Fe-FC concentration below limit of detection, while an asterisk (*) denotes the Fe-FC concentration below limit of quantification. Missing bars at 48–54 h post-infection indicate animal death.

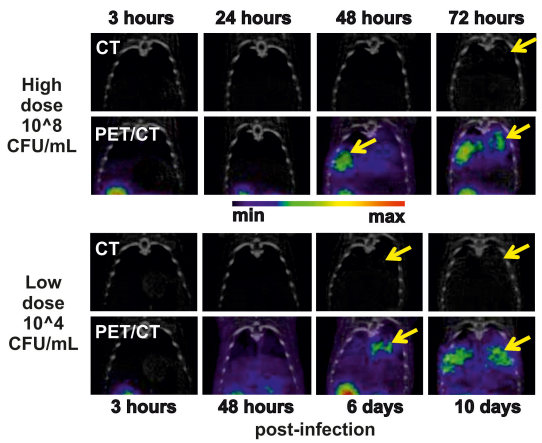

FIGURE 2 | Thoracic coronal CT and PET/CT images of ^{68}Ga-TAFC in infected (yellow arrow indicator) rats over time in experiments using high (top two rows) and low (bottom two rows) doses of conidia. The images in a row indicate the development of infection within a single animal.

analysis was documented with MS, which reflected the disease severity based on the siderophore profile. One HD animal (HD1) tolerated the infection more than the others as reflected both by BDG and MS testing (**Figure 1**).

Low-Dose (LD) Infection Model

Among nine biological replicates in the LD model, three animals (LD7–LD9) did not show any metabolite production and did not develop any signs of infection. One animal (LD6) experienced an exponential increase in metabolites and died on day 6 post-infection (**Figure 3**). All five remaining animals (LD1–LD5) had constant or random FC levels and exhibited few or no symptoms to the end of the experiment (day 10). In the LD model, the TAFC concentration was not statistically significant, while the μPET/CT scan was positive as early as 6 days post-infection (**Figure 2**, bottom). For the LD-infected rats, the SUVs in the lungs of the imaged animals were 0.22 ± 0.02 and 0.38 ± 0.04% ID/g at the beginning and at the end of the experiment, respectively.

Interestingly, the infection foci recorded by μPET/CT increased to the end of the experiment, at which time the animals were without significant infection symptoms. We speculate that fungal mycelium in the lungs may have been able to take up additional ^{68}Ga-labeled tracer independently of the invasion status of the pathogen cells (viable, dormant or dead). This interpretation agrees with the constant or even decreasing siderophore profiles in the urine as revealed by MS. The response of secondary metabolic processes to host defense mechanisms or possible antifungal treatment during fungal invasion thus remains unclear.

The BDG testing in urine was false-positive even for the time points preceding the actual inoculation of two selected rats (LD5 and HD9). Therefore, a comparison of the BDG results with those from MS was impossible (**Supplementary Table S2**). In comparison with MS, the testing of the rat sera for BDG further revealed two false-negative (LD1 and LD2) and two false-positive (LD4 and CTRL1) results. Good mutual agreement between the BDG and MS results was achieved in only the animal with the most highly developed infection (LD6) (**Supplementary Figure S4**).

CONCLUSION

An accurate assessment of the invasive status of aspergillosis and the duration of the illness are required to determine whether siderophore levels correlate with early and/or late infection. In this work, a statistical analysis with adequate technical replication (**Supplementary Table S3**) revealed that the mean Fe-FC concentration in the urine samples of the rat cohort

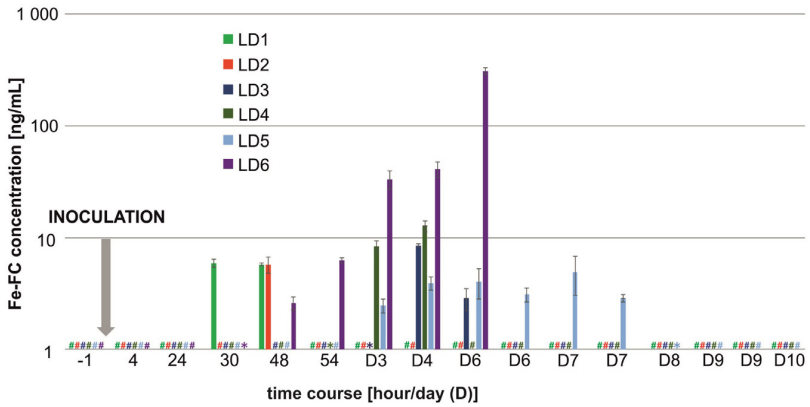

FIGURE 3 | Fe-FC concentration profiles detected in rat urine in the LD model. A hash (#) denotes the Fe-FC concentration below the limit of detection, while an asterisk (*) denotes the Fe-FC concentration below limit of quantitation. Missing bars at D6–D10 days post-infection indicate animal death. From days 6 to 9, two urine samples per day were collected. Rats LD7 – LD9 did not develop any signs of infection and no Fe-FC signal was detected throughout the LD experiment in their urine.

was 4.3 ± 1.3 ng/mL as early as 4 h post-infection in the HD model (**Supplementary Table S4**). The variability in biological replicates was expressed as the coefficient of variation (CV) and standard error of the mean (SEM). Fe-FC was observed to be a better tracer than Fe-TAFC for the *Aspergillus* strain used in our study. The signal originating from the inoculum itself was not significant. The concentrations of both the Fe-FC and Fe-TAFC metabolites in urine increased exponentially during the experiment (**Supplementary Figure S5**).

In the LD model, the signal derived from the inoculum was negligible, and the earliest detected siderophores appeared 30 h post-infection (**Figure 3**). More reliable results (for 1/3 of the animals, namely LD1, LD2, and LD6) were recorded 48 h post-infection and were reflected by the mean Fe-FC concentration of 4.7 ± 1.4 ng/mL. This observation indicates that siderophore detection by MS represents an extremely sensitive tool for early *Aspergillus* detection. Although we did not present sufficient statistics with the animal imaging, we showed that siderophore detection in the urine by MS was faster than the detection of infection by means of PET/CT. However, PET/CT imaging further determines the infection location *in vivo* and allows the visualization of the infection progression.

In the LD model, significant biological variability caused by different responses to the experimental aspergillosis was noted in the infection kinetics. A decline in siderophore production in the later stages of infection was detected, which may have been a response to increasing fungal burden and attenuating the immune system response (Savelieff and Pappalardo, 2017). In a recent study on experimental aspergillosis in mice, the first hyphae appeared after 12 h with an average length of 4.6 μm (Szigeti et al., 2018). The *ex vivo* histological examination showed that lung tissue lysis became visible 48 h post-infection and that the longest hyphae reached a length of 15 μm in infected animals 96 h post-infection.

Although many questions on the application of siderophores in *Aspergillus* detection remain to be answered, both PET and MS *in vivo* tools possess high potential for future human diagnostics. Further studies are needed, for example, to clarify the effect of antifungal prophylaxis on *Aspergillus* secondary metabolite production in human hosts. Moreover, a recent study (Carroll et al., 2016) reported that a subset of samples from patients with suspected invasive aspergillosis was TAFC-positive but galactomannan (GM)-negative. This observation suggested that TAFC is more sensitive than GM and that TAFC secretion may occur early during infection. We conclude that *Aspergillus* siderophore detection is non-invasive, rapid, sensitive, amenable to automation, and quantifiable and as such has great potential for standardization in human diagnostics.

AUTHOR CONTRIBUTIONS

AS performed the LC-MS experiments and prepared the figures. TP performed the LC-MS experiments and performed the statistical evaluation. AP prepared the inoculum and wrote the manuscript. ZN prepared radiolabeled siderophores and collected animal samples. KL wrote the manuscript. MH designed the animal imaging experiments and wrote the manuscript. MP produced the animal IPA model, collected the PET/CT data, and wrote the manuscript. VH conceived the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02356/full#supplementary-material

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