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Detection of azole-susceptible and azole-resistant *Aspergillus* coinfection by *cyp51A* PCR amplicon melting curve analysis

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Background: The AsperGenius® assay is a multiplex real-time PCR test that allows the simultaneous detection of *Aspergillus* species and identification of the most common mutations in the *Aspergillus fumigatus cyp51A* gene conferring resistance (TR_{34}/L98H and TR_{46}/T289A/Y121F) by using melting curve analysis. Mixed infections with azole-resistant and susceptible *A. fumigatus* have rarely been described.

Methods: The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) was used on bronchoalveolar lavage (BAL) samples of 91 consecutive patients with a suspected invasive *Aspergillus* infection at the Erasmus MC University Medical Center, Rotterdam.

Results: In three cases the AsperGenius® assay indicated the simultaneous presence of WT and mutant genes (two patients with TR_{34}/L98H mutation and one patient with TR_{46}/T289A/Y121F mutation) and therefore mixed infections with azole-susceptible and -resistant isolates. In one of the three cases, the mixed infection was confirmed by phenotypic antifungal testing of multiple *A. fumigatus* colonies.

Conclusions: The use of a dedicated *A. fumigatus cyp51A* resistance PCR allowed the detection of mixed infections with azole-resistant and -susceptible *Aspergillus* strains. These mixed infections may remain undiagnosed with conventional phenotypic susceptibility testing.

Introduction

Invasive aspergillosis (IA) is the most frequent pulmonary mould infection in severely immunosuppressed hosts. The introduction of voriconazole has significantly decreased the mortality of IA.[1] However, azole resistance in *Aspergillus fumigatus* is increasingly reported[2,3] and its prevalence ranges from 0.6% to 27.8% across studies.[4,5] The reported mortality of IA caused by azole-resistant strains is very high and varies between 50% and 88%.[2,6] There are several screening assays for azole resistance available (VIPcheck™, Etest) but fungal broth microdilution susceptibility testing is the standard diagnostic technique. However, cultures often remain negative. Recently, a CE-IVD-certified multiplex quantitative PCR (qPCR) was developed (AsperGenius®). It not only demonstrates the presence of *Aspergillus*, but also the presence of certain *cyp51A* mutations that confer resistance of *A. fumigatus* to azoles. *Cyp51A* encodes the cytochrome p450 sterol 14α-demethylase, the target of azoles. There are two main mutation patterns in the *cyp51A* gene that cause azole resistance: TR_{34}/L98H and TR_{46}/T289A/Y121F.[7]

In theory, mixed infections with azole-susceptible and azole-resistant *A. fumigatus* may occur as well but will only be detected if phenotypic testing of multiple colonies is done, a non-standard practice.[8] Here, we describe three cases in which a coinfection was demonstrated using *cyp51A* molecular analysis on bronchoalveolar lavage (BAL) samples.

Methods

The methodology of the AsperGenius® assay (PathoNostics, Maastricht, the Netherlands) has been described elsewhere.[6,9,10] At Erasmus Medical Centre, the AsperGenius® qPCR, a fungal culture (followed by phenotypic

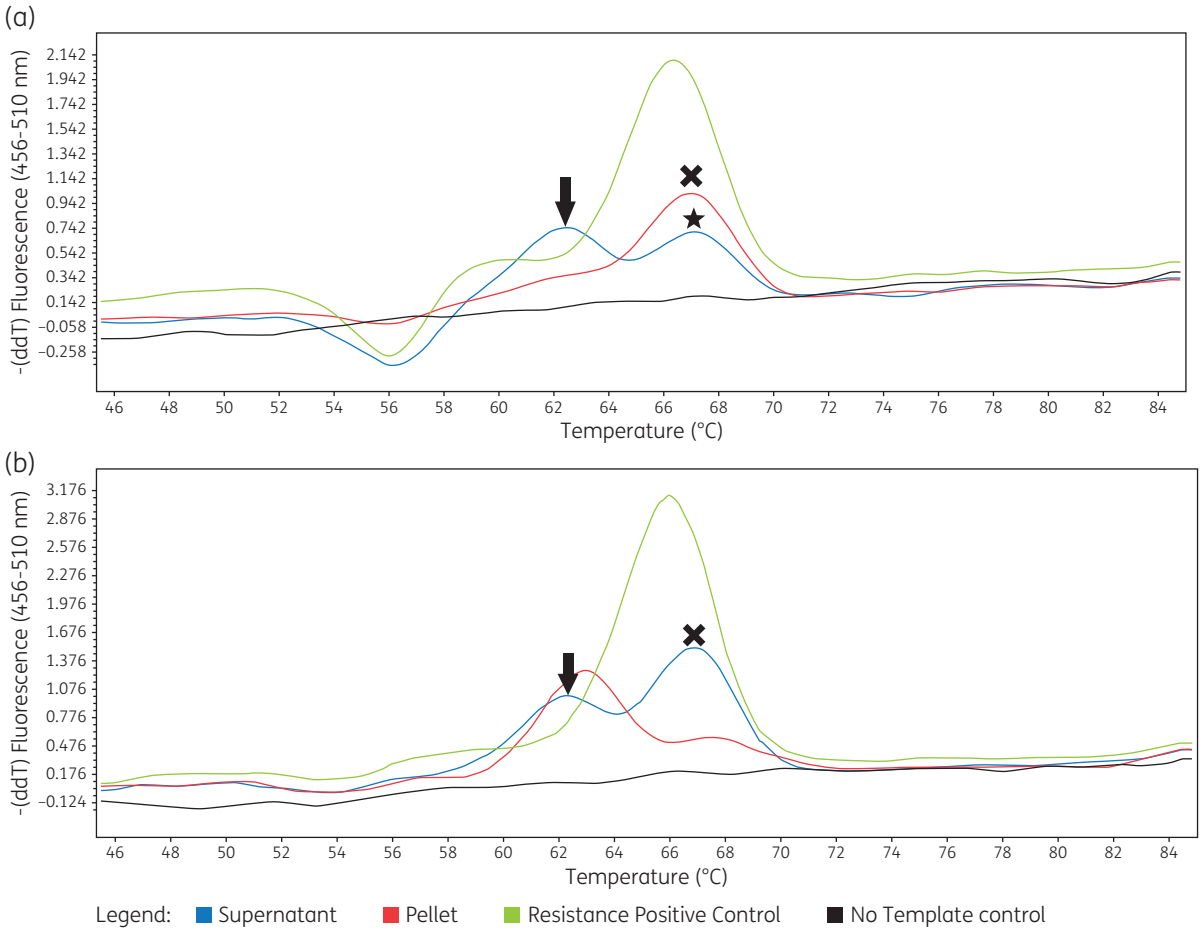

Figure 1. (a) Case 1: melting curves using the L98H mutation probe. Two melting peaks were detected for the supernatant fraction. One peak was specific for WT DNA (marked by an arrow), and the other peak was specific for L98H-mutant DNA (star). A specific melting peak was detected for the pellet extract (cross), and corresponds to the mutant-positive control, indicating L98H-mutant DNA. (b) Case 1: melting curves using the L98H mutation probe on leftover BAL. A double peak for WT (arrow) as well as mutant (cross) is present in the supernatant fraction, indicating that low concentrations of *A. fumigatus* mutant L98H DNA were present in the supernatant of the BAL.

resistance testing if positive) and galactomannan (GM) test, is routinely performed on BAL samples when IA is suspected.

Results

Between December 2014 and February 2017 the AsperGenius® assay was performed on BAL samples with a positive GM assay from 91 patients suspected of having IA. In 79% (72/91) of the patients, DNA of *A. fumigatus* or *Aspergillus* species was demonstrated. In 45 of the 72 patients, the resistance PCR was successful and could therefore differentiate between WT and the presence of resistance-associated mutations (RAMs). TR_{34}/L98H mutations were detected in eight cases and T289A/Y121F mutations were detected in three cases. Interestingly, in three additional cases, the AsperGenius® assay showed the presence of both WT and resistant *A. fumigatus* isolates. So overall, RAMs were detected in as many as 14 of the 45 (31%) patients in which the resistance PCR provided a result.

Case 1

A routine chest CT scan of a 50-year-old lung transplant recipient showed peri-bronchovascular consolidations. A BAL was performed, GM was positive (OD 4.0) and *A. fumigatus* was cultured. The only colony that had grown was susceptible to all antifungals tested (EUCAST). Voriconazole was initiated and therapeutic drug levels were documented.[11] Two weeks later he was admitted to the ICU for respiratory insufficiency and antibiotic therapy was initiated. Again, BAL sampling was performed (GM 0.5 OD, fungal culture negative) and a parainfluenza virus type 1 PCR was positive. The AsperGenius® PCR on the BAL sample showed two melting peaks in the supernatant fraction of the BAL. One peak was located at the melting temperature of WT *A. fumigatus* and the other at the melting temperature of the L98H-mutated *A. fumigatus* (Figure 1a). The patient died 24 days after initiation of voriconazole. The results of the resistance PCR only became available after the patient had died. Retrospectively, the AsperGenius® assay was also

performed on residual BAL fluid from the first BAL sample that had been collected. *A. fumigatus* WT DNA was isolated from both the supernatant and pellet fraction but the resistance PCR also showed a double peak for the L98H probe melting curve analysis (Figure 1b). Therefore, the mutated as well as the WT *A. fumigatus* had been present previously but only WT had been detected by conventional phenotypic analysis.

Case 2

A 60-year-old patient received corticosteroids for 5 months for pyoderma gangrenosum and was admitted for respiratory failure. Ceftriaxone and ciprofloxacin were started. The next day mechanical ventilation was needed. Chest CT scan showed several spherical consolidations. Oseltamivir and antifungal treatment (voriconazole and caspofungin) were added empirically. A BAL showed a GM of 5.6 OD and *A. fumigatus* was cultured. Liver toxicity led to a switch from voriconazole to liposomal Amphotericin B (L-AMB). The phenotypic resistance test (EUCAST) of the *Aspergillus* strains cultured from BAL fluid showed that five of the six *A. fumigatus* cfu had an MIC of 0.25 mg/L for itraconazole, voriconazole and posaconazole, while the MIC of the sixth was >8 mg/L for itraconazole, 4 mg/L for voriconazole and 0.5 mg/L for posaconazole. The qPCR of the BAL sample confirmed the presence of *A. fumigatus* and the resistance PCR showed melting curves specific for the mutant (T289A/Y121F) and WT DNA (Figure S1 available as Supplementary data at *JAC* online), indicating a mixed infection. The patient died of progressive multiple organ failure 10 days after the start of L-AMB.

Case 3

A 7-year-old patient, recently diagnosed with AML, was admitted for dyspnoea. A chest CT scan showed multiple nodular lesions. A BAL was performed and GM was positive (OD 3.9). Combination therapy was initiated with L-AMB and voriconazole and therapeutic voriconazole drug levels were documented. The BAL culture showed 1 cfu of *A. fumigatus* susceptible to all antifungals tested (EUCAST). AsperGenius® qPCR confirmed the presence of *A. fumigatus* DNA and the resistance PCR showed both L98H-mutant DNA and WT DNA (Figure S2). Voriconazole was discontinued after 2 weeks and L-AMB was continued for 12 weeks. A new CT scan, performed 1 month after L-AMB discontinuation, showed that the lesions had increased in size. Pathology of a CT-directed biopsy of one of the lesions showed only chronic interstitial inflammation and cultures remained negative. Unfortunately, the patient died suddenly 13 months later.

Discussion

We describe three patients with an IA infection in which WT as well as mutant *cyp51A* DNA from *A. fumigatus* was detected. In one patient, the mixed infection was confirmed by phenotypic resistance testing of multiple *A. fumigatus* colonies. To the best of our knowledge, this is the first report in which coinfection by azole-resistant and -susceptible *A. fumigatus* was detected by a molecular assay.

A. fumigatus mixed infections are rarely recognized. A recent paper described three cases of culture-confirmed *A. fumigatus* mixed infection with susceptible and resistant isolates.[8] However, the majority of BAL samples from patients with IA are culture negative. Consequently, the presence of azole resistance may remain undetected.[6,12,13] In two of our three cases BAL cultures showed growth of *A. fumigatus* but phenotypic testing failed to show resistance in two of the three cases. As growth of only one colony of *A. fumigatus* was present in Cases 1 and 3, only this colony could be tested phenotypically, which might explain the discrepancy between the resistance PCR and the culture results. Thus, performing a resistance PCR directly on BAL may yield additional information and may avoid the reporting of very major errors (i.e. a susceptible result when resistant *A. fumigatus* is present).

Treatment with voriconazole is associated with a high risk of treatment failure and mortality in patients with azole-resistant *A. fumigatus*.[2,6] Non-culture-based methods of resistance testing therefore have the advantage that appropriate antifungal therapy can be initiated immediately and hopefully reduce the risk of treatment failure.[2,6] *A. fumigatus* is genetically diverse and multiple genotypically different isolates can be obtained from multiple BAL samples of one patient.[14] Recently, a case report described the presence of different *Aspergillus* genotypes in different body compartments.[15] Mixed cultures of *A. fumigatus* strains are present in environmental and clinical samples.[8,16] One of the isolates can be dominant and can disseminate, causing disease. The presence of different isolates with different susceptibility profiles complicates the diagnosis and management of IA.[8,14]

These observations show that even if an azole-susceptible *Aspergillus* isolate is cultured, the patient can still harbour an azole-resistant isolate in regions where TR_{34}/L98H and TR_{46}/T289A/Y121F environmental strains are endemic. As described in Case 1, two BAL samples were performed. The first BAL sample was performed before and the second BAL 2 weeks after azole treatment was initiated. The first BAL sample grew *A. fumigatus* susceptible to voriconazole. The second BAL was culture negative but the PCR analysis showed a mixed infection with TR_{34}/L98H mutated and WT *A. fumigatus*. In retrospect, mixed infection could also be demonstrated on the first BAL sample. We therefore suggest that in regions where azole resistance has been described, at least five and preferably all distinct *A. fumigatus* colonies are phenotypically tested for the presence of azole resistance and, if possible, the BAL sample itself is tested for the presence of known *cyp51A* mutations that confer resistance to azoles. Importantly, it should be noted that AsperGenius® detects only the two most common mutations found in azole-resistant *A. fumigatus* and *A. fumigatus* isolates; other mutations or non-*cyp51A* mutations will remain undetected. Therefore, the assay should be used in addition to conventional susceptibility testing. Furthermore, *in vitro* simulations showed that a ratio of mutant:WT below 1:5 will also remain undetected.

In conclusion, the AsperGenius® assay can detect mixed infections with azole-resistant and azole-susceptible *A. fumigatus* isolates, enabling on-time and targeted therapy. Importantly, it can detect mixed infections when conventional fungal cultures are negative.

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Transparency declarations

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Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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