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 (TR34/L98H and TR46/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 TR34/L98H mutation and one patient with TR46/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 reported²,³ 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: TR34/L98H and TR46/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.⁹,¹⁰ At Erasmus Medical Centre, the AsperGenius® qPCR, a fungal culture (followed by phenotypic
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). TR34/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. 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, indicating that low concentrations of A. fumigatus mutant L98H DNA were present in the supernatant of the BAL.
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 information). Therefore, the assay should be used in addition to conventional susceptibility 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 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. 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. A. fumigatus is genetically diverse and multiple genotypically different isolates can be obtained from multiple BAL samples of one patient. Recently, a case report described the presence of different Aspergillus genotypes in different body compartments. Mixed cultures of A. fumigatus strains are present in environmental and clinical samples. 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.

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 TR34/L98H and TR46/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 TR34/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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Supplementary data
Figures S1 and S2 are available as Supplementary data at JAC Online.

References