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Interspecies discrimination of *A. fumigatus* and siblings *A. lentulus* and *A. felis* of the *Aspergillus* section *Fumigati* using the AsperGenius® assay

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ABSTRACT

The AsperGenius® assay detects several *Aspergillus* species and the *A. fumigatus Cyp51A* mutations TR_{34}/L98H/T289A/Y121F that are associated with azole resistance. We evaluated its contribution in identifying *A. lentulus* and *A. felis*, 2 rare but intrinsically azole-resistant sibling species within the *Aspergillus* section *Fumigati*. Identification of these species with conventional culture techniques is difficult and time-consuming. The assay was tested on (i) 2 *A. lentulus* and *A. felis* strains obtained from biopsy proven invasive aspergillosis and (ii) control *A. fumigatus* (n = 3), *A. lentulus* (n = 6) and *A. felis* species complex (n = 12) strains. The AsperGenius® resistance PCR did not detect the TR_{34} target in *A. lentulus* and *A. felis* in contrast to *A. fumigatus*. Melting peaks for L98H and Y121F markers differed and those of the Y121F marker were particularly suitable to discriminate the 3 species. In conclusion, the assay can be used to rapidly discriminate *A. fumigatus*, *A. lentulus* and *A. felis*.

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1. Introduction

Invasive aspergillosis (IA) is mainly caused by *A. fumigatus*, an *Aspergillus* species in the section *Fumigati* (Escribano et al., 2013; Neofytos et al., 2009). When diagnosis is made early and first line therapy with voriconazole is initiated promptly, a relatively low mortality is observed (Marr et al., 2015; Patterson et al., 2016; Slobbe et al., 2008). However, over the past decade, azole resistance has emerged worldwide and poses a threat as IA with azole-resistant *A. fumigatus* is associated with high mortality of 88% (Chowdhary et al., 2014; van der Linden et al., 2011; Verweij et al., 2016). Resistance in *A. fumigatus* is often caused by 2 mutation combinations (TR_{34}/L98H and TR_{46}/T289A/Y121F) in the *Cyp51A* gene that encodes for lanosterol 14α-demethylase, the target enzyme for azoles (Chowdhary et al., 2014; van der Linden et al., 2011; Verweij et al., 2016). In addition to IA caused by azole-resistant *A. fumigatus* strains, there are also cases caused by species morphologically similar to *A. fumigatus* (Alhambra et al., 2008; Coelho et al., 2011; Escribano et al., 2013; Montenegro et al., 2009; Pelaez et al., 2013; Zbinden et al., 2012). These so called intrinsic azole-resistant 'sibling species' also belong to the *Aspergillus* section *Fumigati* and can be reliable distinguished from *A. fumigatus* by molecular sequencing.

One of these sibling species was described in 2005 and was named *A. lentulus* because of its slow sporulation (Balajee et al., 2005). Subsequently, several reports described patients with IA caused by *A. lentulus* (Alhambra et al., 2008; Escribano et al., 2013; Gurcan et al., 2013; Montenegro et al., 2009; Zbinden et al., 2012). The majority of these reported patients died despite treatment. The *A. lentulus* strains cultured from these patients had higher minimum inhibitory concentrations (MICs) for voriconazole, itraconazole, posaconazole, amphotericin-B and caspofungin in comparison to *A. fumigatus*. The intrinsic low susceptibility for azoles of *A. lentulus* can be partly explained by its *Cyp51A* gene. This hypothesis is supported by (i) the observation that *A. lentulus* without a *Cyp51A* gene has significantly lower MICs for azoles, (ii) *A. fumigatus* transformants harboring the *Cyp51A* gene of *A. lentulus* showed significantly higher MICs than the *A. fumigatus* wild-type (WT) strains (Mellado et al., 2011), (iii) *Saccharomyces cerevisiae* strains expressing the *A. lentulus Cyp51A* gene were significantly less susceptible for azoles than those strains expressing an *A. fumigatus Cyp51A* gene (Alcazar-Fuoli et al., 2011). Moreover, three-dimensional models for the *Cyp51A* proteins of *A. fumigatus* and *A. lentulus* showed that there are differences in the BC loop that affect the lock-up of voriconazole (Alcazar-Fuoli et al., 2011). The *Cyp51A* protein of *A. lentulus* appears to

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have a reduced affinity for voriconazole and therefore could explain the reduced susceptibility to azoles in *A. lentulus* (Alcazar-Fuoli et al., 2011).

Another sibling species in the section *Fumigati* that sporadically causes IA is *A. felis*. To date, only 2 human cases of IA caused by *A. felis* have been reported (Coelho et al., 2011; Pelaez et al., 2013). In both cases, the strains were initially misidentified as *A. viridinutans* and later identified as *A. felis* (Alvarez-Perez et al., 2014; Barrs, 2013). These strains had high MICs to voriconazole and itraconazole, but low MICs to posaconazole and caspofungin, and variable MICs to amphotericin-B (Coelho et al., 2011; Pelaez et al., 2013). The resistance mechanism of *A. felis* remains unclear.

The occurrence of IA due to resistant *A. fumigatus* (sibling) species warrants emphasis on prompt identification of these infecting species and their resistance profile. As in vitro drug susceptibility testing is often not feasible, as cultures remain negative or sibling species fail to sporulate, molecular techniques are an option. The AsperGenius® multiplex real-time polymerase chain reaction (PCR) assay detects the genus *Aspergillus*, and *A. fumigatus* and *A. terreus* to the species level. In addition, it detects the aforementioned 2 most common mutation combinations of *A. fumigatus* that are associated with azole resistance. The assay has been validated in bronchoalveolar (BAL) fluid and serum (Chong et al., 2015; Chong et al., 2016; White et al., 2015). Here, we report 2 patients with proven IA caused by *A. lentulus* and *A. felis* species complex, respectively, and the contribution of the AsperGenius® assay to their identification.

2. Methods

The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, The Netherlands) detects *Aspergillus* species and mutations in the *Cyp51A* gene of *A. fumigatus*. The assay consists of 2 PCRs: species PCR and resistance PCR. The species PCR identifies the fungus by targeting the 28S rRNA multicopy gene. The *Aspergillus* species probe detects *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger*. The *A. fumigatus* probe detects relevant *Aspergillus* of the section *Fumigati*. An internal control is included to monitor for inhibition or manual handling errors. The resistance PCR targets the single copy *Cyp51A* gene of *A. fumigatus* and detects the TR_{34}, L98H, Y121F and T289A mutations to differentiate WT from mutant *A. fumigatus* strains via melting curve analysis. The resistance PCR does not likely detect and identify species outside the section *Fumigati* due to differences in the *Cyp51A* gene nucleotide sequence (Mellado et al., 2001).

We first performed the AsperGenius® assay on cultured sibling strains obtained from the 2 clinical cases to examine (i) if the resistance PCR yielded (characteristic) melting curves and (ii) if melting curve analysis could be a tool for interspecies discrimination of Aspergillus siblings from both WT and mutant A. fumigatus. In addition (iii), to assess the precision of the assay, a larger set of strains was tested: 6 *A. lentulus* strains and 12 *A. felis* species complex strains (5 *A. felis*, 4 *A. parafelis* and 3 *A. pseudofelis*) obtained from the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands) and the 3 control *A. fumigatus* strains (one WT, one TR34/L98H mutant, one TR46/T289A/Y121F mutant). The assay was performed on the strains in one experiment. Historical data were used to assess the precision of the assay for controls. According to the Dutch law on the research on human subjects (WGBO, art., 458) no informed consent was required for the use of clinical data from deceased patients.

The spores of the cultured strains were dissolved and 50 microliter solution was used for DNA extraction. The AsperGenius® assay was performed according to the manufacturer's instructions. Samples were processed as BAL pellets (including bead-beating), as described previously (Chong et al., 2015). The extracted DNA was tested in duplicate and a template control (blank) was included in each run to exclude contamination. For the species PCR, a sample was considered positive when one of the duplicates showed fluorescence above the threshold. For the resistance PCR, the positive control from the assay was used as a standard for the melting peaks and was tested simultaneously to determine if the melting peak represents WT or *Cyp51A* mutations. A Rotor-Gene Q (Qiagen, Hilden, Germany) instrument was used to perform the AsperGenius® assay.

3. Case Reports

3.1. Case Report 1

A 68-year-old man underwent an allogeneic hematopoietic stem cell transplantation (AHSCT) for myelodysplastic syndrome. Fifty-one days after AHSCT, he was admitted to the hospital because of non-neutropenic fever and was diagnosed with possible IA according to criteria of the European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) (De Pauw et al., 2008). Despite antifungal therapy (initially voriconazole and later liposomal amphotericin-B), he showed no improvement. As he had multiple round lung abnormalities with cavitations, a lung biopsy was performed which showed fibrosis with areas of necrosis with fungal septate hyphae. Its culture revealed *Aspergillus* section *Fumigati,* and was sent to a referral laboratory for further identification and sensitivity testing. Antifungal therapy was switched to posaconazole for proven IA with *Aspergillus* section *Fumigati*. Concurrently, patient developed severe Graft-versus-host disease and died 90 days post-transplantation. Post-mortem, the isolate was identified as *A. lentulus.* Susceptibility testing was performed according to the clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The MICs were 2 μg/ml for amphotericin-B, 2 μg/ml for voriconazole, 0.5 μg/ml for itraconazole and 0.125 μg/ml for posaconazole, respectively. The strain was deposited in the CBS-KNAW Fungal Biodiversity Centre culture collection (CBS 141342) after confirmation as *A. lentulus* by sequence analysis of the internal transcribed spacer (ITS) region, and a part of the β-tubulin (*BenA*) and calmodulin (*CaM*) gene. The generated sequences were deposited in GenBank (KX903289, *BenA*; KX903291, *CaM*; KX903293, ITS).

3.2. Case Report 2

A 54-year-old man with a history of pancytopenia and recent use of high dose prednisolone for relapsed chronic lymphocytic leukemia (CLL), was diagnosed with probable IA (specific pulmonary lesions and serum galactomannan of 0.5) according to the revised EORTC/MSG criteria (De Pauw et al., 2008) and treated with voriconazole. Follow-up high resolution computed tomography showed that the initial consolidation of the right upper lobe had regressed, but a new pleural mass was found in the left upper lobe. Biopsy of this mass was performed and cultured *Aspergillus.* Because of its poor sporulation, it was not possible to determine the sensitivity and an *A. fumigatus* sibling species was suspected. The isolate was initially identified as *A. viridinutans* by sequencing of the *BenA* gene. As the culture became positive under voriconazole (no therapeutic drug monitoring performed), the then unidentified *Aspergillus* species, was considered to be azole resistant and antifungal therapy was switched to liposomal amphotericin-B for proven IA. Despite the switch, patient had fever and developed dyspnea due to progressive infiltrates. As there were no therapeutic options to treat the patient for his CLL during an active infection and no improvement was observed after switching antifungal therapy, treatment was discontinued. Patient died of uncontrolled infection shortly thereafter. Postmortem, the infecting strain was re-identified as *A. felis* species complex (CBS 141341) based on sequencing the ITS region, and a part of the *BenA* and *CaM* gene. The generated sequences were deposited in GenBank (KX903288, *BenA*; KX903290, *CaM*; KX903292, ITS). No MICs were available.

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