Chapter 6

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

Objectives

Aspergillus lentulus and *Aspergillus felis* are sibling species within the *Aspergillus* section *Fumigati* and sporadically cause invasive aspergillosis (IA). They are phenotypically similar but can be reliable identified by molecular techniques. The AsperGenius® assay detects (1) the genus *Aspergillus*, *A. fumigatus* and *A. terreus* at the species level, and (2) *Cyp51A* mutations TR_{34}/L98H and T289A/Y121F of *A. fumigatus*. We report two cases with biopsy proven IA caused by *A. lentulus* and *A. felis,* and the contribution of the AsperGenius® assay in their identification.

Methods

A. lentulus and *A. felis* isolates were obtained from biopsy cultures. Identification was conducted using a sequence-based approach. The AsperGenius® assay was tested on these two isolates and several other control *A. fumigatus, A. lentulus* and *A. felis* species complex isolates.

Results

The AsperGenius® resistance PCR did not detect the TR_{34} target in *A. lentulus* and *A. felis* in contrast to *A. fumigatus.* In addition, melting peaks for L98H and Y121F markers differed between *A. lentulus*, *A. felis* and *A. fumigatus*. The melting curves of the Y121F marker were particularly suitable to discriminate the species. This was confirmed by testing additional isolates (three *A. fumigatus*, six *A. lentulus,* twelve *A. felis* species complex).

Conclusion

The AsperGenius® resistance PCR can be used for accurate interspecies discrimination of *A. fumigatus*, *A. lentulus* and *A. felis* of the *Aspergillus* section *Fumigati.* The latter two species are often azole resistant. The ability to identify them to the species level enables improved management.

INTRODUCTION

Invasive aspergillosis (IA) is mainly caused by *A. fumigatus*, an *Aspergillus* species in the section *Fumigati* [1,2]. The standard therapy for IA is voriconazole [3]. A relatively low mortality is observed when the diagnosis is made early and treatment with voriconazole is initiated promptly [4,5]. 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% [6-8]. Resistance in *A. fumigatus* is often caused by two common mutations (TR_{34}/L98H and TR_{46}/T289A/Y121F) in the *Cyp51A* gene that encodes for lanosterol 14α-demethylase, the target enzyme for azoles [6-8]. In addition to infections caused by azole resistant *A. fumigatus* strains, there are also IA cases caused by species morphologically similar to *A. fumigatus*, so called intrinsic azole resistant 'sibling species' [1,9-13]. These 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 as lentulus means 'somewhat slow' in Latin [14]. Subsequently, several reports described patients with IA caused by *A. lentulus* [1,9,11,13,15]. The majority of these reported patients died despite treatment. The *A. lentulus* isolates cultured from these patients had higher minimum inhibitory concentrations (MIC) values for voriconazole, itraconazole, posaconazole, amphotericin-B and caspofungin in comparison to *A. fumigatus*. The intrinsic low susceptibility for azoles of *A. lentulus* can partly be explained by its *Cyp51A* gene. This hypothesis is supported by (i) the observation that *A. lentulus* without a *Cyp51A* gene has significantly lower MIC values for azoles, (ii) *A. fumigatus* transformants harboring the *Cyp51A* gene of *A. lentulus* showed significantly higher MIC values than the *A. fumigatus* wildtype strains [16], (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 [17].

Another sibling species in the section *Fumigati* that sporadically causes IA is *A. felis*. To date, only two human cases of IA caused by *A. felis* have been reported [10,12]. In both cases, the isolates were initially misidentified as *A. viridinutans* and later identified as *A. felis* [18,19]. These isolates had high MICs to voriconazole and itraconazole, but low MICs to posaconazole and caspofungin, and variable MICs to amphotericin B [10,12]. 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*, *A. fumigatus* and *A. terreus* to the species level. In addition, it detects the aforementioned

two most common mutation combinations of *A. fumigatus* that are associated with azole resistance. The sensitivity and specificity of the assay ranged from 84.1% to 84.2% and 79.7% to 91.4% in bronchoalveolar (BAL) fluid, and was 78.8% and 100% in serum, respectively [20-22]. Here, we report two patients with proven IA caused by *A. lentulus* and *A. felis* species complex, respectively, and the contribution of the AsperGenius® assay to their identification.

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 two 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 wild-type (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 [23].

We first performed the AsperGenius® assay on cultured sibling strains obtained from the two 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 *Aspergillus fumigatus*. In addition iii), to assess the precision of the assay, a larger set of strains was tested: six *A. lentulus* isolates and twelve *A. felis* species complex isolates (five *A. felis,* four *A. parafelis* and three *A. pseudofelis*) obtained from the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands) and the 3 control *A. fumigatus* isolates (one WT, one TR_{34}/L98H, one TR_{46}/T289A/Y121F). The assay was performed on the isolates in one experiment. Historical data were used to assess the precision of the assay for controls.

The spores of the cultured isolates were dissolved and 50 microliter solution was used for DNA extraction. Samples were processed as BAL pellets (including bead-beating), as described previously [20]. 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 wildtype or *Cyp51A* mutations. A Rotor-Gene Q (Qiagen, Hilden, Germany) instrument was used to perform the AsperGenius® assay.

CASE REPORT 1

A 68-year-old man underwent an upfront allogeneic hematopoietic stem cell transplantation (HSCT) with a matched unrelated donor for myelodysplastic syndrome, refractory anemia with excess of blasts-1. The allogeneic HSCT was complicated by acute graft-versus-host-disease (GvHD) of the skin grade II, for which he was treated with mycophenolic acid 1080 mg twice daily, prednisolone 1 mg/kg daily and cyclosporine of which the dose was based through levels (therapeutic range 250 to 350 µg/l). After recovery of his GvHD, mycophenolic acid was discontinued and prednisolone was tapered to 10 mg daily. Fifty-one days after the allogeneic HSCT, patient was admitted to the hospital because of fever and renal insufficiency. Physical examination showed a temperature of 38.9 °C, but no other abnormalities. Laboratory tests revealed haemoglobin of 4.3 mmol/l, total leucocyte count of 17.3×10^9/l (absolute neutrophil count of 14.6×10^9/l, no further differentiation), C-reactive protein of 181 mg/l and creatinine of 158 µmol/l. Chest X-ray showed bilateral multiple round densities. High resolution computed tomography (HRCT) of the lungs showed multiple round lung lesions with cavitations, suspect for IA. At admission, patient was treated with piperacillin/tazobactam 4000/500 mg four times daily for non-neutropenic fever. The antibiotics were discontinued after 3 days as blood cultures remained negative and HRCT showed radiological signs consistent with pulmonary IA. Voriconazole was started 400 mg twice daily on the first day and 200 mg twice daily afterwards. A BAL was performed, but cultures remained negative. Furthermore, BAL and serum galactomannan were both negative. Consequently, IA was downgraded to 'possible' according to criteria of the European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) [24]. Despite treatment the patient showed no improvement and continued to have fever. No therapeutic drug monitoring (TDM) of voriconazole was performed. After six days of treatment with voriconazole, antifungal therapy was switched to liposomal amphotericin-B 350 mg daily (5 mg/kg) without success. In the same period, patient developed sepsis with *P. aeruginosa* due to covered perforation based on diverticulitis, for which he received conservative treatment with ceftazidime 2000 mg daily for 17 days. Also, patient had reactivation of Epstein-Barr virus (serum viral load of 6.75×10^3 geq/ml), suspect for post-transplant lymphoproliferative disease (PTLD). As differential diagnosis of the lung abnormalities was invasive fungal disease or PTLD, a biopsy of the lung abnormalities was performed. Lung biopsy showed fibrosis with areas of necrosis with fungal septate hyphae. Culture of the lung biopsy revealed *Aspergillus* section *Fumigati,* and the culture was sent to a

referral laboratory for further identification and sensitivity testing. Patient was switched to treatment with posaconazole 300 mg twice daily for proven IA with *Aspergillus* section *Fumigati*. Concurrently, patient developed progressive renal failure and GvHD of the skin and liver. His clinical condition deteriorated and he died 90 days post-transplantation. Post-mortem, the isolate was identified as *A. lentulus* and had MIC values of 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 isolate was deposited in the CBS-KNAW Fungal Biodiversity Centre culture collection (CBS 141342) after confirrmation 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 (GenBank numbers will follow).

CASE REPORT 2

A 54-year-old man was transferred to our hospital because of neutropenic fever. His medical history revealed relapsed chronic lymphocytic leukaemia (CLL). He had been treated with two regimens of chemotherapy. Initially, he had been treated with fludarabine 40 mg/m^2 and cyclophosphamide 250 mg/m^2 every four weeks, which was discontinued after two cycles because of fludarabine induced pancytopenia. Subsequently, he had been treated with 8 cycles of rituximab 375 mg/m^2, cyclophosphamide 750 mg/m^2, vincristine 2 mg and prednisolone 40 mg/m^2 every three weeks. Shortly after his second-line therapy, patient developed pancytopenia due to refractory CLL. Because initially the pancytopenia presented with only thrombocytopenia, he was treated with prednisolone 1 mg/kg due to suspected autoimmune thrombocytopenia associated with CLL. In the same period, patient was admitted to the general hospital because of polymicrobial sepsis with *Fusobacterium*, *Klebsiella oxytoca* and *Enterococcus faecium*, for which he was treated with cefuroxime 750 mg six times daily, gentamicin 300 mg daily and vancomycin 1000 mg three times daily. In addition, patient was diagnosed with probable IA (lung abnormalities on HRCT and serum galactomannan optical density index of 0.5) for which he was treated with voriconazole 200 mg twice daily. Because of these severe infectious complications, patient was transferred to Erasmus University Medical Center for further treatment of his CLL. On transfer, the prednisolone was already discontinued. Antibiotic treatment for the sepsis and voriconazole were continued. During screening for third-line chemotherapy, patient developed neutropenic fever again. Follow-up HRCT 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 lung biopsy

culture became positive under voriconazole (no TDM performed), the then unidentified *Aspergillus* species, was considered to be azole-resistant and antifungal therapy was switched to liposomal amphotericin-B 290 mg daily (5 mg/kg) for proven IA. Despite the switch to liposomal amphotericin-B, patient continued to have neutropenic 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 therapy to liposomal amphotericin-B, treatment was discontinued. Patient died of uncontrolled infection shortly after discontinuing liposomal amphotericin-B. Postmortem, the infecting isolate 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. No MICs were available for this *A. felis* species complex isolate.

RESULTS

The AsperGenius® assay was performed on isolates obtained from lung biopsy in case report 1 and from pleural mass biopsy in case report 2. Both isolates gave positive signals for the *Aspergillus* species and *Aspergillus* section *Fumigati.* First, it was examined if the resistance PCR yielded (characteristic) melting curves (figure 1) and melting temperature (Tm; table 1) values, respectively, for all *Cyp51A* markers of the case isolates in comparison to *A. fumigatus* WT and mutants (TR_{34}/L98H and TR_{46}/T289A/Y121F). The TR_{34} target was not detected in the *A. lentulus* and *A. felis* species complex strains in contrast to WT or mutant *A. fumigatus*. The L98H target showed lower Tm-values for the *A. lentulus* and *A. felis* species complex compared to WT or mutant *A. fumigatus*. Comparable Tm-values for the T289A target were found for *A. fumigatus*, *A. lentulus* and *A. felis* species complex. The Y121F target showed the most different Tm-values for all three sibling species: the Tm variation was 13.5 °C degrees between *A. lentulus* and *A. felis* species complex, 3.0 °C degrees between *A. fumigatus* WT and *A. lentulus,* and 16.5 °C degrees between *A. fumigatus* WT and *A. felis* species complex (table 1). Thus, the absent TR_{34} melting curve with the species specific Y121F melting curve indicates that melting curve analysis is indeed a tool to discriminate *Aspergillus* siblings from both WT and mutant Aspergillus fumigatus.

To assess the precision of the findings the melting curves of six more *A. lentulus* and twelve more *A. felis* species complex strains were analyzed next to the 3 control WT and mutant *A. fumigatus* strains (figure 2). The results of the TR_{34}, T289A and Y121F targets were confirmed for all strains tested. However, the Tm-values of the L98H target were more variable: three different Tm-regions were found for *A. lentulus* and two different Tm-regions for *A. felis* species complex (table 2). Based on the results, again, two targets, i.e. TR_{34} and Y121F can be used to differentiate the siblings *A. lentulus* and *A. felis* species complex from WT and mutant *A. fumigatus*.

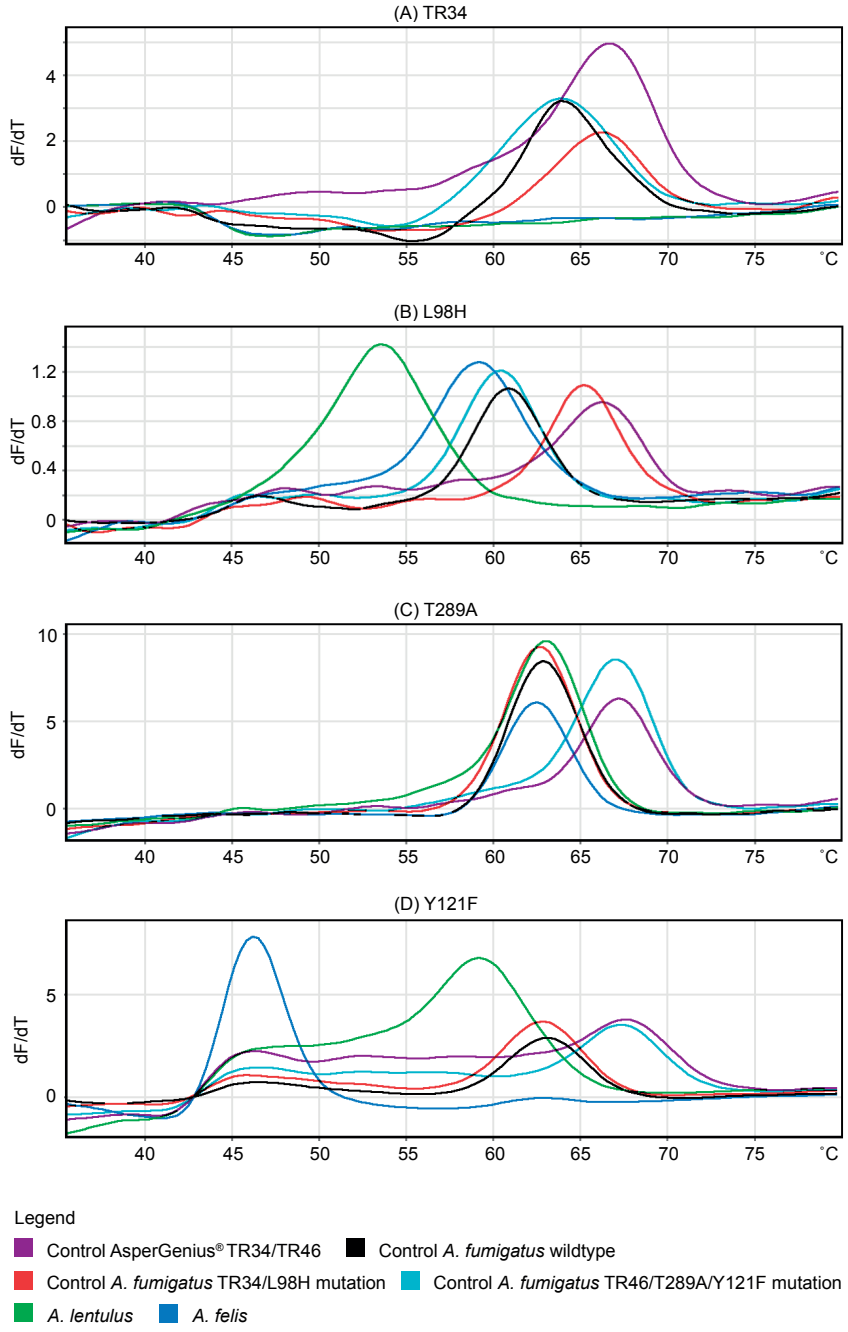

Figure 1. Melting curves of the resistance PCR on lung biopsy cultures of the two sibling species from the cases and control *A. fumigatus* isolates.

Table 1. Melting temperature (Tm) values of the resistance PCR of *A. lentulus*, *A. felis* species complex and wildtype or mutant *A. fumigatus* isolates (controls).

Name	Origen	Tm values			
		TR34	L98H	T289A	Y121F
A. lentulus isolates					
CBS 141342 (Isolate from case 1)	Clinical, NL	ND	54.0	63.0	60.0
CBS 116884	Environmental, Korea	ND	54.0	63.0	59.0
CBS 117887	Clinical, USA	ND	54.0	63.0	59.0
CBS 117886	Clinical, USA	ND	58.0	63.0	59.0
CBS 612.97	Clinical, NL	ND	58.0	63.0	59.0
CBS 117884	Clinical, USA	ND	61.0	63.0	59.5
CBS 117885	Clinical, USA	ND	61.0	63.0	59.0
A. felis species complex isolates					
CBS 141341 (Isolate from case 2)	Clinical, NL	ND	60.5	63.0	46.5
DTO 159-C7 (*A. parafelis*)	Cat, Australia	ND	59.0	62.5	46.0
DTO 176-F1 (*A. parafelis*)	Environmental, DE	ND	59.0	62.5	46.0
CBS 130245^{T} (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130247 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130248 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130249 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130246 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130250 (*A. pseudofelis*)	Cat, UK	ND	60.0	62.5	48.0
DTO 175-H3 (*A. parafelis*)	Environmental, Portugal	ND	60.0	62.5	48.0
CBS 140762^{T} (*A. parafelis*)	Clinical, Spain	ND	60.0	62.5	48.0
CBS 140766 (*A. pseudofelis*)	Clinical, Spain	ND	60.0	62.5	48.0
CBS 140763^{T} (*A. pseudofelis*)	Clinical, Spain	ND	60.0	62.5	48.0
A. fumigatus isolates (controls)					
Wildtype	Clinical, NL	64.5	61.5	63.0	63.0
Mutant TR_{34}/ L98H	Clinical, NL	66.5	65.5	63.0	63.0
Mutant TR_{46}/Y121F/T289A	Clinical, NL	64.5	61.5	67.5	68.0

Note: DE, Germany. NL, the Netherlands. UK, United Kingdom. USA, United States of America. ND, not detected.

To evaluate the precision of the Tm-value differences between wildtype *A. fumigatus, A. lentulus* and *A. felis,* we reviewed Tm-values of nine other clinical WT *A. fumigatus* strains from patients at the Erasmus University Medical Center that we had previously tested with the AsperGenius® assay in the period of January 2015 to June 2016. For the L98H target, the Tm-values of these nine strains ranged from 60.2 to 61.7 °C degrees, which overlapped with the *A. lentulus* and *A. felis.* However, the Tm-values of the Y121F target ranged from 62.5 to 63.3 °C degrees and therefore confirmed that the Tm-values did not overlap with the Tm-values of the *A. lentulus* and *A. felis* species complex isolates.

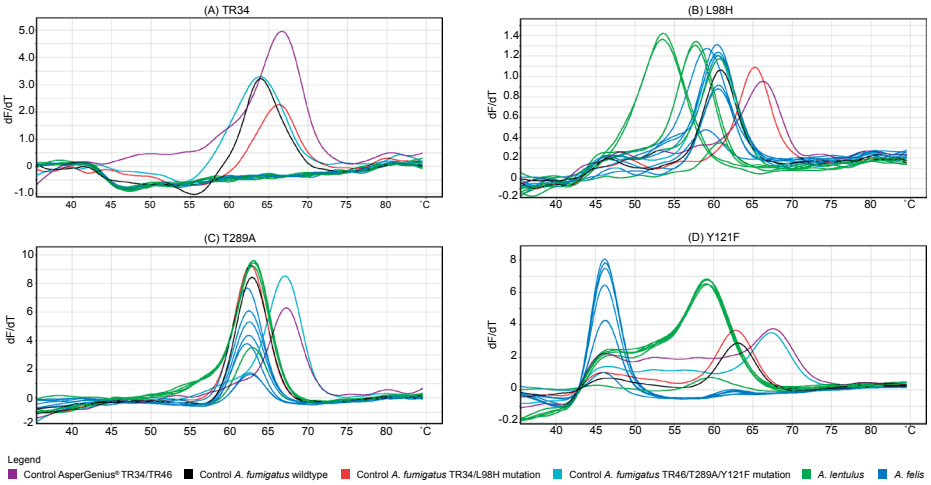

Figure 2. Precision: melting curves of the resistance PCR on the isolates provided by the Fungal Biodiversity Centre.

Table 2. Melting temperature (Tm) and interspecies differentiation of *Aspergilus* section *Fumigati* species with the resistance PCR.

	A. fumigatus WT	*A. fumigatus* TR_{34}/ L98H	*A. fumigatus* TR_{46}/Y121F/ T289A	*A. lentulus*	*A. felis*	Conclusion
L98H	61.5 (historical precision of 60.2 to 61.7)	65.5	61.5	54.0/58.0/61.0	59.0/60.5	Differentiation of *A. fumigatus* TR_{34}/ L98H with other *Fumigati* species. Differentiation of *A. fumigatus* from siblings species not possible.
TR_{34}	64.5 (historical precision of 63.5 to 65.0)	66.5	64.5	ND	ND	Differentiation of *A. fumigatus* TR_{34}/ L98H with *A. fumigatus* WT. Absence of TR_{34} can be indicative for a *Fumigati* species combined with L98H Tm value lower than that of WT (61.5).
T289A	63.0 (historical precision of 62.5 to 63.7)	63.0	67.5	63.0	62.5	Differentiation of *A. fumigatus* TR_{46}/Y121F/T289A with other *Fumigati* species. No differentiation possible of *A. fumigatus* wildtype, *A. fumigatus* TR_{34}/L98H, *A. felis*, *A. lentulus*.
Y121F	63.0 (historical precision of 62.5 to 63.3)	63.0	68.0	59.0	46.0	No differentiation of *A. fumigatus* WT with *A. fumigatus* TR_{34}/L98H. Differentiation possible of all other *Fumigati* species.

Note: ND, not detected. WT, wild-type. Tm, melting temperature.

DISCUSSION

The results reported here showed that the resistance PCR of the AsperGenius® assay not only detects the two most common mutation combinations in the *Cyp51A* gene of the *A. fumigatus* that are associated with azole resistance, but can also be used for interspecies discrimination of the *Aspergillus* section *Fumigati.* Using lung biopsy cultures from two patients with proven IA, we observed that the resistance PCR showed melting curves/Tm-values for *A. lentulus* and *A. felis* species complex that were different from those of *A. fumigatus* WT or *A. fumigatus* with TR_{34}/L98H or TR_{46}/T289A/Y121F mutations. As a result, the resistance PCR could differentiate *A. fumigatus, A. lentulus* and *A. felis* species complex. These results were confirmed on 18 additional isolates.

A. lentulus and *A. felis* species complex belong to *Aspergillus* section *Fumigati* and can sporadically cause IA. They have often high MICs for voriconazole [1,9-13,15]. For *A. lentulus*, its intrinsic low susceptibility for azoles can be explained partly by its *Cyp51A* gene [16,17]. For *A. felis* species complex, its resistance mechanism is unclear. The current results suggest that the *Cyp51A* gene of the *A. felis* is partly alike that of *A. fumigatus* as the resistance PCR gives signals for the L98H, T298A and Y121F targets. Given that voriconazole is the recommended first line therapy for IA [3], a fast identification of *Aspergillus* species that are less susceptible or resistant to voriconazole is important to select the appropriate antifungal therapy. Currently, identification of the two tested sibling species is challenging for two reasons. Firstly, most patients with IA are culture negative and are diagnosed based on a positive galactomannan in serum or BAL fluid [5]. Secondly, if a culture is available, the identification and susceptibility testing of *A. lentulus* and *A. felis* species complex are difficult as they (i) have slow or poor sporulation and (ii) are morphologically similar to *A. fumigatus* except for their inability to grow at 50 °C degrees, and (iii) one or more genes have to be sequenced to confirm their identity [14,19]. Moreover, this identification process is time consuming and often has to be performed in a reference laboratory.

The AsperGenius® assay detects several clinical relevant *Aspergillus* species and differentiates wildtype from azole resistant *A. fumigatus* directly on clinical samples, even in culture negative IA [20,22]. The assay was tested on isolates of two patients with proven IA caused by *A. lentulus* and *A. felis* species complex. As the *A. fumigatus* probe detects these two aspergilli because they are part of the section *Fumigati,* we investigated if the resistance PCR could discriminate the siblings from *A. fumigatus.* This is crucial information, as one would not want to report an isolate as sensitive, when in fact it is resistant to azole treatment (very major error). This would be the case if a sibling would be identified as '*A. fumigatus* by the *A. fumigatus* probe' and sensitive if the resistance PCR of the sibling would be conform the WT melting curve. However, the resistance PCR was able to differentiate between *A. fumigatus, A. lentulus* and *A.*

felis species complex. Not all markers of the resistance PCR were suitable. The T289A marker did not differentiate between the three sibling species. The L98H marker could differentiate WT *A. fumigatus* from TR_{34}/L98H mutant control, but was not able to differentiate *A. lentulus* or *A. felis* species complex. Remarkably, differences were observed in the L98 region between *A. lentulus* strains as well as *A. felis* species complex strains. This is probably caused by small variations in the L98 sequence region. The TR_{34} marker showed no melting curves for *A. lentulus* and *A. felis* species complex in contrast to the wildtype or mutated *A. fumigatus*, which is indicative of a sibling species when the L98H probe produces i) a melting curve and ii) the melting temperature is lower than that of the WT A. fumigatus (63 °C degrees). This is important as the resistance PCR is a single copy PCR and sometimes the TR_{34} might not yield a result whereas the L98H does, which is a known sensitivity issue with low copy numbers. Most importantly, the Y121F marker clearly differentiated *A. lentulus* and *A. felis* species complex from the wildtype as well as the mutant *A. fumigatus*, and was also able to differentiate between *A. lentulus* and *A. felis* species complex. In other words, the resistance PCR can aid in preventing to report a very major error (a sibling species as WT i.e. sensitive to azoles).

The case and control *A. fumigatus, A. lentulus* and *A. felis* species complex strains were tested in one experiment. To assess the precision of this 3 °C degrees difference of the Tm-values from *A. fumigatus* and *A. lentulus*, we reviewed historical data and no overlap was observed for the Y121F. Therefore, when both the melting curve analysis of the TR_{34} and Y121F markers are analyzed, *A. lentulus* and *A. felis* species complex could be differentiated from the *A. fumigatus* WT and *A. fumigatus* with TR_{34}/L98H or TR_{46}/T289A/Y121F azole resistance combinations.

In 2014, Sugui et al. published the results of a phylogenetic analysis on 19 isolates from the section *Fumigati*, of which nine were *A. felis* or *A. viridinutans* [25]. Within these nine isolates, three novel species were distinguished and named *A. pseudofelis*, *A. parafelis* and *A. pseudoviridinutans*. These novel species showed increased MIC values to itraconazole and voriconazole, which is similar to *A. felis* [10,12,25]. The AsperGenius® assay was tested on five *A. felis*, four *A. parafelis* and three *A. pseudofelis* strains. Due to the uncertainty of the taxonomy and no clinical consequences regarding antifungal therapy, we preferred to refer the strains as *A. felis* species complex.

There are a few limitations. First, the resistance PCR detects and identifies polymorphisms in the *Cyp51A* gene as these polymorphisms result in different melting curves. However, susceptibility testing on isolates should be done if possible, to confirm the exact phenotype as well. However, culture-based susceptibility testing is time consuming and often impossible with poorly or non-sporulating species like *A. lentulus* or *A. felis* species complex. Secondly, the resistance PCR was not tested on other siblings in the *Fumigati* section. To date, the *Aspergillus* section *Fumigati* has up to at least 63 species, of which 17 have been reported to be opportunistic pathogens to humans and/or other

mammals [26]. Except for the *A. fumigatus,* the other 16 pathogenic *Aspergillus* species, of which *A. lentulus* and *A. felis* species complex are among the more 'frequent', are described in case reports or as a very small part of collections of clinical isolates [26]. We were unable to test if more sibling species of the section *Fumigati* would have the same melting curves or Tm-values as found in the sibling species in this current report. Further research using the AsperGenius® assay and other sibling species is therefore needed.

We conclude that the AsperGenius® assay accurately detects and differentiates *A. fumigatus* WT, *A. fumigatus* TR_{34}/L98H and *A. fumigatus* TR_{46}/T289A/Y121F from the sibling species *A. lentulus* and *A. felis* species complex using the azole resistance targets TR_{34} and Y121F. *A. lentulus* and *A. felis* species complex are often azole resistant. Retrospectively, the promptly identification of these two sibling species on species/complex level could have enabled improved management in the two cases.

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