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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*.



## 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.

## 4. Results

The AsperGenius® assay was performed on strains obtained from lung biopsy in case report 1 and from pleural mass biopsy in case report 2. Both strains gave positive signals for the *Aspergillus* species and *Aspergillus* section *Fumigati*. First, it was examined if the resistance PCR yielded (characteristic) melting curves (Fig. 1) and melting temperature (Tm; Table 1) values, respectively, for all *Cyp51A* markers of the case strains 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 3 sibling species: the Tm variation was 12.5°C between *A. lentulus* and *A. felis* species complex, 4.0°C between *A. fumigatus* WT and *A. lentulus*, and 16.5°C 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 *A. fumigatus*.

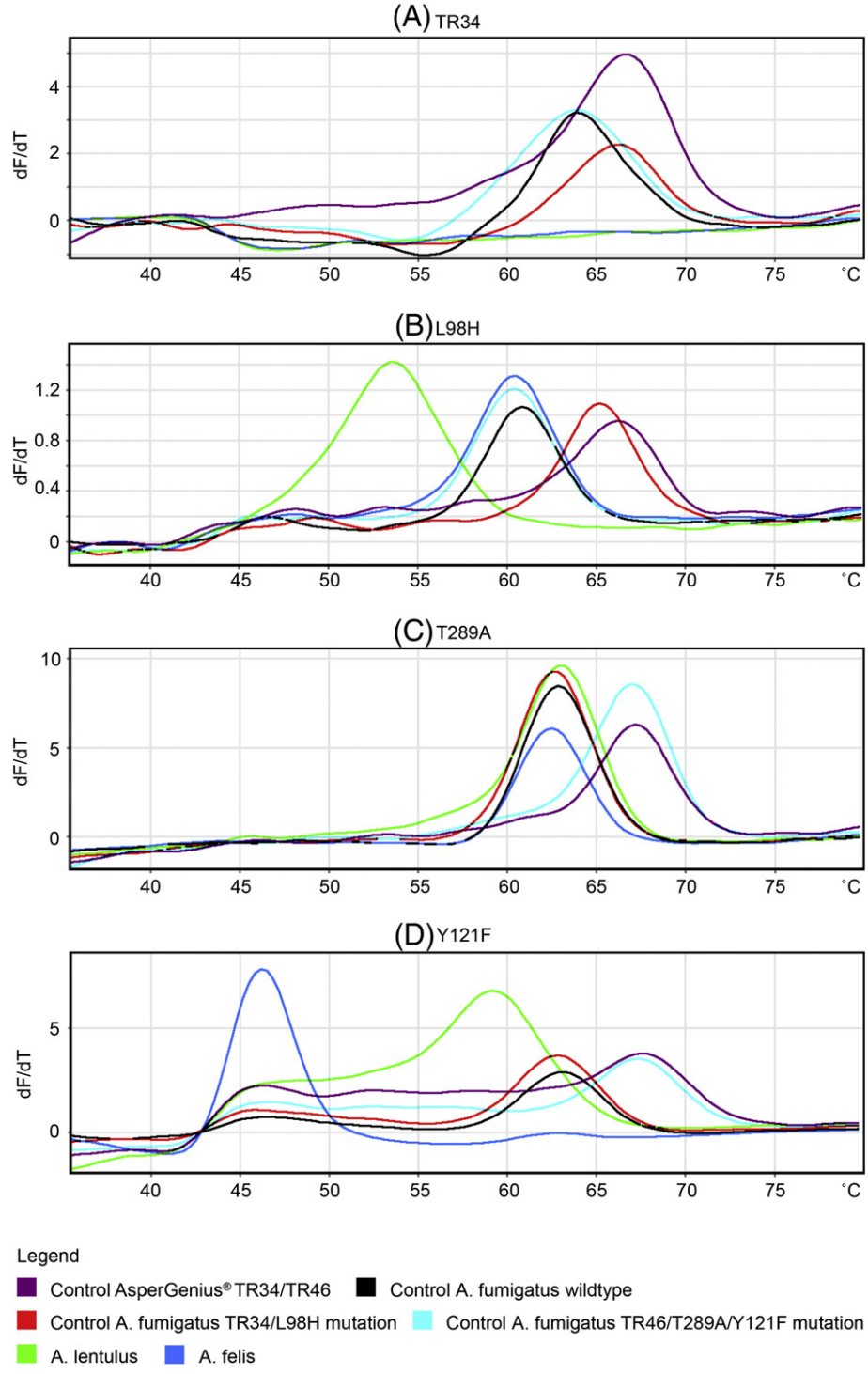


**Fig. 1.** Melting curves of the resistance PCR on lung biopsy cultures of the 2 sibling species from the cases and control *A. fumigatus* strains.

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

| Name | Origen | Tm values (°C) | | | |
|---|---|---|---|---|---|
| | | TR34 | L98H | T289A | Y121F |
| *A. lentulus* strains | | | | | |
| CBS 141342 (strain from case 1) | Clinical, NL | ND | 54.0 | 63.0 | 59.0 |
| 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 strains | | | | | |
| CBS 141341 (strain 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* strains (controls) | | | | | |
| Wild-type | 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 assess the precision of the findings the melting curves of 6 more *A. lentulus* and 12 more *A. felis* species complex strains were analyzed next to the 3 control WT and mutant *A. fumigatus* strains (Fig. 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: 3 different Tm-regions were found for *A. lentulus* and 2 different Tm-regions for *A. felis* species complex (Table 2). Based on the results, again, 2 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*.

To evaluate the precision of the Tm-value differences between WT *A. fumigatus*, *A. lentulus* and *A. felis,* we reviewed Tm-values of 9 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 9 strains ranged from 60.2°C to 61.7°C, which overlapped with the *A. lentulus* and *A. felis.* However, the Tm-values of the Y121F target ranged from 62.5°C to 63.3°C and therefore confirmed that the Tm-values did not overlap with the Tm-values of the *A. lentulus* and *A. felis* species complex strains.

## 5. Discussion

The results reported here showed that the resistance PCR of the AsperGenius® assay not only detects the 2 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 2 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 strains.

*A. lentulus* and *A. felis* species complex belong to *Aspergillus* section *Fumigati* and can sporadically cause IA. They have often high MICs for voriconazole (Alhambra et al., 2008; Coelho et al., 2011; Escribano et al., 2013; Gurcan et al., 2013; Montenegro et al., 2009; Pelaez et al., 2013; Zbinden et al., 2012). For *A. lentulus*, its intrinsic low susceptibility for azoles can be explained partly by its *Cyp51A* gene (Alcazar-Fuoli et al., 2011; Mellado et al., 2011). 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 (Patterson et al., 2016), 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 2

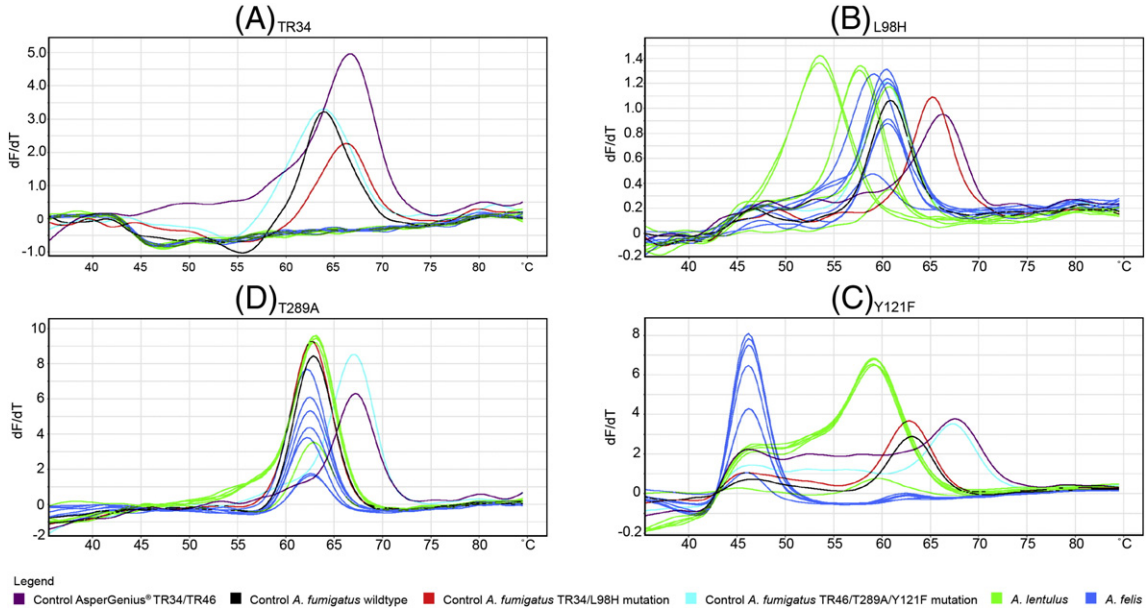


**Fig. 2.** Precision: melting curves of the resistance PCR on the strains provided by the Fungal Biodiversity Centre.

**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* wild-type, *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: Tm values are in degrees Celsius. ND, not detected. WT, wild-type.

tested sibling species is challenging for 2 reasons. Firstly, most patients with IA are culture negative and are diagnosed based on a positive galactomannan in serum or BAL fluid (Marr et al., 2015). The value of galactomannan testing for the diagnosis of *A. lentulus* and *A. felis* is unclear as only very few patients with these infections have been described (Alcazar-Fuoli et al., 2011; Alhambra et al., 2008; Coelho et al., 2011; Gurcan et al., 2013; Pelaez et al., 2013; Zbinden et al., 2012). 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, and (iii) one or more genes have to be sequenced to confirm their identity (Balajee et al., 2005; Barrs, 2013). 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 WT from azole-resistant *A. fumigatus* directly on clinical samples, even in culture negative IA (Chong et al., 2015; Chong et al., 2016). The assay was tested on strains of 2 patients with proven IA caused by *A. lentulus* and *A. felis* species complex. As the *A. fumigatus* probe detects these 2 *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 a strain 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 conform to 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 3 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 WT 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). 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 WT 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 4°C difference of the Tm-values of the Y121F from *A. fumigatus* and *A. lentulus*, we reviewed historical data and no overlap was observed. 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 strains from the section *Fumigati*, of which 9 were *A. felis* or *A. viridinutans* (Sugui et al., 2014). Within these 9 strains, 3 novel species were distinguished and named *A. pseudofelis*, *A. parafelis* and *A. pseudoviridinutans*. These novel species showed increased MICs to itraconazole and voriconazole, which is similar to *A. felis* (Coelho et al., 2011; Pelaez et al., 2013; Sugui et al., 2014). The AsperGenius® assay was tested on 5 *A. felis*, 4 *A. parafelis* and 3 *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 (Frisvad & Larsen, 2015). 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 (Frisvad & Larsen, 2015). 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. The absence of target $TR_{34}$ indicates *A. lentulus* or *A. felis.* Combined with Tm-values of the Y121F target, 59°C indicates *A. lentulus*, while 46°C indicates *A. felis.*

*A. lentulus* and *A. felis* species complex are often azole resistant. Retrospectively, the promptly identification of these 2 sibling species on species/complex level could have enabled improved management in the 2 cases.

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