The development of a real-time PCR assay for the detection of azole resistance in *Aspergillus fumigatus* positive patients

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Introduction

Invasive aspergillosis (IA) is associated with high mortality and morbidity in immunosuppressed patients. IA is difficult to diagnose and is often combatted by the use of prophylactic or empiric anti-fungal therapy, primarily using the azole class of drugs.

Azole resistance in *A. fumigatus*, as a result of mutations in the *Cyp51A* gene, is an emerging clinical problem¹ (Figure 1). The 2017 guidelines for management of invasive fungal infections from the Dutch Working Party on Antibiotic Policy has recommended that PCR is used to test for *Cyp51A* mutations².

Blood culture has limitations on the diagnosis of IA – slow to grow, with poor sensitivity and tending towards positivity late in disease. PCR is becoming a useful aid to the diagnosis of IA .

Fig 1: Epidemiology of Itraconazole Resistance in Dutch *A. fumigatus* isolates¹
Blue bars represent the number of patients with a positive *A. fumigatus* culture (left y-axis) and the red line represents the percentage of those patients with an Azole resistant isolate (right y-axis).

Bruker have launched a CE-IVD *A. fumigatus* azole resistance PCR; Fungiplex® Aspergillus Azole-R, which targets the *Cyp51A* gene, and differentiates the TR₃₄ and TR₄₆ mutations, associated with pan-azole resistance. The validation of this Kit is described herein.

Methods – Experimental Design

Observation of Analytical Specificity was achieved by extracting 10 wild type strains and 11 azole resistant strains of *A. fumigatus* and testing at a concentration of 10,000 genome equivalents per PCR.

Observation of Analytical Sensitivity was determined by spiking various concentrations of genomic DNA from either *A. fumigatus* with a TR34 mutation or *A. fumigatus* with a TR46 mutation into serum, plasma and BAL. Final burdens of 10,000, 1000, 500, 250, 100, 75, 50, 25, 10, 5 and 1 genome / 0.5mL of sample were prepared.

Clinical Performance was assessed using retrospective clinical isolates from cystic fibrosis patients.

All samples were extracted using either a Biomérieux EasyMAG or a Biomérieux Emag producing a final elution volume of 60µL. All PCRs were performed using a Qiagen Rotor-Gene Q platform

For all studies **Fungiplex Aspergillus Azole-R** PCR was performed in duplicate alongside duplicate testing using the **Fungiplex Aspergillus** PCR Kit to establish a relationship between the sensitivities of the two assays.

Results

The results of the analytical specificity study are provided in Table 1. At the high concentration tested, 10,000 ge, no cross reactivity was observed between the resistance mutation assay and the wild type strains.

Table 1: Results of the analytical specificity testing 10,000 ge per reaction

Strains	Detected by Fungiplex Aspergillus Azole-R	Detected by Fungiplex Aspergillus
Wild type <i>A. fumigatus</i> ×10	✗	✓
TR34 mutation ×10	✓	✓
TR46 mutation ×1	✓	✓

The limits of detection (LOD) achieved from the sensitivity study are provided in Table 2 per sample matrix used for preparation of the simulated samples. The reproducible LODs were determined from duplicate testing with positivity in at least 1 replicate considered significant. Non-reproducible detection was observed to 5 ge.

Table 2: Results of the analytical sensitivity testing to define the LODs

Sample	Limit of Detection (6/6 extracts detected)
TR34 marker in serum	50 ge
TR34 marker in plasma	75 ge
TR34 marker in BAL	25 ge
TR46 marker in serum	50 ge
TR46 marker in plasma	50 ge
TR46 marker in BAL	50 ge

The results of testing the retrospective clinical isolates, previously categorised as azole-resistant *A. fumigatus* with the TR₃₄ mutation, are provided in Table 3. The results show the correlation between positivity for the specific Fungiplex Aspergillus Azole-R Kit and the Fungiplex Aspergillus species Kit.

Table 3: Performance of the Fungiplex Aspergillus tests with clinical isolates

TR ₃₄ <i>A. fumigatus</i> Samples	Fungiplex Aspergillus Azole-R TR34 Result	Fungiplex Aspergillus Azole-R TR46 Result	Fungiplex Aspergillus Result
Sample 1	POSITIVE Ct = 20.2	NEGATIVE	POSITIVE Ct = 21.7
Sample 2	POSITIVE Ct = 17.8	NEGATIVE	POSITIVE Ct = 19.5
Sample 3	POSTIVE Ct = 20.7	NEGATIVE	POSTIVE Ct = 22.6

Conclusions

- Fungiplex Aspergillus Azole-R** detects and differentiates the two most common *Cyp51A* mutations associated with pan-azole resistance, TR₃₄ and TR₄₆.
- Analytical specificity is **100 %** and LODs prove it is possible to detect **low fungal burdens**, with this single copy gene.
- Using **Fungiplex Aspergillus IVD** and **Fungiplex Aspergillus Azole-R IVD** offers the potential for early diagnosis of IA and targeted therapy.

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References

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- (2) B.J. Kullberg *et al.*, SWAB Guidelines for the Management of Invasive Fungal Infections, Aug 2017