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Rapid quantification of itraconazole-resistant *Aspergillus fumigatus* in air

Running title: Airborne itraconazole-resistant *A. fumigatus*

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ABSTRACT

Solid-phase cytometry (SPC) was used to determine the total number and the number of itraconazole-resistant *Aspergillus fumigatus* cells in 60 air samples. Of the 570 *A. fumigatus* cells that were recovered, 10 (1.8%) were resistant. SPC proved more specific and rapid than culture and allowed high-troughput susceptibility testing.

MANUSCRIPT TEXT

Resistance of clinical *Aspergillus fumigatus* isolates against azole antifungal drugs has been emerging (Snelders et al., 2008). As part of their use in empiric therapy, it is important to understand whether resistance develops in treated patients or is already present in environmental isolates (Dannaoui et al., 1999).

In order to rapidly quantify *A. fumigatus* cells in air samples we developed a method based on SPC and immunofluorescence labelling as an alternative to culture-based methods (Vanhee et al. 2009). The protocol included impacting air on a water soluble polyvinyl alcohol film (PVA), dissolution of the polymer, filtration of the sample, microcolony formation at 47°C, immunofluorescence labelling, scanning of the filter membrane with a solid-phase cytometer and microscopic validation.

In a first part of the present study, this method was adapted to allow the quantification of airborne, itraconazole resistant *A. fumigatus*. To that end, itraconazole was added to the growth medium during the microcolony formation step and the remainder of the procedure was performed as described previously (Vanhee et al., 2009). In order to determine the optimal concentration of itraconazole that should be added to the medium, the MIC obtained with the CLSI M38-A2 microdilution test for 30 *A. fumigatus* reference strains was compared with the lowest concentration leading to the absence of microcolony formation. The inoculum size with the latter method was 100 cells per filter and ITC concentrations ranged from 0 to 16 µg ml^{-1}.

In a second part, triplicate 1000 l air samples were analysed using the SPC method and a culture-based method for the determination of total and resistant *A. fumigatus* counts. A schematic overview of the SPC and culture-based procedure is given in figure 1. For SPC analysis, air samples were collected on PVA and the

previously described SPC procedure was applied. Two µg itraconazole ml^{-1} was used in the growth medium. To determine the number of culturable, itraconazole resistant *A. fumigatus* cells, the same PVA solution was analysed using membrane filtration and incubation on Sabouraud agar supplemented with 2 µg itraconazole ml^{-1}. Direct collection of air onto Sabouraud agar was used as a reference method to determine the total *A. fumigatus* plate count. Plates were incubated at 47 °C and after four days colonies were confirmed as *A. fumigatus* using light microscopy. For all *A. fumigatus* strains isolated, a CLSI M38-A2 microdilution test to determine itraconazole sensitivity was performed.

The itraconazole concentration inhibiting microcolony formation for all 30 reference strains was one dilution higher than the MIC obtained with the CLSI M38-A2 microdilution assay. As the breakpoint for itraconazole resistance has been set at 1 µg/ml (Rodriguez-Tudela et al., 2008), we decided to use a concentration of 2 µg ml^{-1} in further experiments.

The results obtained after SPC analysis of triplicate air samples from 60 locations are summarized in table 1. For the three air samples collected at 34 locations, no microcolonies were detected, indicating that the number of cells was below the lower limit of quantification (LLQ) (the LLQ of the SPC assay is 4 cells m^{-3} as only one fourth of the sample on which 1000 l of air was impacted, is filtered). *A. fumigatus* cells were found in all air samples for 13 other locations while the number of cells was below the LLQ for at least one air sample for the remaining 13 locations. For two locations (house during removal of a wooden floor and sawmill 3) we were not able to determine the number of *A. fumigatus* cells as too many microcolonies

were present on the filter and therefore the upper limit of quantification (ULQ) was exceeded.

The average number of *A. fumigatus* cells ranged between 1 and 436 cells m^{-3} and a total of 570 *A. fumigatus* microcolonies were detected. The number of airborne *A. fumigatus* cells was variable as considerable differences were observed between replicate air samples. Additionally, no clear correlation between the number of cells and the locations was observed.

At eight locations (bathroom 1, house during removal of a wooden floor, sawmill 3, park 2, forest 1, underneath a bridge 3 and 4, and poultry house 2) a low number of ITC resistant *A. fumigatus* cells was detected. In total, 17 microcolonies of resistant *A. fumigatus* cells were found. Ten of these microcolonies were detected in samples collected at locations for which the total number of *A. fumigatus* cells was enumerated, while an additional seven were retrieved from the two locations for which the total number of *A. fumigatus* cells exceeded the ULQ.

By direct impaction onto Sabouraud agar, *A. fumigatus* was isolated from only three (attic 1, park 2 and pigeonary 2) of the 60 locations examined. Additionally, for 35 locations, no readable results could be obtained because culture plates were overgrown with other fungi. After incubation of a membrane filter on Sabouraud agar supplemented with 2 µg ITC ml^{-1}, five *A. fumigatus* strains resistant to ITC (MIC > 16 µg ml^{-1}) were isolated (attic 1 and park 2), but for nine locations interference of other fungi remained problematic, even on plates with ITC.

Comparison of the results obtained using SPC and culture clearly indicates the important advantages of SPC. The high specificity of the method allowed for the accurate quantification of *A. fumigatus* in air samples, even in the presence of a high number of other fungal cells. Secondly, the short time to result (24 h) makes SPC

superior to culture which requires growth of the fungus (72 – 96 h), isolation, identification and susceptibility testing (an additional 48 h). Finally, the possibility to use SPC to investigate the susceptibility of all *A. fumigatus* cells present in the sample at the same time allows for a large-scale study of the prevalence of resistance among airborne *A. fumigatus*.

In several other studies it was demonstrated that triazole-resistant *A. fumigatus* isolates occur, albeit at a low frequency (± 2%). In some of these studies only clinical samples were investigated (Chryssanthou, 1997; Gomez-Lopez et al., 2003; Verweij et al., 2002) while in others the susceptibility of both clinical and environmental isolates was determined (Arauja et al., 2007; Guinea et al., 2005; Meneau and Sanglard, 2005; Verweij et al., 1998). In the latter studies, no difference in susceptibility between clinical and environmental isolates was observed. We found a similar prevalence of resistance (1.8%) among isolates from air samples, indicating that the environment may serve as a reservoir of itraconazole-resistant *A. fumigatus*, as recently suggested (Snelders et al., 2009).

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125 **Table 1:** Overview of the results obtained after SPC analysis of triplicate air samples
126 from 60 locations expressed as the average total and resistant number of *A. fumigatus*
127 cells per m^3 ± the standard error (SE).

Location	Number of *A. fumigatus* cells m^{-3} (average ± SE)	
	Total	Resistant
False ceiling 1	-[a]	-
False ceiling 2	-	-
Attic 1	-	-
Attic 2	-	-
Bathroom 1	5 ± 4[b]	1 ± 1[b]
Bathroom 2	-	-
Bathroom 3	4 ± 2[b]	-
Bathroom during demolition	-	-
House during removal of a wooden floor	> ULQ[c]	1 ± 1[b]
House after removal of a wooden floor	40 ± 16	-
House during sanding of a wooden floor 1	23 ± 11	-
House during sanding of a wooden floor 2	20 ± 10	-
Demolition of a roof	4 ± 2[b]	-
Sawmill 1 (winter)	13 ± 13[b]	-
Sawmill 2 (winter)	-	-
Sawmill 3 (spring)	> ULQ[c]	8 ± 2
Compost bin (winter)	-	-
Compost bin (spring)	43 ± 19	-
Greenhouse	-	-
Garden centre	3 ± 1[b]	-
Park 1	1 ± 1[b]	-
Park 2	436 ± 55	5 ± 1
Forest 1	5 ± 4[b]	1 ± 1[b]
Forest 2	21 ± 17[b]	-
Mediterranean botanical garden 1	-	-
Mediterranean botanical garden 2	-	-
Subtropical botanical garden	-	-
Tropical botanical garden 1	9 ± 1	-
Tropical botanical garden 2	-	-
Tropical botanical garden 3	7 ± 1	-
Hay barn	-	-
Underneath a bridge 1A	-	-
Underneath a bridge 1B	-	-
Underneath a bridge 2A	-	-
Underneath a bridge 2B	7 ± 5[b]	-
Underneath a bridge 3	17 ± 4	1 ± 1[b]
Underneath a bridge 4	27 ± 5	3 ± 1[b]
Underneath a bridge 5	8 ± 2	-
Underneath a bridge 6	-	-
Pigeonry 1	8 ± 8[b]	-
Pigeonry 2	-	-
Pigeonry 3	43 ± 24	-
Pigeonry 4	-	-
Chicken coop	3 ± 1[b]	-
Poultry house 1	3 ± 3[b]	-
Poultry house 2	11 ± 11[b]	1 ± 1[b]
Horse stable	-	-
Pigs stable 1	-	-

Pigs stable 2	-	-
Cow's stable	-	-
Intensive care unit (ICU) 1A	-	-
ICU 2A	-	-
ICU 3A	-	-
ICU 4A	-	-
ICU 5A	-	-
ICU 1B	-	-
ICU 2B	-	-
ICU 3B	-	-
ICU 4B	-	-
ICU 5B	-	-

[a]: No microcolonies were detected, indicating that the number of cells was below the lower limit of quantification (LLQ = 4 cells m^{-3}) of the SPC assay.

[b]: If for one of the replicates the LLQ was not reached, we calculated the average ± SE by assuming that no *A. fumigatus* cells were present in that sample.

[c]: The upper limit of quantification (ULQ) of the SPC assay was exceeded.

Figure 1: Schematic overview of the SPC and culture-based procedures used in this study.

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