Catching more air

An effective and simple-to-use air sampling approach to assess aerial resistance fractions in *Aspergillus fumigatus*

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Abstract

Triazole resistance in the airborne human fungal pathogen *Aspergillus fumigatus* is a significant human health problem as the environmental use of triazoles has selected for cross-resistance to life-saving clinical triazoles in medicine. Despite the environment being well established as a source of triazole resistant *A. fumigatus* spores, the aerial transmission from the environment to patients remains unclear. This is mainly due to the lack of an affordable, reliable, and simple-to-use method for wide-scale environmental air sampling. Previous methods were ineffective in capturing sufficient *A. fumigatus* colony-forming units (CFUs) to allow the quantitative assessment of aerial triazole resistance fractions. Here we show that 14 days of exposure of sticky seals to the air along with selectively culturing colonies directly from the seals proved key for increasing CFUs per sample. We also tested the use of delta traps for passive outdoor spore capture and show that together with the sticky seals and selective culturing, they are a simple and effective tool for outdoor air sampling. We suggest the use of this cost-effective air sampling technique for wide-scale outdoor sampling to map resistance fractions, assess health risks, and pinpoint environmental resistance hot- and coldspots. Doing so will close the knowledge gap between environmental triazole resistance selection and the transmission to patients.

Introduction

Aspergillus fumigatus is a saprophytic fungus that is ubiquitous in soils rich in organic matter (Kwon-Chung and Sugui 2013). *A. fumigatus* is especially abundant in plant waste heaps where its wide temperature tolerance allows it to grow under the high temperatures generated during the composting process and form extremely high numbers of asexual conidiospores (Altenburger et al. 2014; Zhang et al. 2017; Schoustra et al. 2018). Due to their small size and hydrophobic nature these spores readily disperse through the air when plant waste heaps are manually disturbed (Sewell et al. 2019; Williams et al. 2019). Plant waste heaps are considered the optimal habitat of *A. fumigatus* but it is also an opportunistic pathogen that can infect or colonise the lungs of humans and other mammals (Hohl and Feldmesser 2007; Kwon-Chung and Sugui 2013). Most humans are thought to inhale roughly 100 spores per day (Ascioglu et al. 2002; Kwon-Chung and Sugui 2013). Even though these spores are effectively removed by the immune systems of most individuals, they can cause symptoms ranging from mild allergies to often lethal acute invasive aspergillosis infections, depending on the health status of an individual's lungs and their immunity (Verweij et al. 2009; Kwon-Chung and Sugui 2013). There is a limited pool of antifungals available to treat *A. fumigatus* infections, with triazoles being the most extensively used class.

Triazoles are not only extensively used medically but are also widely utilised as fungicides in the environment, notably for the protection of wood, crops, or ornamentals (Schoustra et al. 2019). The isolation of *A. fumigatus* resistance genotypes from plant waste material and soils that could be linked to those found in patients has been instrumental in establishing the environment as a source for triazole resistance. Crucially the development of selective media such as the flamingo type (Zhang et al. 2021) has not just allowed for the isolation, but also accurate counts of *A. fumigatus* colony forming units (CFU). Plating aliquots of a sample on a selective medium with and without different types of triazoles has enabled researchers to not just qualitatively but also quantitively assess resistance by determining the resistance fractions. Assaying different types of plant waste material in terms of both absolute CFUs and resistance fractions has led to the conceptualisation of triazole resistance hotspots. Such hotspots are defined as environments ideal for *A. fumigatus* growth and reproduction in the presence of triazoles, which drives selection for resistance (Schoustra et al. 2019).

Assessing CFUs and resistance fractions in plant waste material is useful for the identification of sources of environmental resistance, but the direct health risk is posed by inhaled airborne spores. Efforts have been made to assess aerial resistance, predominantly in hospital environments (Reboux et al. 2014; Demuyser et al. 2019; Godeau et al. 2020). The methods vary from air compaction of 250L of air onto agar plates (Reboux et al. 2014), collection by homemade dust vanes (Godeau et al. 2020), and overnight exposure of agar plates (Demuyser et al. 2019). These methods have all proven useful in the qualitative detection of *A. fumigatus* conidiospores and resistance genotypes, and have even been used to quantitively estimate colony forming units (CFUs) per m^3 (Demuyser et al. 2019). However, reported CFUs per sample are generally quite low (<10) in these hospital building studies. When estimating a proportion of a natural population, in our case the fraction of triazole-resistant CFUs, an adequate sample size is key for accuracy. Specifically, because at low (<25) sample sizes sampling error increases rapidly to the point that sample estimates become altogether uninformative on quantitative differences in resistance fractions (Davis 2014; Baveja and Aggarwal 2017). Quantitative assessment of aerial resistance fractions is, however, key in identifying sources of triazole resistance, possible transmission routes to patients, and the health risk they pose across spatial scales. This leads to the question, how can we increase CFUs in air samples to estimate resistance fractions with accuracy?

Increasing the volume of air a plate is exposed to during air compaction could be effective for assessing resistance fractions. Fraaije et al. 2020 reported CFU counts ranging from between five and 69 on triazole-negative plates compacted with 5000L of outdoor air, which is higher than the one to two CFUs on average that were reported by Reboux et al. 2014, where plates were impacted with 250L of air. However, cycling 5000L of air required the machine to run for almost an hour and the CFUs were still not consistently above 25. A second approach could be to simply increase the exposure time of agar plates to increase CFU yield. However, the problem with exposing agar plates for extended periods of time to sample air is that they may dry out and that over time microbes other than *A. fumigatus* will start to grow at ambient temperatures, even on selective medium. This therefore also makes this approach impractical to estimate resistance fractions.

A second issue with air compaction or other types of air sampling machines capable of sampling large volumes of air is that they are costly (Fraaije et al. 2020). This is no major hurdle in longitudinal studies at set locations such as hospitals but combined with their inconsistent CFU yield, makes them impractical and unreliable for more extensive environmental surveys aiming to estimate resistance fractions at varies geographic scales. Shelton et al. 2020, therefore, proposed a (citizen-science) sampling method through which anyone could readily and cheaply collect airborne spores at home. In brief, this method involved attaching two halves of a MicroAmp™ clear adhesive film (Applied Biosystems™, UK) to a windowsill with poster putties and exposing them to the outside air for six to eight hours on a predetermined sampling day. Subsequently, the film would be re-covered and sent back to the lab by Freepost. In the lab, the film would then be pressed onto an agar plate and cultured at 43°C. In principle, this method proved effective at capturing *A. fumigatus* CFUs on a nationwide level, but at ~1.25 CFU per sample, the counts were far too low to assess resistance fractions.

This study aimed to develop and validate a method that is both simple and affordable in its application, yet effective in collecting sufficient *A. fumigatus* CFUs so that local resistance fractions could be reliably determined. Building upon the method of Shelton et al. 2020, we utilised the fact that sticky seals, similar to agar plates, are capable of capturing spores, but are less prone to drying out, weathering, or unwanted microbial growth. The seals may thus remain effective at spore capture for longer exposure times, such as 14 days. However, prolonged exposure also means potential increased exposure to other microbes. Here we tested a culturing method where we used the highly selective Flamingo medium (Zhang et al. 2021) by pouring it directly on top of exposed seals. Besides exposing sticky seals indoors, we also tested whether the use of foldable delta traps could be combined with the use of the sticky seals in a flexible, effective protocol for cheap and large-scale outdoor spore capture, including citizen-science initiatives.

Materials and Methods

Sample processing, culturing, and resistance fraction calculation

All samples were taken with pairs of seals placed side-by-side, one seal was subject to an itraconazole (4mg/L) treatment and the other to an triazole-negative control treatment. Under sterile conditions, the covers were removed from the seals and then individually placed in square Petri dishes with vents (120mmX120mmX17mm), sticky-side-up, again only touching the non-sticky strip with clean hands. Once all seals were placed in this manner, flamingo medium (Zhang et al. 2021) was poured into the plate such that the entire seal and all of the plate was covered with a thin layer of medium. Crucially, the medium was prepared as described in (Zhang et al. 2021) except that the medium, after the autoclaving step, was allowed to cool overnight in an incubator set at 60°C before adding the antimicrobial supplements and pouring the medium. This ensured that the agar was liquid, but still within the temperature tolerance range (Kozakiewicz and Smith 1994) of *A. fumigatus.* The plates were then left to solidify at room temperature for 30 minutes before being incubated at 48°C for a minimum of three days, up to four days for slow-growing colonies on itraconazole plates, for identification of CFUs as *A. fumigatus*. See Figure 1 for a visual representation of the culturing method. CFUs were then counted on all plates and the CFUs on the control and itraconazole plates were used to calculate resistance fractions according to the equation: R-fraction = (# CFU itraconazole plate / # CFU control plate) *100.

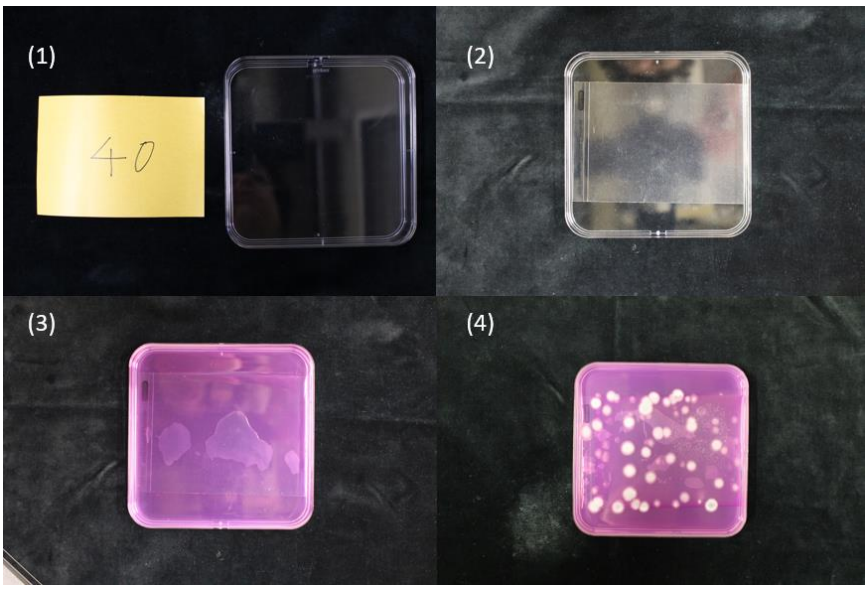

Figure 1: A visual representation of the culturing method used in this study. (1) The cover was removed from each seal. (2) Each seal was then placed in a Square Petri dish, sticky side facing up. (3) Flamingo medium (at 60°C) was now poured over each seal. (4) After the medium had solidified, the plates were incubated for three to four days at 48°C, before CFUs were counted.

Use of delta traps

We tested two types of foldable delta traps, of three brands Biotop (Livron-sur-Drôme, FR), Koppert (Berkel en Rodenrijs, NL), and Biogrow (Leuven, BE). The traps of Biogrow and Koppert were practically identical in design, with part of the bottom folding into the roof of the trap to anchor it. This reduces the size of the openings on the sides of the trap, potentially reducing air flow, but also provides some extra shelter from the elements to the sticky seals inside. We refer to these as *semi-open*. The Biotop traps had a simpler design, with the sides completely open, we refer to these traps as *open* (See Figure 2). The sampling packages given to the participants of this study included both an open and at least one semi-open delta trap to compare their efficacy.

Figure 2: Picture of a semi-open Biogrow trap (left), and an open Biotop trap (right), side by side for visual comparison.

Sampling package

Besides the traps, sampling packages consisted of a zip-lock bag, containing 6 BIORAD microseal 'B' seals (of which the length was cut down to 12cm by removing one of the non-sticky ends) in sets of two per sample (indoors, open trap, semi-open trap) and a piece of paper for labelling, two 30 cm pieces of rope to tie up the delta traps, and a strip of 8 poster putties. The yellow covers of the seals were labelled per set to signify they are part of the same set and for ease of downstream processing. These materials were all packaged within a 22.9 by x 33.4 cm envelope, a size that may readily send as a letter by post.

Third-party sampling instructions

For indoor sampling, the participants were instructed to select an indoor site that was out of direct sunlight and out of reach of pets or children, ideally within a well-ventilated room. The instructions also detailed that the sampling site surface should be cleaned before sticking the non-adhesive side of the seal to it with a putty, and it should subsequently remain undisturbed for the duration of seal exposure. For outdoor sampling, the instructions were to select an unsheltered outdoor location where a delta trap could be securely strung up (e.g. a tree branch, a doorstopper, laundry line, railing of a balcony), out of reach of children or pets. Participants were now directed to expose the seals by first firmly pushing one or two poster putties to the non-adhesive side of the seal. Using the putties, the seals were subsequently to be stuck onto either the desired sampling site indoors or in an unfolded delta trap for outdoor sampling. The seal covers were now to be removed and kept in the clean zip-lock bag in which the seals were handed out. The participants were now directed to hang up their delta traps at the desired outdoor sampling site. The seals were now exposed to the ambient air for ~14 days.

Exposure and sample recovery

For this study sampling was done inside and directly outside the houses of five participants, which were all situated in the centre of the Netherlands, with Wageningen as the focal point. All seals were exposed for 13-15 days between July 2nd and July 19th 2022, at the participant's discretion. After exposure, the participants were asked to gently remove the seals from the delta traps, with the explicit instruction to only touch the non-adhesive strip on the side with washed hands. The covers were now placed back onto the seals with their non-labelled side, which will critically allow the covers to be removed again during sample processing. The re-covered seals were now collected in the zip-lock bag, along with a labelled note with the sampler's postal code and start and end date of sampling. Together with the refolded delta trap, these materials were put back in the C4-size envelope and returned to the lab.

Data analysis

For the analysis of the control CFU count data we used a GLM with a Poisson distribution, the model included sampling method and sampling site as fixed effects. The final model was tested for overdispersion. A *post hoc* test was performed to test the significance of specific contrasts. For the analysis of the resistance fraction we used an ANOVA, including sampling method and sampling site as independent variables. We checked for violations of normality and homogeneity of variance in the data. Statistical analysis and data visualisations were performed in R version 4.1.3 (R Core team 2022), using the following R packages: AER 1.2-10 (Kleiber and Zeileis 2008) emmeans 1.8.1-1 (Lenth 2022) AICcmodavg 2.3-1 (Mazerolle 2020) ggplot2 3.3.6 (Wickham 2016), ggpubr 0.4.0 (Kassambara 2020), dplyr 1.0.9 (Wickham et al. 2022). The R code is made available at: https://git.wur.nl/korte058/catching_more_air

Results

Selective growth of *A. fumigatus* from seals through the medium

Culturing the samples by pouring flamingo media over them proved effective at growing countable *A. fumigatus* colonies (see Figure 3). Some slow-growing colonies, especially on itraconazole plates, may take longer to sporulate (>four days) when cultured this way as they would only do so once they have grown up through the agar. These colonies could however still be readily identified as *A. fumigatus* by their compact and circular morphology. This was because few fungi other than *A. fumigatus* and morphologically distinct *Mucorales* species grew on the medium, as detailed in Zhang et al. 2021.

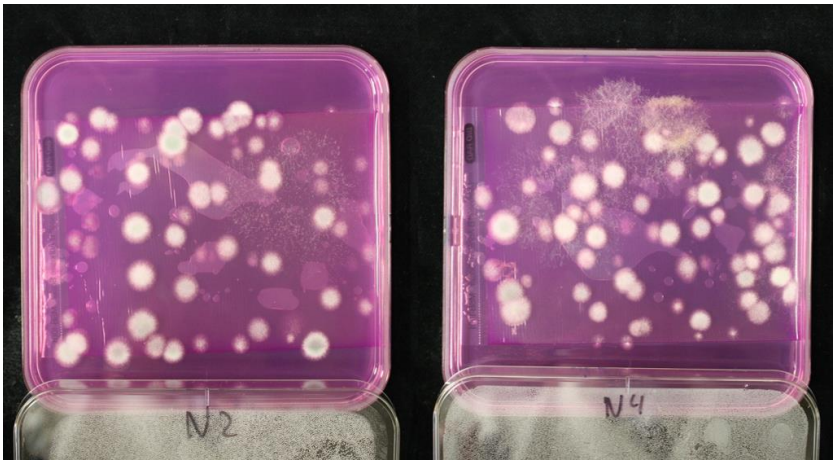

Figure 3: Visual examples of A. fumigatus growth on the sticky seals of outdoor samples covered with Flamingo medium. Plates of most outdoor samples showed similar Mucorales growth but not to a degree that hindered CFU counts. Indoor samples showed even less Mucorales growth.

Effect sampling method on CFU counts

Figure 4 shows CFUs of *A. fumigatus* on the triazole-negative control plate of the samples per sampling method. Per sampling method all samples showed remarkably consistent numbers of *A. fumigatus* CFUs. We did not observe any significant differences in the control CFUs per sampling site. We did, however, find significant differences among all three spore capture methods (see Table 1). The mean number of CFUs captured per control seal outdoors was 44 for the semi-open traps, 32 for the open traps, and 16 for the indoor seals.

Table 1: Multiple comparisons of the means of the CFU counts among the three different spore capture methods tested. P-values were adjusted for multiple testing using the Tukey method.

contrast	estimate	SE	df	Z ratio	Pr(>|z|)
Indoor - Open trap	-0.793	0.1382	Inf	-5.738	<.0001
Indoor - Semi-open trap	-1.029	0.1273	Inf	-8.079	<.0001
Open trap - Semi-open trap	-0.236	0.0949	Inf	-2.481	0.0349

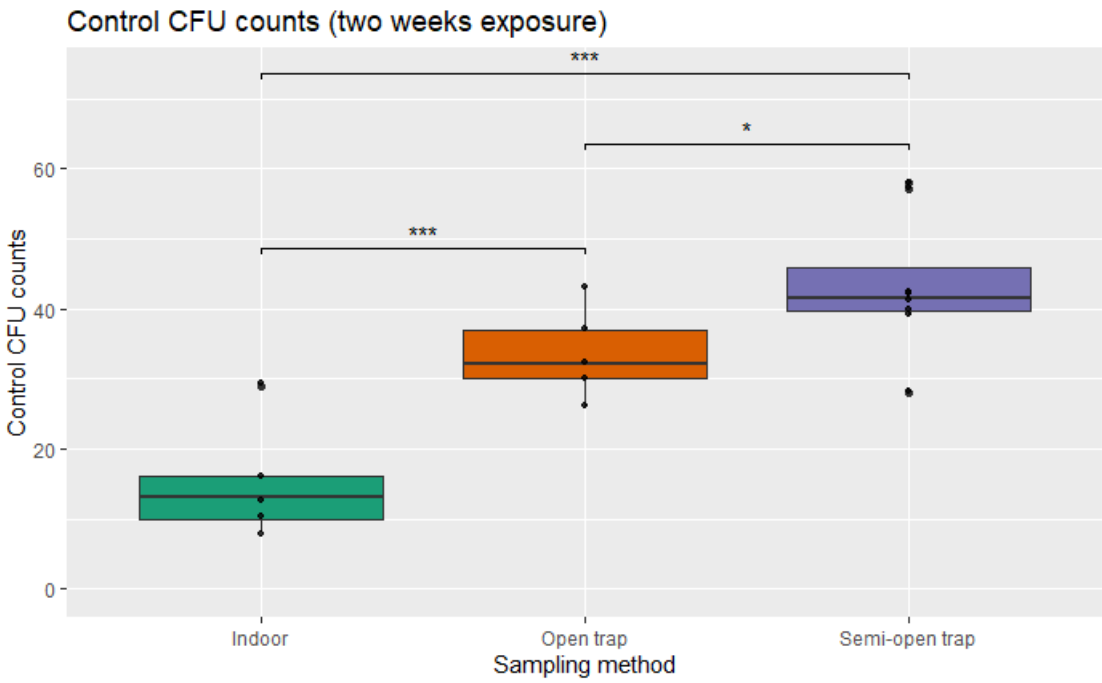

Figure 4: The CFU counts for two weeks of exposure on the negative control plates per sampling method. Significantly fewer spores were captured indoors compared to the two types of outdoor delta traps. The Semi-open trap design, in turn, slightly outperformed the open traps in terms of CFUs captured. (= p<.05, *** = p<.0001)*

All but one of the indoor samples had CFU counts below 25. Therefore, all indoor samples were excluded from the sample estimates of the resistance fractions made for each sample per site (see Figure 5), but they were included in the pooled estimate per site (See Table 2).

Table 2*: A. fumigatus* triazole-negative control CFUs and Itraconazole CFUs pooled per site and including both indoor and outdoor samples, along with the inferred percentage resistance per site and total.

Site	Control CFU's	Itraconazole CFU's	% resistance
A	132	107	81.1
B	100	123	123
C	141	99	70.2
D	68	54	79.4
E	150	94	62.7
Total	**591**	**477**	**80.7**

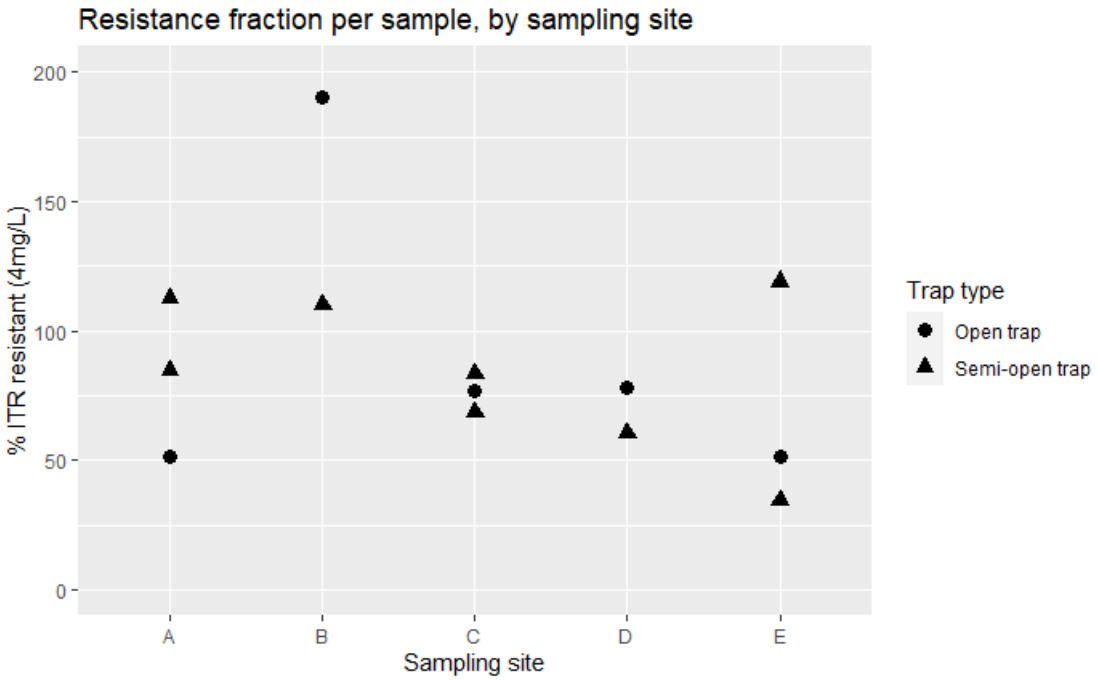

Figure 5: The itraconazole resistance fraction per outdoor sample, grouped by sampling site. Open trap samples are indicated with circles, and semi-open samples are indicated with triangles.

Discussion

This study assessed the efficacy of a novel, simple, and cost-effective combination of spore-capturing and culturing methods to determine triazole resistance fractions among the population of airborne *A. fumigatus* spores. We tested the use of delta traps for outdoor sampling with sticky seals, and the selective culturing of *A. fumigatus* from the exposed seals by pouring selective flamingo medium over them. Outdoor sampling with the sticky seals exposed for 14 days in the open and semi-open delta traps respectively proved two or three-fold more effective at capturing *A. fumigatus* CFUs than indoor sampling. The negative control CFU counts were consistently above the 25 CFU minimal threshold for the estimation of resistance fractions for the outdoor samples, and mostly below it for the indoor samples. Literature on a direct quantitative comparison of *A. fumigatus* CFUs between indoor air in home environments and local outdoor air is sparse. However, our finding that the CFU counts are lower in indoor environments is in line with earlier hospital studies (Sautour et al. 2009). Here it is important to note that lower CFU counts on the indoor seals may be caused by lower spore concentrations, lower airflow, or both. As our sampling method relies on passive spore capture by the seals we cannot readily distinguish between these two.

Rationale for cut-off sample size resistance fraction estimation

When estimating a true population proportion, in our case a triazole resistance fraction, power calculations are key in determining whether sample size will provide the desired precision. For determining the appropriate sample size (*S*) to estimate a proportion of a normally distributed, large population (<20.000) the following equation can be used,

$$S = Z^{2*}P * \frac{1-P}{E^2},$$

(1)

which we can rework to calculate the error size:

$$E = \frac{Z\sqrt{P(1-P)}}{\sqrt{S}}.$$

(2)

The key factors here are the predicated population resistance fraction (*P*), the sampling error (*E*) and the critical value (*Z*) for the desired confidence level of your sample estimate, which is 1.96 for the 95% confidence intervals commonly applied in biology. An informed assessment of expected population proportions requires pilot studies, but an expected P of 0.5 is the conservative assumption here. Adhering to this conservative assumption and working with the common 95% confidence interval we simplify the equation to

$$E = \frac{0.96}{\sqrt{S}}.$$

(3)

This shows us that the sampling error, or how much a sample resistance fraction estimate will differ from the population resistance fraction 95% of the time, will rapidly increase at low values (See figure x). If we aim for our sample resistance fraction to be within 5% of the true population resistance fraction, we ought to capture 385 spores per sample in this case. Reducing the number of spores we capture per sample will increase our sampling error up to ~10% for 100 spores per sample, ~20% per cent for 25, and to >30% for 10.

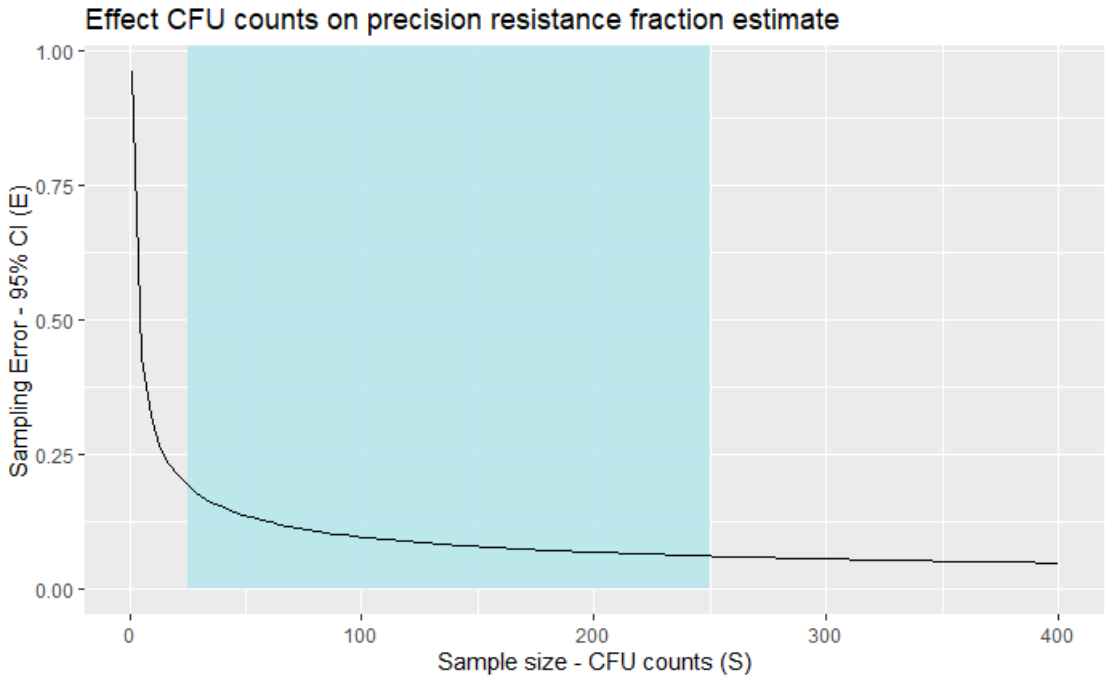

Figure 6: A plot of equation 3, showing the effect of CFU counts or sample size on the 95% confidence intervals, or precision, of the estimated resistance fraction. Where we are under the conservative assumption that the true population resistance fraction is 50%. The commonly accepted range of 25-250 CFUs per sample considered acceptable for numerical assessments of microbial samples (Davis 2014), like the estimation of resistance fractions, is highlighted in blue.

On the one hand, it's important to note that in most cases the resistance fraction of the true population will be closer to 0 or 1. As can be derived from equation 2, this will reduce the expected sampling error. On the other hand, it should be considered that for our method we also have a second type of sampling error. Because we use two adjacent seals to capture spores and determine resistance fractions, we are drawing two samples from a population. This introduces sampling error of the spores captured per seal, on top of the error over the proportion of spores that is resistant. In practice, this will increase our overall sampling error. This highlights that 25 CFUs per sample should be considered the absolute minimum for such estimations using the spore capture method outlined in this paper.

Use of the sticky seals for spore capture

Several factors are key to the efficacy of the described air sampling protocol, in particular the exposure time. *A. fumigatus* spore concentrations in a m^3 of air may readily be four or more orders of magnitude below those found in a gram of plant waste (Schoustra et al. 2019; Fraaije et al. 2020). Increasing the volume of air a seal is exposed to is therefore essential to obtain higher CFU counts. After two weeks we observed no noticeable reduction in the stickiness of the seals, but consistently found higher CFU counts than were described in Shelton et al. 2020 after eight hours of exposure. These findings suggest that spores may effectively be captured and accumulate on the seals up to 14 days of exposure. In the context of third-party sampling a month might also be a good sampling window that is intuitively easy to communicate. Such a larger exposure time may also further increase CFUs per sample along with the accuracy of resistance fraction estimates, potentially allowing for the estimation of resistance fractions from indoor samples. We are still testing whether a month of exposure time will effectively increase the number of CFUs captured. Key here is that spore viability may go down over time which would make CFU counts level off extended exposure times.

Use of the delta traps and its convenience for outdoor sampling

Based on our results we recommend the use of the semi-open delta trap design for spore capture, rather than the open ones. Due to the lower roof and half-closed sides of semi-open traps, the seals in these traps are, relative to the open traps, less exposed to UV light, which is known to kill *A. fumigatus* spores (Hagiwara et al. 2017; Blachowicz et al. 2020), and direct rain which may rinse the spores off the sticky surface. We believe the combination of these factors led to higher CFU counts in the semi-open traps. Especially because air flow is likely to have been higher, rather than lower in the open traps compared to those of semi-open design. Furthermore, the use of delta traps makes sampling more flexible as they can be strung under various common outdoor objects such as tree branches or garden fences, eliminating the need for flat and smooth surfaces to stick the seals onto. The fact that these traps are already commonly used in pest control also means they are readily available and can be ordered in bulk for large-scale sampling efforts at a low price. They weigh roughly 100 grams and can be folded up to fit into any envelope that could hold A4-sized paper, which makes them generally affordable to send by post to and returned by those who are using them. Sampling using the Delta traps is also affordable, with total material costs of to seven to nine euros per sample (in the Netherlands, autumn 2022), including returning the package by freepost, depending on whether resistance fractions for one or two types of triazoles are to be estimated. As the traps can be re-used these costs will decrease for successive sampling rounds. The ease at which the traps and seal can be set up opens up the possibility for sampling aerial resistance fractions at a larger scale than has yet been possible with conventional methods.

Relevance of monitoring triazole resistance through space and time

Our finding that the itraconazole resistance fraction was over 80% across all CFU's counted during this study highlights the urgency to both identify and address the drivers of environmental triazole resistance. Earlier reports have already shown that resistance levels can reach significant levels in plant waste material (Schoustra et al. 2019; Leendertse et al. 2021). However, resistance among airborne spores represents a more direct health risk, as it is these spores that people are exposed to directly through inhalation. Having an efficient and accessible air sampling protocol opens up extensive options for monitoring and research. Hospitals or other institutions housing vulnerable patients may use this method to assess health risks in and around their buildings without the need for costly equipment. The impact of known resistance hotspots on local aerial resistance levels may now also be quantified which is key in prioritising preventative measures. Furthermore, the efficacy of the measures may now be assessed as well. Fundamentally, this method may be used to measure if there are spatial differences in resistance fractions, and if there are, on what scale? This may not just be useful for observing the impact of known resistance hotspots but also for identifying yet unknown triazole resistance hotspots.

Here we present a simple-to-use air sampling method that hold the potential to assess aerial resistance levels at a yet unfeasible spatial scale. Due to its effectiveness and relatively low cost it can help bridge the knowledge gap of understanding transmission a triazole resistant *A. fumigatus* spores from environmental hotspots to patients.

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