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Spore Persistence and Likelihood of Aeroallergenicity of Entomopathogenic Fungi Used for Mosquito Control

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Abstract. Entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, are being researched as alternatives to chemical adulticides to control mosquito vectors of malaria and dengue. Two cited concerns of fungal control include conidial viability and risks fungal entomopathogens pose to human health. We measured spore viability of 10 fungal isolates over 26 weeks and found a range of persistence. Three *B. bassiana* isolates maintained ≥ 50% viability 14 weeks after application. No *M. anisopliae* isolate persisted longer than 1 week. To help assess risk of conidia as potential human allergens, we measured airborne conidia in enclosed environments after simulated biopesticide treatment of *M. anisopliae*. Conidia were detectable immediately after treatment, with concentrations of ~7000/m^3, decreasing over 48 hours to 500 conidia/m^3. At most, *Metarhizium* conidia comprised 2% of total visible particulate matter, falling to 0.1% by 2 days. The implications for viability of biological control of adult mosquitoes are discussed.

INTRODUCTION

Mosquito-borne diseases such as malaria and dengue continue to have a significant impact on human health, and options for control are few. Neither disease has an available vaccine, resistance in *Plasmodium* limits the effectiveness of the most widely available malaria drugs,[1] and insecticide resistance in *Anopheles* spp.,[2] and *Aedes aegypti*[3] is beginning to limit the impact of chemical vector control. Accordingly, alternative methods of control are warranted. One method currently being explored is indoor residual treatments of biopesticides containing spores of entomopathogenic fungi, such as *Metarhizium anisopliae* or *Beauveria bassiana*. Certain isolates of these fungi have demonstrated virulence to *Anopheles gambiae* s.s.[4] and *Ae. aegypti*,[5] together with additional pre- or sublethal effects that act to reduce vectorial capacity.[6] Moreover, experience with fungal entomopathogens for control of agricultural pests indicate the potential for practical delivery of such a technology, including in Africa.[7]

Although the use of fungi for vector control shows promise, concerns have been raised concerning persistence of conidia (spores) after spraying[8] and impact of fungus on human health.[9] Because the conidium is the infective stage, the length of time that fungal spores remain viable in the field is a critical indicator of potential as a biologic control agent. In a study examining *Metarhizium flavoviride* as a potential control agent for locusts, Thomas and others[10] concluded that small changes in spore persistence can have a significant impact on target pest populations. Scholte and others[4] reported that conidia from a strain of *M. anisopliae* pathogenic to mosquitoes decreased in viability over 3 weeks from 95–63%. A laboratory study by Blanford and others[6] indicated that a virulent strain of *B. bassiana* remained equally infectious to adult mosquitoes over 12 days after spraying and that viable spores could be recovered after 6 weeks. Given the importance of spore persistence in determining minimal rate of re-application to sustain effective control and hence the likely operational practicality and final cost of a product, these studies are very limited in extent. One aim of the current study, therefore, was to better understand the limits of persistence and variability between fungal species and strains.

With regard to the potential for fungal entomopathogens to cause infection in human hosts (including immunosuppressed people), we consider this risk extremely small. Numerous studies, including two very recent exhaustive reviews, conclude that microbial insecticides based on fungal entomopathogens are safe to human health.[11–15] We can find a total of 14 references to either *Metarhizium* or *Beauveria* spp. being isolated from human hosts.[16–29] Given these fungi are commonly isolated from insects and soils throughout the world, these cases are very rare compared with numerous other fungi. Moreover, to put this into context, even commonly used fungi, such as brewer's and baker's yeast, can cause very occasional opportunistic infections.[30] Importantly, these types of infections tend to be regulated in the first instance by the innate immune system and not the adaptive immune system. Components of the innate immune system are the first line of defense against opportunistic infections and play a predominant role in clearance of spores in respiratory and gastrointestinal tracts.[30,31] Additionally, the temperature optima for insect pathogenic fungi is typically in the region of 20–25°C and the vast majority fail to grow at 37°C (see reviews of Zimmerman[14,15]). If a fungal strain has no physiologic or morphologic adaptations to grow at 37°C, this will substantially constrain the possibility of infection, irrespective of immune state.

The potential for spores of fungal entomopathogens to act as respiratory allergens is slightly more of an unknown. Ward and others[32] found that mice challenged intratracheally with *M. anisopliae* crude antigen (MACA) exhibited allergic responses. Furthermore, mice primed with *M. anisopliae* mycelium, or MACA, exhibited stronger allergic responses to the model allergen ovalbumin,[33] suggesting that *M. anisopliae* could also serve as an adjuvant to other allergens. Similarly, spores of *B. bassiana* have been found to contain IgE allergens and have been associated with human allergens.[14] However, any small protein-based particles, such as pollen, plant or animal dust, bacterial or fungal spores can act as allergens. Such aeroallergens are ubiquitous, and numerous fungal pathogens, such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., are abundant and widespread.[34,35] Indeed *Beauveria* spp. spores can be naturally present in air samples[36,37] and have

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been found, for example, as common components of the mycobiota isolated from the lungs of small mammals.[38] Thus, the key question is whether use of fungal entomopathogens for mosquito control is likely to increase exposure risk significantly. It has been argued previously that applications of anti-malaria fungal biopesticides are unlikely to add significantly to existing aeroallergen burden within domestic settings.[39] The reason for this is that the proposed delivery systems use oil formulations[4,40] that cause the spores to adhere to the substrate on which they are applied and so should not contribute substantially to spore load in the air. Nonetheless, spore concentrations in the air have not been monitored after treatment so the actual risk of exposure remains untested. The second aim of the current study, therefore, was to monitor spore load in the air following a simulated biopesticide treatment to determine any increase in exposure risk relative to other naturally present spores and total background particulate matter.

MATERIALS AND METHODS

Conidial viability after spraying. *Formulation of fungal isolates*. We examined persistence of 5 isolates of *M. anisopliae* and 5 isolates of *B. bassiana*. These isolates were selected because they had all showed ability to infect adult *Anopheles* and/or *Aedes* mosquitoes in simple laboratory assays[4,6] (and Darbro JM and Thomas MB, unpublished data). Conidia were taken from a potato-carrot agar (PCA) slope and used to prepare a spore suspension of approximately 1×10^6 conidia mL^{-1} in 0.05% w/v Tween 80 in distilled water. This suspension was used to inoculate Oxoid Sabouraud-dextrose agar (SDA) in 9-cm-diameter Petri dishes by pipetting 0.2 mL aseptically onto the surface and spreading evenly over the agar with a glass rod. Inoculated plates were incubated at 25°C for 11 days and conidia were harvested from the plates using a micro spatula. The harvested conidia were then placed in separate, pre-weighed, glass weighing dishes and placed in a glass dome desiccator over dried, non-indicating silica gel at room temperature (~18°C) for 10 days until the conidia were fully desiccated.

Oil formulations were prepared by suspending ~0.1 g of dried conidia in 10 mL of oil formulation comprising 12.5% Ondina EL and 87.5% Shellsol T (standard proprietary mineral oils). The concentrated conidia formulations were resuspended by vortexing, and then sonicated in a bath sonicator for 3 minutes to break up any conidial chains and remove conidia from any remaining mycelial fragments. The formulations were then sieved through a 106-µm sieve to remove any large particles from the formulation. A dilution series was then prepared in the formulating oil and conidial counts performed using an Improved Neubauer hemocytometer. All formulations were adjusted to 1×10^8 conidia mL^{-1} in 10 mL oil formulation.

Biopesticide application. Spray application used a hand-held artist's air brush (Badger Air-Brush Co., Franklin Park, IL) to produce an aerosol of the spore formulation. Eighty-one glass microscope slides (7.62 cm × 2.54 cm) per fungal isolate were treated with 5 mL of a 1×10^8 spores/mL oil formulation. This method resulted in an application rate of 5.5×10^5 conidia/cm^2, or 1.0×10^7 spores/slide.

Slides were allowed to dry for at least 24 hours at room temperature before being used in germination tests. Slides were stored in slide boxes at 26°C, a representative mean temperature for numerous malaria and dengue transmission areas.

Germination tests. One slide was placed spore-side down onto an SDA agar plate. Each plate was wrapped in parafilm and stored in a temperature-controlled incubator. The slide was removed after 20–24 hours and germinated and ungerminated spores were counted along the length of the agar plate. A spore was considered to have germinated if the germ tube was at least twice the length of the conidium. A minimum of 300 spores were counted on a compound microscope at 400× magnification.

Germination tests were performed at 1 day, 1 week, 3 weeks, 6 weeks, 10 weeks, 14 weeks, 18 weeks, 24 weeks, and 28 weeks (9 intervals in all) with three replicates per strain per time point.

Data analysis. Germination rates were plotted over time to visualize the data. Germination rates were standardized to control for small variations in initial germination rate by setting all initial (time = 0) germination rates to 100%, then calculating subsequent relative germination rates based on the standardized initial value. Strains that did not have at least 4 non-zero data points over time (i.e., strains that fell to 0% germination by 3 weeks) were not included for statistical analysis.

Spore persistence values for *B. bassiana* were analyzed using PROC GLM (SAS 9.0, Cary, NC) using standardized germination rates as the dependent variables. Independent variables included in the model were time (days post-application) and isolate, as well as the two-way interaction between time and isolate. Tukey's multiple comparison test was used to detect significant differences between values.

Airborne conidial sampling. *Biopesticide application*. *Metarhizium anisopliae* var *anisopliae* conidia (strain ICIPE 30, as previously tested by Scholte and others[4]) were harvested as above. We applied the oil formulation to four 1 m × 1 m pieces of black cotton sheet using a hand-held artist's air brush with a 20-mL glass jar attached to the spray nozzle. Spores were applied with six spray passes (in sequential rows) of the air brush from a distance of 50 cm, delivering 20 mL of the formulation to give a dose of 2×10^9 spores/m^2. Each treated cloth was then secured to the ceiling of an experimental laboratory room with dimensions of about 3 m × 3 m × 2.5 m, where it covered 13–14% of the total ceiling surface area. This dose and area coverage is broadly equivalent to that used in the village hut trial of Scholte and others.[4]

Sampling schedule. Air was sampled with an Aerotech 6 Sampler, powered by a High Volume AeroLite II Pump (Aerotech Laboratories, Phoenix, AZ). Samples were collected at a flow rate of 28.3 L/min[41] into 9-cm plastic Petri dishes containing SDA agar. Two 3-minute air samples were taken in each room before treatment. Immediately after the treated cloth was placed in each room, two more 3-minute air samples were taken with the intake positioned 120–170 cm directly below the cloth to maximize spore capture. Two 3-minute samples were taken at approximately the same time every 24 hours for the next 3 days (August 13–16, 2007).

The Aerotech II Sampler draws air through 400 holes distributed over the surface of the agar plate, and particulate matter impacts onto the plate in clusters corresponding to each hole. Within 24 hours of collection, plates were examined under an Olympus BH-2 microscope (Olympus America Inc., Hamburg, Germany) to count the number of *M. anisopliae* spores (identifiable by characteristic size and shape) and total particulate matter. We counted *Metarhizium* spores on 40 clusters per plate, corresponding to 10% of the total air drawn in

by the sample. The two samples per treatment were averaged, and spore counts were converted to spores per cubic meter of air. To analyze variation in the proportion of total airborne debris comprised of *Metarhizium* spores, we counted all particulate matter on a subset (N = 10) of the clusters of each sample and estimated proportion by dividing the number of spores in a cluster by the total debris of that cluster.

Plates were then covered, sealed with parafilm, and incubated at 26°C to allow any viable spores to germinate. Plates were assessed after 4 days and the number of colony-forming units (cfu) counted. Colonies were isolated onto new plates, which were sealed with parafilm and incubated at 26°C until they formed spores (usually by 7 days). Spores were examined by microscopy (see above) to determine if they were *M. anisopliae.*

Data analysis. Variation of spore concentrations over time was measured using analysis of variance (ANOVA) in Microsoft Excel 2003 (Microsoft, Redwood, WA). Data for total debris were also analyzed using ANOVA. For all analyses, data were natural log-transformed if necessary to meet assumptions of normality and homogeneity of variance before employing parametric statistics.

RESULTS

Conidial viability. In all five strains of *M. anisopliae* tested, spore viability on glass microscope slides fell to 0% germination by 3 weeks (Figure 1). These data were not analyzed further because of insufficient non-zero values. Strains of *B. bassiana* persisted longer (Figure 2), maintaining ≥ 50% spore viability at 14 weeks (FI-277) to 18 weeks (ARSEF1151, ARSEF5341, IMI 391510) (Table 1). The ARSEF5341 and IMI 391510 persisted the longest of the fungal strains, maintaining ≥ 30% spore viability after 24 weeks and continuing to survive at low levels at the end of the assay (28 weeks). The ARSEF 1151 spore viability fell to zero at 24 weeks, FI-277 fell to zero at 18 weeks, and FI-491 persisted until 14 weeks (Table 1).

Airborne conidial sampling. No *M. anisopliae* spores were detected in the pre-treatment sample. Shortly after putting up the treated cloth, we detected an average of 59 ± 15 spores per plate, which equates to over 7,000 *Metarhizium* spores per cubic meter of air (Figure 3). This amount decreased dramatically over the first 1 and 2 days, resulting in 577 ± 277 spores/m^3 at 3 days.

Metarhizium airborne spore concentration decreased in relative and absolute terms. *Metarhizium* spores made up ~2% of all visible airborne particulate matter on day 0 (Figure 3). This percentage fell to less than 0.4% on day 1, then less than 0.1% on days 2 and 3. According to Tukey's multiple comparison test, the spore percentage of day 1 was significantly more than that of day 3 ($P < 0.015$) and day 4 ($P < 0.004$).

Surprisingly, even though *M. anisopliae* spores were detected on the sample plates, we did not observe any colony-forming units of *M. anisopliae* after 4 days of incubation. However, the average concentration of other species of airborne fungi was 197–397 cfu/m^3 (Figure 4). Individual colonies of these other species were not exhaustively identified but common fungi, such as *Aspergillus* spp. and *Penicillium* spp., were clearly present (based on morphologic characteristics of spores and spore forming structures). Concentrations of these airborne fungi (other than *Metarhizium*) did not vary significantly by day.

DISCUSSION

Under our test conditions, all the *M. anisopliae* strains exhibited low residual persistence, with no spores remaining viable beyond 21 days after spraying. These results contrast with those of Scholte and others[4] who reported that ICIPE 30 (one of the isolates in our study) maintained 63% spore viability after 3 weeks on black cotton inside village huts in rural Tanzania. Comparing persistence across studies is difficult as differences in spore quality (possibly associated with production and storage methods), formulation, application method, substrate, and prevailing environmental conditions (temperature, humidity, and UV) can all affect spore viability. The fact that under real field conditions *M. anisopliae* can persist fairly well, is encouraging, and suggests that our test conditions may have been harsher than those to which spores are exposed in the field. Moreover, this is not an exhaustive test of all isolates and it is possible that other isolates of *M. anisopliae* could persist well even under our conditions. Nonetheless, in the current study the loss of viability of *M. anisopliae* was very rapid.

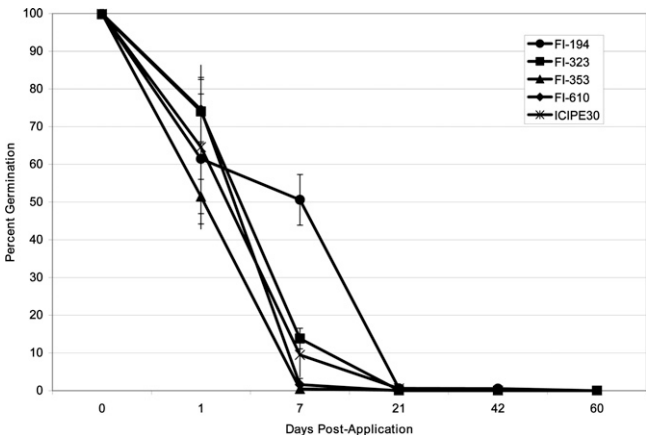

FIGURE 1. Decline in conidial viability of 5 strains of *Metarhizium anisopliae* sprayed in oil formulation onto a glass substrate and maintained at 26°C. Mean ± SE.

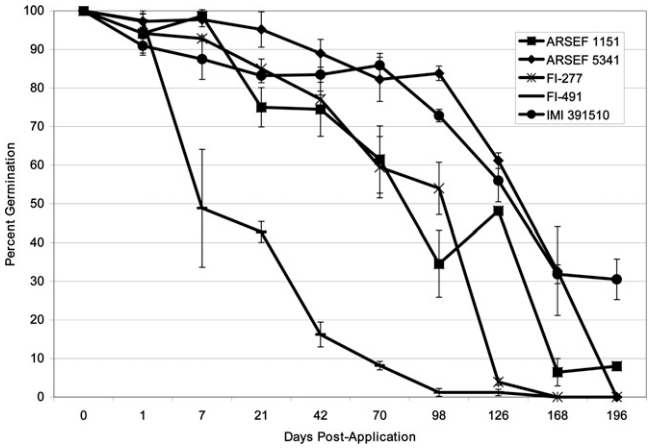

FIGURE 2. Decline in conidial viability of 5 strains of *Beauveria bassiana* sprayed in oil formulation onto a glass substrate and maintained at 26°C. Mean ± SE.

TABLE 1
Germination percentages of *Beauveria bassiana* strains at 3, 14, 18, and 24 weeks

Strain	3 weeks	14 weeks	18 weeks	24 weeks
ARSEF5341	95.2* ± 3.7	83.8* ± 3.7	61.2† ± 3.7	32.6‡ ± 3.7
IMI 391510	83.2* ± 3.7	73.0*† ± 3.7	56.0†‡ ± 3.7	32.9‡ ± 3.7
FI-277	85.0* ± 3.7	54.1†‡ ± 3.7	3.9§ ± 3.7	0§ ± 0
ARSEF1151	75.0*† ± 3.7	34.6‡ ± 3.7	48.2†‡ ± 3.7	6.5§ ± 3.7
FI-491	42.7‡ ± 3.7	1.3§ ± 3.7	0.8§ ± 3.7	0§ ± 0

Least square means ± SE. Means followed by the same footnote symbol are not significantly different (> 0.05 according to Tukey's test for pairwise comparisons).

Beauveria bassiana, on the other hand, tended to persist much better with isolates exhibiting half-lives of ~21–> 126 days and absolute viability in the range 42–> 196 days. Assuming re-treatment levels approximate with the half-life (and acknowledging that we are not testing infectivity but simply spore viability; in reality, it could be that probability of infection falls quicker than the decline in viability, or it could be that infectivity half-life is greater than percentage viability as potentially very few spores are required to infect an insect), these persistence data suggest the possibility of single applications providing control for up to 4–5 months. This would translate to 2 or 3 treatments for year-round efficacy in holoendemic transmission areas, which is comparable to several of the current chemicals used in indoor residual spraying (IRS).[42] In areas with more discrete transmission, even a single application could then deliver effective season-long control. These predictions are supported by a recent modeling study, which indicated there could be a substantial lag-phase in recovery of the density of malaria-infectious mosquitoes even after the infectivity of a fungal biopesticide had declined to zero.[43] Given the, so far unexplored, potential to increase infectivity and effective persistence through application methods, dose, formulation, and isolate selection, these findings suggest considerable promise for developing fungal biopesticides with operationally satisfactory levels of persistence.

With regards to potential aeroallergenicity, spores from the biopesticide-treated cloth were readily detectable immediately after introduction into the experimental rooms. After 24 hours, spore concentrations fell drastically, both in absolute numbers and relative to the other airborne particulates.

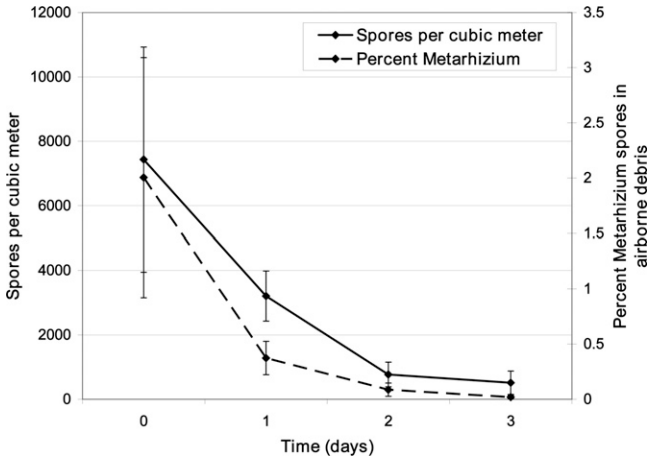

FIGURE 3. Airborne *Metarhizium anisopliae* spore concentrations near treated cotton cloth and percentage of total airborne particles comprised of *Metarhizium anisopliae* spores. The left axis and solid line describe the number of *M. anisopliae* spores per cubic meter, and the right axis and dashed line describe the percentage of total airborne debris comprised by *M. anisopliae* spores.

Our protocol of handling the treated cloth (by affixing it to the ceiling) within a few hours of spraying, followed by immediately sampling the air beneath the cloth in a relatively small room with little ventilation, likely resulted in high initial spore concentrations. The sharp decline in spore concentrations suggests that any airborne spores quickly settle out of the air and are not constantly being liberated, as per the suggestion of Thomas and others.[39]

Despite identifying *M. anisopliae* spores in the air samples, we did not detect any viable colony-forming units of *M. anisopliae* in this study. In contrast, we found between 200–400 cfu of other mold species, including known human allergens such as *Aspergillus* and *Penicillium.* The reasons for the lack of *M. anisopliae* colonies on the sample plates are not clear. It is possible that the spores were of poor quality from the production run, or that the process of spraying and handling the cloth affected viability in some way (this was not anticipated but the results of persistence study for this isolate did indicate rapid loss of viability so this could be a factor). Alternatively, it could be that *M. anisopliae* was out-competed on the plates, or small individual colonies were possibly missed or misidentified because of numerical dominance of other fungi. A study sampling airborne mold in homes in Victoria, Australia, indicated an approximate 10:1 relationship between estimated spore concentrations/m^3 and cfu/m^3.[44] If this is a general relationship then cfu could easily be missed, especially after day 1 where the actual number of *M. anisopliae* spores per plate was extremely small. Moreover, in the current study, the apparent lack of culturable *M. anisopliae* spores is of only secondary concern given that spore viability is not related to possible allergenicity. More important are the spore concentrations (which we were able to estimate) and how these compare with concentrations of other fungi and particulates, both within the study and elsewhere.

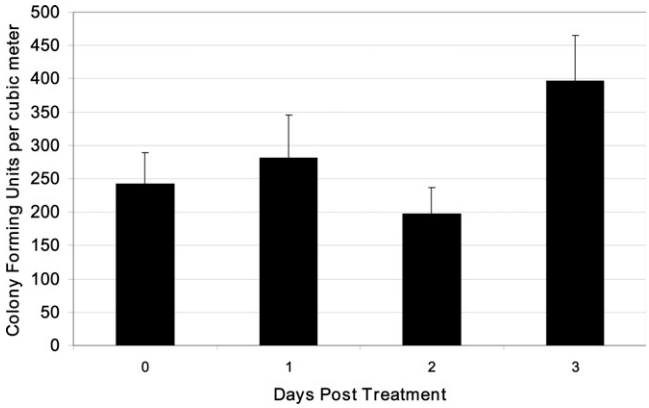

FIGURE 4. Colony-forming units (cfu) of airborne fungi per cubic meter collected in indoor rooms containing *Metarhizium anisopliae*-treated cloth. Mean ± SE.

The aforementioned airborne mold study in Victoria[44] reported median concentrations of 5,111 spores/m^3 in houses in September (a similar time of year to our study), rising to 12,778 spores/m^3 (1,583 cfu/m^3) in March–April. Numerous other studies also report high natural fungal spore loads in indoor and outdoor air, with marked seasonal variation. For example, a study of 15 residences in Canada found seasonal peaks of > 7,000 spores/m^3 indoors and c.15,000 spores/m^3 outdoors.[45] Air samples taken in and around a library in Singapore revealed 34–64 fungal cfu/m^3 indoors but up to c.5,000 cfu/m^3 outdoors.[46] Assessment of airborne fungi from an indoor cattle shed in West Bengal revealed an average concentration range of 233–2,985 spores/m^3.[47] Samples from occupational environments in Calcutta revealed 24 spore types with concentrations of *Aspergillus* spp. in the range of 2,000–8,000 spores/m^3.[48] Outdoor air samples taken in the summer months over 2 years in London revealed over 30 species of fungi with spore concentrations ranging from 38 to 16,119 spores/m^3.[49] Even more extreme, a study of outdoor air quality in Pennsylvania reported seasonal peaks of > 40,000 fungal spores/m^3.[50] Unfortunately, there appears to be few published records of aerial fungal spore concentrations from areas with high prevalence of malaria or dengue. More generally, indoor air pollution is a major concern in many developing countries,[51] and there is no reason to imagine that aeroallergens are naturally low, especially in common-place domestic structures such as village huts with thatched roofs, bare floors, and mud-tiled walls. Overall, therefore, it appears that the transient increase in spore load from the biopesticide treatment falls well within natural variation in fungal load and is dwarfed by concentrations of other particulates such as dust, pollen, bacteria, other spores, and mycelial fragments.

Moreover, there is little evidence correlating airborne spore concentrations of allergenic fungi and human respiratory symptoms. One reason for this may be that allergens in mycelial fragments and other non-spore particles may account for allergenicity, but are typically not detected in microbiologic sampling. In particular, (1→3)-β-D-glucan, which is found not only in spores but also in hyphal fragments, has been linked with indoor, air-related health effects commonly associated with allergies.[52,53] A study sampling air from a domestic residence in Sydney, Australia, detected significantly more hyphal fragments of *Alternaria, Aspergillus/Penicillium,* and *Cladosporidium* than conidia, and 25% of these fragments positively immunostained with allergic serum IgE.[54] Another study reported that detectable fungal fragments of *Avicularia versicolor, Penicillium melinii,* and *Cladosporium cladorsporioides* exceeded fungal spores in the air by 320-fold.[55] Importantly, the fungus-based biopesticides being developed for mosquito control comprise only pure spore powder, and treatment of inert substrates will not result in vegetative growth and production of mycelial fragments in the air.

In summary, the results of these baseline studies indicate considerable inter- and intra-specific variation with regards to conidial persistence after a spray application, but reveal there are strains with potentially adequate persistence for operational use. Further tests are now required to screen more isolates and monitor changes in actual infectivity under a range of conditions considering different substrates, formulations, and abiotic factors representative of "field" environments. On the basis of the current evidence, we also think it unlikely that airborne spores from treated surfaces will represent an additional health risk to household occupants. Of course, our study is limited as we have only considered one experimental application and it is likely that absolute and relative spore loads will vary depending on nature of the formulation, substrate, dose, environment, etc. The use of spore-treated cloth mimicked the delivery system tested in previous semi-field trials in Africa.[4] This delivery system is likely to use fewer spores than something like a full residual spray where all surfaces are treated. However, recent studies have also identified the possibility of delivering spores using treated clay pots, which are attractive to resting mosquitoes both inside and outside domestic dwellings.[56] These highly contained, point source treatments would likely liberate few spores into the air. Given this potential for variation in spore loads as a result of both delivery systems and environmental conditions, it is important that future evaluations of the biopesticide technology include assessment of spores in the local environment to determine possible exposure levels. Any risks then need to be weighed against the anticipated health benefits deriving from reduced incidence of mosquito-borne diseases.

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