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An invertebrate model to evaluate virulence in *Aspergillus fumigatus*: The role of azole resistance

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Abstract

The impact of different mutations in the *Aspergillus fumigatus* ergosterol biosynthesis pathway on pathogenesis has been evaluated using a simple invertebrate mini host, the caterpillar *Galleria mellonella*. A set of strains that includes clinical isolates and isogenic mutants with mutations at the *cyp*51A gene conferring azole resistance were studied. All strains demonstrated a similar *in vitro* growth pattern and are equally virulent against the insect larvae. These results suggest that in *A. fumigatus* acquisition of this particular azole-resistance mechanism would not imply any significant change in virulence. *G. mellonella* may provide a convenient and inexpensive model for the *in vivo* prescreening of mutants of *A. fumigatus*, contributing to the generation of a hypotheses that can be further tested in refined experiments in mammalian models.

Key words: nonmammalian model, isogenic mutants, *cyp*51A gene.

Introduction

The impact of acquired resistance mechanisms in *Aspergillus fumigatus* in terms of virulence and fitness is not yet understood. In fact, it has been noted that persistence of drug-resistant organisms in nature depends on the relative fitness of resistant and susceptible genotypes [1].

The main mechanism of azole resistance in *A. fumigatus* is via alterations, either point mutations or overexpression, of the target enzyme Cyp51A [2–7]. Evidence that isolates with acquired resistance mechanisms are just as pathogenic as wild-type strains is growing [8]. However, there are few comparative analyses of the pathogenic potential of different *Aspergillus* species and drug-resistant mutants [9]. In this context, Mellado et al. found that a *cyp*51A gene knockout strain (lacking the azole target gene) was morphologically indistinguishable from the wild type and also was able to retain the ability to cause pulmonary disease in a neutropenic mice model [5].

Different aspects of pathogenesis, innate and acquired host responses, and therapy have been extensively

Table 1. Azoles susceptibility results (MIC in mg/L) obtained by EUCAST methodology for wild type (Wt) strain and derived mutants.

Strain identification	Oorigin	*Cyp*51A protein	Itraconazole, MIC in mg/l	Voriconazole, MIC in mg/l	Posaconazole, MIC in mg/l
CNM[a] CM-237	Clinical strain	Wt	0.25—0.5	0.12—0.25	0.06—0.12
CNM CM-2266	Clinical strain (sputum)	G54W	>8	0.5	>8
CR056	Isogenic strain	G54E	>8	0.25	1
CR061	Isogenic strain	G54R	>8	0.25	1
CR058	Isogenic strain	M220K	>8	1	1
CR059	Isogenic strain	M220V	>8	1	0.5
CR060	Isogenic strain	M220T	>8	1	0.12–2
CR019	Isogenic strain	TR/L98H	>8	4	0.5
CM-A8	Isogenic strain	Δ_*cyp*51A	0.06	0.25	0.03

MIC, minimal inhibitory concentration.
[a]CNM-CM. Filamentous Ffungi Collection of the Spanish National Center for Microbiology.

investigated using laboratory animal models of invasive aspergillosis. These models have been important for the advancement of therapy and for expanding our understanding of pathogenesis and host resistance. Rodent models of aspergillosis, particularly murine models, are often used, and there is particular interest in developing nonmammalian host models in order to address the bioethical impact of the classic animal experimentation [10]. Alternative invertebrate hosts, such as *Galleria mellonella* (Lepidoptera; commonly known as the greater wax moth), may also be useful.

Recent evidence suggests that innate immune responses involved in the defense against most pathogens are phylogenetically ancient and are present in both higher and lower animals [11,12]. For these reasons, analysis of the immunological responses of insects may provide relevant insights into defense mechanisms against fungi [13]. The caterpillar *G. mellonella* has several favorable attributes for assessing fungal virulence and antifungal activity including ease in handling, low cost, and low maintenance [14,15]. Several recent studies have suggested that *G. mellonella* infection models can be used in *A. fumigatus* pathogenesis studies [16,17]. *A. fumigatus* can proliferate in *G. mellonella*, leading to the eventual death of the caterpillar.

Here, we explore the impact of different *Cyp51A* mutations, which are the azole target, on the pathogenesis of *A. fumigatus* using a simple invertebrate minihost, the caterpillar *G. mellonella*. Further investigations focused on the *in vitro* growth patterns of the isolates were also performed.

Material and methods

Strains

Nine *A. fumigatus* strains were used in this work. They included an azole-susceptible CNM CM-237 strain (wild-type *cyp*51A gene) and seven azole-resistant (isogenic strains with altered *cyp*51A alleles introduced into the CM-237 wild-type strain by electroporation). In addition, a clinical strain isolated from the sputum of a patient with aspergillosis that showed a point mutation in the target *cyp*51A was included. Descriptions of the strains are provided in Table 1. Briefly, two mutants (CR056, CR061) and one clinical strain (CNM CM-2266) had one mutation at position glycine 54 including amino acid changes G54E, G54R, and G54W [3]. A second group of mutants (CR058, CR059, CR060) included a point mutation at methionine 220 and the following amino acid changes: M220K, M220V, and M220T [4]. Two additional strains included CR019 (gene replacement of the TR/L98H fragment from strain CM-2627 into azole-susceptible strain CM-237) [6] and the *cyp*51A-deleted strain (CM-A8) [5]. *In vitro* susceptibility testing was performed following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [18]. The minimal inhibitory concentrations to itraconazole, voriconazole, and posaconazole are summarized in Table 1.

Growth kinetic assay

Growth kinetics using a fixed inoculum size (2.5×10^6 conidia/ml) on two solid media, potato dextrose agar (PDA; Becton Dickinson, Madrid, Spain) and minimal essential medium (Sigma Aldrich, Madrid, Spain), were estimated. A single spot (0.1 ml, 2.5×10^5 conidia/spot) was dispensed on each agar plate and incubated for 3 days at 37°C; the zone diameter was measured after 24, 48, and 72 h. Experiments were repeated at least two times on different days. The average diameter was used to determine the radial growth rate (Kr), which is calculated using the linear regression of the diameter vs. time. Differences of Kr were assessed among strains.

Fungal growth rate in liquid medium

Fungal growth in a microbroth kinetic system (iEMS Reader MF spectrophotometer; LabSystems, Thermo Fisher Scientific, Madrid, Spain) was measured in order to evaluate differences in the growth curves of strains. These studies were conducted in Roswell Park Memorial Institute medium supplemented with 2% glucose (Sigma Aldrich) in 96-well microdilution plates inoculated with 10^5 conidia/ml and incubated at 37°C. The optical density (OD) of each well in the plate was monitored at 405 nm (OD_{405}) every 30 min over a 24-h period [19]. Several experiments were performed in order to study the reproducibility of growth curves.

For each time point, *t*, the average OD of two consecutive measurements until the $t = +2$ time point was calculated and subtracted from the average OD of the previous two measurements from the $t = -2$ time point. Lag phase of growth (defined as a delay in OD changes due to germination of spores) was calculated as time during which OD_{405} does not increase. The beginning of the growth phase was defined as a change in $OD_{405} > 0.001$ U (the lower detectable OD). Exponential-phase broth cultures were marked by a specific growth change of >0.002 U/h.

Data were exported to GraphPad Prism (Graph Pad v5 for Windows, La Jolla, CA, USA); parameters related to growth patterns were evaluated for all strains. For each curve, the coefficient of determination (r^2) for the adjustment of the curve to the model was obtained.

Experimental model

Larvae of *G. mellonella,* approximately 0.3–0.5 g body weight (Vanderhorst Wholesale, Inc., St. Marys, OH, USA, and Mous Livebait R.J., The Netherlands) were used (10 per group). Each larva was infected with conidia of wild-type *A. fumigatus* or mutant strains. Preliminary experiments were performed with the wild-type strain in order to determine the appropriate range for the inoculum that would cause median survival (50% mortality) at 72–96 h post infection (data not shown). An inoculum of 2×10^5 conidia per larva was established to cause 50% death 3–4 days post infection.

Then, 10 µl of both wild-type *A. fumigatus* and mutant strain conidia (2×10^7 conidia/ml) prepared in phosphate-buffered saline (PBS) + 0.01% Tween 20 were injected into the hemocoel of each larva via the last left proleg using a 26-gauge, 16-mm needle with Hamilton syringes (Teknokroma, S.C.C.L., Barcelona, Spain). Before injection, the proleg area was cleaned with a swap soaked in 70% ethanol. The inoculum was verified with quantitative counts. In addition, a group of larvae inoculated with conidia of *A. fumigatus* wild-type strain killed with heat (a suspension of 2×10^7 conidia/ml boiled at 90°C for 1 h, injected 10 µL) were included as negative control of infection. These conidia are not able to grow on solid media and are intended to demonstrate nonvirulent behavior in the model used.

Larvae were incubated in 90-mm plastic petri dishes (Soria Genlab, S.A., Madrid, Spain) at 37°C in a humidified chamber and monitored daily. Caterpillars showing signs of severe morbidity (e.g., no response to touch or change of body color) were sacrificed using cold treatment at −20°C or water immersion. Three additional types of controls were used in each assay to ensure that neither the injection procedure nor the incubation period were responsible for observed mortality. In the untouched control, the larvae were not handled and were incubated at the same temperature as infected larvae. The second set of controls consisted of larvae that were injected with a sterile syringe into the last left proleg, but no conidia (or PBS) were inoculated. A third PBS control consisted of larvae injected with 10 µl of sterile PBS to monitor killing due to physical injury. Three independently conducted experiments were performed for each strain. Survival curves were plotted, and differences between groups were analyzed with Kaplan-Meier statistics using the program Graph Pad Prism (version 5).

Estimation of fungal burden

In order to investigate the time course of fungal infection, we quantified fungal burden after infection using a specific real-time polymerase chain reaction (RT-PCR). Three whole inoculated larvae were homogenized in 5 ml of sterile PBS. Then 200 µl were processed for DNA extraction using the QiAmp Tissue DNA Mini Kit (Qiagen, Izasa, Madrid, Spain) following the manufacturer's instructions. A specific and validated RT-PCR assay was performed with each extracted sample to quantify the fungal density at selected time intervals (1 h and 72 h after infection). Fungal burden data were expressed as nanograms of fungal DNA per gram of larva. All samples were tested in duplicate, and an average of fungal burden was evaluated. Controls were used throughout the process (for both DNA extraction and PCR amplification). Larvae inoculated with heat-killed conidia were also included as control for RT-PCR assay. In addition, at different time points after infection, selected larva were formalin-fixed and embedded in paraffin. Sections approximately 5 µm in size were prepared, stained with periodic acid-Schiff and methenamine silver staining, and examined by light microscopy.

The ability of conidia to germinate into larva was also determined using injected spores (10 µl, 2×10^7 conidia/ml) previously stained with 10 µg/ml of calcofluor-white (Sigma-Aldrich) for 30 min at 37°C. Hemolymph was recollected after 2 h and 72 h of incubation. Conidia were then

Table 2. Growth characteristics of strains included in the study.

	Growth parameters (solid media)[a]				Growth parameters (liquid media)[b]		
	Colony growth rate Kr (slope $\log_{10}$ mm/h)		Maximal growth (diameter size in $\log_{10}$)		Parameters of growth in liquid media after 24 hour of incubation (best fit values)		
Strains	PDA	MM	PDA	MM	Preferred model (R^2)	Lag (hour)	Mue (OD_{450} /per h)
CNM CM-237	0.014	0.016	1.843	1.594	Gompertz (0.997)	6.138	0.0061
CNM CM-2266	0.011	0.009	1.886	1.556	Gompertz (0.996)	7.471	0.0059
CR056	0.013	0.014	1.782	1.526	Gompertz (0.995)	8.163	0.0068
CR061	0.014	0.013	1.846	1.559	Gompertz (0.997)	8.176	0.0093
CR058	0.014	0.014	1.840	1.604	Gompertz (0.996)	6.697	0.0069
CR059	0.011	0.013	1.688	1.453	Gompertz (0.996)	6.959	0.0079
CR060	0.013	0.011	1.833	1.515	Gompertz (0.999)	4.722	0.0063
CR019	0.014	0.017	1.843	1.627	Gompertz (0.996)	8.583	0.0080
CM-A8	0.014	0.014	1.819	1.491	Gompertz (0.997)	5.508	0.0053

Kr, radial growth rate; MM, minimal essential medium; PDA, potato dextrose agar.

[a] Solid media includes MM and PDA.

[b]The Gompertz model is a type of mathematical model [Y = N0 + C∗exp(−exp((2.718∗mue/C)∗(Lag - X) + 1))] for a time series, where growth is slowest at the start and end of a time period (sigmoidal). Lag, delay before growth, time units; mue, maximum specific growth rate.

microscopically examined using a Leica DMI 3000B fluorescence microscope (Leica Microsystem, Barcelona, Spain) to check formation or extension of germ tubes.

Hemocyte density

Additional experiments were performed to better characterize the interaction between *G. mellonella* and *A. fumigatus* strains. Fluctuations in hemocyte numbers have been shown to occur after infections with a range of microorganisms. Consequently, checking hemocyte numbers after infection may provide clues on differential virulence. Hemocyte density was assessed by counting the number of cells. Groups of larvae (three per time point) were infected with 2×10^5 conidia/larvae of each strain studied. At different time points (2 h, 4 h, and 24 h), yellow hemolymph was collected (this was done by bleeding larvae with a sterile needle) and diluted in PBS containing 0.37% 2-mercaptoethanol (v/v); hemocyte density was assessed by counting the number of cells using a hematocytometer. All determinations were performed on three independent experiments. An untouched group of larvae was included in order to determine whether a change in circulating hemocyte density can be an indication of the protective effect observed in infected larvae vs. those left untouched. Results were expressed as cells per milliliter hemolymph.

Statistical analysis

All data analyses were performed by using the software package GraphPad Prism software (v. 5). The level of statistical significance was set at a $P < 0.05$.

Results

Fungal growth rate in liquid and solid media

There were only small and statistically insignificant differences ($P > 0.8$) between the Kr on solid media among strains (Table 2). Monitoring of fungal growth in the microbroth kinetic system showed a sigmoid-shaped pattern, with the length of the lag phase largely independent of the strain (Fig. 1). Differences in the duration of this lag phase were less than 4 h (range, 4.7–8.5 h) [19]. All data fit a Gomperzt model curve with a $R^2 > 0.99\%$. Characteristic growth parameters are shown in Table 2.

In vivo evaluation of pathogenic potential of *A. fumigatus* strains and fungal burden estimation

Using the experimental conditions described in this study, all *A. fumigatus* strains (wild type and mutants) were able to kill *G. mellonella* larvae. Based on these data, the 50% lethal dose (LD_{50}) was 2×10^5 conidia per larva (Fig. 2). There was no significant difference in mean survival between larvae infected with the wild-type strain and those infected with any of the isogenic strains (Mantel-Cox test, $P > 0.2$) regardless of the *cyp51A* mutations (G54, M220, TR/L98, or *cyp*51A deleted strain). We also found that the heat-killed *A. fumigatus* wild-type strain was not lethal (Fig. 2).

The RT-PCR results demonstrate progressive increases in the *Aspergillus* burden during the time of infection. Immediately after infection (1 h post infection), the estimated fungal burden from larval homogenate was low

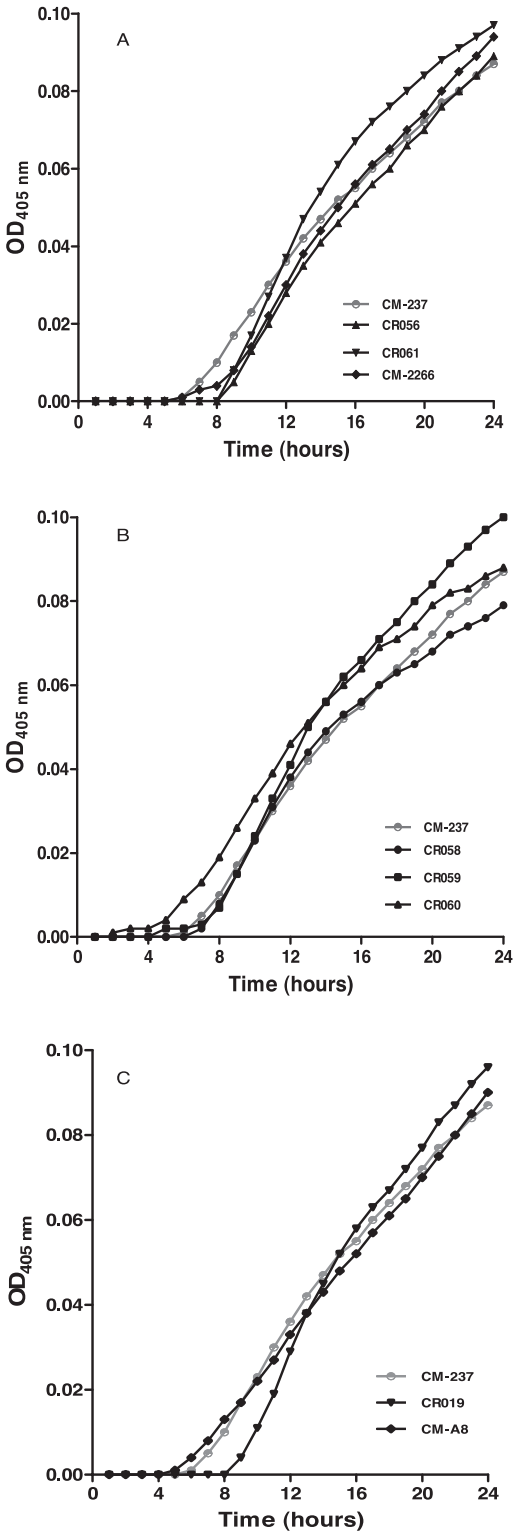

Figure 1. Growth rate of *Aspergillus fumigatus* strains expressed as optical densities (ODs) in liquid media. ODs were normalized for each growth curve by subtracting the background OD at the beginning of measurement. The wild-type strain CM-237 is included as a comparator. (A) Isogenic and clinical strains including *cyp*51A mutation at position glycine 54; (B) isogenic strains including *cyp*51A mutation at methionine 220; and (C) additional strains including TR/L98H strain and c*yp*51A deleted strain.

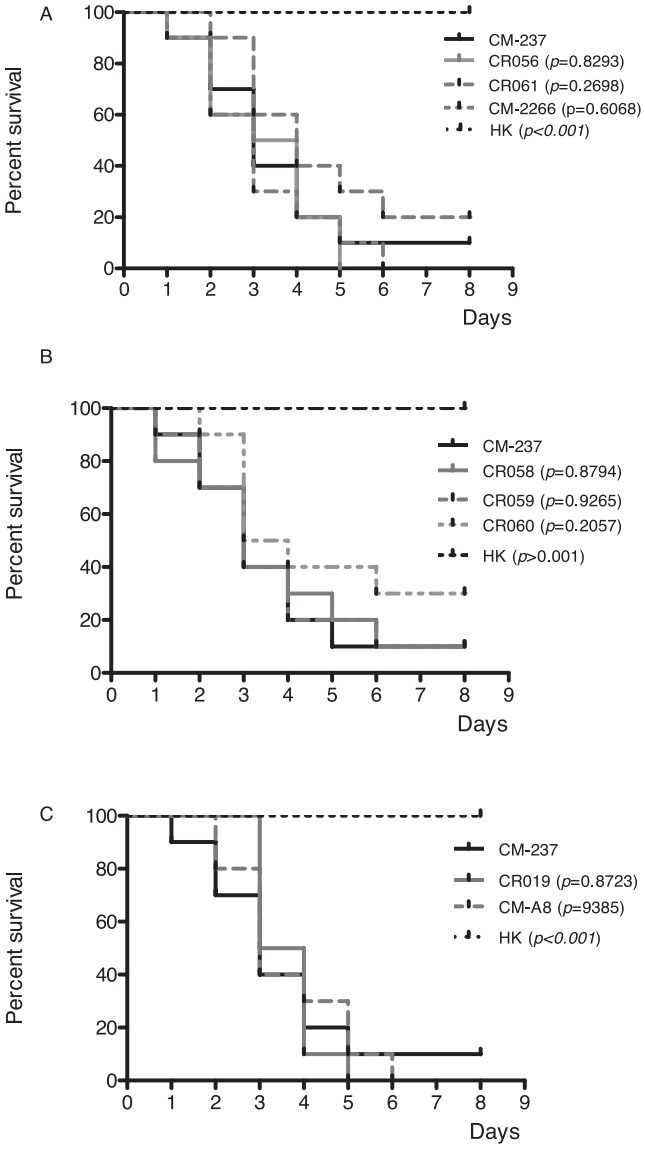

Figure 2. Survival curves of *Galleria mellonella* infected with 2×10^5 spores per larva of each strain included in the study. The survival data were grouped taking into account Cyp51A protein characteristics. (A) Isogenic and clinical strains including *cyp*51A mutation at position glycine 54; (B) isogenic strains including *cyp*51A mutation at methionine 220; and (C) additional strains including TR/L98H strain and *cyp*51A deleted strain. Wild-type strain (CM-237) was included in all analyses as a comparator. Differences in survival were analyzed using the Kaplan-Meier test.

(range, 0.8–2.1 ng DNA/g), whereas the burden obtained after 72 h was significantly higher for all strains studied (one-way analysis of variance, $P < 0.05$; Fig. 3). Fungal burden at this time point ranged from 27.7 to 53.9 ng DNA/g, with no statistically significant differences ($P > 0.05$) among strains (mutants and wild type). The exception was larvae infected with heat-killed conidia for which fungal burden remained low throughout the study ($P > 0.05$).

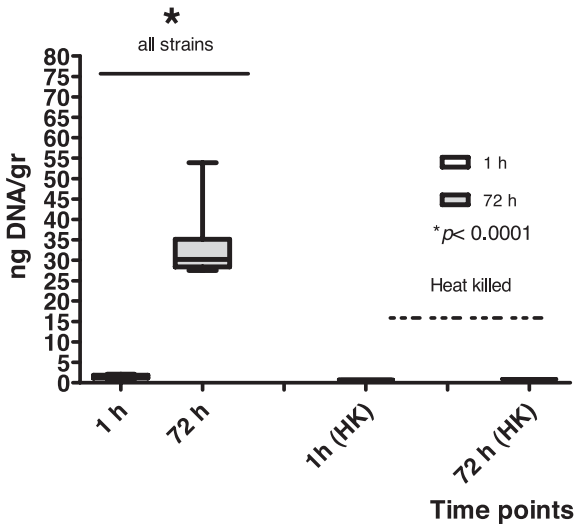

Figure 3. Estimated fungal burden from larval homogenate by a specific real time-polymerase chain reaction. All infected larvae were evaluated together. Larvae infected with heat-killed CM-237 conidia were used as a comparator.

Conidia were observed to be engulfed by hemocytes after injection and proliferated in the insect hemolymph after incubation (Fig. 4).

Hemocyte density after *A. conidia* injection

The magnitude of larval immune response to *Aspergillus* infection was also quantified for all strains. Fluctuations in hemocyte numbers have been shown to occur following infections with a range of microorganisms. At an early time point (4 h post infection), the number of hemocytes increased in the infected larvae (mutants and wild type) compared with those that were untouched (Fig. 5), denoting an early response in the larva against invading microorganism. However, at later time points, these differences were not as evident, probably because the infection had overcome the activated immune response. Of note, no statistically significant differences among mutants or wild-type–infected larvae on immune response (hemocyte count) were found at any of the time points evaluated.

Discussion

Although the molecular mechanisms of *A. fumigatus* azole resistance is well characterized (*cyp*51A point mutations), there are few measurements of the impacts these mechanisms have on pathogen fitness in different environments. The most commonly reported mechanism of azole resistance is modification of the target site encoded by the *cyp*51A gene, which leads to the impaired interaction between the azole and the target enzyme [20,21]. These modifications have been demonstrated to be advantageous in the presence of the drug since the mutants are able to grow while the wild type is not. Results presented here demonstrate that in the absence of antifungal exposure, *A. fumigatus* mutant strains (isogenic and clinical isolates) have a pattern of growth that is comparable to that of the wild type in a range of environments (solid media, liquid media, and *in vivo* mini host). Consequently, the acquisition of these particular resistance mechanisms does not appear to have an impact on growth or virulence. For CM-A8 strain, which lacks the target gene (*cyp*51A), virulence assessment in *G. mellonella* has been found to correlate closely with historic data generated using a neutropenic mice model. The mutant was morphologically indistinguishable from the wild-type strain and demonstrated an infection rate that was comparable to that in neutropenic mice [5]. Also, Mavridou et al. suggested that the virulence of *A. fumigatus* isolates with an azole-resistant phenotype due to mutations in the *cyp*51A gene was not diminished compared with

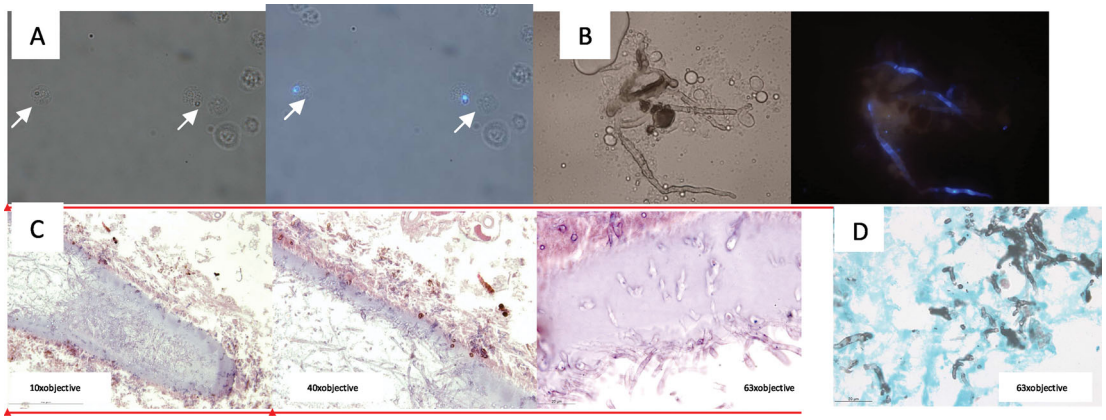

Figure 4. Light microscopy (A, left; B, left) and fluorescence examination (A, right; B, right) of homogenized larvae demonstrated the ability of *Aspergillus* spores to be engulfed (A) and proliferate (B) in the larvae. Histological sections demonstrating hyphal growth into the larvae: periodic acid-Schiff (C) and methenamine silver staining (D). This Figure is reproduced in color in the online version of *Medical Mycology*.

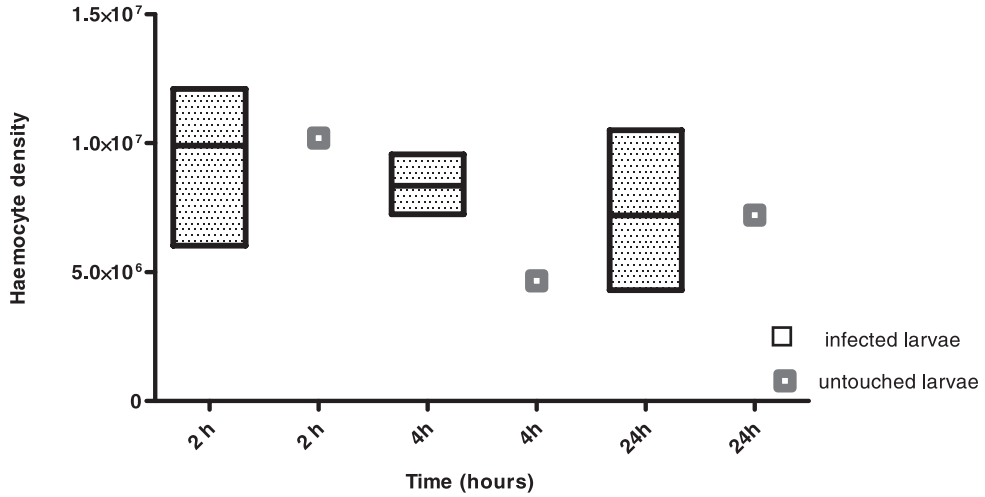

Figure 5. Hemocyte fluctuation following infections with strains included in the study. A group of untouched larvae (not handled and incubated at the same temperature as infected larvae) was included as a comparator (data point represents average density from three experiments). All infected larvae were evaluated together (floating bars represent data from mutant and wild type from three independent experiments).

wild-type isolates [22] in a nonneutropenic murine model of disseminated aspergillosis. In contrast, other investigators have suggested that the acquisition of azole resistance in *A. fumigatus* may be associated with a significant change of virulence [9]. Concerning this controversy, Anderson et al. suggested that the fitness impact of a resistance mechanism could be attributed to several complex changes in gene expression that accompany single mutations. The totality of these changes may explain the contradictory results that have been published concerning this issue [1].

Our study includes a set of strains (isogenic isolates and wild-type and clinical strains) with different azole susceptibility profiles, providing a unique opportunity to investigate the impact of azole resistance on growth and virulence of *A. fumigatus*. In general, the acquisition of a specific resistance mechanism may result in an alteration of phenotypic characteristics such as germination rate, hyphal growth, or growth rate of the resistant isolates. These changes, in turn, may result in a significant loss of virulence. Relative to this particular point, our results demonstrated that all *A. fumigatus* strains, both wild type and mutant (isogenic strains and clinical isolates differing in their azole resistance mechanism and antifungal susceptibility pattern), had comparable *in vitro* growth profiles in different environments. Therefore, a common *in vivo* pathogenic pattern might be expected. Supporting these results are data from molecular epidemiology of *Aspergillus* azole resistance that show that this phenomenon evolved in infecting strains within the lung, confirming that the ability of mutant strains to cause infection is maintained even after resistance is developed [8].

On the other hand, the possibility of using insect models instead of mammalian models for *in vivo* pathogenicity testing and screening offers a number of advantages. Invertebrates have highly efficient innate immune responses against an array of pathogens that are present in their natural environment. The *G. mellonella* model of invasive aspergillosis appears to be a useful tool for studying pathogenicity, especially because there appears to be a clear correlation with findings from mammals [23]. Conserved aspects of the innate immune response to microbial infection in insects and mammals may explain this correlation [24,25]. As a result of data presented here, the model is capable of showing either no virulence (heat-killed conidia) or equal virulence, as shown in wild-type and mutant strains. However, it is reasonable to think that the described experimental model is unable to predict reduced virulence since no strain with any kind of virulence defect was included throughout the experiments. Reports have shown the ability of a model like this to demonstrate difference in virulence. Slater et al. demonstrated that this model is also applicable when detecting differences in virulence in a wide range of *A. fumigatus* mutant strains [23].

Another point to consider is the experimental conditions selected for our study (37°C of incubation); we attempted to reproduce conditions that naturally exist during human infections, with the intention of bridging the results to the latter hosts. Therefore, other conditions were not considered in this particular work, although experimental temperature has been found critical in defining the pathogenic potential of several fungi [26–28]. In addition, an impairment of the immune response of the larvae at physiological temperatures have been suggested [29]. Concerning results found in the present study, the use of alternative experimental conditions may not have had a serious impact on the final results, since both *in vitro* and

in vivo data strongly suggest comparable behavior among strains.

In other terms, measuring changes in hemocyte density and fungal load have provided useful information on the immune response of the invertebrate to *Aspergillus* spp. [30]. The number of hemocytes appears to be a marker of the extent of the response of *G. mellonella* to fungal infections. The initial increase in the hemocyte number (compared with untouched larva) may be the result of an early response to the invading microorganism. The subsequent decline in the number of hemocytes (observed in all infected larvae) may be associated with the ability of the fungus to evade hemocyte neutralization and thus proliferate and cause larval death, as has been demonstrated by means of the fungal burden quantification (specific RT-PCR). These results have been described previously, since pathogenic isolates overcome immune response leading to a decline in hemocyte numbers and an increase in fungal cells in the hemolymph [30].

Our results suggest that acquisition of these particular azole-resistance mechanisms in *A. fumigatus* does not appear to have any impact in terms of virulence. The results also highlight the fact that insects may provide valuable early insights into virulence and pathogenicity and, in particular, may contribute to the generation of hypotheses that can be further tested in refined experiments in mammalian models.

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Declaration of interest

In the past 5 years, M.C.E. has received grant support from Astellas Pharma, bioMèrieux, Gilead Sciences, MSD, Pfizer, Schering Plough, Soria Melguizo SA, European Union, the ALBAN Program, Spanish Agency for International Cooperation, Spanish Ministry of Culture and Education, Spanish Health Research Fund, Instituto de Salud Carlos III, Ramon Areces Foundation, and the Mutua Madrileña Foundation. M.C.E. has also been an advisor/consultant to the Pan-American Health Organization, Gilead Sciences, MSD, Pfizer, and Schering Plough and has been paid for talks on behalf of Gilead Sciences, MSD, Pfizer, and Schering Plough. S.J.H. has received research grants from Astellas; support grants from Gilead, Pfizer, and the Fungal Research Trust; travel grants from Astellas and Schering-Plough; equipment grants from the Fungal Research Trust; and has been paid for talks on behalf of Pfizer and Astellas. W.H. has received research support and acted as a consultant for Astellas, Pfizer, Gilead, Merck, and F2G. S.H. has received research grants from Astellas; support grants from Gilead, Pfizer, and the Fungal Research Trust; travel grants from Astellas and Schering-Plough; equipment grants from the Fungal Research Trust; and has been paid for talks on behalf of Pfizer and Astellas. Other authors report no conflicts.

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