An alternative host model of a mixed fungal infection by azole susceptible and resistant *Aspergillus* spp strains

L Alcazar-Fuoli, Mj Buitrago, A Gomez-Lopez, and E Mellado

Mycology Reference Laboratory; Centro Nacional de Microbiologia; Instituto de Salud Carlos III; Madrid, Spain

**Keywords:** *Aspergillus* spp, *cytP51A*, *Galleria mellonella* infection model, mixed infection, one-step single tube real time PCR

---

*Aspergillus fumigatus* is the most common mold involved in human infections. However, the number of non-*fumigatus* species able to cause disease is continuously increasing. Among them, *Aspergillus lentulus* is reported in hematological and cystic fibrosis patients and in those treated with corticosteroids. *A. lentulus* differs from *A. fumigatus* in some clinically relevant aspects such as virulence and antifungal susceptibility, showing high MICs to most antifungals. Previous studies proved that *A. lentulus* was pathogenic in immunocompromised mice, although the course of the infection was delayed compared to *A. fumigatus*. These differences could explain why *A. lentulus* is mostly found in mixed infections with *A. fumigatus* challenging the diagnosis and treatment. We used the alternative model host *Galleria mellonella* to compare virulence, host interaction, fungal burden and antifungal response when larvae were infected with *A. fumigatus* or *A. lentulus* alone, and with a mixture of both species. *A. lentulus* was pathogenic in *G. mellonella* but infected larvae did not respond to therapeutic doses of voriconazole. We were able to simultaneously detect *A. fumigatus* and *A. lentulus* by a multiplex Nested Real Time PCR (MN-PCR). Comparative analysis of larvae histological sections showed melanization of both species but presented a different pattern of immune response by haemocytes. Analysis of fungal burden and histology showed that *A. lentulus* survived in the *G. mellonella* despite the antifungal treatment in single and mixed infections. We conclude that the simultaneous presence of antifungal susceptible and resistant *Aspergillus* species would likely complicate the management of these infections.

---

**Introduction**

The incidence of invasive fungal diseases (IFD) together with the number of fungal species pathogenic for humans has increased in the past decade.1-3 *Aspergillus fumigatus* continues to be the most common fungi isolated from *Aspergillus* related mycosis. However, the epidemiology of these infections is changing with new emerging cryptic species becoming isolated and occasionally causing disseminated infections in immunocompromised hosts.5,4 The risk factors associated with these cryptic species are still undefined but it is known that they show remarkable high MICs to multiple antifungal agents.5,6 The reasons for this change in fungal populations, as well as the emergence of drug resistance, are still poorly understood.

*Aspergillus lentulus*, one of these sibling species belonging to the section *Fumigati*, can be considered as an uncommon fungal pathogen for humans. However, it has been increasingly reported in severely immunocompromised patients such as hematological and cystic fibrosis patients, those suffering from chronic obstructive pulmonary disease (COPD) or other populations receiving high doses of corticosteroids.7-10 Invasive aspergillosis caused by *A. lentulus* is associated with high mortality and clinical isolates of this species show high MICs to azoles, echinocandins and amphotericin B.6,9,11 Resistance to voriconazol (VRC) is especially worrisome considering that this antifungal is the primary drug used to treat invasive aspergillosis. *A. lentulus* cannot be distinguished by classical morphological methods from other *Fumigati* section species including *A. fumigatus*. To solve this problem, molecular methods based on amplification and sequencing of the β-tubulin, rodlet or calmodulin genes are currently used in order to differentiate them at species level. Colonies of *A. lentulus* appear to be less sporulated and are white colored compared to *A. fumigatus*. Pathogenicity of *A. lentulus* has been proven in immunocompromised mice although the course of infection was delayed compared to *A. fumigatus* when the same intranasally administered inocula size was used.12 This difference in the developmental fungal growth in vivo might explain why *A. lentulus* is frequently isolated in mixed infections with *A. fumigatus* or other *Aspergillus* spp.1,9,11 The simultaneous co-infection by multiple fungal species could represent a possible threat as *A. lentulus* is often misidentified and shows resistance to most antifungals in clinical use. In addition, population dynamics on a fungal mixed infection and species interaction in the host and in the environment remains entirely unexplored.

Correspondence to: Laura Alcazar-Fuoli; Email: lalcazar@isciii.es
Submitted: 12/03/2014; Revised: 02/13/2015; Accepted: 02/25/2015
http://dx.doi.org/10.1080/21505594.2015.1025192
To date, murine models are considered to be the gold standard to study fungal pathogenesis and analyze efficacy of antifungal treatment. However, economic and logistical factors, together with ethical considerations, are limiting the use of mammals in infection experiments. To overcome these limitations, an alternative approach using different invertebrate infection models has been developed, including amoebae, Caenorhabditis elegans, Drosophila melanogaster, and larvae of the wax moth Galleria mellonella. All of them are inexpensive, ethically approved and do not require specialized facilities for maintenance. Compared to other invertebrates, G. mellonella offers several advantages that include: the size of the larvae which facilitates easy handling; and the possibility of injecting a defined inoculum and drug concentration. Furthermore, in contrast to other invertebrate hosts, G. mellonella larvae can be maintained at temperatures up to 37°C, equivalent to the temperature in mammalian hosts.

In this study we first optimized a one-step diagnostic tool based on multiplex nested PCR (MN-PCR) for simultaneous detection of A. fumigatus and A. lentulus that allowed fungal quantification from tissue samples. We then established a host model of mixed infection with A. fumigatus and A. lentulus in the alternative host G. mellonella. The model was used to evaluate differences in virulence, fungal burden, pathogenesis and antifungal drug response in presence of Aspergillus species mixtures in vivo.

Material and Methods

Strains, media and growth conditions

For the standardization and validation of the one step multiplex nested-PCR (MN-PCR) a total of 20 clinical strains for each species (20 A. fumigatus and 20 A. lentulus) were employed. All strains belonged to the Mycology Reference Laboratory collection, except the strain AF14 that was kindly donated by Dr. J.A. Calera. The specificity of the technique was assessed with other fungal species with clinical origin: Aspergillus flavus (CM-2669), Aspergillus terreus (CM-2013), Fusarium verticillioides (CM-2975), Fusarium oxysporum (CM-2914), Scedosporium apiospermum (CM-3169), Cryptococcus neoformans (CL-2132), Rhizopus oryzae (CM-3020), Rhizomucor variabilis (CM-2437), Candida albicans (ATCC64551) and Candida glabrata (CL-5533). In addition, human and murine genomic DNAs (Promega, Madrid, Spain) were included.

Primers and molecular beacon probes were designed on the basis of the nucleotide sequence of the cyp51A gene from 10 strains of each A. fumigatus and A. lentulus. The Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA) was employed to design primers and probes for the MN-PCR assay. Selected primers and probes were subjected to a blast search into the GenBank sequence database (http://www.ncbi.nih.gov/Genbank/) to avoid cross-homology with other organisms. The primers for the first PCR were designed with a primer-annealing temperature higher than the primers for the second PCR segment (Table 1). The first PCR segment at that temperature did not allow the primers for the second PCR to anneal to the template. The outer

Table 1. Primers and Probes Sequences designed for the Multiplex Nested Real Time PCR assay.

| Primer Name                  | Sequence (5'-3')*          |
|------------------------------|----------------------------|
| OliNestCyp51A (Forward, First PCR) | CTYGTKCATAATGTYGTATTTATCAAYATTCTTCTYCGG |
| OliNestCyp51A2 (Reverse, First PCR) | CGCMGACTGATGTYAAGCCTACTGTAAGCTCTC |
| OliCyp51 int-3 (Forward, second PCR) | CAGAACCGCAGAATGCTC |
| OliCyp51 int-2 (Reverse, second PCR) | GACRTCTCGACTGATGCCC |
| Cyp51 int-AP (A. fumigatus probe) | FAM-CGGGATCAAGCTTGATGTAAGGATTGATCAGCTT |
| Cyp51 int-AL (A. lentulus probe) | HEX-CGGGATCAAGCTTGATGTAAGGATTGATCAGCTT |

*Underlining regions indicates the PCR target sequence

DNA extraction

Genomic DNA from Aspergillus spp and other fungal strains were isolated using a rapid extraction procedure. Two µL of extracted DNA were used for each PCR reaction. DNA extraction from lung tissue samples used to validate the PCR assay, was performed using the QiAmp DNA Kit (Qiagen, Izasa, Madrid, Spain) following manufacturer recommendations. Elution was performed in 50 µL of elution buffer. DNA from G. mellonella was isolated from 0.85% saline homogenized larvae as follows: DNA from 25 mg of homogenized larvae was isolated using the QiAmp DNA Kit for tissues, following manufacturer’s instructions and eluting in 50 µL. All samples were stored at –20°C and they were allowed to thaw at room temperature before testing.

Multiplex Nested-PCR assay

Primers and Probes design

Primers and molecular beacon probes were designed on the basis of the nucleotide sequence of the cyp51A gene from 10 strains of each A. fumigatus and A. lentulus. The Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA) was employed to design primers and probes for the MN-PCR assay. Selected primers and probes were subjected to a blast search into the GenBank sequence database (http://www.ncbi.nih.gov/Genbank/) to avoid cross-homology with other organisms. The primers for the first PCR were designed with a primer-annealing temperature higher than the primers for the second PCR segment (Table 1). The first PCR segment at that temperature did not allow the primers for the second PCR to anneal to the template. The outer
primers were degenerated to hybridize with all species belonging to the *Fumigati* section to be used in future assays. The Molecular Beacon probes were designed based on the species specific cyp51A intron sequences (Supplementary Fig. 1) and were labeled with 5′-6-carboxyfluorescein (FAM) for *A. fumigatus* and 4,7,2′, 4′, 5′, 7′-hexachloro-6-carboxyfluorescein (HEX) for *A. lentulus* (Sigma-Genosys, Spain). Both were labeled with Black Hole Quencher1 (BHQ1) in the 3′ end as quencher.

**PCR conditions and standardization in vitro**

PCR reactions were undertaken in a LC480 unit (Roche Diagnostics, Madrid, Spain). The SensiMix (dT) kit 2x (Bioline, Ecogen, Spain) was used following manufacturer’s instructions. The PCR was performed in a total volume of 20 μL. The PCR mixture contained 0.05 μM each of the first-PCR primers, 0.5 μM each of the second-PCR primers, 0.4 μM of both probes, 3mM MgCl₂ and a 2 μL aliquot of DNA from the extracted sample. Cycling conditions included a first step for pre-incubation (activation of the enzyme) and denaturation of the template DNA at 95°C during 10 minutes. The first PCR consisted on only 10 cycles which included 25s at 95°C, 30s at 58°C and 5s at 72°C. The first PCR was immediately followed by the second PCR: 40 cycles which included 25s at 95°C, 30s at 52°C and 5s at 72°C. Quantification standards were run in conjunction which each set of samples as well as negative controls.

Standard curves were constructed with PCR results from 5 repetitions of different dilutions of *A. fumigatus* (CM-237) and *A. lentulus* (CM-1290) genomic DNA. Dilutions ranged from 20 ng to 2 fg DNA/20 μL of reaction. A regression line (Supplementary Fig. 2) was constructed by plotting the Log10 concentration of DNA vs. its corresponding threshold cycle value (Ct). If this curve exhibited a linear regression coefficient value of >0.980, the standard curve was then used to determine the efficiency of the PCR, sensitivity and reproducibility of the assay. In order to evaluate the specificity, 2 ng of DNA/20 μL from the species mentioned before (see Control strains) as well as human and mouse genomic DNA were included in the PCR assay.

Data were analyzed using LC480 software (Roche Diagnostics, Madrid, Spain) and a color compensation experiment was created to avoid signal overlapping between both fluorophores. Each PCR run contained both positive and negative controls consisting of water and different concentrations of genomic DNA from *A. fumigatus* or *A. lentulus*.

**Murine infections**

Murine models for lung sample collection were performed according to the models previously described. Murine infections were performed in compliance with the guidelines of the Animal Welfare Act of 1966 (USDA and NIH). The animals were monitored daily to determine clinical signs of infection. The primary scoring of the infected murines and the control groups was performed by a review committee. Treatment and follow-up procedures were in agreement with the Guide for the Care and Use of Laboratory Animals 1985 (NIH, USA). The surviving mice were sacrificed by an overdose of anaesthesia (Ketamine). The lungs were collected and homogenized in saline. An aliquot of this homogenate was plated onto Sabouraud plus chloramphenicol and gentamicin for fungal infection validation. The rest of the homogenate was stored at −20°C until DNA extraction was performed.

**Histopathological analysis of infected *G. mellonella***

Larva histology was performed as described before. Three larvae per group were longitudinally sectioned and fixed for 24 h in 4% buffered formalin and dehydrated with increasing concentrations of ethanol (70%, 80%, 90%, 96% and 100%). The samples were then treated with xylene and paraffin embedded. Tissue sections of 5 microns were stained with periodic acid Schiff (PAS) and sections examined with a Leica DMI 3000B microscope.

**Fungal burden quantification in *G. mellonella***

In order to assess fungal burden as a quantitative criterion for virulence and antifungal response, the MN-PCR assay described before was performed on total genomic DNA extracted from homogenized *G. mellonella* at different time points (2, 4 and 6 d post infection). DNA was extracted as previously described. Fungal burden was expressed as ng of fungal DNA / gram of larva.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism, version 5 Project (GraphPad Software, San Diego, CA). The statistical significance of variances between fungal burdens was
calculated by using a nonparametric Mann-Whitney t test. A p value <0.01 was considered significant. Kaplan-Meier survival curves were analyzed by using a log-rank (Mantel-Cox) test for significance. A p value <0.01 was considered significant.

Nucleotide sequence accession numbers

The full nucleotide sequences of the cyp51A genes used in this work appear in the GenBank nucleotide sequence database under Accession Ns. GU479991 for A. lentulus cyp51A and Accession Ns. AF338659 was the reference sequence used for A. fumigatus.

Results

Detection of A. fumigatus and A. lentulus by one step Multiplex Nested Real Time PCR

As accurate species identification could influence the infection outcome, a one-step diagnostic tool based on a multiplex nested PCR (MN-PCR) was optimized for differential detection of A. fumigatus and A. lentulus. Two sets of primers were designed for the first and second round of amplification including 2 degenerated outer primers, 2 specific inner primers and 2 Molecular Beacon probes labeled with FAM and HEX specific for A. fumigatus and A. lentulus, respectively. The design was based in a region of the cyp51A gene that contained a common intron of 71 bp. This region allowed the design of common primers for all the Aspergilli of the section Fumigati and specific probes for each species within the group (Fig. S1).

The detection limit of the designed PCR was calculated at 200 fg of DNA in 20 μl of PCR reaction mixture for both A. fumigatus and A. lentulus. The efficiency for both PCR amplifications was higher than 86% with coefficients (r2) of 0.99 (Fig. S2). The averages of coefficients of variation for all DNA concentrations were 3.4% for A. fumigatus and 2.7% for A. lentulus. Once the PCR conditions were optimized, the developed MN-PCR assay was validated on 40 clinical strains of A. fumigatus and A. lentulus with a specificity of 100%. Sequencing of all PCR amplified fragments confirmed the specificity. No signal was detected when 2 ng of DNA from other fungi (different species of Aspergillus, Fusarium, Candida, mucorales etc...) and genomic DNA from human and mouse were also tested. In addition, no cross-reaction was observed between A. fumigatus and A. lentulus.

In order to validate the MN-PCR assay in vivo, first we used an experimental model of pulmonary aspergillosis using cyclophosphamide treated mice. Mice were infected with A. fumigatus (CM-237) or A. lentulus (CM-1290) and the infection was confirmed with positive cultures from lung tissues. A total of 8 mice inoculated with A. fumigatus had proven lung infection and positive PCR results from lung tissue. Similarly, PCR was positive in 13 of 14 lung samples (93%) from mice with proven infection with A. lentulus. The average of DNA concentration detected per μL in samples was 1.5 × 107 fg.

A second type of cortisone immunosuppressed murine model, closer to the cystic fibrosis patients, was performed. In this model the PCR assay was able to detect 100% of samples from mice with proven infection infected with A. fumigatus (10/10) or A. lentulus (10/10).

All together, the MN-PCR technique was positive in 41 of 42 samples (98%) and was able to distinguish both species in the immunosuppressed mice models.

Differential pathogenesis of A. lentulus and A. fumigatus in G. mellonella

Prior to any assay in vivo, the growth rate and the antifungal susceptibility profile of A. fumigatus versus A. lentulus strains were compared in vitro. There were no differences in terms of colony size and radial growth between both species. As shown before, A. lentulus was resistant to VRC (MIC of 4 μg/L) while A. fumigatus was susceptible (MIC 0.5 μg/L) (Fig. S3). The virulence in the alternative host Galleria mellonella was determined by infecting the larva with an inoculum of each Aspergillus spp (1.5 × 103 cfu/larva). When A. fumigatus was used, 100% of infected larvae died between the 3rd and 4th day post infection. However, virulence of A. lentulus was slower and only 80% of larvae had died by day 10 post infection (Fig. 1A). The survival pattern of infection mimics that observed using the same inocula of Aspergillus spp in immunocompromised mice12 (Fig. S4).

In order to explore the effect of Aspergillus infection on G. mellonella physiology, the infected larvae were fixed and paraffin-embedded sections were stained with PAS and evaluated for histological changes at different time points post infection (Fig. 1B). Histological analysis revealed differences between the A. fumigatus and A. lentulus infection pattern. Larvae showed branched invasive hyphal growth of A. fumigatus while A. lentulus was encapsulated by immune cells. Hemocytes with clearly visible nodule melanization were observed in nodules attached to organ structures and the fungus (Fig. 1B).

Voriconazole efficacy in an in vivo model of infection with Aspergillus spp using G. mellonella

The G. mellonella alternative model of infection was used to compare the efficacy of antifungal agents to treat A. fumigatus or A. lentulus infected larva. Groups of 20 larvae were infected with 1.5 × 105 spores per larvae and treated groups were inoculated with therapeutic doses of VRC (4 μg/larva equivalent to 10 mg/Kg/day) within 2 hours of infection. Virulence was reported as survival rate against time (Fig. 2A).

The results showed that at therapeutic doses of VRC, larvae infected with azole susceptible A. fumigatus (akubKU80) strain significantly improved survival in relation to the untreated group (P < 0.0002). In contrast, when G. mellonella were infected with the azole resistant A. lentulus strain (CM-1290), the percentage of survival showed no significant differences between this group and the respective untreated group (p = 0.7) (Fig. 2B).

To test VRC efficacy of A. fumigatus or A. lentulus in vivo, we used the developed MN-PCR to examine the fungal burden of G. mellonella infected with each Aspergillus strains. Time points were set at 2 and 4 d post infection. The analysis identified a significant decrease (P < 0.01) in the fungal burden retrieved from VRC treated larvae infected with A. fumigatus compared to A.
lentulus infected larvae, and in comparison with untreated animals (Fig. 2C) at both time points.

Histopathology analysis showed that when the larvae were infected with *A. fumigatus* and treated with VRC, the fungus was almost cleared with tissues appearing healthy. In contrast, *A. lentulus* CM-1290 strain persisted in the tissues even after 6 d of infection (Fig. 3).

**Mixed infection model**

A model of *A. fumigatus* and *A. lentulus* mixed fungal infection (1:1) in *G. mellonella* was used to evaluate the influence of intrinsically resistant species on the antifungal treatment outcome. Using the previously described model, larvae were infected with an equal amount of spores of both species (5 × 10^4 spores per larva of each *Aspergillus* species). VRC response (4 μg/larva equivalent to 10 mg/Kg) of the *G. mellonella* larvae infected with the mixture of *A. fumigatus* and *A. lentulus* was monitored by larvae survival rate and fungal burden using MN-PCR.

When the larvae were infected with a combination of both species, survival of infected larvae was intermediate to those of each single species infection respectively (Fig. 4A). In addition, *Aspergillus* spp mixed infected *G. mellonella* did not respond to therapeutic doses of VRC (p = 0.08) (Fig. 4B).

Analysis of fungal burden showed no differences of fungal DNA per larvae between 2 and 4 d for both *Aspergillus* species (Fig. 4C). When VRC was administered fungal burden dropped for *A. fumigatus* at both, 2 and 4 d post infection, compared to the untreated group (P < 0.01) (Fig. 4C and D). In the case of *A. lentulus* this decrease was statistically significant at 2 d (p = 0.002) but not at 4 d (p = 0.35) (Fig. 4C and D). The fungal burden was maintained at 6 d with no differences when it was compared to fungal burden of VRC treated larvae at 4 d post infection (p = 0.44) (Fig. 4D).

**Discussion**

Mixed infections are expected to occur in nature where interaction between co-infecting microorganisms will depend on the host environment. The clinical relevance and the epidemiology of fungal mixed infections is relatively unknown but recent reports highlight the fact that these might be underestimated.27 Mixed infections that include more than one fungal species are
recently published. Several case reports have described invasive and *A. fumigatus* also, a report of a mixed infection of the lung with *A. fumigatus* and *Candida* species in the section *Fumigati* has high MICs to multiple antifungals including VRC and AmB. A correct and early diagnosis leading to a prompt antifungal therapy is one essential step to improve clinical outcome. In this sense, molecular techniques have been proven to be very useful for the diagnosis of fungal infections, and most of them are based on the detection of the Internal transcriber Spacer (ITS) regions of the rDNA. However, ITS sequences cannot be used to differentiate species within the *A. fumigatus* complex. This is a great disadvantage as, until now, only single locus genes have been able to discriminate between those species. Among them, the more usual targets have been β-tubulin, calmodulin, and rodlet A genes. The use of single copy genes shows better discriminatory power but have the inconvenience of lack of sensitivity when clinical samples such as blood or BALs are used. In order to improve this, we developed a multiplex quantitative one-step nested PCR (MN-PCR) targeting the intron sequences of the azole target, 14-sterol demethylase (*cyP51A*).

The developed MN-PCR is useful for the correct identification of *A. fumigatus* and *A. lentulus*. Moreover, it allows discrimination of *A. fumigatus* and closely related species and it can be implemented for the correct identification of other *Aspergillus* species in the section *Fumigati* in clinical specimens (under way in our laboratory). Also, the assay has been validated in cultured strains and in lung samples from 2 experimental mice models of invasive aspergillosis with excellent sensitivity (98%) and specificity (100%).

The *G. mellonella* model was used to explore *Aspergillus* spp fungal virulence and the in vivo correlation with in vitro antifungal susceptibility data. Results showed a high correlation in terms of survival rate between the murine and the wax moth model of aspergillosis (Supplementary Fig. 4). Similarly to the murine model, *A. lentulus* was pathogenic in *G. mellonella*, although survival of infected larvae was prolonged compared to larvae infected with *A. fumigatus*. Those differences in the virulence pattern would suggest a more rapid or invasive growth characteristic of *A. fumigatus* in vivo.

Also, comparative analysis of tissue sections of *G. mellonella* infected with each *Aspergillus* spp showed a different pattern.

![Figure 2](image-url)
Larvae melanization occurred with both species at all time points, but recruitment of haemocytes differed. Branched invasive hyphal growth of *A. fumigatus* was observed in untreated larvae while the pattern of infection with *A. lentulus* was encapsulated hyphal by immune cells producing nodules. This data suggests that *A. fumigatus* and *A. lentulus* might use different strategies to respond to host defenses. While *A. fumigatus* escapes from haemocytes, germinates and quickly develops in the host, *A. lentulus* is encapsulated by immune cells.

No statistical differences were found in terms of haemocyte density between the 2 species in any of the time points, although it seems that there is a delay of the immune response by haemocytes against *A. lentulus* compared to *A. fumigatus* (data not shown). Changes in haemocyte density following microbial challenge have been previously observed correlating high haemocyte concentration with longer survival rates.

*Galleria mellonella* presents certain benefits in comparison with other non-mammalian models of infection. Previous studies have documented that *G. mellonella* is a valid model to evaluate the microbial virulence and/or the efficacy of different antimicrobial agents in infections caused by different bacteria and fungi, such as *C. neoformans*, *Candida* spp., *Histoplasma* spp., and *A. fumigatus*. In addition, the *G. mellonella* model appears to be a promising approach to evaluate fitness of drug-resistant strains.

Since VRC is the first line antifungal used to treat aspergillosis, we also analyzed the in vivo response of *G. mellonella* infected with *A. fumigatus* or *A. lentulus* and treated with this antifungal. *G. mellonella* was a useful model in the evaluation of the efficacy of VRC at concentrations equivalent to the therapeutic doses used in humans. The results correlated very well with the antifungal susceptibility phenotype shown in vitro. While VRC was capable of improving the survival of larvae infected with azole susceptible *A. fumigatus*, this drug was ineffective for larvae infected with azole resistant strains (*A. lentulus*).

To further investigate the effect of antifungals during infection, we analyzed fungal burden of *Aspergillus* infected larvae by the MN-PCR described before. MN-PCR showed progressive increases in the *Aspergillus* burden during the time of infection at 2 and 4 d post infection, although it was not significant, when larvae were not treated with VRC. Antifungal treatment significantly reduced...
A. fumigatus DNA per gram of larvae at 2 d of infection. Reduction of fungal burden at 4 d of infection was observed for larvae infected with A. fumigatus and A. lentulus although that decrease was more prominent for A. fumigatus than for A. lentulus (P < 0.002 and P < 0.01 respectively) (Fig. 2C). These results suggest that isolates with higher VRC MICs might show a poorer response to antifungal treatment in vivo.

Following the same steps, we used the model of G. mellonella to evaluate the efficacy of VRC in the mixed infection model. A similar approach to evaluate a mixed infection of Rhizopus oryzae and A. fumigatus using corticosteroid-immunosuppressed mice has been used to explore if the exposure to antifungals could provoke a selection pressure for breakthrough mucormycosis during infection. Results of survival analysis and fungal burden of mice infected with both species showed that VRC pre-exposure in vitro promotes breakthrough infection in vivo with R. oryzae and increases mortality due to R. oryzae.44

In our model, the results show that at therapeutic doses VRC did not significantly improved larval survival when they were co-infected with equal amounts of A. fumigatus and A. lentulus. In addition, quantitative analysis of fungal burden at 6 d post infection demonstrated A. lentulus selection by VRC in the mixed infection. This suggests that in a mixed infection with A. fumigatus and A. lentulus the treatment with VRC may facilitate invasive growth of A. lentulus. The eventual domination of A. lentulus over A. fumigatus is consistent with concepts of antimicrobial selection, where suppression of more susceptible populations prepares the emergence of resistant pathogens with reduced fitness cost.45 Similarly, with malaria it has been proposed that when sensitive and resistant parasites co-infect the same host individuals, drug use would further increase the relative fitness of drug-resistant clones by removing drug-sensitive competitors.46

It may seem that there is a discrepancy between the decrease in fungal burden and the lack of response in survival of G. mellonella infected with A. lentulus in the single and the mixed infection. It has been described that A. lentulus VRC MICs range from 4 to 6 mg/L.6 Therefore, the reduction of fungal burden of G. mellonella infected with A. lentulus and treated with VRC compared to the untreated ones might be explained because VRC is able to eliminate or to reduce fungal growth to some extent but not enough to improve survival of the Galleria at the VRC concentration that was tested. We cannot discard that A. lentulus would respond in vivo if higher concentrations of VRC are used.

Altogether, this data indicates that G. mellonella is a simple model that can also be used to evaluate competition and in vivo fitness in mixed infections by fungi. Moreover, we were able to simultaneously detect 2 species closely related such as A. fumigatus and A. lentulus by using real time PCR, showing a promising tool for clinical detection of Aspergillus section Fumigati in single or mixed infections. Given the differences in antifungal susceptibility between both species, antifungal pressure against A. fumigatus might be relevant for selective infection by A. lentulus. In conclusion, the presence of antifungal resistant species would likely complicate the management of fungal infections, thus representing a threat for patients under long-term azole treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Gema del Rio for technical assistance.

Funding
EM was supported by Fondo de Investigacion Sanitaria (FIS: PI12_02376). LA-F was funded by Fondo de Investigacion Sanitaria with a Miguel Servet fellowship (FIS:CP11/00026).

Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.
13. Desalermos A, Fuchs BB, Mylonakis E. Selecting an invertebrate model host for the study of fungal pathogenesis. PLoS Pathog 2012; 8: e1002451; PMID:22319439; http://dx.doi.org/10.1371/journal.ppat.1002451.

14. Fuchs BB, Mylonakis E. Using non-mammalian hosts to study viral virulence and host defense. Curr Opin Microbiol 2006; 9: 345-51; PMID:16814595; http://dx.doi.org/10.1016/j.mib.2006.06.004.

15. Fuchs BB, Mylonakis E. Galleria mellonella as a model host to study virulence of Candida. Virulence 2014; 5: 237-9; PMID:23484470; http://dx.doi.org/10.4161/viru.27434.

16. Vicentenfranquiza R, Moreno MA, Leal F, Calera JA. Tip1 and zeef genes of Aspergillus fumigatus encode the zinc transporter proteins of a zinc uptake system induced in an acid, zinc-depleted environment. Eukaryot Cell 2005; 4: 837-48; PMID:15879518; http://dx.doi.org/10.1086/428753.

17. da Silva Ferreira ME, Kress MR, Savoldi M, Goldman GH. The akuB(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity and decreased virulence. Antimicrob Agents Chemother 2014; 58: 1071-83; PMID:24295973; http://dx.doi.org/10.1128/AAC.00946-13.

18. Peleas Garcia F, Gama B, Garcia-Gil Y, Alcazar-Fuoli L, Escribano R, Goyo JL, Moro P, Mellado E, Bouza E. Incidence of mixed invasive aspergillosis in a general hospital in Madrid: An underestimated entity? In 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 10-13 May 2014. Abstract P0005A.

19. David R.Soll. Mixed Mycotic Infections, In: Broden KA GJ, ed. Polymeric Diseases. Washington (DC): ASM Press, 2002.

20. Yau MC, Chai P, Desalermos A, Fuchs BB, Mylonakis E. Selecting an invertebrate model host to study virulence of Candida albicans mutants in mice and Galleria mellonella larvae. FEMS Immunol Med Microbiol 2002; 34: 157-63; PMID:12138147; http://dx.doi.org/10.1111/j.1574-696X.2002.tb00617.x.

21. Brennan M, Thomas DY, Whiteway M, Kavanagh K. Correlation between virulence of Candida albicans mutants in mice and Galleria mellonella larvae. FEMS Immunol Med Microbiol 2002; 34: 157-63; PMID:12138147; http://dx.doi.org/10.1111/j.1574-696X.2002.tb00617.x.

22. Meyers JD. Fungal infections in bone marrow transplant patients. Semin Oncol 1990; 17: 10-3; PMID:2353204.

23. Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, et al. Aspergillus flavus and Aspergillus fumigatus virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect Immun 1993; 61: 1650-6; PMID:5940632; http://dx.doi.org/10.1128/IAI.61.5.1650-6.1993.

24. Galleria mellonella. BMC Microbiol 2014; 14: 2536-8; PMID:15917566; http://dx.doi.org/10.1186/1471-2180-14-2536.

25. Serrano R, Gusmao L, Amorim A, Araujo R. Rapid identification of Aspergillus fumigatus within the section Fumigati. BMC Microbiol 2011; 11: 82; PMID:21530879; http://dx.doi.org/10.1186/1471-2180-11-82.

26. Bergin D, Brennan M, Kavanagh K. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of Galleria mellonella. Microbes Infect 2003; 5: 1389-95; PMID:14670452; http://dx.doi.org/10.1016/j.micinf.2003.09.019.

27. Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, et al. Aspergillus flavus and Aspergillus fumigatus virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect Immun 2003; 71: 3842-50; PMID:15972460; http://dx.doi.org/10.1128/IAI.73.8.3842-3850.2005.

28. Mellado E, Garcia-Effron G, Buitrago MJ, Alcazar-Fuoli L, Escribano R, Goyo JL, Moro P, Mellado E, Bouza E. Invasive aspergillosis caused by cryptic Aspergillus species: a report of two consecutive episodes in a patient with leukaemia. J Med Microbiol 2005; 54: 2605-9; PMID:19332683; http://dx.doi.org/10.1099/jmm.0.044867-0.

29. Meyers JD. Fungal infections in bone marrow transplant patients. Semin Oncol 1990; 17: 10-3; PMID:2353204.

30. Junttila MR, Mylonakis E. Using non-mammalian hosts to study fungal virulence and host defense. Curr Opin Microbiol 2006; 9: 345-51; PMID:16814595; http://dx.doi.org/10.1016/j.mib.2006.06.004.

31. Desalermos A, Fuchs BB, Mylonakis E. Selecting an invertebrate model host for the study of fungal pathogenesis. PLoS Pathog 2012; 8: e1002451; PMID:22319439; http://dx.doi.org/10.1371/journal.ppat.1002451.

32. Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, et al. Aspergillus flavus and Aspergillus fumigatus virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect Immun 1993; 61: 1650-6; PMID:5940632; http://dx.doi.org/10.1128/IAI.61.5.1650-6.1993.

33. Vicentenfranquiza R, Moreno MA, Leal F, Calera JA. Tip1 and zeef genes of Aspergillus fumigatus encode the zinc transporter proteins of a zinc uptake system induced in an acid, zinc-depleted environment. Eukaryot Cell 2005; 4: 837-48; PMID:15879518; http://dx.doi.org/10.1086/428753.

34. da Silva Ferreira ME, Kress MR, Savoldi M, Goldman GH. The akuB(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity and decreased virulence. Antimicrob Agents Chemother 2014; 58: 1071-83; PMID:24295973; http://dx.doi.org/10.1128/AAC.00946-13.

35. Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, et al. Aspergillus flavus and Aspergillus fumigatus virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect Immun 1993; 61: 1650-6; PMID:5940632; http://dx.doi.org/10.1128/IAI.61.5.1650-6.1993.

36. Meyers JD. Fungal infections in bone marrow transplant patients. Semin Oncol 1990; 17: 10-3; PMID:2353204.

37. Serrano R, Gusmao L, Amorim A, Araujo R. Rapid identification of Aspergillus fumigatus within the section Fumigati. BMC Microbiol 2011; 11: 82; PMID:21530879; http://dx.doi.org/10.1186/1471-2180-11-82.

38. Bergin D, Brennan M, Kavanagh K. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of Galleria mellonella. Microbes Infect 2003; 5: 1389-95; PMID:14670452; http://dx.doi.org/10.1016/j.micinf.2003.09.019.

39. Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, et al. Aspergillus flavus and Aspergillus fumigatus virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect Immun 1993; 61: 1650-6; PMID:5940632; http://dx.doi.org/10.1128/IAI.61.5.1650-6.1993.

40. Meyers JD. Fungal infections in bone marrow transplant patients. Semin Oncol 1990; 17: 10-3; PMID:2353204.