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# *Caenorhabditis elegans-Aspergillus fumigatus* (Nematode-Mould) Model for Study of Fungal Pathogenesis

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** *Caenorhabditis elegans* nematode pathosystem has been used to study both bacterial and fungal pathogenesis. Apart from *Candida* and *Cryptococcus*, studies using this model for other fungal infections especially filamentous fungi however, are still lacking. This work aimed at developing a *C. elegans-Aspergillus fumigatus* (nematode-mould) killing assay model.
**Study Design:** Infection model of *Caenorhabditis elegans* with *Aspergillus fumigatus*.
**Place and Duration of Study:** Centre for Molecular Microbiology and Infection, Imperial College London, London SW7 2AZ, United kingdom, between October 2011 and April 2012.
**Methodology:** Double mutant *glp-4;sek-1* strain of *C. elegans* worms were propagated and maintained on nematode growth medium (NGM) with *Escherichia coli* non-pathogenic strain HB101 used as food prior to a fungal killing assay. L4 stage of the worms were infected with spores of *A. fumigatus* wild-type strain AF293, and incubated in 30% brain heart infusion (BHI) in M9 buffer at room temperature for 72 h. The survival of the worms was studied within this period.
**Results:** The scenario presented after killing of the worms by *A. fumigatus* appears to be the same

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as previously reported for *Candida albicans*, except for the position of the protruded filaments on the worms.
**Conclusion:** This model provides a platform for future studies of fungal pathogenesis, "curing" experiments, as well as for discovery of new antifungal agents.

*Keywords: Caenorhabditis elegans; Aspergillus fumigates; fungal pathogenesis.*

## 1. INTRODUCTION

*Caenorhabditis elegans* (usually measuring 825-1400 µm in length and 45-90 µm in width) is a soil-dwelling, genetically tractable, transparent nematode which has been used as a host model for infections to study virulence of various human pathogens including fungi and bacteria, and these pathogens have been shown to infect and kill the nematode [1,2,3]. Several studies have demonstrated that using this roundworm pathosystem, virulence genes could be determined through high-throughput screening [4,5].

Studies in this model could well be extrapolated to applications in mammal. For example, SEK-1, a mitogen-activated protein kinase kinase (MAPKK) and NSY-1, a MAPKK kinase (MAPKKK) both signal via the PMK-1 p38 MAPK required for innate immunity in *C. elegans* [6]. ASK1 which is a mammalian homologue of the NSY-1, has been recently shown to perform similar function to that of SEK-1 and NSY-1 [7]. In mice, ASK1 is required for the activation of p38 MAPK and downstream immune effectors while responding to bacterial pathogen-associated molecular pattern lipopolysaccharide [6]. *Candida albicans* and *Cryptococcus neoformans* infections have been studied with this model, and potential antifungal compounds have also been identified using the same model [3,8,9]. However studies employing this model with other medically important fungi such as *Aspergillus* spp are still lacking.

Since the 1990s, there has been increasing cases of mycoses in severely immunocompromised patients [10-12], and *Aspergillus* spp are the most frequently identified aetiologic agents. In haematopoietic stem cell transplant patients, the mortality rates in cases of invasive aspergillosis have surpassed 90% [13].

In this work we sought to establish that *A. fumigatus* is able to kill the worm *C. elegans* in a manner that affords the use of this nematode-mould model to study fungal pathogenesis.

## 2. MATERIALS AND METHODS

### 2.1 Choice of Strains

*A. fumigatus* wild-type strain AF293 and *C. elegans* strain *glp-4;sek-1* double mutant were used throughout our work. The *glp-4(bn2)* mutation renders this strain of the nematode incapable of producing progeny at 25ºC [14,15]; production of progeny will interfere with the assay and may thus affect reproducibility. *sek-1(km4)* encodes for a mitogen-activated protein kinase which is vital asset for the resistance to various pathogens, as a result the worms become more susceptible to infection [16], and thus reduces the assay time. *Escherichia coli* strain HB101 was used as food for the worms.

### 2.2 Preparation of *A. fumigatus* spores

A heavy loopful of *A. fumigatus* was well streaked out all over the surface of 90 mm acidovorax complex media (ACM) agar plate and incubated for 48 h. Thirteen ml of M9 buffer ($KH_2PO4$, 3 g; $Na_2HPO4$, 6 g; NaCl, 5 g; distilled $H_2O$, 1 L; 1 ml of 1M $MgSO_4$) was poured on the fungal culture and the spores were carefully suspended by gentle scraping with a plastic loop (care was taken not to scrape the agar particles). A layer of Miracloth (Calbiochem, LaJolla, CA, USA) was used to filter the suspension to separate the hypae (most of which remained in the layer), and obtain homogenized suspension of spores. Ten ml of the spore suspension was transferred into a 50 ml Falcon tube, and centrifuged at 3500 rpm for 10 min. The spores were concentrated by slowly inverting the tube to remove the supernatant and quickly returning to upright position (a very tiny portion of the supernatant remained in the tube with the spores). The spores were resuspended as a seemingly thick spore suspension by continuously tapping the bottom tip of the tube. There is no need for evaluation of the spore concentration since the intent will be to allow the worms to get infected while feeding on the spores as food.

### 2.3 Infection of *C. elegans* by *A. fumigatus*

The worms were propagated, maintained and synchronised as previously described [17]. L4-stage worms were washed out from the NGM growth plates into 50 ml tube using M9 buffer. The worms were allowed to sediment for about 10 min, and the supernatant suctioned out down to about 500 µl. Three more washes were performed to free the worms of most bacteria carry-over from the plates. Each of the washing was done by first, adding M9 buffer up to the 40 ml level, allowing to sediment, and suctioning out the supernatant (as described above). The M9 was always added slowly from the side of the tube to minimise trauma to the worms. A wide-tip pipette was used to pipette out 150 µl of worms in M9 (from the bottom of the tube). This was placed as a droplet of M9 containing the worms on a spot on the agar surface of a 90 mm NGM agar plate containing ampicillin (100 µg $ml^{-1}$), streptomycin (100 µg $ml^{-1}$) and kanamycin (45 µg $ml^{-1}$). The worms were usually trapped in the M9 droplet due to surface tension. A plastic loop was used to make about 5 different smears from the edge of the droplet, across the agar surface. This helps break the tension and release the worms on the plate surface. The plates were left at room temperature for 20-30 min. The worms were seen spreading over the entire agar surface. Then 100 µl of the thick spore suspension was placed on the NGM agar surface. The plate was carefully transferred to an incubator for 12 hours at room temperature. The worms gradually migrated to feed on the spores. M9 buffer and NGM plates were prepared as previously described [17].

### 2.4 Transfer of Infected Worms to Assay Medium and Killing by *A. fumigatus*

After the overnight incubation for infection, M9 buffer was used to wash out the worms from the plate into a 50 ml Falcon tube. Care was taken not to pour the M9 directly on the spot where the spores were initially placed, as this will liberate more of the unwanted spores with the infected worms. The worms were washed for 8 times (as described above) to get rid of most of the carry-over spores from the infection plate before transferring to the killing assay media. As the washing is done, there was need to remove clogs of spore from the bottom of the tube with a pipette, as they sediment much quicker than the worms. If these clogs were not gotten rid of properly, they tended to form clusters of heavy mycelial growth in the assay medium, which made visualization of worms difficult. After the washes, the worms were concentrated to 500 µl in the 50 ml tube. Trickles of M9 buffer were slowly added to adjust the concentration of the infected worms to roughly 2 worms $µl^{-1}$. Each time the concentration was checked by gently tapping the bottom of the tube until a homogenous suspension is formed, immediately manually pipetting 10 µl into a clear area of a Petri dish (before the worms resettle), and counting under a dissecting microscope. This method was preferred over that previously described [17], as it greatly reduced trauma, and thus the amount of dead worms observed on day 0 (day of transfer to assay medium), which tended to complicate the assay. Ten µl of the suspension (20 infected worms) were transferred to 190 µl of the assay medium in each of 12 wells in a 96-well plate and incubated for 72 h. The assay (liquid) medium was 30% brain heart infusion (BHI) medium in M9 buffer containing ampicillin (200 µg $ml^{-1}$), streptomycin (200 µg $ml^{-1}$) and kanamycin (90 µg $ml^{-1}$). The worms were observed every 4 h for 3 d. Dead *C. elegans* worms appear rod shaped or straightened out or slightly bent (with *A. fumigatus* filament growing out mostly from the distal portion, and in some cases from the sides of the worms), whereas live worms assumed sinusoidal shape and movement. A detailed observation of the worms was done using done using a digital microscope (Nikkon, Japan, model: Eclipse TS100-F). The killing assay was also performed in a 6-well plate with roughly 30 worms and 3 ml of assay medium in each well.

### 2.5 Exposure of Worms to Cell-free Culture Supernatant

A loopful of *A. fumigatus* spores was inoculated into 50 ml of the assay medium in a 250 ml Erlenmeyer flask and incubated at 180 rpm shaking at room temperature for 3 d. This was also repeated at 37ºC. The cultures were filtered with 0.22 µm Millipore filters to obtain cell-free supernatants. Twenty L4 stage worms were placed in 190 µl of the cell-free supernatant in each of 12 wells of a 96 well plate, and incubated at room temperature for 3 d.

## 3. RESULTS AND DISCUSSION

### 3.1 Optimisation of Infection Assay

The *C. elegans* nematode host model has been adapted for use to study bacterial and fungal

pathogenesis. To further expand the use of this model as a pathosystem to study virulence in filamentous fungi, we developed a killing assay using *A. fumigatus* and L4 stage *glp-4;sek-1 C. elegans* mutant strains.

Developing the infection model as previously described [9] using the co-infection (co-exposure) method which entailed adding the worms and spores together in the liquid assay medium, was not successful. According to Okoli et al. [9], *C. albicans* cells are ingested within 2 – 4 hours and filament within 2-3 days, resulting in killing of *C. elegans*. When this approach was replicated for *A. fumigatus* and it was immediately evident, due to germination of *A. fumigatus* spores, that the assay would not be practical due to hyphal growth in the assay medium (30% BHI in M9 buffer). BHI is provided as a nutrient favourable to both *C. elegans* and infecting pathogen.

It was notable that, unlike *C. albicans* yeast cells, which are ingested within 2 hours of co-exposure – as measured by the presence of cells within worms [3], *A. fumigatus* spores are unlikely to become ingested as readily. This is unlikely to be due to insufficient encounters between worms and spores, as the same inoculums size (multiplicity of infection, same volume of medium also) as previously used for *C. albicans* infections was adopted here. It is possible that either the worms avoid ingestion of spores and/or are incapable of ingesting spores under these conditions.

### 3.2 Worms Plus Spores on Solid Agar for 12 hours

Initial attempts to co-incubate worms and fungus were met with failure due to rapid inactivation of worms upon fungal exposure. To circumvent this problem, ingestion was facilitated by inoculating remote sites on the solid agar medium with about 50 µL drops of spore suspension and facilitating worm migration to eventually reach the spore inculated site (i.e. co-exposure on a soild medium), as well as an extended incubation (12 hours) of worms and spores – in a medium which limits germination.

The infection setup of our work, especially preparation of the spores in the M9 buffer, permitted extremely low germination rate of the spores and facilitated ingestion by the worms, at least within the 12 h exposure to the worms. Observation of samples of the spores after the infection, prior to transfer of the worms to the assay media showed that more than 98% of the spores left over in the infection plate were ungerminated.

### 3.3 *C. elegans* killing by *A. fumigatus*

The infection and killing pattern of *A. fumigatus* in *C. elegans* is much similar to that previously described for *Candida albicans* [3,9], except that for *A. fumigatus*, the protrusion of the filament from the inside to the outside of the worms appears to be mostly initiated from the distal end of the worm (Figs. 1A, 1B and 1C).

This observation was made following the first 12-14 h after transferring the infected worms to a pathogen-free assay medium. At this stage the activity of most of the worms is greatly decreased, and they appear to be slowing down. Few of the worms show signs of advanced infection and were slowing down for death. This advanced stage of the infection is mostly evident from 24-48 h during incubation, and is characterised by extended filament protrusions concentrated at the distal portion of most of the worms (Figs. 2A, 2B, 2C and 2D).

Worm viability was assessed on a two-point scale including a) lack of mobility and b) rod-shaped morphology (Fig. 2C). The survival curve of the worms is shown in Fig. 3.

The duration of the experiment was 72 h, at this point an average of about 80% of all worms were dead. The whole killing assay experiment was repeated trice and all readings in Fig. 3 represent averages in the three experiments. Readings for worms showing signs of infections were taken at points 0 h, 24 h, 48 h and 72 h in the three experiments and the Standard Deviation (SD) for each of those points were 0, 5, 2.88, 5 respectively. Also the survival of the worms were read at 0 h, 24 h, 48 h and 72 h in the three experiments, and SD for each of those points were 0, 2.88, 5, 5.77 respectively. Death of the worms was attributed to the infection by *A. fumigatus*. Worms incubated in the cell-free culture supernatant appeared healthy and active; also previously, cell-free culture supernatant of *A. fumigatus* was shown to actually improve the survival of *C. elegans* in the case of *Candida albicans* infection [18].

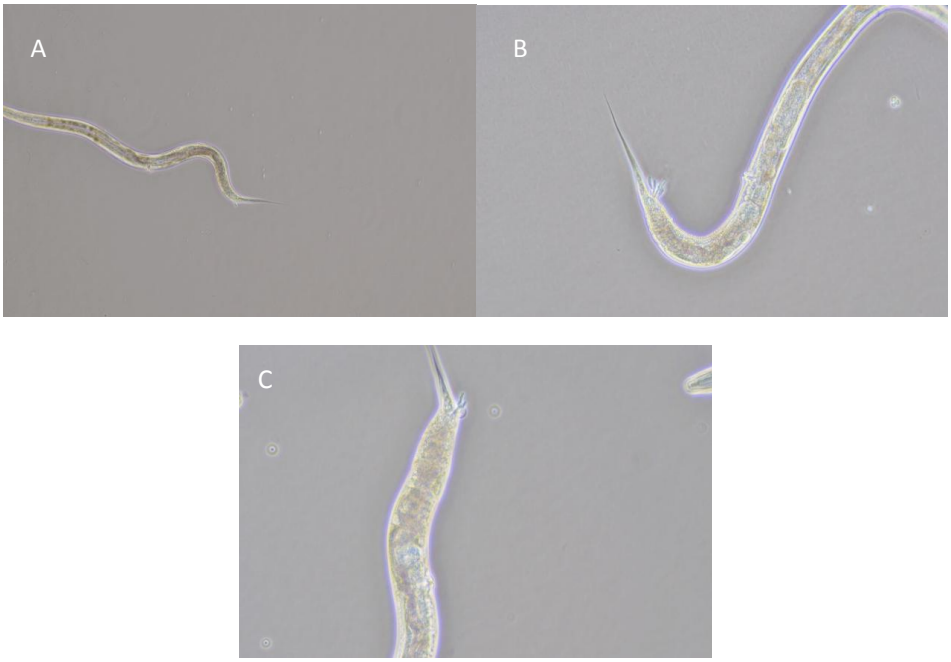


**Fig. 1A, 1B and 1C. Initial penetration of *A. fumigatus* through the cuticle of worm to the surrounding medium about 24 h from time of exposure to spores**

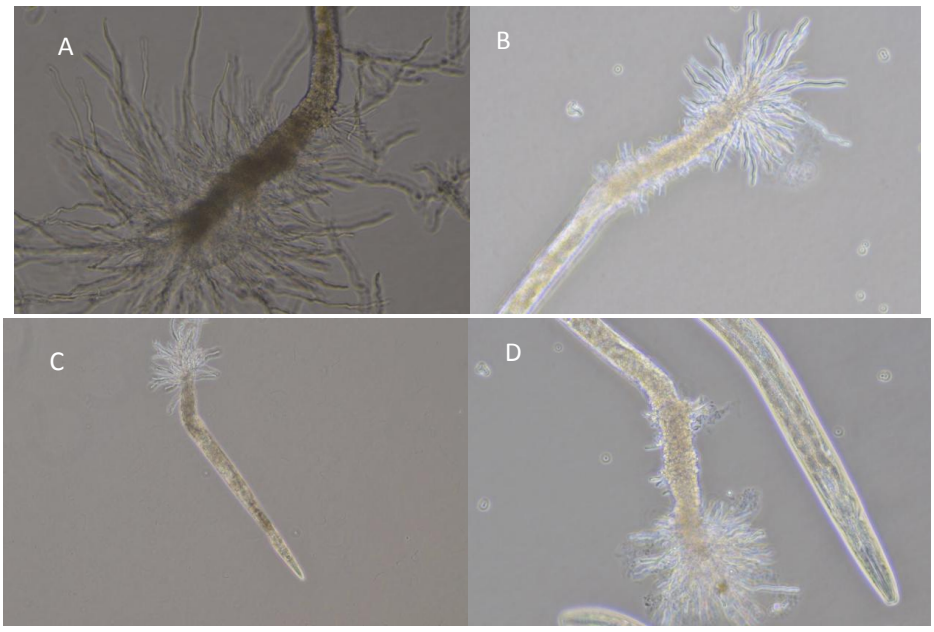


**Fig. 2A, 2B, 2C and 2D. Worms in the advanced stage of *A. fumigatus* infection, 24-48 h after infected worms are transferred to pathogen-free medium**

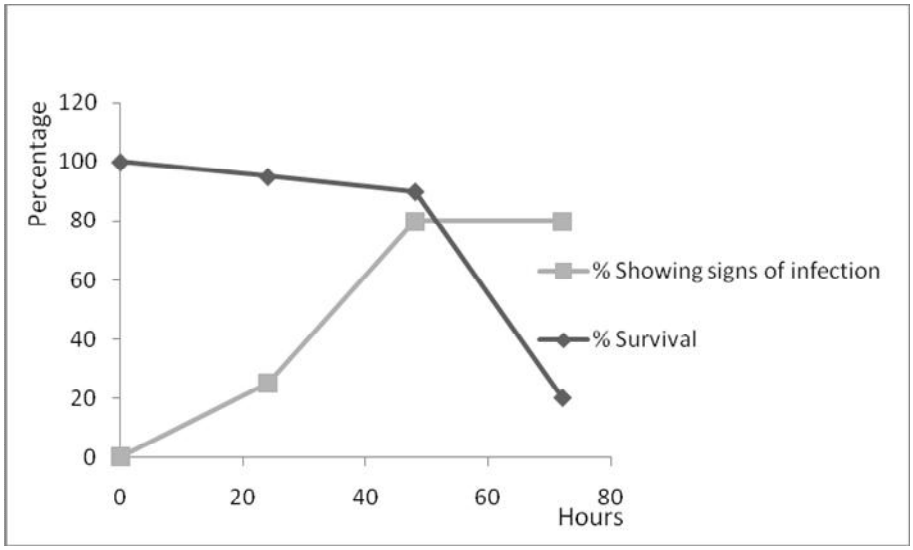


**Fig. 3. Survival curve of *Caenorhabditis elegans* worms infected with *Aspergillus fumigatus* spores**

## 4. CONCLUSION

We have developed a nematode-mould pathosystem for the study of fungal infection via killing of *C. elegans* by *A. fumigatus*. Though this model has not been optimised for high throughput studies, it provides at least a starting point in a whole animal nematode worm-mould model to study fungal pathogenesis. More work will still need to be done in future for example in studying/comparing the virulence of different strains of *A. fumigatus* with different genetic background, and studying variations in gene expressions of fungal pathogens using *in vivo* model.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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