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Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella*

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Abstract

Aspergillus fumigatus is a pathogenic fungus capable of causing both allergic lung disease and invasive aspergillosis, a serious, life-threatening condition in neutropenic patients. Aspergilli express an array of mycotoxins and enzymes which may facilitate fungal colonisation of host tissue. In this study we investigated the possibility of using the insect, *Galleria mellonella*, for *in vivo* pathogenicity testing of *Aspergillus* species. Four clinical isolates of *Aspergillus fumigatus* and a single strain of *Aspergillus niger* were characterised for catalase and elastase activity and for the production of gliotoxin. Gliotoxin is an immunosuppressive agent previously implicated in assisting tissue penetration. Results illustrated a strain dependent difference in elastase activity but no significant difference in catalase activity. Gliotoxin production was detected *in vitro* and *in vivo* by Reversed Phase-High Performance Liquid Chromatography, with highest amounts being produced by *A. fumigatus* ATCC 26933 (350 ng/mg hyphae). Survival probability plots (Kaplan–Meier) of experimental groups infected with *Aspergillus* conidia indicate that *G. mellonella* is more susceptible to fungal infection by *A. fumigatus* ATCC 26933, implicating a critical role for gliotoxin production rather than growth rate or enzymatic activity in the virulence of *A. fumigatus* in this model.

Key words: gliotoxin, *Aspergillus fumigatus*, *Galleria mellonella*, fungal infection, immunosuppression

Abbreviations: *Aspergillus fumigatus*, *A. fumigatus*; *Galleria mellonella*, *G. mellonella*; Minimal Essential Medium Eagle, MEM; High Performance Liquid Chromatography, HPLC; Malt Extract Agar, MEA.

Introduction

Fungal infections are of increasing importance in severely neutropenic and immunosuppressed patients because of their high incidence and the associated high mortality rate. Despite the development of triazole anti-fungals and amphotericin B formulations the mortality associated with fungal infection remains high at 60–90%. Aspergillosis is one such opportunistic infection, which can occur in immunosuppressed patients and is most commonly caused by the fungus *Aspergillus fumigatus* [1].

A. fumigatus, as well as some *Candida* [2] and *Penicillium* [3] species, produce gliotoxin a member of the epipolythiodioxopiperazine class of fungal metabolites [4]. The activity of gliotoxin is attributable to a reactive disulfide bond component in the molecule, which can undergo redox cycling, resulting in the production of oxygen radicals. A broad spectrum of biological activity has been reported for gliotoxin which include the prevention of macrophage adherence, followed by the induction of apoptosis [5, 6]. Inhibition of phagocytosis by rodent macrophages and mitotic stimulation of lymphocytes has been shown, an effect unrelated to induction of apoptosis [7] but which may assist fungal colonization. Other activities associated with gliotoxin include inhibition of fungal and bacterial growth [8] and viral replication [9]. From a clinical perspective, gliotoxin is important since it has been reported as being produced in tissues of mice and turkey poults [10] following development of invasive aspergillosis.

Models of invasive aspergillosis were first developed in experimental murine systems [11], with evidence suggesting that the innate mammalian immune response is involved in the defence against fungal pathogens. Key features involved in innate immunity are found to be homologous in insects and vertebrates [12, 13]. The insect cellular processes such as phagocytosis [14], production of reactive oxygen species [15] and anti-microbial peptides, bear strong similarity to those produced by phagocytes of mammals, giving evidence of an ancient immune system found in the ancestor of all bilaterian animals. Most immunological data for insects have been reported from *Drosophila melanogaster* [16] however in the Greater Wax moth *Galleria mellonella*, various immunorelevant protein molecules have been described and this system is being increasingly used as a model for assessing the virulence of a range of microorganisms [17, 18]. For example, larvae of *G. mellonella* have been successfully used to evaluate the pathogenicity of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* [19] and a good correlation exists between the virulence of *P. aeruginosa* in Galleria larvae and in mice. Larvae of *G. mellonella* have also been used to assess the virulence of *Candida albicans* and strong agreement has been established between the results obtained in insects and mice [20].

This paper describes the use of *G. mellonella* larvae as a screening mechanism for testing the virulence of *A. fumigatus*. Studies confirm the production of gliotoxin by four clinical isolates of *A. fumigatus* and a single isolate of *A. niger* and demonstrate that *G. mellonella* is more susceptible to fungal infection by strains of *Aspergillus* producing elevated levels of gliotoxin.

Material and methods

Fungal strains and culture conditions

Clinical isolates of *Aspergillus fumigatus* used in this study included ATCC 26933, 16424, 13073 and 14109 (obtained from the American Type Culture Collection, Maryland, USA), one strain of *Aspergillus niger* (environmental isolate) and the yeast *Saccharomyces cerevisiae* YJM 128 (A gift from Dr. K. Clemons, San Jose, USA) [20]. *Aspergillus* spores were grown in Minimal Essential Medium Eagle (Sigma Aldrich Chemical Co., Dublin, Ireland) supplemented with 5% (v/v) Foetal Calf Serum (Sigma Aldrich) at 37 °C and 200 rpm, for up to 4 days. Stocks were maintained on Malt Extract Agar (MEA) (Oxoid Ltd.). *S. cerevisiae* was grown to stationary phase (approximately 1×10^8/ml) in YEPD broth (2% (w/v) glucose, 2% (w/v) bactopeptone (Difco), 1% (w/v) yeast extract (Oxoid) at 30 °C and 200 rpm. Stocks were maintained on YEPD agar (2% (w/v) Agar, Difco).

Growth curve for Aspergillus

MEA plates containing sporulating *Aspergillus* cultures were washed with 10 ml of 0.1% (v/v) Tween 80 (Merck) in Phosphate Buffered Saline (PBS, pH 7.2) (Sigma Aldrich) to isolate conidia. Conidia were washed twice in sterile PBS, centrifuged (1,500 g, 5 min in a Beckman GS-6 centrifuge) and counted using an haemocytometer. Flasks containing MEM (100 ml) were inoculated with 1×10^5 *Aspergillus* conidia and incubated at 37 °C and 200 rpm. A flask was removed every 24 h and the contents filtered through a Whatman No. 1 filter in a Buchner funnel and air-dried. A growth curve was constructed of dry fungal biomass versus incubation time.

Characterisation of Aspergillus strains

Catalase activity of the five *Aspergillus* strains was determined using *Aspergillus niger* catalase (Sigma Aldrich) as a standard, the difference in absorbance (ΔA_{240}) per unit time being a measure of the catalase activity [21]. Elastase activity was quantified using the elastase-specific substrate *N*-succinyl-alanyl-alany-prolyl-leucine *p*-nitroanilide (Sigma Aldrich) [22]. Substrate (final concentration, 2.5 mM) was added to fungal hyphae (final concentration, 0.33 mg/ml protein) in buffer (100 mM Tris-HCl, 200 mM NaCl, 0.05% (v/v) Triton X-100, pH 7.8) in a 96-well, flat bottomed microtiter plate at 37 °C. The A_{405} was read every 60 s for 10 min.

Preparation of extracts for detection of gliotoxin by Reversed Phase-HPLC

Hyphae of *Aspergillus* were removed from the MEM culture medium by filtration and an equal volume of chloroform (Hyper Solv, BDH) added to the filtrates (10 ml). Following continual mixing for 30 min, the chloroform fraction was collected and evaporated to dryness. For detection of gliotoxin, dried extracts were dissolved in 200 μl methanol (Hyper Solv, BDH) and stored at -70 °C until assayed. Gliotoxin was detected by Reversed Phase-HPLC (Spectra-Physics).

The mobile phase was 34.9% (v/v) acetonitrile (Hyper Solv, BDH), 0.1% (v/v) trifluoroacetic acid (Sigma Aldrich) and 65% (v/v) deionized-distilled water. The gliotoxin extract (20 μl) was injected onto a C18 Hewlett Packard column. A standard curve of peak area versus gliotoxin concentration was constructed using gliotoxin standards (50, 100 and 200 ng/ml dissolved in methanol, (Sigma Aldrich). The amount of gliotoxin in the samples was calculated from the standard curve.

Inoculation of insects with conidia of Aspergillus

Sixth instar larvae of *G. mellonella* were obtained from the Meal Worm Company (Sheffield, England). Larvae were stored in wood shavings in the dark at 15 °C prior to use. All larvae were used within 3 weeks of receipt. Larvae between 0.2 and 0.4 g in weight were employed in all assays. Larvae were injected with 1×10^4 conidia in 20 μl PBS into the haemocoel through the last left pro-leg as described previously using a Myjector U-100 Insulin needle (Terumo) [17]. Larvae were placed in a sterile petri dish and incubated in the dark at 30 °C. Mortality rates were determined over a 5 day period. Larval death was assessed by the absence of movement and discolouration of the cuticle [17, 20]. The extraction of gliotoxin from tissue samples infected with *Aspergillus* was as described by Richard et al. [10]. In brief, 100 infected larvae were homogenized in 5 ml of H_2O. The homogenizer was rinsed twice with 10 ml of 6N HCL and pooled to give 25 ml. Chloroform (200 ml) was added and the mixture stirred at room temperature for 30 min. The sample was poured into a separatory funnel and the gliotoxin extracted in the lower chloroform layer. Detection and quantification of gliotoxin was as described above.

Statistical analysis

Survival of larvae after injection was analysed by Kaplan–Meier survival analysis. Catalase and elastase activities were analysed by Student's two tailed t-test.

Results

Growth curves for the five *Aspergillus* isolates (Figure 1) showed the exponential growth phase began at 12 h and extended until 48 h. The stationary phase

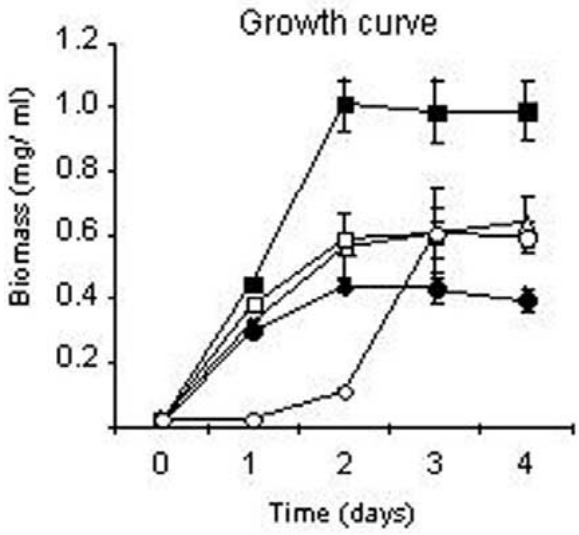

Figure 1. Growth curves. Growth curve of *Aspergillus* isolates in MEM at 37 °C, ● = ATCC 26933, ○ = *A. niger*, ■ = 16424, △ = 13073, □ = 14109.

was reached at approximately 72 h with a greater biomass being obtained for *A. fumigatus* ATCC 16424 (1.0mg/ml of culture).

Characterization of the different *Apergillus* strains showed them to vary in enzymatic activity. Quantification of elastase activity demonstrated that *A. niger* and *A. fumigatus* ATCC 14109 exhibited elevated levels of activity (25–30 U/mg hyphae), whereas isolates ATCC 26933, 16424 and 13073 produced between 10 and 20 U of elastase activity per mg of fungal hyphae (Figure 2a). Between the different *Aspergillus* isolates no significant difference was found in the level of catalase activity, with a standard level of approximately 0.25 U/min/mg hyphae activity calculated (Figure 2b).

Production of gliotoxin by *A. fumigatus* has previously been detected at temperatures ranging from 30 °C [23] to 37 °C [24]. The conventional method of Reversed Phase-HPLC quantification was used for the determination of gliotoxin production [25]. All isolates of *Aspergillus* were found to produce gliotoxin with production starting after 24 h (Figure 3). Strain ATCC 26933 produced more gliotoxin than the other 4 strains of *Aspergillus* (350 ng/mg hyphae, $P < 0.001$), with maximum gliotoxin production occurring at approximately 72 h incubation. A subsequent decline in levels was observed which may have been due to catabolism or instability of the compound.

An investigation into the pathogenicity of the five strains of *Aspergillus* was performed using the *G. mellonella* virulence model. Figure 4a shows the mor-

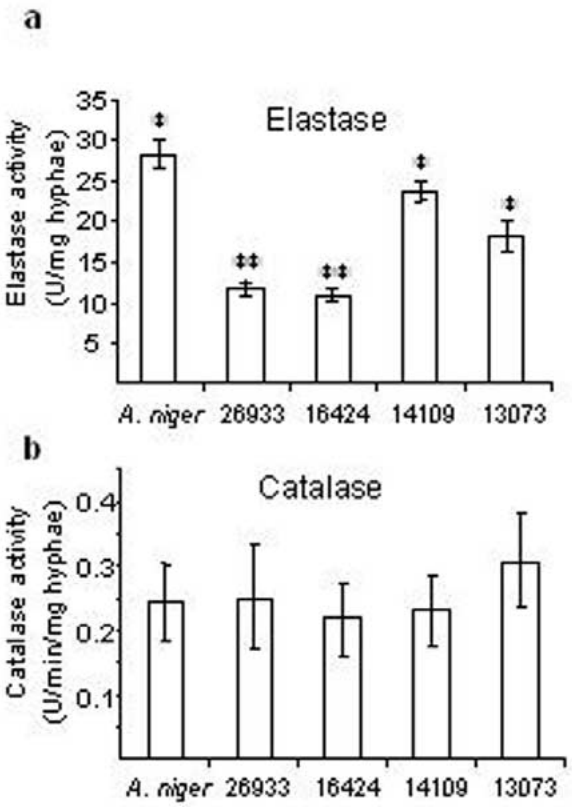

Figure 2. Enzymatic activity. (a) Elastase activity of *A. niger* and four strains of *A. fumigatus*. ∗∗ = no significant difference between isolates ATCC 26933 and 16424 ($P = 0.1$), ∗ = significant difference between *A. niger*, ATCC 14109 and 13073 relative to 26933 and 16424 ($P < 0.01$). (b) Catalase activity of the 5 isolates of *Aspergillus* ($P = 0.09$). All results are means ± s.e.m. from three separate measurements.

tality of *Galleria* following injection with *Aspergillus* conidia. The control, a non-virulent strain of *S. cerevisiae* [17] and *A. fumigatus* strains ATCC 13073 and 16424 had minimal effect on larval mortality. This was in marked contrast to the *A. fumigatus* strain ATCC 14109 and *A. niger* which killed approximately 20% of the infected larvae within 24 h after infection with 1×10^4 conidia.

The results reveal elevated mortality rates for larvae inoculated with the clinical isolate *A. fumigatus* ATCC 26933. Furthermore, within 48 h all larvae inoculated with this strain had been killed. This strain was found to produce the highest levels of gliotoxin (Figure 4b), indicating a likely correlation between gliotoxin production and virulence in *G. mellonella*.

In order to establish a more direct link between gliotoxin production and virulence, further studies were made on the occurrence of gliotoxin in larvae of *G.*

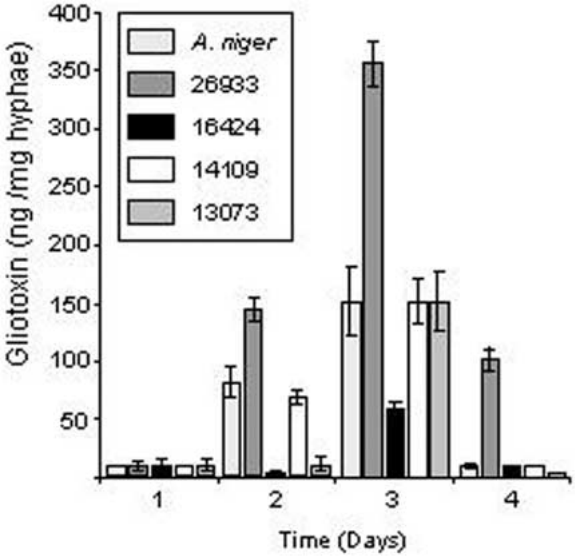

Figure 3. Quantification of gliotoxin production. Gliotoxin production as a function of fungal hyphal biomass. All results are means ± s.e.m. from three measurements. A statistically significant difference was found for ATCC 26933 compared to all other strains at days 2, 3 and 4 ($P < 0.001$).

mellonella infected with the fungus. Results showed that the toxin was formed during growth of *A. fumigatus*, as gliotoxin was found in larvae infected with the gliotoxin-producing strains and was not found in tissues from healthy uninfected larvae. As depicted in Figure 4b various levels of gliotoxin was found in *G. mellonella* tissue samples following inoculation with 1×10^4 conidia of the different *Aspergillus* isolates. The levels of gliotoxin ranged from 250–2400 ng/g of insect mass; two *Aspergillus* isolates, ATCC 16424 and ATCC 13073 produced no detectable levels. The highest concentration of gliotoxin detected followed inoculation with *A. fumigatus* ATCC 26933 (2400 ng/g) at a time corresponding to 50% larval death (Figure 4a). Infection with 1×10^4 *A. fumigatus* ATCC 14109 or *A. niger* resulted in similar (250–280 ng/g) gliotoxin concentrations. Collectively these results indicate a likely correlation between gliotoxin production and virulence of *A. fumigatus* ATCC 26933 in *G. mellonella*.

Discussion

The fact that there are many airborne fungi, not implicated in disease suggests that *A. fumigatus* may produce specific virulence factors that are important in helping the pathogenic fungus to colonize tissue. Several lines

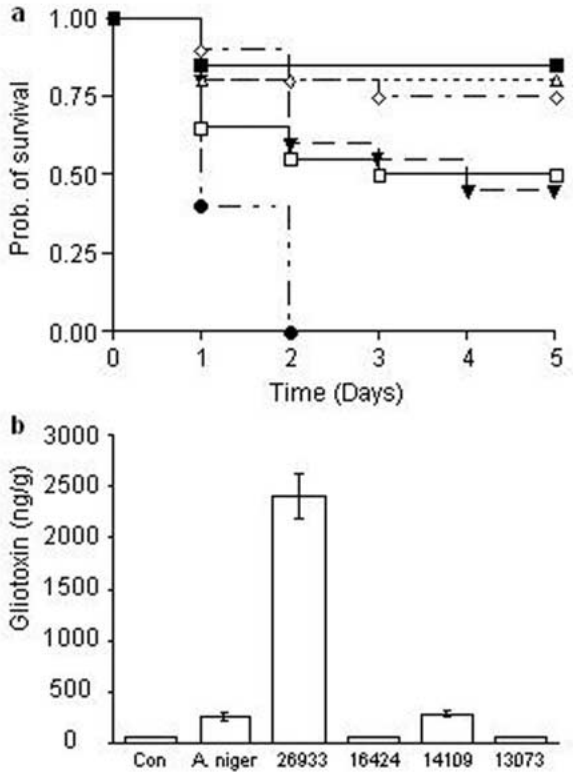

Figure 4. Susceptibility of *G. mellonella* larvae to fungal infection. (a) Survival probability plots (Kaplan–Meier) of larvae injected with 1×104 conidia of the *Aspergillus* isolates ($n = 20$). The rates of decreased probability of survival of larvae injected with *A. fumigatus* ATCC 26933 was significantly different from all other isolates ($P < 0.05$). ● = ATCC 26933, □ = *A. niger*, ▼ = 14109, ◊ = 16424, △ = 13073, ■ = *S. cerevisiae*. (b) *In vivo* production of gliotoxin as a function of larval biomass. Larvae of *G. mellonella* were injected with 1×10^4 conidia of *A. fumigatus* ATCC 26933, 16424, 14109, 13073, and *A. niger* and gliotoxin production compared to uninfected larvae (Con).

of evidence suggest a role for gliotoxin in the pathogenesis of aspergillosis, as this metabolite is produced by over 95% of pathogenic isolates of *A. fumigatus*.

Neutrophils and alveolar macrophages are the main cellular components of the vertebrate immune system responsible for the defence against *C. albicans* [26] and *A. fumigatus* [27]. They eliminate pathogens by phagocytosis, the release of reactive oxygen intermediates and activation of proteolytic enzymes [26, 28]. Similar cellular processes have been described for phagocytes of insects, with receptors on the surface of plasmatocytes and granulocytes exhibiting striking similarity to receptors on mammalian phagocytes [14]. *A. fumigatus* can escape local host defences by synthesizing metabolites that inhibit these immune defences. Müllbacher and Eichner described the inhibition of phagocytosis by gliotoxin [6], while others have demonstrated that gliotoxin causes morphological alterations to macrophages [29]. The wax moth *G. mellonella* has previously been used to test the virulence of a variety of microorganisms [17, 18, 20] and in this study we employed *G. mellonella* to differentiate between pathogenic *Aspergillus* species and to determine if the production of an immunosuppressive agent, such as gliotoxin, could further compromise the infected host. On comparing the lethality of different strains, it should be noted that the growth rate of each strain might have the potential to affect pathogenesis. It was observed that the *Aspergillus* isolate ATCC 16424 which possessed the fastest growth rate, exhibited a virulence level equivalent to that of the non-virulent isolate of *S. cerevisiae* [20].

Catalase is an antioxidant metalloenzyme, which is almost ubiquitous among aerobic organisms and protects cells against oxidative damage caused by hydrogen peroxide. It has been reported that oxidative mechanisms are important in the killing of *A. fumigatus* by polymorphonuclear cells and that this fungus is sensitive *in vitro* to oxygen metabolites [30]. The catalase activities of *Aspergillus* isolates employed in this study were quantified. As all isolates possessed similar activity, an anti-virulent role for catalase in protection against oxidative killing by reactive oxygen intermediates produced by haemocytes cannot be assessed using the particular *Aspergillus* strains of this study.

It has been speculated that extracellular proteinases play a role in virulence, by allowing tissue invasion by fungal cells and also in resistance to phagocytic killing by the host [31]. A number of isolates of *A. fumigatus* obtained from a hospital environment produced extracellular elastolytic activity and immunogold localization of the elastolytic enzyme showed that *A. fumigatus* germinating and penetrating into the lungs of neutropenic mice secreted the elastolytic protease [32]. Elastase activity of the five *Aspergillus* isolates used in this study was quantified and although various levels of activity were observed, those strains illustrating increased elastase activity, demonstrated low levels of virulence as measured in *G. mellonella*.

Gliotoxin production by *A. fumigatus* has been estimated to be in the range of 20 to 80 μg/ml culture after approximately 4–7 days [6] and this concentration has been shown to inhibit the growth of several Gram-negative bacteria and fungi, and viral replication *in vitro* [33]. In this study *A. fumigatus* ATCC 26933 produced the highest concentration of gliotoxin

(350 ng/mg of hyphae) after three days growth at 37 °C. Interestingly a lower concentration of gliotoxin (20-50 ng/ml) has been shown to have an inhibitory effect on phagocytosis of carbon particles by peritoneal exudates [7]. An additional observation was the detection of maximum gliotoxin production after three days of growth *in vitro* with maximum larval death occurring two days post infection. This temporal discrepancy may indicate that the high concentration of gliotoxin detected *in vitro* may not be required *in vivo* to obtain the level of killing observed.

The results indicate the existence of a hierarchy among the *Aspergillus* isolates studied here with respect to their ability to kill larvae of *G. mellonella*, i.e. ATCC 26933 > ATCC 14109 = *A. niger* > ATCC 16424 = ATCC 13073. Consistently, *Aspergillus* species killed larvae to a greater extent than the non-pathogenic *S. cerevisiae* and the differences in relative pathogenicity between the *Aspergillus* isolates, in particular *A. fumigatus* ATCC 26933, may be explained by the increased levels of gliotoxin produced by this isolate.

The correlation between gliotoxin production and virulence in *G. mellonella* was further strengthened by the observation of the occurrence of gliotoxin in larvae infected with the fungus. Recoveries of gliotoxin have been made from bovine samples and from lung tissue of turkeys (10) inoculated via the posterior thoracic air sac with *A. fumigatus* conidia, with levels of gliotoxin ranging from 183–6849 ng/g, a concentration similar to what was found in the *Galleria* tissue in this study. Preliminary spiking studies indicated, approximately 40% of the gliotoxin was recovered from normal larval tissue samples to which purified gliotoxin was added at known concentrations, and therefore the relevant adjustment in calculations were made. Relative to the other *Aspergillus* strains, *A. fumigatus* ATCC 26933 produced higher concentrations of gliotoxin *in vivo* (Figure 3) than *in vitro* (Figure 4b), which may by due to the ability of this strain to produce increased levels of the immunosuppressive toxin when challenged by cells of the host immune response. In addition, the growth environment within the larvae may facilitate elevated gliotoxin production compared to that in culture.

Inoculation of *Galleria* with similar concentrations of purified gliotoxin did not appear to induce the same pathogenic effect as the growing fungus (result not shown). It is possible therefore, in cases of aspergillosis that the toxin produced by the fungus is concentrated in the tissue adjacent to the colony and assists escape from local host defences via its reported immunosuppressive role [6, 29, 34, 35].

In conclusion, the work presented here demonstrates differences in the virulence of clinical isolates of *Aspergillus*, as detected in larvae of *G. mellonella*. While the insect and mammalian immune systems differ, they share a high degree of similarity in terms of their innate immune responses [12] which is an important line of defence against fungal infection [36]. The response of insects to infection shows strong similarities to that found in mammals [17, 18]. The information generated by the use of low gliotoxin producing strains of *Aspergillus*, e.g. the reduced mortality of larvae infected with ATCC 13073 compared to the high mortality of larvae infected with the high gliotoxin-producing strain ATCC 26933, suggests that gliotoxin production is a significant contributor to the pathogenicity of *A. fumigatus* in this model. We have not excluded the presence of other factors that may explain the difference in virulence between the *Aspergillus* isolates however with the data obtained, gliotoxin is a likely candidate, further strengthening its role in the pathogenesis of *A. fumigatus*-induced diseases.

Acknowledgements

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