Genetic and virulence variation in an environmental population of the opportunistic pathogen
*Aspergillus fumigatus*

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Environmental populations of the opportunistic pathogen *Aspergillus fumigatus* have been shown to be genotypically diverse and to contain a range of isolates with varying pathogenic potential. In this study, we combined two RAPD primers to investigate the genetic diversity of environmental isolates from Manchester collected monthly over 1 year alongside Dublin environmental isolates and clinical isolates from patients. RAPD analysis revealed a diverse genotype, but with three major clinical isolate clusters. When the pathogenicity of clinical and Dublin isolates was compared with a random selection of Manchester isolates in a *Galleria mellonella* larvae model, as a group, clinical isolates were significantly more pathogenic than environmental isolates. Moreover, when relative pathogenicity of individual isolates was compared, clinical isolates were the most pathogenic, Dublin isolates were the least pathogenic and Manchester isolates showed a range in pathogenicity. Overall, this suggests that the environmental population is genetically diverse, displaying a range in pathogenicity, and that the most pathogenic strains from the environment are selected during patient infection.

**INTRODUCTION**

*Aspergillus fumigatus* is an opportunistic fungal pathogen that can cause aspergillosis in immunocompromised patients, such as those with neutropenia or on immuno-suppressive therapy following solid organ transplantation (Abad et al., 2010; Ben-Ami et al., 2010; Dagenais & Keller, 2009; Latgé, 1999; Rementeria et al., 2005). The principal route of infection is through the inhalation of airborne spores, which due to their small spore size are able to reach the alveoli (Dagenais & Keller, 2009; Ibrahim-Granet et al., 2003; Latgé, 1999; O’Gorman, 2011; Philippe et al., 2003). While other aspergilli can cause opportunistic infections (such as *Aspergillus terreus* and *Aspergillus flavus*), *A. fumigatus* accounts for ~90% of all cases despite airborne spore numbers only accounting for <1% of all *Aspergillus* spores (Rementeria et al., 2005). A number of studies have shown a high degree of genetic variation within the *A. fumigatus* population, and whilst some studies have indicated potential geographical subgroups, comparisons between clinical isolates and environmental isolates have largely been unable to distinguish these populations (Araujo et al., 2010; Auffaure-Brown et al., 1992; Balajee et al., 2008; Chazalet et al., 1998; Denning et al., 1990; Duarte-Escalante et al., 2009; Leenders et al., 1999; Menotti et al., 2005). Moreover, epidemiological studies have largely failed to match genotypes in infected patients with genotypes found in hospitals, in part due to the high level of genetic diversity in the environmental populations (Araujo et al., 2010; Bart-Delabes et al., 1999; Chazalet et al., 1998; Guinea et al., 2011; Leenders et al., 1999; de Valk et al., 2007), and similar findings have been reported in avian populations (Arné et al., 2011; Lair-Fulleringer et al., 2003; Olias et al., 2011). Whilst genotyping of isolates from some individual patients has revealed infection with multiple isolates, most patients appear to be colonized by only one genotype despite exposure to a genetically diverse population (Alvarez-Perez et al., 2010; Bart-Delabes et al., 1999; Chazalet et al., 1998; Guinea et al., 2011; Menotti et al., 2005; Tang et al., 1994; de Valk et al., 2007). This has led to the suggestion that the lung environment selects for a strain or strains from the environment that are the most adapted and therefore pathogenic (Cimon et al., 2001; Guinea et al., 2011; de Valk et al., 2007).

Animal models are used widely to determine microbial pathogenicity, but their use is constrained by high costs and ethical considerations. As a result, a number of insect models have been developed as potential alternatives for studying the pathogenicity of microbes and the efficacy of antimicrobial agents (Desbois & Coote, 2012; Mak et al., 2010; Papaioannou et al., 2013; Thomas et al., 2013) as they contain functional and structural features similar to the innate immune system of mammals (Arvanitis et al., 2013; Browne et al., 2013). The greater wax moth larva (*Galleria mellonella*), in particular, has been used successfully to study pathogenicity for a range of microbial pathogens, including *Aerococcus baumanii*, *Enterococcus faecium*,...
Escherichia coli, Legionella pneumophila, Listeria monocytogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Candida albicans, Candida tropicalis, Cryptococcus neoformans, A. flavus, A. fumigatus and Fusarium spp. (Andrejko et al., 2009; Coleman et al., 2011; Desbois & Coote, 2012; Dunphy et al., 1986; Evans & Rozen, 2012; Fallon et al., 2011; Fuchs et al., 2010; Harding et al., 2012; Harrison et al., 2006; Joyce & Gahan, 2010; Lefebreton et al., 2011; Leuko & Raivio, 2012; Mesa-Arango et al., 2013; Miyata et al., 2003; Mylonakis et al., 2005; Mukherjee et al., 2010; Peleg et al., 2009; Scully & Bidochka, 2009; Soukup et al., 2012). Moreover, virulence in these larvae has in many instances been shown to be similar to that in mammals, and to involve similar pathogenicity and virulence determinants (Brennan et al., 2002; Cook & McArthur, 2013; Dunphy et al., 2003; Fuchs & Mylonakis, 2006; Jander et al., 2000; Mukherjee et al., 2010; Wand et al., 2011), including for A. fumigatus (Amich et al., 2013; Jackson et al., 2009; Li et al., 2011; O’Hanlon et al., 2011; Slater et al., 2011; Soukup et al., 2012).

In this study, we investigated the genetic variation in airborne environmental A. fumigatus strains isolated over a period of 12 months from the outdoor environment in Manchester, Dublin environmental strains isolated from the outdoor environment at one sample time and clinical isolates using two RAPD primers. In addition, we also compared the relative pathogenicity of environmental and clinical isolates in a Galleria larva model, and report that clinical isolates were at the most pathogenic end of the spectrum whilst environmental isolates were found throughout the spectrum from high to low, suggesting clinical isolates represent the most pathogenic strains present in the environment.

**METHODS**

**Strains and maintenance.** Clinical isolates were obtained from the A. fumigatus culture collection, Wythenshawe Hospital, Manchester, UK. Manchester environmental isolates were collected monthly over a period of 12 months from the air at the University of Manchester campus, between March 2009 and February 2010 (Alsahreef & Robson, 2014), and 60 strains were selected randomly for use in this study. Dublin environmental isolates were kindly supplied by H. Fuller (University of Dublin, Dublin, Ireland) and were isolated on a single day from a single site at the Belfield Campus, University College Dublin, Ireland. Isolates were grown on potato dextrose agar (PDA; ForMedium) in tissue culture flasks and incubated at 37 °C until confluent growth was obtained. Spores were harvested by gentle agitation of the mycelial surface with a sterile loop with 20 ml 0.05 % (w/v) Tween 20, filtered through four layers of lens tissue (Whatman) to remove phialides and spore aggregates, and washed two times in sterile water by centrifugation at 1500 g and resuspending the spores in 20 ml sterile water. Spores were enumerated using a modified Neubauer haemocytometer, and viability determined by serial dilution and c.f.u. determination on PDA plates after 48 h incubation at 37 °C. All strains had a spore viability > 95 %. Spores were kept for up to 1 week at 4 °C, for long-term storage, spore suspensions were mixed with an equal volume of sterile glycerol and stored at ~80 °C. For DNA extraction, mycelium was grown in 50 ml potato dextrose broth in 250 ml conical flasks for 48 h at 37 °C in an orbital shaker (200 r.p.m.).

**Virulence determination in Galleria larvae.** Galleria larvae (Live Food Direct) were stored in wood shavings in the dark at 4 °C for up to 10 days and transferred to room temperature for 2 h before use. Galleria larvae in groups of 30 were inoculated with 10 μl A. fumigatus spore suspension into the haemocoel through the last right or left proleg, using a Hamilton 1 ml gas-tight syringe (Fisher Scientific) with a 10 μl repeating dispenser attachment (Jaytee Biosciences). Spore concentrations of 1 × 10^6 to 1 × 10^7 ml^-1 were used to infect larvae, which were incubated at 37 °C in the dark in Petri dishes (10 larvae per plate) and the mortality monitored daily for 7 days. Mortality was assessed by lack of movement in response to stimulation and by discoloration (melanization) of the cuticle.

The survival of the Galleria larvae was analysed using the Mann–Whitney U test (Stats Direct) with P<0.05 considered significant, and differences in the survival rates of larvae inoculated with different strains were compared. A non-parametric estimate of the mean survival time for Galleria larvae was obtained as the area under the Kaplan–Meier estimate of the survival curve.

**DNA extraction.** Mycelium was harvested on muslin cloth, washed briefly with sterile water, and ground to a fine powder in liquid nitrogen using a mortar and pestle. Ground mycelium was added to 50 ml Falcon tubes (on ice) to the 10 ml mark and an equal volume of pre-heated DNA extraction buffer [0.7 M NaCl, 1 M Na2SO3, 0.1 M Tris/HCl (pH 7.5), 0.05 M EDTA, 1 % (w/v) SDS, 65 °C] was added and mixed thoroughly with a pipette tip, and incubated at 65 °C for 30 min to terminate nuclease activity and induce protein denaturation. A NanoDrop 1000 spectrophotometer (Thermo Scientific) was used to measure DNA concentrations in samples.

**RAPD.** In total, 106 isolates were analysed using RAPD. The primers used for RAPD were R108 (5’-AGTGCACACC-3’), UBC90 (5’-GG-GGTTAGG-3’), R151 (5’-GCTGTAGTG-3’) and RC08 (5’-AG-GATGTGCAGA-3’) (Eurofins) as these have been used to successfully fingerprint A. fumigatus populations in the past (O’Gorman et al., 2009). The samples were run on 2.5 % (w/v) agarose gels in 40 mM Tris/acetate EDTA buffer (pH 8.0). Only primers R108 and C08 were used to compare all isolates, and RAPD bands included in the analysis were scored as either absent (0) or present (1). The data from all primers were pooled for each isolate and a pair’s similarity matrix was calculated with Jaccard’s coefficient with the program FREETREE v. 0.9.1.50. A bootstrapped dendrogram with 1000 resamplings was produced with NJPlot.

**RESULTS**

**RAPD fingerprinting revealed genetic diversity in the environmental population and clustering of clinical isolates**

Four primers were used initially for RAPD fingerprinting (R108, RC08, R151 and UBC90) with seven A. fumigatus isolates, and fingerprinting was repeated at least two times to evaluate reproducibility. The four primers produced a range of RAPD bands, from 0.4 to 6 kb. All four primers gave several strong bands that were reproducible, along with many fainter bands (data not shown). Of the four primers tested, R108 and RC08 gave the most reproducible
results and had the greatest discriminatory power (0.992 and 0.868, respectively) (Bikandi et al., 2004) in a pilot study, and were selected for fingerprinting the remaining isolates. Only the strongest and reproducible bands were included in the analysis, and fainter and variable bands were excluded. To increase discrimination, RAPD data from both primers were pooled prior to analysis. As the number of isolates exceeded the maximum number of wells on a gel, strain AF293 was run on every gel as an internal control.

RAPD analysis of the Manchester environmental isolates showed a high genetic diversity in the population (Fig. 1). With few exceptions, isolates collected at the same time points could be distinguished from each other, indicating multiple sources of airborne spores. In addition, comparison of Manchester isolates collected monthly for 12 months indicated that the source of airborne spores varied at each time point. When environmental isolates were analysed along with clinical isolates, clinical isolates displayed a degree of clustering with 21 of the 25 clinical isolates forming three clusters accounting for 12, 5 and 4 isolates, although, with the exception of isolates 22178 and 22636 and isolates 17871 and 16258, they could be distinguished from each other.

**Virulence testing in Galleria larvae demonstrated a broad range in pathogenicity in the environmental population**

The virulence of ten randomly selected Manchester environmental isolates, ten Dublin environmental isolates, ten clinical isolates, and sequenced strains AF293 and AF1163 was determined in Galleria larvae inoculated with $1 \times 10^3$ to $1 \times 10^6$ spores per larva by monitoring survival over 7 days. Initial spore inoculum size had a large effect on Galleria larvae survival in all strains, with all strains showing the greatest survival after 7 days with $1 \times 10^3$ spores and lowest survival with $1 \times 10^6$ spores (data not shown). The greatest discrimination in survival between the different strains was seen at an inoculum level of $1 \times 10^5$ spores per larva (Fig. 2). In order to compare the relative pathogenicity of the Manchester, Dublin and sequenced strains, survival over 7 days for each spore inoculum for each group was combined and subjected to Mann–Whitney and Kaplan–Meier analyses (Fig. 3). The clinical isolates were significantly more pathogenic than the Dublin and Manchester isolates ($P<0.0001$) at the four different doses. Moreover, the Manchester isolates were significantly more pathogenic than the Dublin isolates ($10^6$ and $10^5$, $P<0.0001$; $10^4$, $P=0.0135$; $10^3$, $P=0.0043$). This was also apparent when the survival of each group 4 days after inoculation was compared at initial spore concentrations from $1 \times 10^3$ to $1 \times 10^6$ spores per larva, confirming that the greater pathogenicity of the clinical isolates was across the spectrum of spore inoculum concentrations (Fig. 4). When the percentage survival values after 4 days for each individual strain at an inoculum of $1 \times 10^3$ spores per larva were compared, the clinical isolates clustered together as the most pathogenic, the Dublin isolates the least pathogenic and the Manchester isolates showed a broad spectrum of pathogenicity (Fig. 5).

**DISCUSSION**

A variety of molecular typing methods have been developed over the past 20 years to investigate genetic diversity in the population of a wide variety of fungal species. Many of these techniques have also been used to study genetic diversity in the *A. fumigatus* population. The methods include RAPD (Anderson et al., 1996; Aufauvre-Brown et al., 1992; Mondon et al., 1995; O’Gorman et al., 2009), RFLP (Spreadbury et al., 1990), restriction enzyme analysis (Denning et al., 1990), amplified fragment length polymorphism (Vos et al., 1995) and sequence-specific DNA primer analysis (Mondon et al., 1997), and more recently microsatellite length polymorphism (Balajee et al., 2008; Bart-Delabesse et al., 1998; de Valk et al., 2007), MLST (Bain et al., 2007) and variable number of short tandem repeat typing (Vanhee et al., 2009). These methods have also demonstrated that there is a large genetic diversity within the *A. fumigatus* population (Balajee et al., 2008; Chazalet et al., 1998; Leenders et al., 1999; de Valk et al., 2009; Vanhee et al., 2009; O’Gorman et al., 2009) and have largely been used to investigate the epidemiology of isolates following clinical outbreaks of disease in hospitals (Alvarez-Perez et al., 2010; Bart-Delabes et al., 1999; Vanhee et al., 2010; Guinea et al., 2011), as well as to study genotypic diversity of strains in avian disease (Arné et al., 2011; Lair-Fulleringer et al., 2003; Olias et al., 2011). The recent discovery of a functional sexual cycle in *A. fumigatus* probably accounts for the genetic diversity observed in the population (O’Gorman et al., 2009), and much of this diversity appears to be concentrated in discrete genomic islands within the DNA that encode for proteins involved in heterokaryon incompatibility and cell-wall-associated proteins that may be involved in virulence (Fedorova et al., 2008). In addition, whilst the macromorphology of *A. fumigatus* strains...
appears to be largely similar within the population, the micromorphology (such as conidia and vesicle size and shape), pathogenicity and enzyme secretion have been found to be variable between isolates (Alp & Arikan, 2008; Aufauvre-Brown et al., 1998; Ben-Ami et al., 2010; Birch et al., 2004; Chamilos et al., 2007, 2010; Mondon et al., 1995, 1996; Olias et al., 2011; Rinyu et al., 1995). Limited studies on enzyme secretion have demonstrated a range of expression levels and isoenzymes from different isolates (Alp & Arikan, 2008; Birch et al., 2004; Blanco et al., 2002; Lin et al., 1995; Rinyu et al., 1995; Rodriguez et al., 1996).

Fig. 2. Percentage survival of wax moth larvae inoculated with various strains of A. fumigatus over 7 days. Thirty Galleria larvae were inoculated with isolates of A. fumigatus at 1 × 10⁵ c.f.u. per larva and incubated at 37 °C for 7 days. The number of surviving Galleria larvae was determined daily. Controls were inoculated with sterile 0.05% (v/v) Tween 20 (×). (a) Manchester environmental strains: Mar13 (Δ), Mar15 (○), Mar20 (●), Mar32 (■), Apr12 (▲), Apr15 (●), May1 (grey circle), Jun4 (grey square), Jun5 (grey triangle) and Jul1 (grey diamond). (b) Dublin environmental strains: RB11 (Δ), RB12 (○), RB13 (●), RB14 (■), RB15 (▲), RB16 (●), RB17 (grey circle), RB18 (grey square), RB19 (grey triangle) and RB20 (grey diamond). (c) Clinical strains 17871 (Δ), 15562 (○), 177796 (●), 16258 (■), 16795 (▲), 15819 (●), 17406 (grey circle), 17768 (grey square), 17882 (grey triangle) and 16916 (grey diamond). Sequenced strains AF293 (○) and AF1163 (□) were included as internal controls.

Fig. 3. Mann–Whitney analysis of the mean survival of G. mellonella inoculated with Manchester environmental (black bars), Dublin environmental (white bars) and clinical (grey bars) strains of A. fumigatus at spore concentrations of 1 × 10³, 1 × 10⁴, 1 × 10⁵ and 1 × 10⁶ spores per larva after 7 days. Controls were inoculated with sterile 0.05% (v/v) Tween 20 and retained 100% viability over 7 days. Error bars represent SEM.
In our study, RAPD typing also demonstrated a large degree of genetic variability within the airborne population of \textit{A. fumigatus} collected monthly over a period of 12 months from the same site in Manchester (Fig. 1). Moreover, isolates typed from the same time point showed several genotypes, indicating a mixture of sources contributed to the airborne population at any one time. This contrasted with the small number of isolates from Dublin, which were also collected at a single time point from a single site (H. Fuller, personal communication), that with one exception showed identical genotypes with both primers (Fig. 1), indicating that the majority of airborne spores at the time of sampling may have arisen from a single source. However, other isolates collected from Dublin at different time points also showed a range of phenotypes, indicating variable sources over time (O’Gorman \textit{et al.}, 2009). That a range of genotypes was found at each monthly sampling point in this study is not unexpected as \textit{A. fumigatus} spores are dispersed readily in the atmosphere due to their small size and samples were taken in the open outdoor environment. Other studies that have typed \textit{A. fumigatus} spores collected from the environment have also shown a high degree of genotypic diversity in the population, including the enclosed environments of hospitals where it might have been expected that the number of contributing sources would have been smaller (Araujo \textit{et al.}, 2010; Debeaupuis \textit{et al.}, 1997; Guinea \textit{et al.}, 2011; Menotti \textit{et al.}, 2005).

Previous studies attempting to identify environmental sources of patient infection in hospital environments have generally found only a low number of patient isolate genotypes and only a small number could be matched with an environmental phenotype, probably due to insufficient environmental samples being collected to capture the high level of genotype diversity reported (Araujo \textit{et al.}, 2010; Guinea \textit{et al.}, 2011). In addition, in those studies where environmental samples were taken at different time periods or locations within the same building, in only a few instances was an identical genotype recovered, indicating a high degree of variability occurs at the same location over time and at any one time in different locations (Araujo \textit{et al.}, 2010; Guinea \textit{et al.}, 2011). Many aspects of the pathogenesis of aspergillosis remain to be clarified, including the contribution of the place of acquisition, incubation period, virulence variation in different \textit{Aspergillus} isolates and the possible contribution of more than one clone in human infections (Alvarez-Perez \textit{et al.}, 2010). Interestingly, in our study there was some genetic clustering amongst the clinical isolates where 21 of the 25 isolates clustered into three groups that also contained some environmental isolates, suggesting that some traits were detected that may be associated with a higher pathogenicity (Fig. 1).

Whilst many studies have used mammalian models to investigate the impact of specific gene function on pathogenicity (Askew, 2008; Rementeria \textit{et al.}, 2005), there have been few studies that have compared the pathogenicity of different isolates of \textit{A. fumigatus}, and those that have reported a range of pathogenicity between isolates (Jackson \textit{et al.}, 2009; Lionakis \textit{et al.}, 2005; Reeves \textit{et al.}, 2004). Invertebrate models of pathogenicity offer a cheaper and quicker alternative to animal models, enabling large

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\caption{Percentage survival of \textit{Galleria} larvae 4 days after inoculation with $1 \times 10^5$ spores per larva of various strains of \textit{A. fumigatus}: clinical (black bars), Manchester environmental (white bars) and Dublin environmental (grey bars). Controls were inoculated with sterile 0.05 % (v/v) Tween 20.}
\end{figure}
numbers of isolates to be compared (Chamilos et al., 2010; Kavanagh & Reeves, 2004). In *A. fumigatus*, these models have included *Galleria* larvae and Toll-deficient mutants of *Drosophila* (Jackson et al., 2009; Lionakis et al., 2005; Reeves et al., 2004), and relative pathogenicity has been correlated with pathogenicity levels in mammalian models (Slater et al., 2011; Chamilos et al., 2010). In this present study, a range in pathogenicity against *Galleria* larvae was apparent in both clinical isolates and environmental isolates (Fig. 2). Moreover, as a group, the clinical isolates were significantly (*P*<0.005) more pathogenic than the environmental isolates (Fig. 3) with the exception of the clinical isolates and the Manchester environmental isolates at an inoculum level of 1 × 10⁶ spores per larva. However, the environmental isolates showed an overlap in pathogenicity with the clinical strains (Fig. 5), suggesting that clinical isolates may represent the most pathogenic of the environmental isolates and that the infection process in patients may select for the most pathogenic strains present in the environment. Selection for the most pathogenic strains from the environment has been suggested previously as an explanation for why patients are often infected with only one strain and in those cases where coinfection is present, only two or three genotypes have been observed despite a highly diverse environmental population (Alvarez-Perez et al., 2010; Debeaupuis et al., 1997; Menotti et al., 2005; Guinea et al., 2011; de Valk et al., 2007; Tang et al., 1994). Moreover, in one study, whilst several genotypes were recovered from the respiratory tract of a patient, only one was recovered from invasive disease from solid organs, suggesting further selection for invasion (de Valk et al., 2007). It is now clear that the pathogenicity of this opportunistic pathogen is polygenetic rather than being dependent on any one virulence factor (Askew, 2008; Rhodes, 2006), with a broad range of factors being implicated as playing a role in the pathogenicity, including extracellular hydrolases, nutrient acquisition and metabolism, toxin production, melanin and transcription factors, and secondary messenger pathways with broad genotypic and phenotypic effects (Amich et al., 2013; Amich & Krappmann, 2012; Jackson et al., 2009; Richie et al., 2009; Kim et al., 2013; Krappmann et al., 2004; Li et al., 2011; O’Hanlon et al., 2011; Schrettl & Haas, 2011; Soukup et al., 2012). Differing levels of expression of these factors in the population may therefore give rise to the range in pathogenicity observed in the *A. fumigatus* population, and the ability to adapt and infect the lung. In addition, infection also depends on host factors that will vary in differing individuals (Hartmann et al., 2011; Sales-Campos et al., 2013). Thus, selection in the lung of *A. fumigatus* from a diverse range of genotypes will be multifactorial dependent on the interplay between *A. fumigatus* and host genotype.

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