Major variations in *Aspergillus fumigatus* arising within aspergillomas in chronic pulmonary aspergillosis


School of Translational Medicine, Manchester Academic Health Science Centre, University Hospital of South Manchester, Manchester, The University of Manchester, Manchester, UK

**Summary**

Aspergillomas develop from progressive layers of mycelial growth on the walls of pulmonary cavities over months. Aspergillomas are characteristic of chronic pulmonary aspergillosis and are a risk factor for azole resistance. We investigated genotypic and phenotypic alterations in *Aspergillus fumigatus* recovered from aspergillomas. Aspergillomas were removed from three patients (two at surgery, one at autopsy) and dissected. Overall 92 colonies of *A. fumigatus* were isolated. Microsatellite typing was conducted to determine genetic type. Itraconazole, voriconazole and posaconazole susceptibilities were performed. The *cyp51A* gene was sequenced in 22 isolates. Isolates from Patient 1 (*n* = 25) were azole susceptible and resistant, although all *cyp51A* sequences were wild type, the isolates split into two distinct clades. In Patient 2, isolates were less variable (*n* = 10), all were azole susceptible. In Patient 3 only azole-resistant strains (*n* = 57) were isolated, with M220K or M220T Cyp51A alterations, and microevolution was indicated. Marked diversity was observed in isolates from these patients; revealing differences in azole susceptibility, mechanism of resistance and genetic type. Importantly, routine sampling from respiratory specimens proved suboptimal in all cases; azole resistance was missed (Patient 1), cultures were negative (Patient 2) and high-level posaconazole resistance was not detected (Patient 3).

**Key words:** *Aspergillus*, aspergilloma, azole resistance.

**Introduction**

The term aspergilloma refers to the presence of solid material containing primarily *Aspergillus* hyphae within an intrathoracic or paranasal cavity or bronchus.1,2 First described as a ‘mégyunycéme intrabronchectasique’ by Dévé in 1938 France,3 the term aspergilloma seems to have entered common parlance in the early 1950s.4 Aspergilloma is a minority feature of chronic pulmonary aspergillosis (CPA) and may be simple and amenable to resection, or be a component of chronic cavitary pulmonary aspergillosis (CCPA) generally requiring long-term antifungal therapy.

The symptomatology in individual patients with CPA and pulmonary aspergillomas is highly variable. Most patients with simple pulmonary aspergillomas are asymptomatic initially; in contrast symptoms are often severe in cases of CCPA, and prognosis is significantly poorer.5,6 A characteristic feature is haemoptysis which varies from trivial to fatal in severity. Haemorrhage occurs from ulceration into a plexus of newly formed small arteries fed from the remnant bronchial artery circulation. In association with haemoptysis, most patients have cough and productive sputum. *Aspergillus* precipitins (IgG antibody) are detectable in over 90% of
patients with aspergilloma and CPA. Furthermore, about 40–65% of patients are sensitised to *Aspergillus fumigatus* by specific IgE and skin prick testing. Spontaneous resolution of aspergillomas is recognised to occur in 10% of cases within 3 years and may occur occasionally with antifungal treatment.

Aspergillomas are most commonly seen in CCPA – about 25% in our experience. Any bullous or cavitary pulmonary disease (i.e. tuberculosis, sarcoidosis, prior pneumothorax) provides the risk for an aspergilloma, although localised invasion of Aspergillus can produce cavitaries which increases in size and form aspergillomas (see Patient 8 in reference). In patients with pulmonary sarcoidosis, 10 of 19 (53%) patients with cysitic parenchymal damage had aspergillomas compared with none of 81 patients with non-cystic pulmonary sarcoidosis. Following tuberculosis, 11–20% patients with cavities of 2.5 cm or larger go on to develop CPA. Patients are typically in the 4–6th decade of life, and more men than women are affected.

Aspergillomas are formed when spores germinate on the bronchial or cavity wall, where mycelia and debris attach to form an amorphous mass. They form over weeks or months in abnormal and ectatic bronchi, further enlarging them to form a cavity, or directly in a cavity in continuity with the airways, usually with surrounding fibrosis. Often an affected pulmonary cavity wall has a cobblestone or shaggy appearance, sometimes with ulceration, but it may be smooth, as is typical in the paranasal sinuses. Sometimes aspergillomas are closely adherent to the cavity wall, but often they are mobile in the cavity. Many patients have multiple cavities, but multiple aspergillomas are uncommon. Very few recent studies of the structure and mycology of aspergillomas have been done. Histologically, aspergillomas show layers (lamelliform) of hyphal growth with a variable dense-layered appearance. Sometimes conidiophores are present. Much other dense material is present, consistent with extracellular matrix (polysaccharide) and extracellular DNA. Isolates obtained from the sputum of patients with aspergillomas are often highly atypical, growing slowly and poorly sporulating.

In this study we sought to investigate genotypic and/or phenotypic changes associated with antifungal susceptibility and genetic type between multiple *A. fumigatus* isolates recovered from aspergillomas in three patients; by performing susceptibility testing, microsatellite genetic typing, and *cyp51A* sequencing (the most commonly described causative mechanism ofazole resistance).

**Patients and methods**

**Patients**

A single aspergilloma was removed from Patients 1 and 2 during surgery, whereas samples were taken from multiple aspergillomas and surrounding areas (cavity, etc.) from Patient 3 at autopsy. A brief case report for each patient follows. Figure 1 shows radiological and gross pathological images from all three patients.

Patient 1 – This 43-year-old Pakistani woman resident in the UK developed a severe chest infection with haemoptysis following a trip home, which resolved slowly with antibiotics. A chest radiograph showed a large infected ‘cyst’ in the right lung. She had another similar episode a year later. She reported having been told that she has a bulla in the lung 15 years earlier shortly after emigrating to the UK, with no specific therapy advised. There was no history of tuberculosis. When seen at Wythenshawe Hospital, she looked well, but reported tiring easily and an intermittent cough and frequent episodes of ‘flu’. She was a life-long non-smoker. Her *Aspergillus* precipitins were positive undiluted. She underwent a right lower lobectomy. A cavity measuring 5 × 6 × 6 cm was found containing grey spongy tissue (fungal ball) measuring 3 cm across. Histology confirmed chronic inflammation and local fibrosis in the cavity wall, and necrotic fungal hyphae, without tissue invasion. *A. fumigatus* was cultured from the fungal ball and was reported to be fully susceptible to itraconazole, voriconazole and amphotericin B.

Patient 2 – This 25-year-old woman, originally from Pakistan, presented with significant haemoptysis 5 years after suffering from pulmonary tuberculosis. She had a single large, left upper lobe cavity containing an aspergilloma. She was infected with hepatitis B (HBsAg positive). Her *Aspergillus* precipitin titre was 1:128. After rigid bronchoscopy, she underwent a routine left posterolateral thoracotomy. The cavity was stuck to the chest wall in the left apex and very vascular. The left upper lobe was mobilised with blunt dissection and removed, without spillage of the cavity contents, after complex dissection along the pulmonary artery and vein to the hilum. She recovered well postoperatively except for prolonged duration of action of mivacurium, probably attributable to an interaction with voriconazole given perioperatively. She also developed some scar and left arm pain which persisted at least 18 months postoperatively. Histopathology of the operative specimen showed a 3-cm cavity within the apex of the left upper lobe lined by fibrin overlying an area of chronic inflammation and fibrosis, without...
granulomata. Silver staining showed branching septate hyphae, many filaments being necrotic. Sputa were negative prior to surgery, but a fully susceptible A. fumigatus was grown from her aspergilloma.

Patient 3 – This 47-year-old woman with CPA had pulmonary tuberculosis in 1986 and suffered smoke inhalation in 1989 (Patient 8 in reference12). In March 1994 she became ill with three cavities in her left upper lobe and fungus balls consistent with aspergillomas in two cavities. Aspergillus precipitins were detectable in blood (2+) and sputum cultures grew A. fumigatus. She declined surgical treatment, and returned to clinic in November 1997 when her condition had markedly deteriorated. Her chest X-ray showed complete opacification of the left lung, with multiple cavities, at least two of which contained an aspergilloma, widespread consolidation and a small pleural effusion. Itraconazole 200 mg bd was started in 1998 without benefit and intravenous amphotericin B (1 mg kg$^{-1}$) was then given, and a left bronchial artery embolisation was performed. A percutaneous lung biopsy of the left lower lobe revealed fibrotic lung tissue containing carbon pigment and focal chronic inflammation without Aspergillus. She also received subcutaneous gamma IFN. She was found to have profound mannose-binding protein deficiency. Apart from one further bronchial artery embolisation, she remained stable on itraconazole. In April 2005, her general practitioner substituted generic itraconazole, at the same dosage. Itraconazole levels were undetectable in June 2005 and were 4.2 mg $l^{-1}$ (by bioassay) 5 months later, having had therapeutic levels previously. Raised precipitins and IgE levels indicated ongoing active disease. Culture from sputum performed in December 2005 revealed azole resistant A. fumigatus, whereas multiple specimens had been culture negative for years before this. She tried a low dose of voriconazole in February 2006 [250 mg daily (6.5 mg Kg$^{-1}$ d$^{-1}$); she weighed only 38 Kg], but was unable to tolerate this (insomnia and heavy head feeling) and stopped it after 2 weeks. Renal dysfunction was present, making amphotericin B treatment difficult. In June 2006 she started posaconazole 200 mg four times daily. Four weeks later she was admitted with a severe septic episode with renal failure and ileus with an...
abdominal compartment syndrome. She underwent a laparotomy, which relieved her splinted diaphragm, but she died of unrelenting sepsis within 24 h. Autopsy showed multiple cavities (n > 15) and fibrosis replacing the left lung with multiple aspergillomas. One closed cavity contained caseous material with acid fast bacilli observed on microscopy and grew *Mycobacterium tuberculosis* on culture.

**Isolates**

Multiple colonies were isolated from throughout the dissected aspergillomas, from these 92 *A. fumigatus* were selected for study with individual colony picking; 25 from Patient 1, 10 from Patient 2 and 57 from Patient 3. Isolates from Patient 3 were individually cultured from the main (largest) aspergilloma, a smaller aspergilloma and surrounding cavities/tissue. Isolates are held in the Mycology Reference Centre Manchester (MRCM) culture collection. All were identified using macro- and micromorphological techniques. Isolates which did not sporulate sufficiently, or were not able to be recovered from long-term storage at –80 °C (several from Patients 1 and 2), were not studied. A selection of the total isolate population was studied, related to differing colony morphologies, minimum inhibitory concentrations and location of the isolate in the aspergilloma(s). Data from the first aspergilloma informed the second (poorly growing) and third, in terms of isolate selection for additional study.

**Susceptibility**

Minimum inhibitory concentrations were determined for itraconazole (Sigma, Poole, UK), voriconazole (Pfizer Ltd, Sandwich, UK) and posaconazole (Schering-Plough, Summit, NJ, USA) in 72 isolates by modified European Committee for Antibiotic Susceptibility Testing (mEUCAST) method,\(^{21}\) using a slightly lower inoculum of 0.5 × 10^5 cfu ml^-1, as some of this work was conducted prior to publication of the standard. Epidemiological cut-offs applied to this data set were as follows: itraconazole and voriconazole >2 mg l^-1, and posaconazole >0.5 mg l^-1.\(^{22}\)

**Sequencing of *cyp51A* gene**

The entire coding region of the *cyp51A* gene was amplified in 22 isolates, as previously described,\(^{23}\) including someazole resistant and susceptible isolates from each patient (where applicable). Alterations were identified by alignment against the sequence from anazole susceptible strain (GenBank accession number AF338659). GenBank accession numbers for the mutated *cyp51A* sequences in Table 1 are as follows: JX283443 (F13560), FJ548885 (A8), FJ548886 (A12), JX283444 (A21), FJ548889 (A22) and JX283445 (A30).

**Microsatellite typing**

Microsatellite typing was conducted for selected isolates from each patient using the technique by de Valk et al.\(^{24}\) M3 and M4 primer sets were used, with one primer of each pair labelled with a fluorescent dye (VIC, NED or PET). We chose not to include the M2 markers in this study, as they add minimal additional discriminatory power, and suffer from more difficulties in interpretation (i.e. stutter peaks). PCR reactions were carried out individually, and products examined on an ethidium-stained agarose gel. Products were pooled by locus (M3 and M4), with 5 μl of each product being added to either of the two pools. Pooled samples were sent to The University of Warwick for capillary electrophoresis using an ABI 3130 × 1 genetic analyser. Output trace files were analysed using Peak Scanner v1.0 (Applied Biosystems, Carlsbad, CA, USA). Amplicons were adjusted using a correction factor based on previously sequenced alleles.\(^{25}\)

Microsatellite types were defined by the number of repeats at each of the loci, therefore isolates with the identical number of repeats at a given loci were defined as the same. Distance matrices between the microsatellite types were generated using the SplitsTree MLST data analysis tool on the PubMLST website (http://pubmlst.org). Distance matrices were imported in NEXUS format into SplitsTree v.4.11.3 (http://www.splitstree.org), and non-rooted trees were generated using the Neighbour-Joining algorithm BioNJ. In addition to the 39 typed isolates from this study, the data set was populated with the data from an additional 68 MRCM clinical and reference strains, and 99 isolates from de Valk et al.\(^{24}\) Locus 3B was excluded from this analysis due to the known presence of a 3-base addition in the non-repeat DNA sequence;\(^{25}\) rendering the interpretation of fragment sizes unreliable. Fragment sizes for locus 4C tended to fall between values for two numbers of repeats in this study: this made the comparison of repeat numbers with those from other studies problematic without access to the original data. Therefore, this locus was also omitted from downstream analysis. 3B and 4C were amongst the least variable loci in this collection, therefore their exclusion had minimal effect on the findings.
Results

Morphology

The macromorphology of isolates was frequently atypical; with reduced growth rate compared with the wild type, unusual spore colour (variants of green/brown/etc.) and/or poor sporulation (data not shown). Interestingly some of these atypical features were not observed when isolates were resurrected from storage at −80 °C. Notably, however, morphology did not appear to be associated with susceptibility, type or resistance mechanism.

Susceptibility

Isolates tested from Patient 1 were predominantly (75%, 6/8) azole susceptible, with the exception of two isolates (25%) which showed high-level cross-resistance between all three azoles tested (Table 1: showing key isolates). All isolates from Patient 2 were azole susceptible. Whereas all isolates from Patient 3 were azole resistant; all 57 were resistant to itraconazole, 12/57 (21%) were cross-resistant to voriconazole and 14/57 (25%) were cross-resistant to posaconazole. Interestingly, only high-level posaconazole resistance (>8 mg l⁻¹) was detected in the main aspergilloma of Patient 3, and not in the smaller fungus ball/cavities/surrounding tissue or routine respiratory samples.

Cyp51A sequencing

Isolates from Patient 1 revealed wild-type cyp51A sequences, including the azole-resistant strains. A single isolate was sequenced from Patient 2 (as all were azole susceptible), interestingly this isolate revealed a non-wild-type cyp51A sequence. An amino acid substitution was observed at position 172 (M172V), plus three synonymous mutations at G89G, L358L and C454C. Isolates from Patient 3 had two different amino acid alterations, both at position 220 (M220K and M220T). The main fungus ball contained a mix of the two mutations, whereas only M220T was found in the periphery (in smaller aspergillomas and cavity, etc.). All isolates with high-level posaconazole resistance had a M220K mutation.

Typing

The neighbour-joining tree generated 146 distinct 4-allele types, which grouped into 8 rooted clusters, 6 of which also split into distinct branches at varying distances along their lengths (Fig. 2). The isolates from the different aspergilloma patients did not cluster together. In isolates from Patient 1, significant genetic differences were observed in 5 of 6 microsatellite loci, splitting the isolates into at least 2 clades. Locus 3A was most variable, followed by 3C, 4A, 3B and 4B, respectively, with no variation in 4C. Most of these isolates were distributed broadly across several branches within 1 clade. Isolates were less variable in Patients 2 and 3, with only microvariation seen in 3A and 3B, and 3A, microsatellite loci respectively.

Discussion

A limited amount is known about the biology of aspergillomas, but they represent an extreme manifestation of biofilm formation in Aspergillus. Substantial extracellular matrix surrounds hyphae and contains some novel polysaccharides including galactosaminogalactan and the galactomannan. Recently, A. fumigatus has been shown to produce extracellular DNA similar to neutrophil nets, and it is likely that this material is present in aspergillomas as well. Previous studies in patients with aspergilloma showed marked phenotypical diversity in isolates recovered from fungal balls, although few studies have focussed on the genetic diversity within aspergillomas. In a study from 1991 multiple genetic types were observed from a patient with aspergilloma. Again, several types were identified from an aspergilloma patient using random amplification of polymorphic DNA (RAPD). However, a more recent study, again using RAPD, revealed only identical genetic types in isolates from a single patient with aspergilloma, although genetic variation was observed in the cyp51A gene sequence and expression of genes encoding efflux pumps and Cyp51A, all causative.
mechanisms of azole resistance. Indeed, aspergillomas have been linked with an increased risk of the development of azole resistance.

Random amplification of polymorphic DNA is a highly sensitive technique that is able to detect small changes in genetic variation, however, it produces banding patterns which makes comparing data between studies difficult. Here, we used highly polymorphic microsatellites to determine genetic change/type. De Valk et al. described nine novel markers which offer higher throughput, speed, reduced cost, and higher discriminatory power (as the loci are quicker to evolve) than other methods such as multilocus sequence typing (MLST).

Both azole susceptible and resistant isolates were recovered from the aspergilloma of Patient 1. Although the mechanism of resistance remains ill defined as no cyp51A mutations were detected – the most commonly reported mechanism of azole resistance in Aspergillus currently. Likely alternative mechanisms are upregulation of efflux transporters or overexpression of the cyp51A gene. Importantly resistance was only observed when the aspergilloma was dissected, and not via diagnostic specimens. Significant genetic variation was observed in these strains, with differences in multiple microsatellite loci (all except 4C). However, there was no clear separation in type between these isolates, and the variation observed could be accounted for by
extensive progressive sequential changes (stepwise addition of tandem repeats) as the repeat numbers were close. The two resistant isolates differed by three microsatellite loci, and shared identical alleles with susceptible strains (suggesting that genetic type was not linked with susceptibility changes in these strains).

Importantly the MLST-like analysis used here does not account for how related different types might be (e.g. an allele size of 163 might be more closely related to 165 than 167), it simply distinguishes whether it is the same or not. Although isolates with the same allele size might not necessarily be related, they could have been evolved independently. Furthermore, changes in nine arbitrary loci may not be indicative of other changes in the genome.34 Moreover, some allele sizes are more common than others, and some loci are more variable than others. To our knowledge, however, there is no analysis that accommodates these additional levels of complexity.

The presence of a non-synonymous mutation in a susceptible isolate from Patient 2 indicates that changes at this position may not be associated with azole resistance, for example could be epidemiological. However, it does not rule out the association entirely, as it could potentially be a contributing factor that requires additional alteration/s to confer resistance. Isolates from Patients 2 and 3 were significantly less variable, and could both be attributed to microevolution of a single strain.

Different amino acid substitutions were linked with different patterns of cross-resistance in isolates from Patient 3. Mutations at codon 220 are well characterised.35 Interestingly, the mutation M220K (which was more highly cross-resistant) was only found in the main aspergilloma, whereas M220T mutations were distributed throughout the autopsy-sampled lung and isolated from respiratory samples whilst the patient was alive – suggesting that the M220T mutation was an alteration earlier in infection development, whereas M220K was a subsequent event limited to the primary aspergilloma. Unfortunately the same base was altered (ATG to ACG and AAG), therefore it was not possible to determine if the alterations had occurred independently or sequentially, so the path of resistance evolution could not be confirmed. Importantly, however, this meant that high-level posaconazole resistance (>8 mg l⁻¹) was not detected in this patients diagnostic specimens.

In summary, these aspergilloma isolates revealed extensive genetic changes, even within the same fungal ball. Azole resistance was detected, only some as a result of cyp51A mutations. Microevolution of microsatellite loci was detected in both Patients 2 and 3, furthermore, the significant microsatellite in Patient 1 also cannot rule out progressive genetic alterations at these highly polymorphic markers. The typing data revealed that isolates from different patients did not cluster. The ability for A. fumigatus to change genetically to such an extent within the lung is remarkable. And finally, the limitations of diagnostic specimens without biopsy in patients with aspergillomas were highlighted in all three cases, and is of concern.

Acknowledgments

Thanks to The University of Manchester sequencing facility for conducting sequencing reactions, and The University of Warwick for performing the capillary electrophoresis. We also thank surgeons Mark Jones and Rajesh Shah for harvesting the aspergillomas, and Paul Bishop the histopathologist for conducting the autopsy in Patient 3. These data have been presented in part at the 5th Advances Against Aspergillosis meeting 2012 Istanbul, and in references.23,36 This work was funded by David Denning’s endowment fund.

Conflicts of interest

Susan Howard has received research grants from Astellas, support grants from Gilead, Pfizer and the Fungal Research Trust, travel grants from Astellas and Schering-Plough, equipment grants from the Fungal Research Trust and has been paid for talks on behalf of Pfizer and Astellas. Alessandro Pasqualotto has received research grants and given paid talks on behalf of Pfizer, MSD, Astellas, United Medidal (Gilead) and Bagó. The other authors report no conflicts of interest.

References

6 Nam HS, Jeon K. Um SW et al. Clinical characteristics and treatment outcomes of chronic necrotizing pulmonary
Aspergillus fumigatus variation in aspergillosis


