Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009

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Objectives: Resistance to azole antifungal drugs in *Aspergillus fumigatus* is now a major clinical problem in some locations. Here we update our previous experience with data from 2008–09.

Methods: We tested all *A. fumigatus* isolates submitted to the Mycology Reference Centre Manchester in 2008 and 2009 for susceptibility to itraconazole, voriconazole and posaconazole. We undertook CYP51A sequencing for most of the azole-resistant isolates.

Results: Of 230 isolates, 64 (28%) were azole resistant. In 2008 and 2009, 14% and 20% of patients had resistant isolates, respectively. During this period 62 of 64 (97%) were itraconazole resistant, 2 of 64 (3%) were only voriconazole resistant and 78% of cases were multi-azole resistant. Forty-three percent of isolates did not carry a *cyp51A* mutation (previously the most common azole resistance mechanism), indicating that other mechanisms must be responsible and are increasing in frequency.

Conclusions: Azole resistance is evolving and growing in frequency. Established and novel mechanisms may be responsible.

Keywords: antifungal drug resistance, sterol 14-alpha demethylase CYP51A, *cyp51A* mutation

Introduction

Previously, we reported a rising frequency of azole resistance in *Aspergillus fumigatus* to the end of 2007.[1] Despite itraconazole resistance being reported in *A. fumigatus* for the first time in 1997 (in isolates obtained in the late 1980s), this was a rare phenomenon until 2004 in our centre and also in the Netherlands.[2] In contrast, relatively few reports of azole resistance have been documented elsewhere. In this report we update our azole resistance experience to include 2008 and 2009 data, and provide important mechanistic information.

Currently the most commonly reported mechanism conferring azole resistance in *A. fumigatus* is mutation in the *cyp51A* gene, leading to alterations in the target protein. In the Netherlands an alteration at codon 98 (L98H), in combination with a tandem repeat in the promoter region, is the most common *cyp51A* mutation. In Manchester, however, this mutation was much less common and 18 other non-synonymous alterations were found,[1] some of which have not yet formally been demonstrated to confer resistance.

Breakpoints or epidemiological cut-offs have been proposed for itraconazole, voriconazole and posaconazole in *A. fumigatus*.[3–5] We have used these cut-offs in our interpretation.

Materials and methods

Isolates

Isolates of *A. fumigatus* submitted to the Mycology Reference Centre Manchester [formerly the Regional Mycology Laboratory Manchester (RMLM)] for susceptibility testing in 2008 and 2009 were identified and tested for susceptibility to the three licensed triazoles active against *Aspergillus* spp. Laboratories submitted isolates selected by the clinician responsible for the patient, and those submitted represent a limited number of those cultured. Identification was performed using macro- and micromorphological characteristics, with confirmation by internal transcribed spacer (ITS) sequencing of all resistant isolates. Resistant aspergilli were subcultured onto Sabouraud glucose agar (Oxoid, Basingstoke, UK) for 48 h at 50°C to eliminate non-*fumigatus* species.

Susceptibility testing

Susceptibilities were determined using a modified European Committee on Antimicrobial Susceptibility Testing (EUCAST) method, as previously described,[1] except for a lower final inoculum concentration (0.5×10^5 as opposed to 1×10^5–2.5×10^5 cfu/mL). The breakpoint used for resistance was ≥ 4 mg/L for itraconazole and voriconazole, and ≥ 1.0 mg/L for posaconazole.

CYP51A sequencing

DNA was extracted using the MycXtra kit (Myconostica, Manchester, UK). The entire coding region of the CYP51A gene was sequenced as previously described,[6] and aligned against the sequence from an azole-susceptible strain (GenBank accession no. AF338659) using the software tool AlignX (VectorNTI; Invitrogen, Paisley, UK). Mutations were confirmed by repeating the DNA extraction, PCR and sequencing both strands.

Patient analysis

Repeat resistant isolates from the same patient were removed from the patient analysis (but not isolate analysis), even if isolated >1 year apart, unless their susceptibility profiles differed by interpretative breakpoint for at least one azole. In the case of mutation analysis, if at least one isolate from the same patient had a *cyp51A* mutation that confers resistance then that patient was included as a patient with a *cyp51A* mutation.

Results and discussion

In 2008, 92 *A. fumigatus* isolates were tested for triazole susceptibility, of which 21 (23%) were resistant to at least one azole. In 2009, 43 of 138 (31%) isolates tested were azole resistant (Figure 1). Of these resistant isolates, 3 of 21 (14%) and 9 of 43 (21%) were resistant to itraconazole only in 2008 and 2009, respectively. Only 2 (10%) isolates from 2008 were voriconazole resistant. The remainder (16 in 2008 and 34 in 2009) were multi-azole resistant. Thus during this period, 62 of 64 (97%) were itraconazole resistant, 2 of 64 (3%) were only voriconazole resistant and 50 of 64 (78%) cases were multi-azole resistant. Using patients rather than isolates as the denominator (discounting additional isolates from patients with the same susceptibility pattern), the frequency of resistance remains high in the cases referred to our laboratory during 2008–09 at 14% (9 of 64 patients) and 20% (19 of 93 patients), respectively. In 2007 we found a sharp rise from 5%–7% azole resistance in 2004–06 to 17%[1] (Figure 1). This high frequency of resistance continued in 2008 (14%) and 2009 (20%). The increasing number of

isolates submitted reflects referral of patients to the National Aspergillosis Centre and increasing awareness of resistance.

Most remarkable is the increasing frequency of azole-resistant isolates without *cyp51A* mutations. These isolates have been rarely reported elsewhere.[1,7] Prior to 2007 very few resistant isolates in our centre had a wild-type CYP51A sequence. In 2008, of the 13 resistant isolates studied, 1 had a M220K mutation, 3 had the F46Y/G89G/M172V/L358L/E427K/C454C combination (which is probably not linked to resistance) and the remaining 9 isolates had no CYP51A mutations. In 2009, 10 of 31 (32%) isolates tested had a wild-type CYP51A sequence (Figure 2). For patients, the frequency of mutations found in at least one isolate was 22% and 58% in 2008 and 2009, respectively (Figure 2). Thus 43% of isolates and 54% of patients did not have a CYP51A mutation known to confer resistance (including two isolates that were voriconazole resistant only). Interestingly, three patients had serial resistant isolates, some with *cyp51A* mutations, others with wild-type sequences. Efflux-mediated resistance could be responsible, as it is a common mechanism in yeasts, although it has been mooted rarely in *Aspergillus*.[8,9] Up-regulation of the CYP51A gene has also been implicated in azole resistance, but has yet to be found in isolation.[10] It is not certain whether all the *cyp51A* mutations found in Manchester confer resistance, so other mechanisms could also be contributory in these isolates.

Despite the influence of other mechanisms, cross-resistance patterns appeared to remain closely linked with the Cyp51A amino acid substitution. Isolates with G54R, P216L and G448S mutations are all associated with itraconazole and posaconazole resistance, whilst remaining susceptible to voriconazole. We found isolates with five different amino acid substitutions at position M220, namely isoleucine (I), lysine (K), valine (V), arginine (R) and tryptophan (W), of which M220R and M220W have not been previously reported to our knowledge. All alterations at codon 220 are associated with itraconazole and posaconazole resistance, but result in variable voriconazole MICs (typically raised). A novel finding is that two patients had one isolate
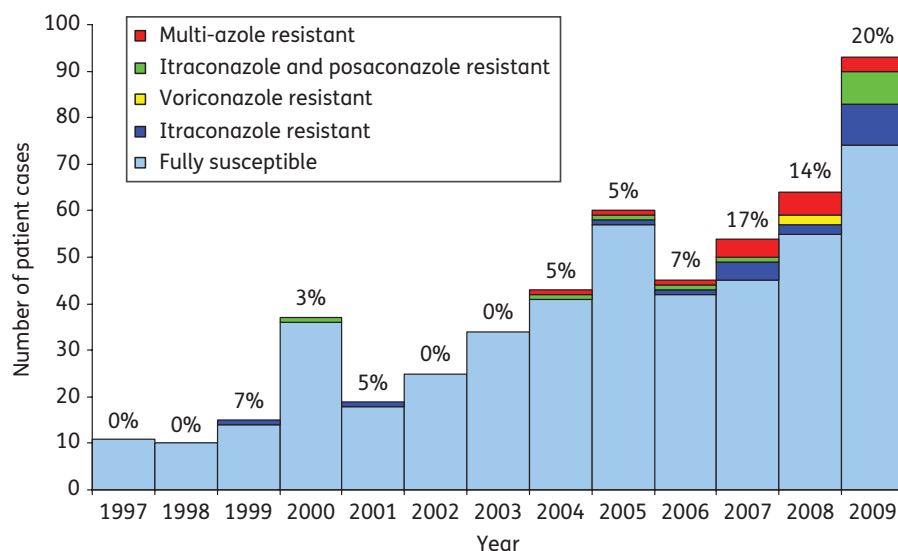
Figure 1. Azole resistance frequency in *A. fumigatus* by patient 1997–2009. Overall azole resistance for each year is shown above each column as a percentage. The data from 1997 to 2007 have been published previously.[1]
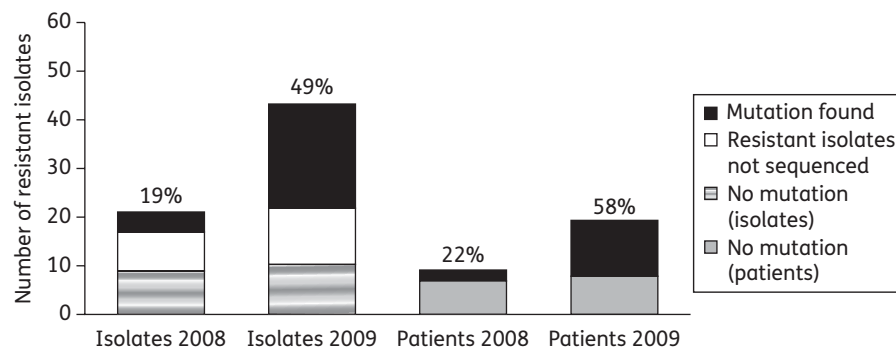
Figure 2. Percentage frequency of mutations conferring resistance in 2008 and 2009. Overall percentage of mutation found [G54R, H147Y, M220 (I, K, V, R, W), P216L, A284T, G448S] in isolates and patients for each year is shown above each column. The percentage figure above each column refers to those with demonstrated mutations (single nucleotide polymorphisms).

each with a *cyp51A* A284T mutation (alanine to threonine substitution) conferring reduced susceptibility to itraconazole, voriconazole and posaconazole. Two patients yielded an isolate each with F46Y/G89G/M172V/L358L/E427K/C454C mutations, one of which also had the mutations N248T and D255E. However, it is likely that these mutations are not associated with resistance, as they have been described previously in both susceptible and resistant isolates.[1,5]

These results highlight the continuing increasing frequency and evolution of resistance mechanisms in *A. fumigatus*, in both azole-naive and azole-treated patients (data not shown). The increasing rate of resistance is of concern. Furthermore the emergence of alternative mechanisms of resistance other than *cyp51A* mutations, including isolates resistant only to voriconazole with no target mutations detected, implies a quite distinct mechanism compared with previously reported resistant isolates. There appear to be differences in the geographical distribution of azole resistance in *A. fumigatus*, which cannot be explained by differences in methodology (as excellent concordance has been shown between CLSI and EUCAST methods). Since not all centres monitor the susceptibility of aspergilli to azoles the true incidence is unknown. Nonetheless, resistance has now been reported from many countries in Europe, China, Canada and the USA, as well as particularly high frequencies from the Netherlands and the north-west of the UK.

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