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Deciphering *Aspergillus fumigatus cyp51A*-mediated triazole resistance by pyrosequencing of respiratory specimens

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Background: Infections caused by triazole drug-resistant *Aspergillus fumigatus* are an increasing problem. The sensitivity of standard culture is poor, abrogating susceptibility testing. Early detection of resistance can improve patient outcomes, yet tools for this purpose are limited.

Objectives: To develop and validate a pyrosequencing technique to detect resistance-conferring *cyp51A* polymorphisms from clinical respiratory specimens and *A. fumigatus* isolates.

Methods: Method validation was performed by Sanger sequencing and pyrosequencing of 50 *A. fumigatus* isolates with a spectrum of triazole susceptibility patterns. Then, 326 *Aspergillus* quantitative PCR (qPCR)-positive respiratory samples collected over a 27 month period (January 2017–March 2019) from 160 patients at the UK National Aspergillosis Centre were assessed by *cyp51A* pyrosequencing. The Sanger sequencing and pyrosequencing results were compared with those from high-volume culture and standard susceptibility testing.

Results: The *cyp51A* genotypes of the 50 isolates analysed by pyrosequencing and Sanger sequencing matched. Of the 326 *Aspergillus* qPCR-positive respiratory specimens, 71.2% were reported with no *A. fumigatus* growth. Of these, 56.9% (132/232) demonstrated a WT *cyp51A* genotype and 31.5% (73/232) a resistant genotype by pyrosequencing. Pyrosequencing identified the environmental TR34/L98H mutation in 18.7% (61/326) of the samples in contrast to 6.4% (21/326) pan-azole resistance detected by culture. Importantly, pyrosequencing detected resistance earlier than culture in 23.3% of specimens.

Conclusions: The pyrosequencing assay described could detect a wide range of *cyp51A* polymorphisms associated with triazole resistance, including those not identified by commercial assays. This method allowed prompt recognition of resistance and the selection of appropriate antifungal treatment when culture was negative.

Introduction

Aspergillus fumigatus is the most common filamentous fungus associated with acute and chronic pulmonary infections and fungal asthma.[1,2] The various forms of pulmonary aspergillosis affect many millions of people annually.[3,4] Acute invasive pulmonary aspergillosis (IA) is estimated to develop in 200000 patients annually and has a mortality rate of approximately 50%[5] if not diagnosed and treated promptly. In contrast to rapidly progressing IA, chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA) are insidious, progressive conditions leading to the gradual destruction of the airways.[1,6] The estimated global burden of CPA is over 200000 in Europe alone, with a 5 year mortality of 50%–85% despite treatment.[7,8] The estimated global burden of ABPA is higher (1062000 cases in Europe), but the mortality rate is lower.[9]

Triazole-class antifungal drugs are used as first-line treatment for pulmonary aspergillosis due to their good efficacy and tolerability. The broadly active, but generally less well-tolerated, amphotericin B is the second-line treatment choice.[10] However, the rising trend of aspergillosis caused by triazole-drug-resistant *A. fumigatus* provides an increasing challenge to the management of aspergillosis patients.[11–15] The current incidence of *A. fumigatus* triazole resistance is largely unknown due to continued reliance on

culture-based susceptibility testing and poor sensitivity of standard culture methods. The SCARE-network study demonstrated a prevalence of 3.2% across 19 countries.[16] Compounding the spectre of resistance is the paucity of antifungal agents available for clinical use.[7,17]

The development of triazole resistance is frequently associated with polymorphisms in the promoter region and ORF of the *A. fumigatus cyp51A* gene, which encodes lanosterol 14α-demethylase, the target of triazole drugs.[18] Azole exposure is the main driver for resistance, either in the patient during long-term triazole therapy or in the environment as a response to the agricultural use of azoles.[19] The former is associated with specific *cyp51A* point mutations, with codons 54, 138, 220 and 448 implicated predominantly.[20,21] Patients treated for CPA or ABPA with long courses (months to years) of triazole antifungals are particularly susceptible to this mechanism of resistance.[22,23] Acquisition of an azole-resistant environmental strain of *A. fumigatus*, associated with a tandem repeat (TR) within the promoter region (TR_{34}, TR_{46} and TR_{53}) combined with specific *cyp51A* point mutations, is more frequently seen in IA patients, but also in CPA and ABPA patients.[19,24,25]

Rapid detection of triazole resistance can provide critical guidance in early, effective treatment and reduces the mortality of aspergillosis.[26] Culture is still the mainstay of detecting resistance, although 85% of cultures of respiratory specimens from patients with aspergillosis are negative for *A. fumigatus*.[27–29] When culture is positive, susceptibility testing may only be available at reference centres, whereby the total turnaround time, from sampling to an MIC result, is typically 1–2 weeks.[30,31] Direct screening of the *cyp51A* region via molecular methods can overcome these issues and is becoming increasingly accessible,[32,33] with the availability of commercial quantitative PCR (qPCR) platforms, such as Pathonostics AsperGenius, Ademtech MycoGENIE and Bruker Fungiplex® Aspergillus Azole-R IVD. These assays combine detection of *A. fumigatus* and, if the signal strength is high enough, some *cyp51A* polymorphisms.[27,34–36] However, this confined scope of detectable mutations limits their ability to determine some key resistance mutations associated with long-term azole therapy. Sanger sequencing provides a well-established method for gene characterization, yet there are difficulties associated with insensitivity, laboratory workflow and therefore turnaround time.[37] A more accessible and efficient sequencing technology is required for this purpose.

We report the design and validation of a pyrosequencing method to determine resistance-conferring polymorphisms within the *cyp51A* region of *A. fumigatus*[38] from isolates and clinical respiratory samples. This assay was then used to screen respiratory samples from patients attending the UK National Aspergillosis Centre (NAC) to determine its utility to detect triazole resistance compared with high-volume culture (HVC)[29] and routine susceptibility testing.

Materials and methods

Study design and ethical considerations

A pyrosequencing method was developed to detect polymorphisms within the *cyp51A* region of *A. fumigatus*. This method was validated against Sanger sequencing using 50 *A. fumigatus* isolates obtained from the Mycology Reference Centre Manchester (MRCM) culture collection (Table 1). These isolates originated from respiratory samples collected from 24 patients with CPA or ABPA as part of their standard care at the NAC. A spectrum of isolates with different triazole resistance patterns were collected from patients who demonstrated signs of clinical failure despite good serum antifungal drug levels. Then, 326 *Aspergillus* qPCR-positive respiratory samples from 160 patients who attended the NAC over a 27 month period (January 2017–March 2019) were screened for *cyp51A* polymorphisms by pyrosequencing. The pyrosequencing results were compared with those from HVC and standard susceptibility testing to determine resistance patterns and assay utility. This study was assessed through the NHS Health Research Authority system (HRA)[39] and was found to meet the UK NHS definition of a retrospective service evaluation for which formal ethical review was therefore not required.

Isolates and susceptibility testing

Aspergillus isolates and reference strains (ATCC 46645 and AF293) were obtained from the MRCM depository. *A. fumigatus* species complex isolates with a spectrum of macro- and micro-morphological characteristics and susceptibility profiles were selected. The identification was confirmed by internal transcribed spacer (ITS), β-tubulin (bt2) and calmodulin (cam) Sanger sequencing or microsatellite typing[22] of 17/50 isolates (Table 1). Isolates were grown on Sabouraud chloramphenicol dextrose agar (Oxoid, Basingstoke, UK) at 37°C for 3 days. Spore suspensions were made in PBS with 0.5% Tween 20 (Sigma–Aldrich) for DNA extraction. Respiratory samples, comprising sputa, bronchial washes and bronchoalveolar lavages, were processed using HVC as described previously.[29] Susceptibilities were determined using the EUCAST broth microdilution method.[40] Routine isavuconazole susceptibility testing was initiated at the MRCM in July 2015 and therefore was not performed for the older isolates tested in this study.

Detection of Aspergillus DNA

DNA was extracted from fungal isolates using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems). Bead beating was performed with a MagNA Lyser instrument (Roche Life Sciences). Extraction of DNA from respiratory samples (minimum 1.0 mL) was performed using a prelysis step with the EXTRAblood Prelysis Kit (ELITechGroup); sputum, but not bronchoalveolar lavage, samples were liquefied prior to this step using dithiothreitol. Automated extraction following liquefaction was performed with the ELITe STAR instrument and ELITe STAR 200 Extraction Kit (ELITechGroup) according to the manufacturer's instructions. The presence of *Aspergillus* spp. was confirmed by qPCR using the *Aspergillus* spp. ELITe MGB Kit (ELITechGroup) on the 7500 Fast Dx Real-Time PCR instrument (Applied Biosystems) as per the manufacturer's instructions.

Design of the pyrosequencing assay

The pyrosequencing method was designed to encompass the 5′ untranslated region and codons of interest within the *cyp51A* locus and all polymorphisms known to date (Figure 1) using the published reference genomes of *A. fumigatus* strains AF293 and A1163 (CBS144.89).[41] The method consists of two steps: amplification of the four fragments (Figure 1a) using biotinylated primers (Table S1, available as Supplementary data at *JAC* Online) and pyrosequencing of each fragment separately (Figure 1b–j) using sense or anti-sense sequencing primers (Table S2).

Amplification

Amplification was performed on extracts with more than 460 copies of 18S ribosomal DNA (rDNA), confirmed by *Aspergillus* spp. ELITe MGB qPCR, using a Q-Sat 96 thermal cycler (Hain Lifescience, now part of Bruker Corporation) and the PyroMark PCR Kit (QIAGEN). The number of polymorphisms screened per fragment dictated the volume of master mix required, with a

Table 1. Comparative *cyp51A* sequencing and MIC values for *A. fumigatus* species complex clinical isolates

	cyp51A genotype		Isolate MIC (mg/L)				
Isolate	Sanger sequencing	pyrosequencing	ITC	AMB	VRC	POS	ISA
1	no polymorphisms	no polymorphisms	0.5	0.5	0.25	0.25	NA[a]
2	no polymorphisms	no polymorphisms	0.5	1	0.5	0.125	NA
3	no polymorphisms	no polymorphisms	0.25	0.5	1	0.125	NA
4	no polymorphisms	no polymorphisms	0.25	0.5	1	0.125	1
5	no polymorphisms	no polymorphisms	0.5	1	0.5	0.125	1
6	no polymorphisms	no polymorphisms	0.125	0.25	0.25	0.03	0.5
7	no polymorphisms	no polymorphisms	0.5	0.5	0.25	0.125	NA
8[b]	no polymorphisms	no polymorphisms	>8	0.25	2	1	>8
9	no polymorphisms	no polymorphisms	0.25	2	1	0.06	NA
10	D262Y[c]	no polymorphisms	0.25	0.5	0.5	0.06	NA
11	no polymorphisms	no polymorphisms	0.5	0.5	0.5	0.125	NA
12	G448S	G448S	>8	0.5	8	0.5	8
13	A284T[c]	no polymorphisms	>8	0.5	>8	>8	>8
14	TR34/L98H	TR34/L98H	>8	1	4	0.5	8
15[b]	no polymorphisms	no polymorphisms	>8	1	4	1	8
16	F219V	F219V	>8	0.5	0.5	1	NA
17	G54W	G54W	>8	0.5	1	>8	NA
18	G54W	G54W	>8	0.5	1	>8	NA
19	no polymorphisms	no polymorphisms	0.5	0.5	1	0.25	NA
20	P216H	P216H	>8	0.25	0.5	0.125	1
21[d]	G448S	G448S	>8	2	>8	>8	>8
22[b]	no polymorphisms	no polymorphisms	>8	0.5	0.5	1	NA
23	no polymorphisms	no polymorphisms	1	0.5	1	0.25	NA
24	M220T	M220T	>8	0.25	1	0.5	2
25[b,d]	no polymorphisms	no polymorphisms	>8	1	4	2	4
26	A284T[c]	no polymorphisms	>8	0.5	8	>8	>8
27[d]	no polymorphisms	no polymorphisms	0.5	1	0.25	0.125	NA
28	TR34/L98H	TR34/L98H	>8	1	2	0.5	8
29	TR34/L98H	TR34/L98H	>8	0.5	4	1	8
30	no polymorphisms	no polymorphisms	0.5	0.5	2	0.125	NA
31[e]	no polymorphisms	no polymorphisms	NA	NA	NA	NA	NA
32[e]	no polymorphisms	no polymorphisms	NA	NA	NA	NA	NA
33[f]	TR34/L98H	TR34/L98H	>8	0.5	>8	1	NA
34[f]	M220T	M220T	>8	1	0.5	0.25	NA
35[f]	M220K	M220K	>8	2	2	1	2
36[f]	G54E	G54E	>8	2	0.5	>8	0.5
37[f]	G54R	G54R	>8	1	0.25	2	NA
38[f]	G54R	G54R	>8	2	0.25	1	NA
39[f]	M220T	M220T	>8	1	2	0.5	2
40[f]	G54V	G54V	>8	0.5	0.5	1	NA
41[f]	TR34/L98H	TR34/L98H	>8	0.25	4	0.5	NA
42[f]	G54E	G54E	>8	1	0.25	1	NA
43	F219I	F219I	>8	0.5	0.5	1	2
44	TR34/L98H	TR34/L98H	>8	0.25	4	1	8
45	G448S	G448S	2	1	4	0.25	>8
46[d,g]	TR46/Y121F/T289A	TR46/Y121F/T289A	>8	1	>8	1	NA
47[d,g]	TR46/Y121F/T289A	TR46/Y121F/T289A	>8	1	>8	1	NA
48[b,d]	no polymorphisms	no polymorphisms	>8	0.5	8	2	>8
49	TR34/L98H	TR34/L98H	>8	0.5	4	1	8
50[d]	TR34/L98H	TR34/L98H	>8	1	4	0.5	4

ITC, itraconazole; AMB, amphotericin B; VRC, voriconazole; POS, posaconazole; ISA, isavuconazole.
[a]Susceptibility results are not available.
[b]Isolates that demonstrate no *cyp51A* polymorphisms, but demonstrate phenotypic triazole resistance.
[c]Pyrosequencing is not yet available for this position.
[d]Isolate confirmed as *A. fumigatus* by ITS, bt2 and cam Sanger sequencing.
[e]Reference *A. fumigatus* isolates ATCC 46645 and AF293, respectively.
[f]Sanger sequencing result from a previous publication; isolates confirmed as *A. fumigatus* by microsatellite typing;[22] also pyrosequenced in forward and reverse directions.
[g]Sanger sequencing result from a previous publication.[59]

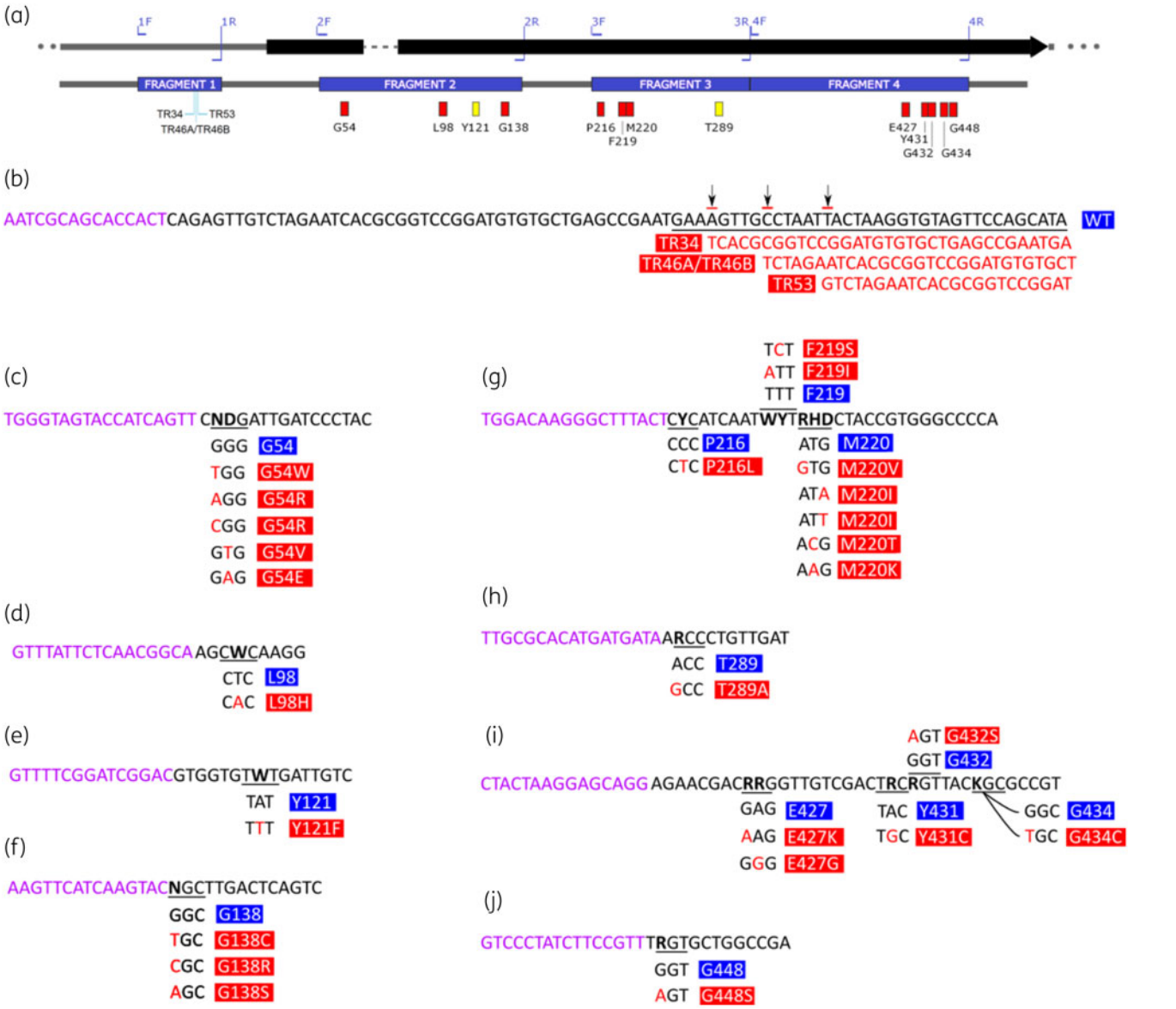

Figure 1. Overview of the *cyp51A* gene and the targeted regions and reads. (a) Diagram of *A. fumigatus cyp51A* demonstrating all the sites monitored by pyrosequencing. Upper diagram shows PCR priming sites. TR sites are shown beneath fragment 1. SNPs associated with resistance are indicated by red rectangles. SNPs associated with TR_{46}-mediated resistance are indicated by yellow rectangles. (b) Expected sense-direction pyrosequencing read for the TR region shown (black text) preceded by the pyrosequencing primer (purple text). TR insertion sites are indicated by arrows, with the expected alternative sequences aligned below (red text). (c to j) Expected sense-direction pyrosequencing reads for amino acid substitution 'hotspots' associated with triazole resistance. WT nucleotide sequences are indicated in black, polymorphism(s) are indicated in red and pyrosequencing primers are indicated in purple: (c) glycine 54 (G54); (d) leucine 98 (L98); (e) tyrosine 121 (Y121); (f) glycine 138 (G138); (g) phenylalanine 216 (P216) to methionine 220 (M220); (h) threonine 289 (T289); (i) aspartic acid 427 (E427) to glycine 434 (G434); and (j) glycine 448 (G448). Underlined text indicates the codon target of interest, with variations shown below. Bold or red text indicates a nucleotide position associated with resistance polymorphisms.

minimum of 25 μL required per assay. For each 25 μL reaction volume, 12.5 μL of PyroMark PCR Master Mix, 0.2 μM forward and reverse primer (one of the pair was biotinylated), 2.5 μL of CoralLoad dye, 5 μL of QIAGEN Q solution and 4 μL of template DNA were used. Thermal cycling was performed with a hot-start step of 95°C for 15 min, followed by a touch-down phase consisting of 10 cycles of denaturation at 95°C for 30 s, annealing between 58 and 56°C (0.2°C decrease with each cycle) for 30 s and elongation at 72°C for 45 s. A subsequent 40 cycles of 30 s at 95°C, 30 s at 56°C and 45 s at 72°C followed, before a 10 min extension step at 72°C. PCR products were analysed by 2% agarose gel electrophoresis.

Pyrosequencing

Amplicons were prepared for pyrosequencing on the PyroMark Q24 Advanced platform (QIAGEN) using PyroMark Q24 Advanced Reagents as per the manufacturer's instructions. Codons for glycine 54 (Gly-54), leucine

98 (Leu-98), tyrosine 121 (Tyr-121), proline 216 (Pro-216), phenylalanine 219 (Phe-219), methionine 220 (Met-220), threonine 289 (Thr-289) and the region comprising promoter-associated TRs were analysed for polymorphisms (Figure 1) from January 2017. Codons glycine 138 (Gly-138), aspartic acid 427 (Asp-427), tyrosine 431 (Tyr-431), glycine 432 (Gly-432), glycine 434 (Gly-434) and glycine 448 (Gly-448) were screened from mid-September 2018. Individual assays were designed using the QIAGEN Assay Design software (QIAGEN) to span each polymorphism by *de novo* sequencing (SEQ) or allele quantification (AQ) depending on the multiplicity of the polymorphisms. SEQ assays were used for positions where multiple nucleotide polymorphisms were possible within one codon, whereas semi-quantitative AQ assays were designed for codons with multiple alleles at a single base-pair position (Figure 1 and Table S2). Both sense and anti-sense primers were used for pyrosequencing of 10 isolates during the validation phase, with results compared against Sanger sequencing of the same region.

Sanger sequencing

DNA extracts from isolates and respiratory samples were prepared as above for Sanger sequencing, except that amplification was performed with HotStarTaq Plus Master Mix (QIAGEN) using overlapping sequencing primers (Table S1). Purification of amplicons was performed with the QIAGEN PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Sanger sequencing (of both sense and anti-sense strands) was performed by Eurofins Genomics Germany GmbH (Ebersberg, Germany).

Data analysis

The pyrosequencing and Sanger sequencing methods were assessed by comparing the amplification efficiency, which was calculated as the percentage of the actual amplicons produced divided by the total number of amplicons expected. Furthermore, the readability of both sequencing techniques was assessed by calculating the percentage number of reads yielding sequence data divided by the total number of reads. Pyrosequencing data (exemplified in Figure S1) was analysed with use of PyroMark Q24 Advanced software version 3.0.0 (QIAGEN) and compared against known sequences of strain A1163.[41] Sequence quality was checked with use of the software's quality grading system in addition to manual interpretation. Triazole susceptibility was inferred from the sequencing results by comparison with a reference table of *A. fumigatus cyp51A* polymorphisms.[42]

Results

Pyrosequencing validation

The pyrosequencing method was as effective as Sanger sequencing for producing readable sequences. A comparison of both sequencing methods revealed that the amplification efficiency was greater for Sanger sequencing than for pyrosequencing (99% versus 96%, respectively). Similarly, the readability of sequences was 99% effective for Sanger sequencing versus 94% for pyrosequencing.

The two sequencing methods produced matching *cyp51A* genotypes for all the isolates analysed (Table 1), with the exception of three isolates that harboured non-synonymous polymorphisms not currently screened by pyrosequencing: two isolates with alanine 284 to threonine (A284T) and one isolate with glutamic acid 262 to tyrosine (D262Y). A284T has been described to confer reduced susceptibility to itraconazole, voriconazole and posaconazole,[43] confirmed in this study along with isavuconazole resistance. D262Y is clinically insignificant,[44] also confirmed here.

When the genotypic profiles obtained by pyrosequencing were compared with phenotypic susceptibilities, 16 (32%) isolates had no detectable *cyp51A* polymorphisms associated with triazole resistance, which corresponded to their phenotypic susceptibility profiles (Table 1). However, five (10%) isolates, which had demonstrated triazole-resistant phenotypes, were found to have no *cyp51A* polymorphisms by either sequencing method. Twenty-seven (54%) isolates harboured a *cyp51A* genotype associated with resistance, confirming the phenotypic susceptibility patterns (Table 1). The remaining two isolates contained new polymorphisms, proline 216 to histidine (P216H) and phenylalanine 219 to valine (F219V), both of which coincided with itraconazole-resistant phenotypes. The most numerous polymorphisms were seen at codons glycine 54 (14%), methionine 220 (8%), leucine 98 (16%, combined with the TR_{34} insertion) and glycine 448 (6%). For 10 isolates, bi-directional pyrosequencing confirmed the reproducibility of forward and reverse reads (Table 1).

Screening human respiratory specimens

A total of 335 respiratory samples were analysed during the study period. Of these samples, 40 were selected randomly for method validation and showed consistent agreement between both sequencing techniques. Pyrosequencing was more sensitive than Sanger sequencing for detecting single, as well as mixed, polymorphisms and for producing complete resistance genotypes (Table S3). Moreover, Sanger sequencing failed to detect one instance each of TR34/L98H and glycine 54 to arginine (G54R) polymorphisms, which were detected by pyrosequencing. Conversely, Sanger sequencing detected one instance of methionine 220 to lysine (M220K) not detected by pyrosequencing. Nine of 335 samples (2.7%) were culture positive for other *Aspergillus* spp. (Table 2) and were excluded from further analysis as pyrosequencing is limited to *A. fumigatus* species complex (Figures S2 and S3). Notably, pyrosequencing results were obtained in eight of these specimens, including one case of a glycine 54 to tryptophan (G54W) polymorphism in a background of *Aspergillus niger* growth (Table S4), indicating that genotyping was possible when *A. fumigatus* growth was not detected.

Of all the respiratory specimens analysed (Tables S3 and S4), only 28.8% (94/326) reported *A. fumigatus* growth after 14 days despite the use of higher culture volumes (Table 2). Pyrosequencing demonstrated a WT *cyp51A* genotype in 40.5% (132/326) and resistant genotypes in 22.4% (73/326) of samples. In contrast, HVC showed WT phenotypic susceptibility in 12.6% (41/326) and resistance in 16.3% (53/326) of samples (Table 2). A wide range of genotypes were identified, including multiple resistant genotypes in five specimens (Tables 2, S3 and S4). Agreement between inferred resistance patterns deduced by pyrosequencing and susceptibility testing was only 56.4% (53/94). Intriguingly, 11 respiratory samples were found to have a pan-azole-resistant or resistant genotype, but demonstrated susceptible MICs (Tables S3 and S4), suggesting phenotypic testing missed the *cyp51A* resistance detected by pyrosequencing. In contrast, 23 samples demonstrated a resistant phenotype, which pyrosequencing failed to detect, either outright (11/23) or due to paucity of pyrosequencing results, because amplicons could not be generated for the whole *cyp51A* region (12/23). Nevertheless, resistance was demonstrated by pyrosequencing before HVC results were available in 23.3% (76/326) of cases (Table 2).

Table 2. Summary of the results of *cyp51A* pyrosequencing and susceptibility data obtained from respiratory specimens

Respiratory specimens[a]	*n*	%
Patients	160	
resistant genotypes in		
CPA/CCPA	47	
APBA/CPA	10	
ABPA	12	
Aspergillus bronchitis with/out fungal sensitization	4	
resistant phenotypes in		
CPA/CCPA	21	
APBA/CPA	5	
ABPA	6	
Aspergillus bronchitis with/out fungal sensitization	0	
Samples	335	
A. fumigatus species complex	326	
confirmed non-*A. fumigatus*	9	2.7
HVC results		
positive samples	94	28.8
negative samples	232	71.2
resistant *A fumigatus* (of positive cultures)	53	56.4
resistant *A fumigatus* (of all samples)		16.3
susceptible *A fumigatus* (of positive cultures)	41	43.6
susceptible *A fumigatus* (of all samples)		12.6
WT susceptibility inferred by pyrosequencing (of all samples)	132	40.5
Pan-azole resistance		
found by HVC, but not pyrosequencing	21	6.4
found by pyrosequencing, but not HVC	61	18.7
found by pyrosequencing or HVC	82	25.1
Resistance to at least one azole		
found by HVC, but not pyrosequencing	23	7.1
found by pyrosequencing, but not HVC	73	22.4
found by pyrosequencing or HVC	96	29.4
Resistance matched by culture and pyrosequencing (of HVC positives, *n* = 94)	25	26.6
Agreement of all phenotypes between culture and pyrosequencing	53	56.4
No susceptibility results by pyrosequencing or HVC	43	13.2
Resistance results obtained by pyrosequencing before HVC results	76	23.3
Polymorphisms found by pyrosequencing:		
G54E, R, V, W	28	8.6
TR34/L98H	59	18.1
M220I, K	4	1.2
P216L	2	0.6
F219I	2	0.6
WT	99	30.4
partial WT[b]	77	23.6
mixed[c]	5	1.5
Success rate with 460 copies (*n* = 326)[d]	275	84.4
Success rate with 1000 copies (*n* = 229)[d]	208	90.8

[a]Respiratory specimens consisted of 331 sputa, 3 bronchial washes and 1 bronchoalveolar lavage.
[b]Pyrosequencing results were obtained from at least one of four fragments, together representing 90% coverage of the *cyp51A* gene length.
[c]Polymorphisms found in the same sample: G54R and M220V, I from two CPA patients; M220V, K and L98H from two other CPA patients; and G54W and L98H in an ABPA patient.
[d]Pertains to the pyrosequencing amplification success rate when the minimum yield of *Aspergillus* spp. qPCR is 460 or 1000 18S rRNA copies, respectively.

The respiratory specimens analysed in this study came from 160 patients, of which 95 (59.4%) were diagnosed with CPA (including chronic cavitary pulmonary aspergillosis, CCPA), 28 (17.5%) were diagnosed with ABPA, 20 (12.5%) were diagnosed with both ABPA and CPA, 2 (1.3%) had sub-acute invasive aspergillosis, 7 (4.4%) had *Aspergillus* bronchitis, 4 (2.5%) had severe asthma with fungal sensitization, 2 (1.3%) had *Aspergillus* bronchitis with or without fungal sensitization and 2 (1.3%) had cystic fibrosis. In pyrosequenced samples, resistance was found most often in CPA patient specimens (Table 2), with 29.5% (28/95) demonstrating the pan-azole-resistant genotype and 14.5% (14/95) the glycine 54 polymorphisms, and secondly in ABPA patient specimens, with 28.6% (8/28) and 14.3% (4/28) demonstrating the pan-azole-resistant genotype and the glycine 54 polymorphisms, respectively. Resistance determined by culture was significantly lower in all patient groups (Tables 2, S3 and S4) and no resistance was found, by either pyrosequencing or culture, in patients with sub-acute invasive aspergillosis, severe asthma with fungal sensitization or cystic fibrosis. Mixed resistant genotypes determined by pyrosequencing were found in five patients: four with CPA and one with ABPA (Table 2); for none of these cases were there cultures available for susceptibility testing. Finally, concomitant presence of WT and resistant genotypes occurred in 33 samples, predominantly from CPA patients, with ABPA/CPA and APBA patient specimens demonstrating similar patterns in 9 and 8 specimens, respectively.

Discussion

The rising incidence of aspergillosis caused by triazole-resistant *A. fumigatus* is an alarming and significant global issue. Mortality and morbidity from both invasive and chronic forms of aspergillosis could be greatly reduced by rapid detection of triazole resistance and prompt revision of antifungal treatment.[1,3,6] As triazole resistance in *A. fumigatus* is frequently associated with polymorphisms in the *cyp51A* gene, molecular methods targeting this region offer a new approach to diagnostic antifungal stewardship. Screening for *cyp51A* genotype prevalence can also provide essential epidemiological data for local empirical guideline development.[26] The pyrosequencing assay described here was shown to be able to detect a wide range of *A. fumigatus cyp51A* polymorphisms associated with triazole resistance, including those not identified by commercial assays. This method allowed prompt recognition of resistance and the selection of appropriate antifungal treatment when culture was negative.

Pyrosequencing, unlike Sanger sequencing, was able to detect both susceptible and resistant genotypes when concomitantly present in a clinical sample. Polyclonal colonization is particularly common in patients with chronic or allergic conditions, but is seen in patients with invasive disease as well. In patients on antifungal therapy, this polyclonal colonization is likely to lead to dynamic ratios of the various *A. fumigatus* genotypes.[17,22,36,45] The PyroMark platform excels in detecting the characteristics of mixed populations when assays are designed appropriately.[46] In our study, where possible, AQ assays were used to allow the detection and semi-quantification of mixed templates (Figure S1), although

detection (but not quantification) is also possible using SEQ assays. The AQ assay could theoretically be used to track the advancement of a resistant *A. fumigatus* population when performed regularly on serial samples. In practice, the emergence of a resistant *cyp51A* genotype could be monitored before phenotypic resistance was detected, as evidenced in over 20% of the samples analysed. Consequently, the ability to detect subtleties in mixed samples could permit modification of patient treatment before a resistant phenotype became dominant within an infected patient. The advantage of this technique is the duality of its application. The presence of a resistant genotype(s) in an IA patient can be confirmed rapidly and direct urgent therapy. In contrast, pyrosequencing is ideal for long-term monitoring for CPA and ABPA patients whose mycobiomes may evolve more slowly and for whom, in particular, being able to monitor WT/resistant organisms is key for therapeutic success.

Pyrosequencing was significantly more effective than susceptibility testing for assessing the presence of triazole resistance, because of the low rate of culture positivity, even when using HVC. Yet, when culture was available, resistance was detected by phenotypic means more frequently than by pyrosequencing. This paradox may be due to the fact that selection of isolates for susceptibility testing is based on their typical morphological phenotype rather than on molecular confirmation of *A. fumigatus*. It is well known that isolates from patients with a long history of antifungal therapy and antifungal resistance are often phenotypically atypical and appear similar to cryptic species. Consequently, a closely related species, such as *Aspergillus fischeri* or *Aspergillus lentulus*, with some level of intrinsic resistance to triazoles, may have been reported as azole-resistant *A. fumigatus*,[47] but they would not yield any polymorphisms by pyrosequencing, because the resistance mechanism is not *cyp51A* based. Also, as pyrosequencing is specific to *A. fumigatus*, it would fail on cryptic species. Moreover, phenotypic resistance may have originated from efflux, overexpression of *cyp51B*,[48,49] uncharacterized *cyp51A* mutations or polymorphisms in gene(s) other than *cyp51A*,[50–52] none of which this pyrosequencing assay can identify.

The findings in our study highlight the significant flexibility of pyrosequencing compared with qPCR-based commercial assays. For example, pyrosequencing identified the A284T polymorphism[43] together with an undescribed P216H polymorphism associated with itraconazole resistance in pan-azole-resistant isolates recovered from a single patient. Other than the insertion and ORF polymorphisms associated with the environmental pan-azole resistance mutations, and recently evidence for glycine 54 and methionine 220 polymorphisms,[27] detection of other *cyp51A* polymorphisms is lacking in commercial assays. Moreover, pyrosequencing yields *cyp51* sequence data, which can distinguish other members of the species complex from *A. fumigatus*, which is not possible using qPCR assays. In addition, assays for detecting novel mechanisms can be incorporated into the Q24 Advanced PyroMark workflow with relative ease. For example, either the tyrosine 433 to asparagine (Y433N) in *cyp51A*[53] or the lysine 363 to arginine (K363R) *erg5* polymorphism[52] may explain the pan-triazole and amphotericin B resistance found in the isolates in this study lacking *cyp51A* polymorphisms. The advantage of pyrosequencing over newer sequencing approaches is the determination of definitive sequence information rather than a reliance on the restriction pattern differences generated by surveyor nucleases and detected by gel electrophoresis.[54]

The main limitation of this method is the challenge of amplifying low numbers of the single-copy *cyp51A* gene, as evidenced by an 84% amplification success rate from respiratory samples and 96% when using isolates. In addition, fragment amplification efficiencies varied; for example, fragment 3 (which contains hotspot amino acids proline 216 to methionine 220) is the most successful, while the promoter region (fragment 1) proved the most difficult to amplify, regardless of the iterations of primer sequences assayed during method development. These differences could be explained by varying amplification efficiencies or primer complementarity, amplification insensitivity, PCR inhibition and inferior DNA quality due to the lengthy extraction process required for respiratory samples[55] compared with fungal cultures.[29] As a consequence of these limitations, we determined that there should be greater than 1000 *Aspergillus* spp. rDNA copies in a respiratory sample to improve downstream pyrosequencing. Considering the 18S rDNA:single gene ratio in *A. fumigatus* varies from 38:1 to 91:1,[56] this cut-off translates to a limit of detection of 11–26 genomes, in line with probe-based qPCR resistance assays.[34,57,58] Consequently, a 1000 copy cut-off improved the pyrosequencing success rate from 84.4% to 90.8%. Although use of an *A. fumigatus*-specific probe to further discriminate non-*Aspergillus* templates would not completely rule out those respiratory specimens containing templates of members of *A. fumigatus* species complex (see Figure S2), it would reduce testing of samples containing other *Aspergillus* species (such as *Aspergillus flavus* and *A. niger*; Figure S3) and improve the success rate of the method even further.

In our centre, *cyp51A* findings via this method are routinely discussed with clinicians and compared against other laboratory findings to inform decisions on revision of antifungal treatment. A significant level of resistance was not unexpected in this group of patients, consisting mainly of ABPA and CPA patients, who are known to acquire *A. fumigatus cyp51A* polymorphisms over the course of long-term triazole therapy. Our *cyp51A* pyrosequencing screening approach is an effective means of deciphering resistance mechanisms from a range of clinically relevant samples containing *A. fumigatus* and is particularly effective when phenotypic testing is not possible. This technique is easily adaptable, requires low computing power and can be implemented easily within a molecular-based clinical laboratory workflow.

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Transparency declarations

L.N.-F. has received travel grants from and has been paid for talks by Gilead Sciences. C.B.M. has received grant support from the Fungal Research Trust and Pfizer, has received travel grants from Astellas and Gilead, and has been paid for lectures on behalf of Pfizer. M.D.R. has received grant support from Gilead Sciences, Pfizer and MSD, acts as a consultant and speaker for Gilead Sciences and Pfizer, is co-founder of Richardson Bio-Tech (Guangzhou) Limited and is a long-standing member of the European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines, Mucormycosis and Rare Moulds writing group. D.W.D. holds founder shares in F2G Ltd, a University of Manchester spin-out antifungal discovery company, acts or has recently acted as a consultant to Scynexis, Pulmatrix, Pulmocide, Zambon, iCo Therapeutics, Roivant, Mayne Pharma, Biosergen and Fujifilm, has been paid for talks on behalf of Hikma, Gilead, Mylan and Pfizer (in the last 3 years), was co-applicant of the original University of South Manchester NHS Foundation Trust/NHS England business case that secured funding for this project and is a long-standing member of the Infectious Diseases Society of America Aspergillosis Guidelines group, the European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines group and the British Society for Medical Mycology Standards of Care committee. R.R.-R. has been paid for lectures on behalf of Astellas, Gilead, Pfizer, MSD and Basilea. All other authors: none to declare.

Author contributions

L.N.-F. conceived the project. L.N.-F., S.P.A.-H. and D.H. designed the validation study. S.P.A.-H., D.H. and R.M. performed the experiments. L.N.-F. compiled the audit data. L.N.-F., S.P.A.-H. and D.H. analysed the results. S.P.A.-H. and L.N.-F. co-wrote the manuscript. C.B.M., M.D.R., R.R.-R. and D.W.D. gave clinical and conceptual advice and contributed to writing the paper.

Supplementary data

Tables S1 to S4 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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