Antifungal resistance in *Aspergillus fumigatus*

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Content

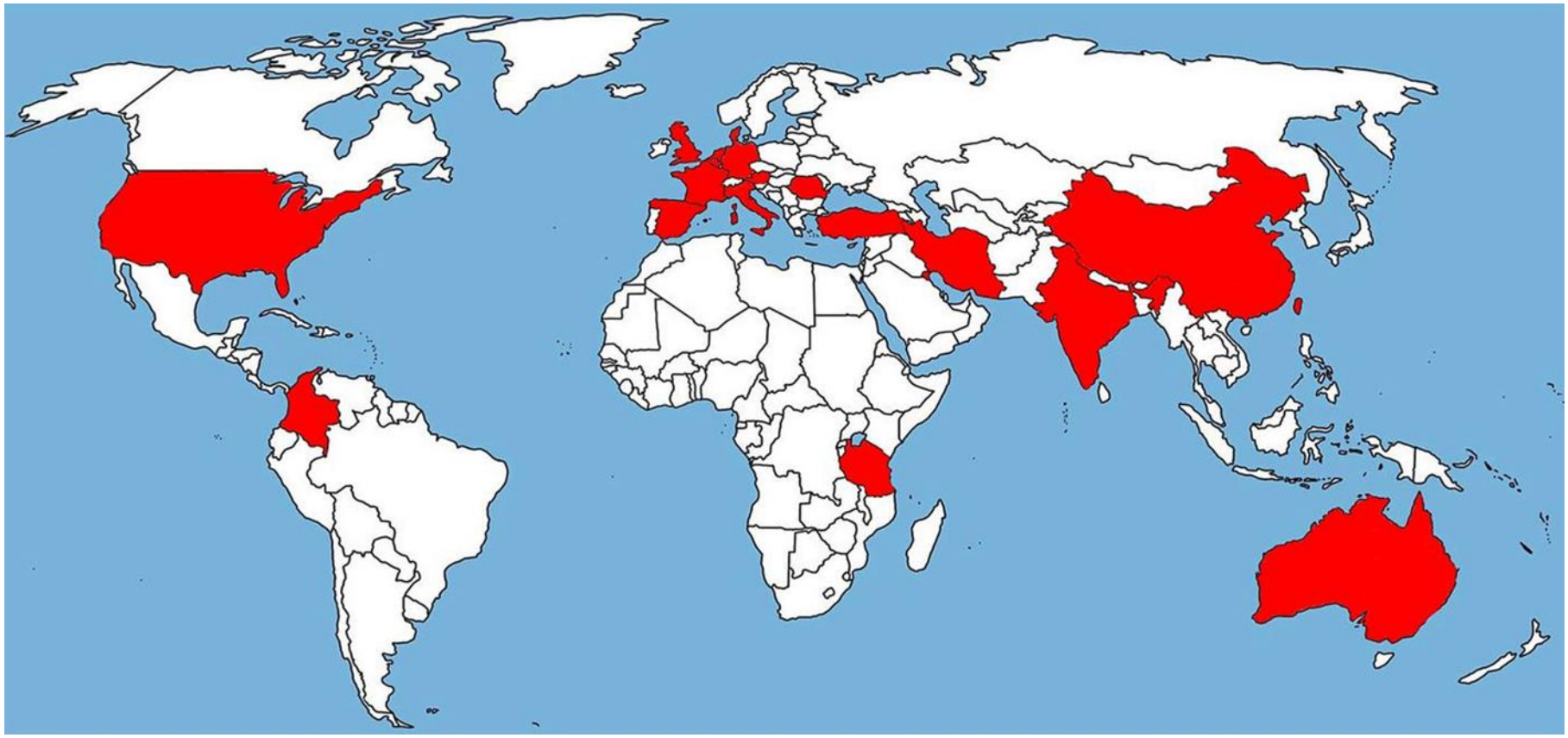
- Antifungal drugs, use and activity
- Global resistance problem
- How triazole resistance develops
- Resistance mechanisms
- Scale of the problem in the UK
- Different methods for monitoring resistance
- How to monitor resistance using pyrosequencing
- How this test may improve patient care
- Future developments

Antifungal drugs for *Aspergillus*

Class	Drug	Route of administration	Indication with respect to *Aspergillus* diseases
Triazole	Itraconazole	Intravenous/oral	Treatment of chronic *Aspergillus* diseases Salvage therapy
	Voriconazole	Intravenous/oral	Primary therapy of invasive aspergillosis (IA) Salvage therapy
	Posaconazole	Oral	Prophylaxis of invasive fungal disease Salvage therapy
	Isavuconazole	Intravenous/oral	
Polyene	Lipid formulations of amphotericin b	Intravenous	Primary therapy of invasive aspergillosis (IA) as an alternative choice for voriconazole Salvage therapy
Echinocandin	Caspofungin	Intravenous	Prophylaxis of refractory invasive fungal disease Salvage therapy
	Anidulafungin	Intravenous	
	Micafungin	Intravenous	

Modified from Kleinkauf et al 2013. European Centre for Disease Prevention and Control. Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species. Stockholm: ECDC
Walsh et al 2008. Treatment of Aspergillosis: Clinical Practice Guidelines of the Infectious Diseases Society of America. Clinical Infectious Diseases 46:327–60
Denning et al 2015. Chronic pulmonary aspergillosis: rationale and clinical guidelines for diagnosis and management. Eur. Resp. J. 47(1): 45-68
Morris & Villmann 2006. Echinocandins in the Management of Invasive Fungal Infections. Am J Health Syst Pharm 63(18):1693-1703
Mikulska & Viscoli 2011. Current Role of Echinocandins in the Management of Invasive Aspergillosis. C. Curr Infect Dis Rep (2011) 13: 517

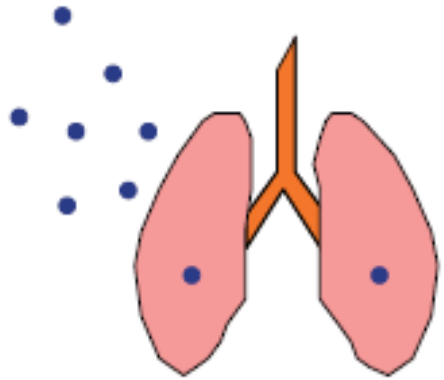
Resistance – a global problem



Current global prevalence of azole resistance is estimated at between 0.3 and 28%

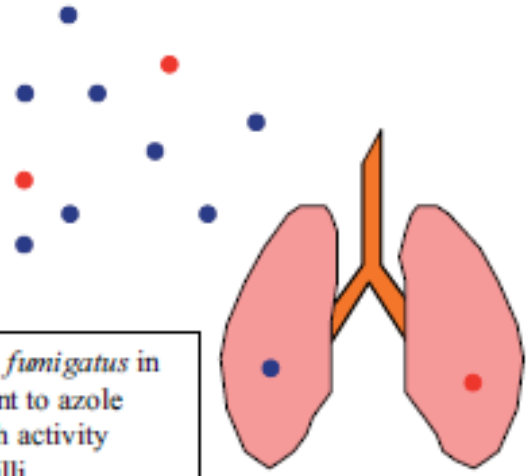
Reviewed in Hayes & Novak Frazer 2016. Chronic Pulmonary Aspergillosis—Where Are We? and Where Are We Going? Journal of Fungi 2(2): 18
Verweij et al 2015. Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles? Clin Infect Dis. 62(3): 362-368
Rivero-Menendez et al 2016. Triazole resistance in *Aspergillus*: a worldwide problem? Journal of Fungi 2(3): 21

Development of resistance



Patient route

Characteristics
- Long term azole therapy
- Mainly chronic cavitary pulmonary aspergillosis
- Point mutations in the Cyp51A-gene or unknown resistance mechanisms
- Multiple resistance mechanisms may be found in different colonies from a single specimen

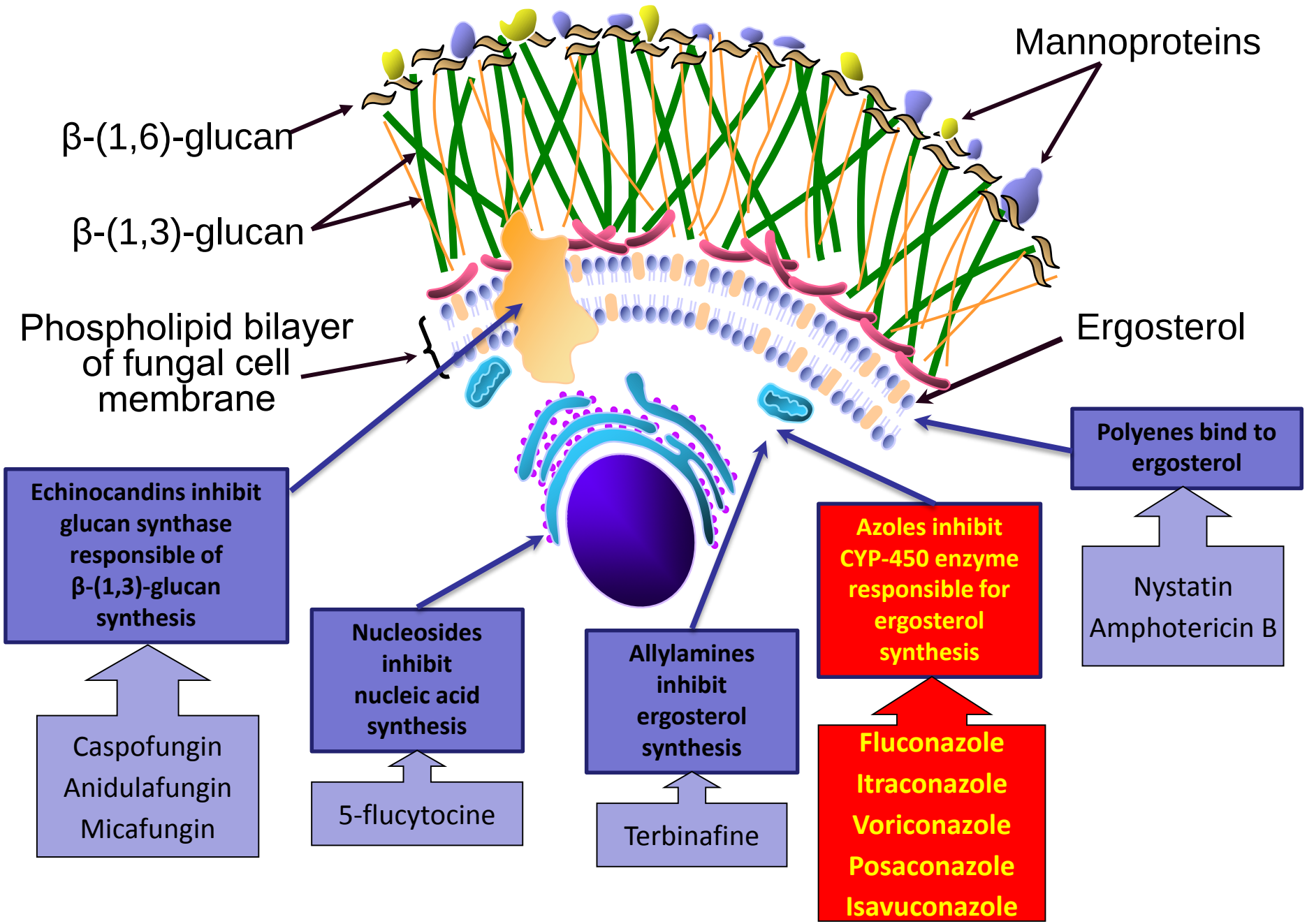


Environmental route

Characteristics
- Majority of patients are azole naïve
- Patients with invasive aspergillosis and chronic aspergillus diseases
- Only a few resistance mechanisms described
- Resistance mechanisms consist of Cyp51A-substitution with transcriptional enhancer

Exposure of *A. fumigatus* in the environment to azole fungicides with activity against aspergilli

Verweij et al 2015. Drug Resistance Updates 21-22: 30-40
Kleinkauf et al 2013. European Centre for Disease Prevention and Control. Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in Aspergillus species. Stockholm: ECDC



Courtesy Prof MD Richardson

Mechanisms of triazole resistance

Resistance mechanisms in *Aspergillus fumigatus*:

- Target enzyme of tri-azoles: lanosterol 14α-demethylase, ***cyp51A***
- Target pathway: ergosterol biosynthesis, resulting in ergosterol depletion and accumulation of toxic sterols
- Mutations in target gene result in decreased drug binding and effectiveness

Other mechanisms:

- Overexpression of efflux pumps which clear the drug
- Mutations in gene transcription (e.g. *Hap*, Aft1) leading to overexpression of *cyp51A*

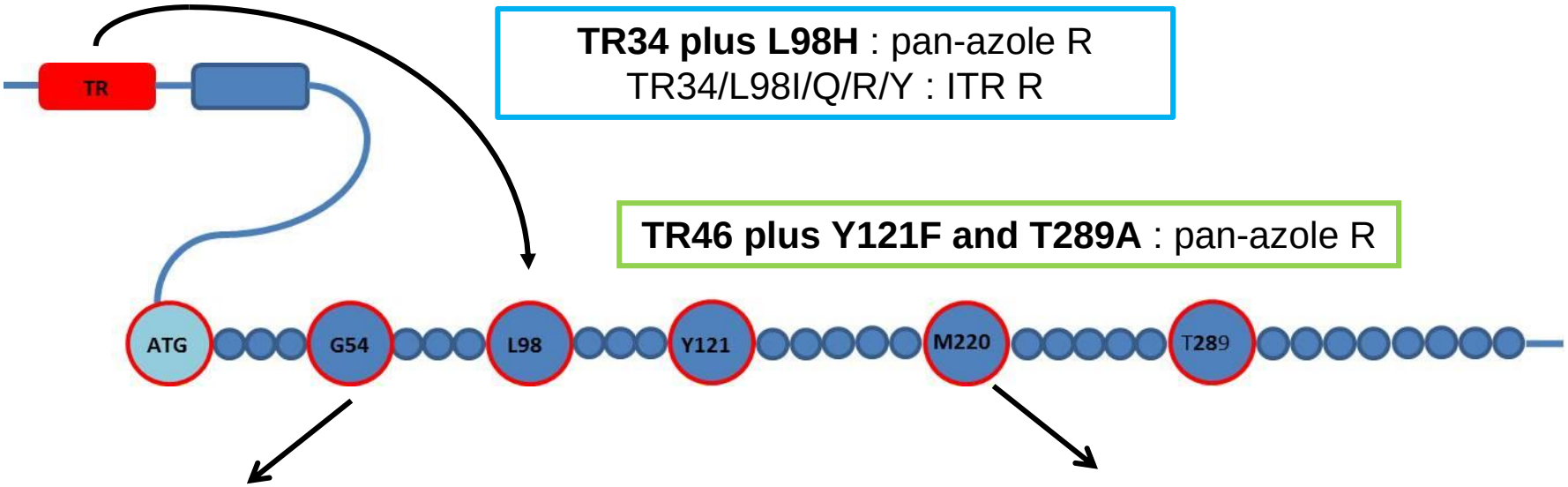
- Unknown…

Reviewed in Stensvold et al 2012. Azole-Resistant Invasive Aspergillosis: Relationship to Agriculture. Current Fungal Infection Reports 6(3):178–191

Resistance markers in *cyp51A*

ITR = itraconazole
VOR = voriconazole
PSC = posaconazole

Aspergillus fumigatus cyp51A



TR34 plus L98H : pan-azole R
TR34/L98I/Q/R/Y : ITR R

TR46 plus Y121F and T289A : pan-azole R

G54E/K : ITR R
G54R/V/W : ITR R and PSC R

M220I/V : ITR R
M220R/K/T/W : ITR R and PSC R

Modified from Garcia-Effron *et al*. 2008. Rapid Detection of Triazole Antifungal Resistance in *Aspergillus fumigatus*. Journal of Clinical Microbiology 46(4):1200-1206
See Stensvold et al 2012. Azole-Resistant Invasive Aspergillosis: Relationship to Agriculture. Current Fungal Infection Reports 6(3):178–191
and Lelièvre et al 2013. Azole resistant *Aspergillus fumigatus*: An emerging problem. Médecine et Maladies Infectieuses 43:139–145

cyp51A mutations in Europe

Acquired resistance mechanisms from each country in *cyp51A* gene in 47 *Aspergillus fumigatus* isolates with an azole-resistant phenotype				
Country	**No. azole-resistant isolates, n = 47**	**TR_{34}/L98H or TR_{46}/Y121F/T289A mechanism (no. isolates)**	**Other mutations (no. isolates)**	**No. isolates without *cyp51A* -mutations**
Austria	2	TR_{34}/L98H (2)	0	0
Belgium	8	TR_{34}/L98H (7)	F46Y/M172G (1)	0
Denmark	6	TR_{34}/L98H (4)	0	2
France	4	TR_{34}/L98H (1)	G54W (1)	2
Italy	5	TR_{34}/L98H (5)	0	0
The Netherlands	7	TR_{34}/L98H (4), TR_{46}/Y121F/T289A (3)	0	0
Spain	1	No isolates	0	1
Sweden	1	No isolates	F46Y/M172G	0
United Kingdom	13	No isolates	P381R/D481E, L329V, M220K, L77V/L399I/D481E, M220I (3), M220R, G54R, G54E, G54W	2
Resistant isolates, %	100	55.3	29.8	14.9

Prospective multicentre international surveillance study in which a total of 3,788 *Aspergillus* isolates were screened in 22 centres from 19 countries.

Prevalence of 3.2% azole-resistance in *A. fumigatus* isolates in a period of 8 months to 1 year.

van der Linden et al 2015. Prospective Multicenter International Surveillance of Azole Resistance in Aspergillus fumigatus. Emerg Infect Dis. 21(6): 1041–1044

Patient case 1

- Male, early forties, admitted to Burns Centre in April 2016 following self-inflicted burns (44% total body surface area)
- Works in UK marble plant, resizing imported marble from Spain and Italy
- Last travel to Spain was ~3 months prior, no history of prior azole use
- Prior to day 47, 12 respiratory samples: all negative for fungi
- Day 47 – *Aspergillus fumigatus* isolated from non-directed BAL – resistant to itraconazole, voriconazole, posaconazole and isavuconazole; also on days 53, 57, 69 and 74
- Isolates from days 47 and 57: sequencing revealed a TR46 repeat insertion, and also mutations Y121F and T289A
- All *A. fumigatus* isolates from air samples were susceptible to all azoles

- **The first case of a pan-azole resistant *A. fumigatus cyp51A* TR46/Y121F/T289A mutant in the UK**

The National Aspergillosis Centre

- 457 referrals, 111 new cases in 2015/16
- Chronic Pulmonary Aspergillosis (CPA) patients, ~10-15% annual mortality
- 346 additional referrals in 2015/16:

  Allergic Bronchopulmonary Aspergillosis (ABPA)

  Severe Asthma with Fungal Sensitisation (SAFS)

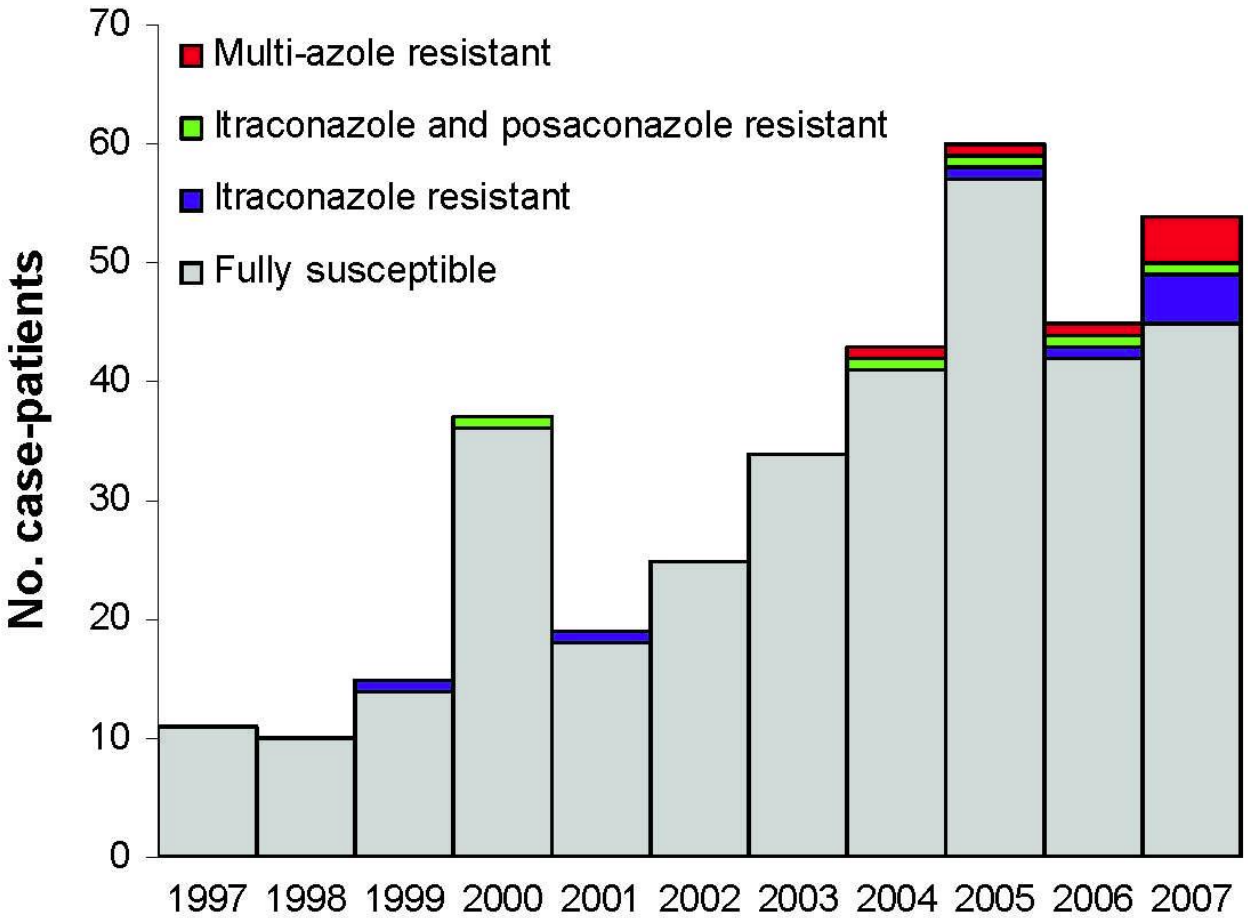
  Cystic Fibrosis (CF)

  Fewer cases of invasive aspergillosis (IA)

  Rhinosinusitis and *Aspergillus* bronchitis
- *Globally: 100,000 IA, 3 million CPA, 7.5 million allergic*

The National Aspergillosis Centre Annual Report 2015-2016
http://www.life-worldwide.org/assets/files/get_the_facts.pdf
Denning & Perlin 2011. Azole resistance in *Aspergillus*: a growing public health menace. Future Microbiology 6:1229–1232

Scale of the problem in the UK*



13	**16**	**24**	**47**	**83**	**61**	**Number of patients monitored**

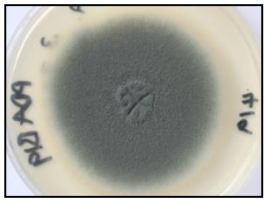
Since 2007: patients monitored
2009 – 112
2011 – 136
2013 – 218

Resistance levels stabilised to 18-20%
Increased voriconazole and itraconazole resistance in 2012-13

*National Aspergillosis Centre
Howard et al 2009. Frequency and Evolution of Azole Resistance in *Aspergillus fumigatus* Associated with Treatment Failure. Emerging Infectious Diseases 15:1068-1076

Monitoring infection & resistance

Sputum
BAL
blood



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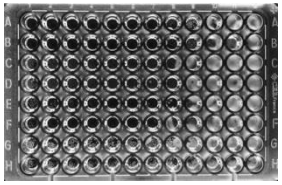
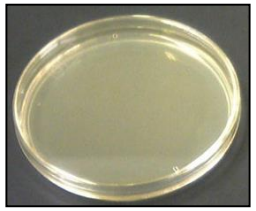


EUCAST: Antifungal drug susceptibility testing plate
All 4 azoles
Amphotericin b
Micafungin

Susceptibility results: S, I, R
Guidance for clinicians

Patient responds to recommended treatment

Pro-active therapeutic drug monitoring



No growth ?
Typically only 2.1 patients of 10 000 admissions grow *Aspergillus fumigatus*
HVC: 50-70% culture negative

* High volume culture

Bouza et al 2005. Workload due to *Aspergillus fumigatus* and significance of the organism in the microbiology laboratory of a general hospital. J Clin Microbiol 43: 2075–9
Fraczek et al 2014. Volume dependency for culture of fungi from respiratory secretions and increased sensitivity of *Aspergillus* quantitative PCR. Mycoses 57: 69–78

Monitoring infection & resistance

Sputum
BAL
blood

DNA extraction
4-6 h

ASPERGILLUS qPCR
(quantitative PCR, 2 h)

PCR negative – other cause?

PCR positive
Patient responds to recommended treatment

PCR positive >> **Patient does not respond**

Pro-active therapeutic drug monitoring

Monitoring resistance: the demand*

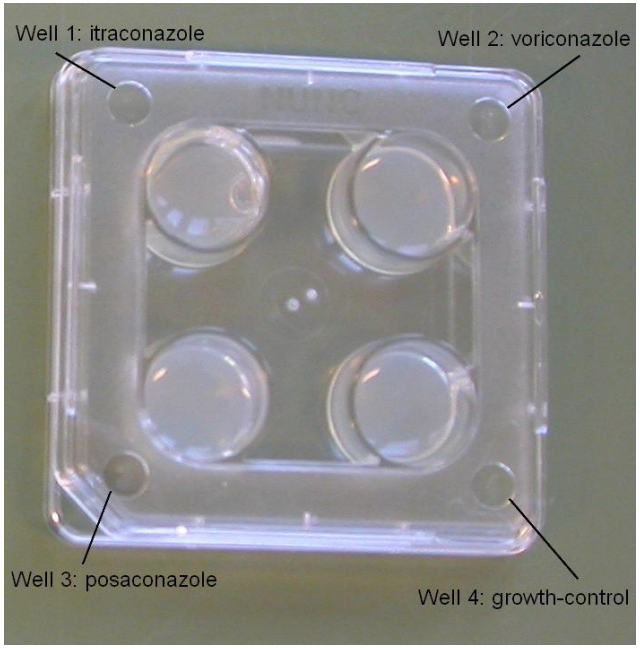
Monthly:

- 80-90 positive cultures - susceptibility testing
- 250-300 respiratory samples are culture negative > processed by qPCR
- A quarter of PCR samples (60-75) test positive for *Aspergillus* spp.
- Aim: minimum of 600 samples per annum monitored for resistance (two thirds)
- Future: process all new patients at diagnosis

*National Aspergillosis Centre

Monitoring resistance

- 2nd Duden Conference/1st ISHAM/ECMM *Aspergillus* Resistance Surveillance working group meeting, 20-21 January 2017, Berg en Dal, Nederland
- VIPcheck™ azole resistance detection
- Pathonostica AsperGenius® PCR
- In-house qPCRs
- Sanger sequencing



Monitoring resistance by pyrosequencing

- Discovered in 1990s, up to 150 base pyrosequencing
- DNA extract directly from patient sample, polymerase chain reaction (PCR) (6h)
- PCR and pyrosequencing, time to result: 6h
- Determination of mutations in *cyp51A* associated with azole resistance:

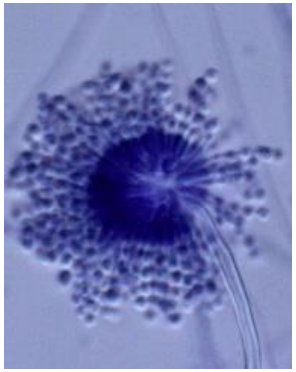
TR34/L98H, TR46/Y121/T289, G54, M220

Funding: January 2016

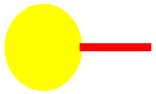
Dedicated personnel: July 2016

First patient samples processed: December 2016

Pyrosequencing in a nutshell (1)



First polymerase chain reaction (PCR):
Amplify *Aspergillus fumigatus cyp51A* gene from the patient sample.



ATCGATGAAGGGTTCAT**GTG**CATGCTAGATATC

EXAMPLE: Section of the *cyp51A* gene containing the **Met220** amino acid sequence

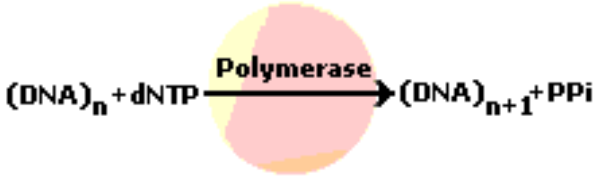
Second PCR: Amplify short sections of the *cyp51A* gene with biotinylated primers. This enables purification of the single strands of DNA of interest.

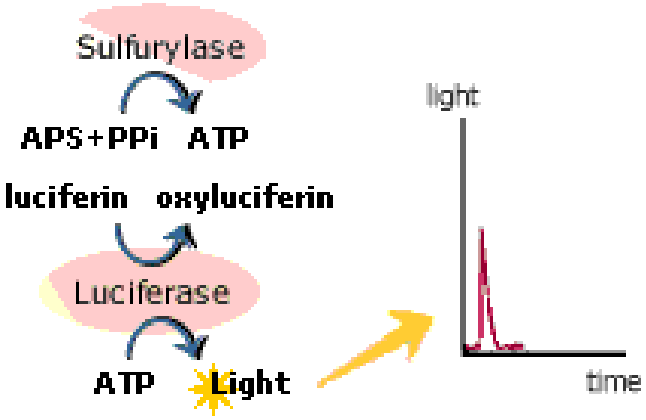
Pyrosequencing

Pyrosequencing in a nutshell (2)

All components included to make an exact copy of the patient *cyp51A* sequence.

Components are added one at a time and in a known sequence so that they can be monitored and checked by the software.



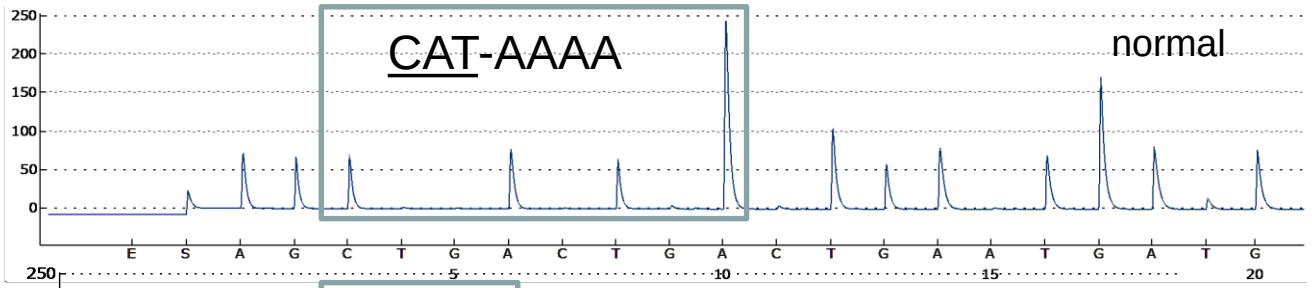


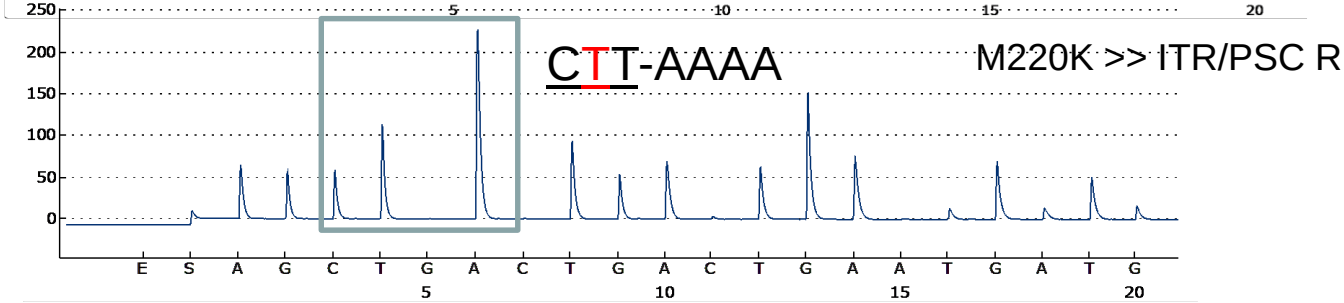
nucleotide incorporation generates light seen as a peak in the pyrogram

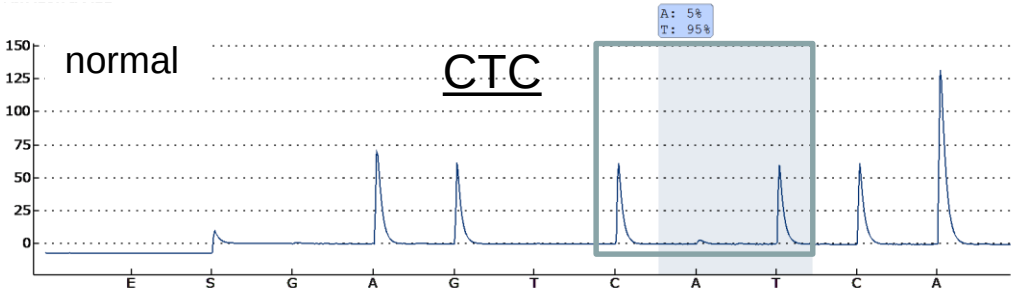
Software then compares the new, patient pyrosequence to the normal *cyp51A* sequence: mismatches (or mutations) can be identified by sequence comparison.

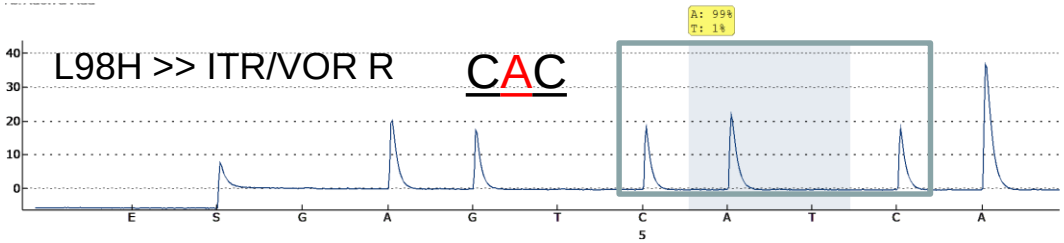
Courtesy http://genoseq.ucla.edu

Pyrosequencing output









Can we improve patient care?

- Assess whether therapy failure is associated with a *cyp51A* mutation
- Predict whether a patient may fail therapy by detecting a *cyp51A* mutation
- Alternative azole therapy

  e.g. G54R/V/W ITR R / PSC R

  e.g. M220I/V ITR R
- Alternative therapy if tri-azole resistance is detected

  e.g. M220R/K ITR/PSC R, VOR elevated MICs
- Combination therapies?
- Surgery

Patient case 2

- Female, mid sixties, first diagnosed with CPA in 2010, prescribed itraconazole in August
- TDM demonstrates maintenance of high serum itraconazole levels
- Switched to voriconazole in 2012
- Susceptibility testing of *A. fumigatus* isolates reveals:
  - April 2012: resistant to itraconazole and voriconazole, susceptible to posaconazole
  - June 2012: resistant to itraconazole, voriconazole, posaconazole intermediate
  - August 2012: resistant to itraconazole, voriconazole, and posaconazole
- Continued sampling: no growth in culture but PCR positive, *Aspergillus fumigatus species complex* confirmed by sequencing

- Surgery suggested: left upper lobectomy in February 2014, full recovery, no symptoms
- Fungal cultures are negative, GM negative, PCRs negative
- Discharged from service in March 2015

Future Prospects: resistance monitoring

Other resistance mechanisms in *Aspergillus*:

- *Increased expression resulting in decreased cytosolic drug levels and stress response proteins*
  - *Efflux pumps (MDR1 or CDR1/2)*
  - *ATP-binding cassette transporters*
  - *Other regulatory elements, e.g. SrbA*

- Other pyrosequencing targets: expression changes leading to resistance but via nucleotide substitutions

  HapE (P88L), transcription factor complex subunit

  Presence of Aft1 transposon (inserted 370 bp upstream of the *cyp51A* start codon)
- Resistance to other antifungal drugs, in other fungi/yeasts, bacteria/antibiotics

Camps et al. 2012. Discovery of a hapE mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing. PLoS ONE 7(11): e50034
Hey et al 2008. Characterisation of Aft1 a Fot1/Pogo type transposon of *Aspergillus fumigatus*. Fungal Genetics and Biology 45:117–126
Zoll et al. 2016. Next-Generation Sequencing in the Mycology Lab. Current Fungal Infection Reports 10:37-42

Summary

- Antifungal resistance is on the rise in the UK and globally
- Early and pro-active monitoring of triazole resistance can improve:
  - Patient outcome > the right drug, right time
  - Patient well-being and experience
  - Antifungal stewardship
- Save costs

Acknowledgements

- Staff in the:
- Mycology Reference Centre Manchester
- National Aspergillosis Centre
- Profs Denning and Richardson
- NHS England

UHSM Your Hospital

Mycology Reference Centre Manchester

ECMM CEMM European Confederation of Medical Mycology Confédération Européenne de Mycologie Médicale

National Aspergillosis Centre

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