What’s new in antifungal susceptibility testing? Molecular detection of antifungal drug resistance

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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*

<table>
<thead>
<tr>
<th>Class</th>
<th>Drug</th>
<th>Route of administration</th>
<th>Indication with respect to <em>Aspergillus</em> diseases</th>
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<tbody>
<tr>
<td>Triazole</td>
<td>Itraconazole</td>
<td>Intravenous/oral</td>
<td>Treatment of chronic <em>Aspergillus</em> diseases&lt;br&gt;Salvage therapy</td>
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<td>Voriconazole</td>
<td>Intravenous/oral</td>
<td>Primary therapy of invasive aspergillosis (IA)&lt;br&gt;Salvage therapy</td>
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<td>Posaconazole</td>
<td>Oral</td>
<td>Prophylaxis of invasive fungal disease&lt;br&gt;Salvage therapy</td>
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<td>Isavuconazole</td>
<td>Intravenous/oral</td>
<td>Salvage therapy</td>
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<tr>
<td>Polyene</td>
<td>Lipid formulations of amphotericin b</td>
<td>Intravenous</td>
<td>Primary therapy of invasive aspergillosis (IA)&lt;br&gt;as an alternative choice for voriconazole&lt;br&gt;Salvage therapy</td>
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<tr>
<td>Echinocandin</td>
<td>Caspofungin</td>
<td>Intravenous</td>
<td>Prophylaxis of refractory invasive fungal disease&lt;br&gt;Salvage therapy</td>
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<tr>
<td></td>
<td>Anidulafungin</td>
<td>Intravenous</td>
<td></td>
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<tr>
<td></td>
<td>Micafungin</td>
<td>Intravenous</td>
<td></td>
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Current susceptibility testing methods

• EUCAST
• CLSI
• ETEST
• Sensititre One
• Vitek 2
Etest - isavuconazole

Wild type *Aspergillus fumigatus*

*Aspergillus fumigatus* Cyp51 TR46/Y121F/T289A
Resistance – a global problem

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

Echinocandins inhibit glucan synthase responsible of $\beta$-(1,3)-glucan synthesis

Nucleosides inhibit nucleic acid synthesis

Allylamines inhibit ergosterol synthesis

Azoles inhibit CYP-450 enzyme responsible for ergosterol synthesis

Polyenes bind to ergosterol

Nystatin
Amphotericin B

Mannoproteins

Phospholipid bilayer of fungal cell membrane

Ergosterol

$\beta$-(1,6)-glucan

$\beta$-(1,3)-glucan

Terbinafine

Fluconazole
Itraconazole
Voriconazole
Posaconazole
Isavuconazole

5-flucytocine

Caspofungin
Anidulafungin
Micafungin

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…

Resistance markers in *cyp51A*

Modifications in *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

ITR = itraconazole
VOR = voriconazole
PSC = posaconazole


cyp51A mutations in Europe

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.


<table>
<thead>
<tr>
<th>Country</th>
<th>No. azole-resistant isolates, n = 47</th>
<th>TR34/L98H or TR46/Y121F/T289A mechanism (no. isolates)</th>
<th>Other mutations (no. isolates)</th>
<th>No. isolates without <em>cyp51A</em>-mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>2</td>
<td>TR34/L98H (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Belgium</td>
<td>8</td>
<td>TR34/L98H (7)</td>
<td>F46Y/M172G (1)</td>
<td>0</td>
</tr>
<tr>
<td>Denmark</td>
<td>6</td>
<td>TR34/L98H (4)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>France</td>
<td>4</td>
<td>TR34/L98H (1)</td>
<td>G54W (1)</td>
<td>2</td>
</tr>
<tr>
<td>Italy</td>
<td>5</td>
<td>TR34/L98H (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>7</td>
<td>TR34/L98H (4), TR46/Y121F/T289A (3)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Spain</td>
<td>1</td>
<td>No isolates</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sweden</td>
<td>1</td>
<td>No isolates</td>
<td>F46Y/M172G</td>
<td>0</td>
</tr>
</tbody>
</table>

Resistant isolates, %

|                      | 100 | 55.3 | 29.8 | 14.9 |

Acquired resistance mechanisms from each country in *cyp51A* gene in 47 *Aspergillus fumigatus* isolates with an azole-resistant phenotype
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:
  – >100 new cases annually
  – ~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
Antifungal resistance

• Intrinsic resistance (eg. *Aspergillus calidoustus*)
• Resistance that develops during treatment (azoles and low levels/long-term therapy) **Patient route**
• Infection caused by resistant isolates from the environment (agricultural use of azole fungicides) **Environmental route**
• Transient resistance caused by polyploidy (*Candida* and *Cryptococcus* during azole therapy)
Antifungal resistance – The Manchester experience

A

- Multi-azole resistant
- Itraconazole & posaconazole resistant
- Itraconazole & voriconazole resistant
- Voriconazole resistant
- Itraconazole resistant
- Fully susceptible

B

Monitoring infection & resistance

Sputum BAL blood

High volume culture

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

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

Patient responds to recommended treatment

Pro-active therapeutic drug monitoring

Susceptibility results: S, I, R
Guidance for clinicians

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 sensitivity/resistance: the demand

Monthly:

- 100 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
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.

**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.

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ATCGATGAAGGGTTCATGTGCATGCTAGATATC

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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.

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

- CAT-AAAA
- CTT-AAAA
- L98H >> ITR/VOR R

M220K >> ITR/PSC R

normal
Can we improve patient care?

- Assess whether therapy failure is associated with a \textit{cyp51A} mutation
- Predict whether a patient may fail therapy by detecting a \textit{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

More future

- Flow cytometry
- MALDI-TOF mass spectrometry
- X-plate technology
- Porous aluminum oxide-based culture
- Isothermal microcalorimetry
Antifungal susceptibility testing in practice
ISHAM Working Group: Digital Mycological Education

FungiMICs

Continue

ISHAM
International Society for Human and Animal Mycology

EUCAST
European Committee on Antimicrobial Susceptibility Testing
Aspergillus fumigatus: Isavuconazole

MIC Breakpoint (mg/L)

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% microorganisms

MIC (mg/L)
Aspergillus fumigatus: Amphotericin B

Clinical Breakpoint
ECOFF
Histogram
Box Plot

MIC Breakpoint (mg/L)

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<tr>
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% microorganisms

MIC (mg/L)

Susceptible Intermediate Resistant
Aspergillus fumigatus: Isavuconazole

MIC Breakpoint (mg/L)

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<tr>
<td>MIC</td>
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Susceptible | Intermediate | Resistant

% microorganisms vs. MIC (mg/L)
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