Molecular Diagnostics in Fungal Infections

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Diagnosis of IFD

- IFDs associated with considerable morbidity & mortality
  - 30-70% mortality due to aspergillosis
  - 40-50% mortality due to candidiasis

- Early initiation of therapy is important & relies on early, rapid & accurate identification of the pathogen & detection of resistance

- Timely diagnosis limited by:
  1. Current diagnostic methods
  2. Diagnostic uncertainty of disease
Culture Independent Diagnosis

• Detection of circulating surrogate markers
  1. Serological tests – detect fungal antigens
  2. PCR based assays – detect fungal DNA
  3. Mass spectrometry – characterise fungal protein

• Ideally ‘new’ tests will:
  1. Confirm disease in patients with signs/symptoms
  2. Provide early clues of impending infection when used as a screening tool
  3. Rapid, sensitive and specific, high NPV
  4. Easily integrated into clinical workflow
  5. Detect drug resistance
1. Rule out particular IFD
   - Screening test in asymptomatic patients
   - Utilizes a high NPV
   - Pre-emptively diagnose in high-risk patient (not cost effective in patients with lower incidences)
   - Requires frequent testing e.g. blood
   - Ideally TAT 24-48h; short enough to impact patient management

2. Rule in a diagnosis
   - Enable a definite diagnosis in patients with signs and symptoms of infection
   - Pre-test probability increased

Halliday, Kidd et al., Pathology, 2015
Barnes et al., Med Mycol., 2018
Aspergillus PCR Assays

• Not included in EORTC/MSG diagnostic criteria, 2002
  ➢ Potential for false positive results
  ➢ Lack of standardised commercial testing platform
  ➢ No standard
  ➢ Validation limited clinically

• EAPCRI working group formed in 2006 (www.eapcri.eu)

• Develop standard for incorporation into EORTC/MSG definitions III (expected in 2019)
EAPCRI Recommendations

• DNA extraction is most critical step
  – Ensures sufficient target DNA available
  – DNA contains minimal inhibitory compounds

• *Aspergillus* PCR
  – Multi-copy gene target best (18S, 28S and ITS regions); 28S gene targets offer best analytical specificity
  – Should utilize a probe
  – Pan-*Aspergillus* assays may amplify *Penicillium* spp.
  – Pan-*Aspergillus* assays preferable & more reliable to detect low DNA concentrations despite potential cross reactivity

Barnes *et al.*, Med Mycol., 2018
Morton *et al.*, Med Mycol., 2017
Commercial *Aspergillus* PCR Assays

- Numerous commercial assays available
  - MycAssay Aspergillus (Microgen Bioproducts)
  - AsperGenius (Pathonostics)
  - Mycreal Aspergillus (Ingenetix)
  - Affigene Aspergillus (Cepheid)
  - Fungiplex Aspergillus IVD (Bruker)
  - *Aspergillus* spp. Q-PCR Alert (Nanogen)
  - Septi-Fast (Roche)

- Allow inter-laboratory standardization
- Reduced preparation time in diagnostic labs
- Independent QC of reagent
- Favourable clinical validity; limited clinical utility
Candida PCR Assays

- Blood culture is gold standard for diagnosis of IC (~20-50%)
- PCR not standardised or part of EORTC/MSG

- Systematic review & meta-analysis of PCR diagnosis of IC (Avni et al., JCM, 2011)
  - 54 studies, 4694 patients (963 proven/probable or possible)
  - Pooled sensitivities & specificities, 95% and 92%, respectively
  - Diagnosis available up to 4 weeks earlier than culture &/or clinical signs
  - Effect on clinical outcome unknown – Need RCT

<table>
<thead>
<tr>
<th></th>
<th>Positivity Rates</th>
<th>Range</th>
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<tbody>
<tr>
<td>PCR +ve</td>
<td>85%</td>
<td>78-91%</td>
</tr>
<tr>
<td>BC +ve</td>
<td>38%</td>
<td>29-46%</td>
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</table>
T2 Candida Assay

- FDA approved in 2014

- Fully automated, no extraction, 3-5 h, LOD 1-3 CFU/ml (C. albicans/C. tropicalis; C. glabrata/C. krusei & C. parapsilosis)

- Mylonakis et al., CID, 2015
  - DIRECT multicentre trial in 1801 patients
  - Overall sensitivity: 91%
  - Specificity, 98.1%
  - Negative result: 4.2 ± 0.9 h
  - Positive result, including species ID: 4.4 ± 1.0 h

- Diagnose(n=15) or rule out (n=9) candidaemia from paediatric patients using < 2 ml; 100% sensitivity and specificity (Hamula et al., Am J Clin Pathol, 2016)
T2 Candida Assay

Clancy et al., CID, 2018

- DIRECT2, prospective multi-centre trial to determine sensitivity of T2Candida in hospitalized patients with candidaemia
- 1ª endpoint: clinical sensitivity of T2Candida at time of positive companion BC (cBC)
- 2ª endpoint: T2Candida performance in patients with -ve cBC
- 45% (69/152) T2Candida +ve vs 24% (36/152) cBC
- Clinical sensitivity of T2Candida 89% (32/36)
- Antifungal Tx, neutropenia & C. albicans independently associated with T2Candida +ve, cBC –ve (P values <.05)

Limited data on utility of T2Candida for non-candidaemia IC (Zacharioudakis et al., J of Fungi, 2018)
T2 Candida Assay

• Potential to improve patient care, USD 250/test in 2016

• Cost-effectiveness analysis (Walker et al., JCM, 2016)
  - < costly than BC-directed & empiric echinocandin therapy
  - For suspected candidaemia: clinically more effective than BC; slightly < effective than empiric

• Optimal use?
  - Clinical Trials underway
  - Moderate-risk setting (IC prevalence of 5%) where empiric & prophylactic therapy routinely prescribed
  - Negative T2Candida = withhold/stop empiric therapy (estimated PPV: 71.6%; NPV: 99.5%)
**Pneumocystis PCR**

- Immunofluorescence = ‘Gold Standard’
  Requires expertise, lacks sensitivity & specificity

- PCR
  Highly sensitive & specific
  Excellent NPV
  Detects true infection, colonisation & sub-clinical infection

- Numerous Commercial assays
  MycAssay Pneumocystis (Myconostica)
  FTD Pneumocystis jirovecii (FastTrack Diagnostics)
  PneumoGenius (PathoNostics)
  Pneumonia/Atypical Pneumonia (AusDiagnostics)
Is *Pneumocystis* truly ubiquitous?
3% respiratory specimens positive (44/1457)

“a useful tool to diagnose PCP in non-HIV patients”
(Robert-Gangneux et al., J Clin Microbiol, 2014)

“Correlation with clinical & radiologic findings affords discrimination of early true disease vs rarer instances of colonization” (Doyle et al., OFID, 2017)
Mucorales PCR

• Numerous in-house PCR assays available
  – Serum, BAL, fresh & FFPE tissue
  – Promising potential for diagnosis; differentiate *Aspergillus*
  – Used for screening high risk patients > targeted treatment > improve outcome
    (Springer et al., J Med Microbiol, 2016; Gholinejad-Ghadi et al., 2018; Baldin et al., J Clin Microbiol., 2018)

• MucorGenius (PathoNostics)
  – multiplex real-time PCR
  – Targets 28S rDNA, 2.5 h after DNA extraction
  – BAL, fresh and FFPE tissue
Panfungal PCR Assays

• Impractical to develop assays for all fungal pathogens
  – Panfungal PCR & DNA sequencing
  – Target genome conserved within all fungi (e.g. 28S or ITS)

• Detect new & emerging pathogenic species & low target numbers from viable & non-viable cells

• Complement culture-based methods but detect commensal organisms (e.g. BAL)

• Accurate species identification relies on quality of sequences in databases
Triazole Resistance in *A. fumigatus*

- Increasing concern in *A. fumigatus*
  - 1st choice for prophylaxis & treatment
  - Emerges through spontaneous mutation or recombination & selection during exposure/treatment to antifungal, ie acquired
  - Commonly found in *Cyp51A* gene (SNPs &/or tandem repeats), & usually modify binding site, restricting entry of the azole
  - Most common SNPs: G54, M220, G138 & G448
  - TR: TR$_{34}$/L98H, TR$_{46}$/Y121F/T289A, TR53, TR$_{46}^3$, TR$_{46}^4$
  - Strong association between *Cyp51A* mutations & azole-resistant phenotypes ➔ ideal target for molecular diagnostics

Sharpe *et al.*, Med Mycol, 2018
Perlin & Wiederhold, JID, 2017
Triazole Resistance in *A. fumigatus*

- Several real-time multiplexed PCR assays
  (Perlin & Wiederhold, JID, 2017)
  - Work well on cultures
  - High fidelity vs DNA sequencing
  - Nested PCR increases sensitivity in specimens
    (Zhao et al., J Antimicrob. Chemother., 2013)
• Detects *A. fumigatus*, *A. terreus*, *Aspergillus* spp & prevalent azole resistance mutations (TR$_{34}$, L98H, Y121F and T289A) in BAL

• Good performance in haematology & ICU patients; correlated with azole treatment failures (Chang et al., J Antimicrob. Chemother., 2016)
Antifungal Resistance in *Candida* spp.

- Rapid diagnosis & detection of resistance essential for early, appropriate treatment

- Prevalence of *C. glabrata* increasing (2-fold rise in frequency)

- Azole resistance &/or echinocandin resistance is ↑
  - Azole resistance in *C. glabrata*: up to 30%
  - Echinocandin resistance in *C. glabrata*: up to 23% resistance
  - Azole resistance in *C. tropicalis*: 8-16.7%  
    (Chakrabarti *et al*., Intensive Care Med., 2015)
  - 41% of *C. auris* (n=54) resistant to ≥2 classes of drugs  
    (Lockhardt *et al*., CID, 2017)
Echinocandin Resistance Mechanisms

1. Point mutations in 2 ‘hotspot’ regions of \( FKS1 \)
   - HS1: region 640-650
   - HS2: 1345-1365
   - Most frequently Ser641 & Ser645 in \( C. \) albicans & Ser629 in \( C. \) glabrata

2. Mutations in \( FKS2 \) (\( C. \) glabrata only)
   - Most frequently Ser663 and Phe659
   (Pfaller et al., Am J Med, 2012; Dudiuk et al., JCM, 2014; Lackner et al., Curr Fun Infect Reports, 2015)

- For \( C. \) glabrata, mutations result in higher MICs to \( \geq 1 \) echinocandin & clinical failure
  (Alexander et al., CID, 2013)
Echinocandin Resistance

- Relatively low in most *Candida* species
  - <3% in *C. albicans*  
    (Castanheira *et al.*, AAC, 2010)
  - Rising for *C. glabrata* (4.9 to 12.3% in 10 yrs in 1 US hospital)  
    (Alexander *et al.*, CID, 2013)

- *C. parapsilosis* cx & *C. guilliermondii* demonstrate higher echinocandin MICs
  - Naturally occurring polymorphisms in *FKS* genes  
    (Sanglard *et al.*, Frontiers in Med., 2016)
  - Not associated with treatment failure  
    (Perlin, CID, 2015)
Detecting Echinocandin Resistance

- *In vitro* MIC testing cannot distinguish wild-type from echinocandin-resistant (*FKS* mutant) isolates
  - Isolates falsely classed as ‘resistant’
  - Overcome by molecular testing

- Molecular testing to detect *FKS* mutations
  - DNA sequencing – accurate but slow, multiple PCR assays, $$
  - Real-time PCR
  - NGS – high concordance between MICs & genomic variation
Detecting Echinocandin Resistance

• Dudiuk et al., JCM, 2014

- Multiplex PCR to detect resistance mutations in *C. glabrata*
- 98% concordance (n=50) between mutation detection & echinocandin MICs
- Cheap, simple, quick (<4 h)
- Unable to detect less common mutations in *FKS2*
Detecting Echinocandin Resistance

- NGS to analyse *FKS* mutations in clinical isolates of *C. glabrata* (n=12)
- Included 3 strain pairs from 3 patients that developed resistance during treatment (2 strains >60 fold ↑ in MIC for all echinocandins, 3rd strain developed 16-64 X ↑ in MICs to 5FC and all azoles)
- Detected mutations in either *FKS1* (S629P) or *FKS2* (S663P) genes of echinocandin-resistant isolates
- Also detected SNPs associated withazole resistance (*CgPDR1*, *CgCDR1*)
Can Genetic Technologies Help?

Table 1 Genetic mechanisms leading to resistance by antifungal compound in *Candida* species and molecular detection tools (modified from Perlin [32] and Cuenca-Estrella [24])

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Genetic target</th>
<th>Availability of reliable molecular tools</th>
<th>Correlation measured MIC with presence of gene mutation</th>
<th>Integration into routine daily use</th>
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<tbody>
<tr>
<td>Amphotericin B</td>
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<td>MDR1</td>
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Independent risk for treatment failure

Conclusions

• IFD diagnosed pre-mortem (vs post mortem) has increased from 16 to 51% (Lewis et al, Mycoses, 2013)

• Early recognition & diagnosis key to improved patient management

• PCR-based assays lack standardisation, subject to wide intra- and inter-laboratory variability
  ➢ FPCRI: Candida, Mucorales, FFPE, PJP
Conclusions

- Culture independent tests to simultaneously identify fungal pathogen & detect resistance exist

- More work needed to:
  - Determine clinical utility
  - Develop new assays to detect mechanisms other than point mutations associated with echinocandin & azole resistance
  - Correlate results of molecular tests with clinical outcome to justify routine use
  - Make tests available to non-reference laboratories
  - Develop point of care tests