Molecular Diagnostics in Fungal Infections

Catriona Halliday

Clinical Mycology Reference Laboratory, CIDMLS, Westmead Hospital

www.wslhd.health.nsw.gov.au/CIDM-PH

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

PCR-based Assay Strategy

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

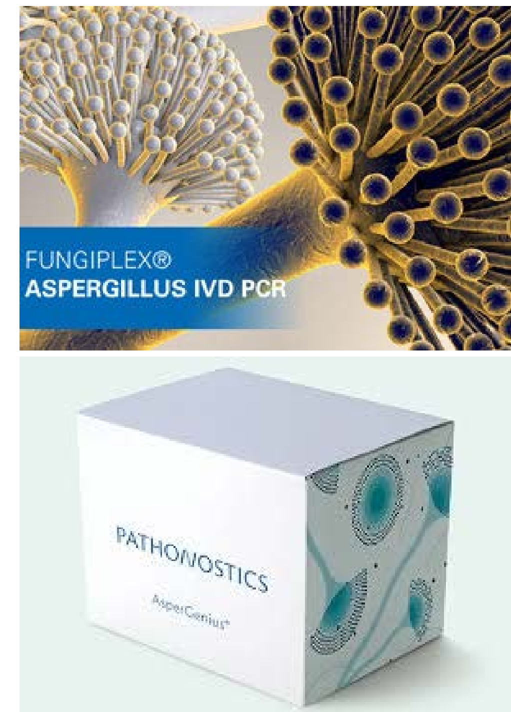
- DNA extraction is most critical step
  - Ensures sufficient target DNA available
  - DNA contains minimal inhibitory compounds

- *Aspergillus* PCR
  - Multi-copy gene target best (18*S*, 28*S* and ITS regions); 28*S* gene targets offer best analytical specificity
  - Should utilize a probe
  - Pan-*Aspergillus* assays may amplify *Penicillium* spp.
  - Pan-A*spergillus* 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

	Positivity Rates	Range
PCR +ve	85%	78-91%
BC +ve	38%	29-46%

  - ➢ Diagnosis available up to 4 weeks earlier than culture &/or clinical signs
  - ➢ Effect on clinical outcome unknown – Need RCT

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
  - 1º endpoint: clinical sensitivity of T2Candida at time of positive companion BC (cBC)
  - 2º 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 T2*Candida* = withold/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

  Fan et al., PLoS One, 2013; Alanio et al., Clin Microbiol Infect, 2011; Fauchier et al., J Clin Microbiol, 2016
- Numerous Commercial assays
  - MycAssay Pneumocystis (Myconostica)
  - FTD Pneumocystis jirovecii (FastTrack Diagnostics)
  - PneumoGenius (Patho*N*ostics)
  - Pneumonia/Atypical Pneumonia (AusDiagnostics)

Pneumocystis PCR

Open Forum Infectious Diseases

MAJOR ARTICLE

Pneumocystis PCR: It Is Time to Make PCR the Test of Choice

Laura Doyle, Sherilynn Vogel, and Gary W. Procop

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 (Patho*N*ostics)
  - multiplex real-time PCR
    Pan-*Mucorales, Cunninghamella* spp, *Lichtheimia* spp., *Mucor* spp., *Rhizopus* spp. & *Rhizomucor* spp.
  - 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. 28*S* 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
  (Lau et al. J Clin Microbiol, 2007; Babouee et al., Clin Microbiol. Infect, 2013; Lass-Florl et al, J Clin Microbiol, 2013)

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

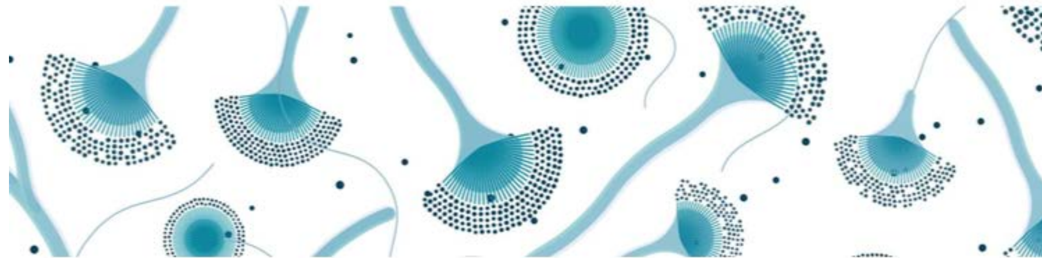
Triazole Resistance in *A. fumigatus*

- 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)

AsperGenius®

AsperGenius® | Instructions For Use | Version 2

PATHONOSTICS



AsperGenius®

- 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 ≥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 (Garnaud *et al*., *J.* Antimicrob. Chemother., 2015; Biswas *et al*., CMI, 2017)

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

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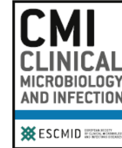

Research note

Identification of genetic markers of resistance to echinocandins, azoles and 5-fluorocytosine in *Candida glabrata* by next-generation sequencing: a feasibility study

C. Biswas [1, 2, *, †], S.C.-A. Chen [1, 2, 3, †], C. Halliday [1], K. Kennedy [4], E.G. Playford [5], D.J. Marriott [6], M.A. Slavin [7], T.C. Sorrell [2, 3], V. Sintchenko [1, 2, 3]

- 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 echincocandins, 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 with azole 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 Cuence-Estrella [24])

Antifungal agent	Genetic target	Availability of reliable molecular tools	Correlation measured MIC with presence of gene mutation	Integration into routine daily use
Amphotericin B	No	No	No	No
Azoles	*MDR1*	Yes	Uncertain	No
	CDR1	Yes	Uncertain	No
	CDR2	Yes	Uncertain	No
	ERG11	Yes	Uncertain	No
	ERG3	Yes	Uncertain	No
	TAC1	Yes	Uncertain	No
Echinocandins	*FKS1*	Yes	Yes	Strongly considered
	FKS2	Yes	Yes	Strongly considered
	FKS 3	Yes	Yes	Strongly considered

} Independent risk for treatment failure

Halliday, Slavin & Chen, Curr. Fungal Infection Rep, 2017

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