Updates on Molecular Tests & PCR

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Disclosures

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Culture Independent Diagnosis

- Fungal biomarkers – surrogate markers of IFD
  1. Fungal DNA – PCR based assays
  2. Fungal antigens – β-D- glucan, *Aspergillus* galactomannan

- Useful adjunct for early diagnosis

- Incorporated into care pathways & diagnostic algorithms
  - Steward & monitor antifungal therapies
  - Predict treatment outcomes
  - EORTC/MSGERC consensus definitions (Donnelly, CID, 2020)

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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 8–10% incidence (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. Support in a diagnosis ("Upgrade" category of IFD)
   - Enable a definite diagnosis in patients with signs and symptoms of infection
   - Pre-test probability increased

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Kidd *et al.*, *Front Microbiol*, 2020; Barnes et al., *Med Mycol.*, 2018

Aspergillus PCR for Diagnosis

- Accepted as mycological criterion for probable IA
  - Blood (serum, plasma, whole blood); ≥2 consecutive PCR +ve tests
  - BAL fluids; ≥2 duplicate PCR +ve results
  - ≥1 PCR +ve blood (serum/plasma/whole blood) AND 1 PCR +ve BAL fluid

(Donnelly et al, *CID* 2020; White et al *CID* 2021)

- Provides robust diagnostic test for:
  - Screening patients at moderate-high risk of IA
  - Confirming diagnosis of *Aspergillus* infection

(Cruciani et al. *Cochrane Databases Syst Rev* 2019, 9:CD009551)

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Aspergillus PCR Technical Considerations

- Specimen volume, nucleic acid extraction protocol & elution volume critical to PCR assay performance
  - ≥3 mL whole blood; ≥0.5 mL serum/plasma
  - Mechanical disruption of cell wall required for efficient NA extraction
  - Elution volume ≤100 µL

- PCR assay not rate-limiting to success
  - Multi-copy gene target enhances sensitivity (28S rRNA or ITS)
  - Pan-*Aspergillus* target preferred
  - Recommend PCR testing in duplicate
  - qPCR minimises contamination

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Commercial *Aspergillus* PCR Assays

- Numerous commercial assays available (Rath & Steinmann, Front Microbiol, 2018)
  - Provide standardised methodology & independent QC of reagents
  - Significantly lower sensitivities in serum vs respiratory specimens
  - Limited data on clinical utility & head-to-head comparisons
  - Some assays detect prevalent *cyp51A* gene mutations conferring azole resistance

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Aspergillus PCR

- Negative PCR result can exclude IA in antifungal drug naïve patients
- Positive PCR result is useful for diagnosis
  - Positive result from BALF cannot distinguish colonisation from IA (PPV 72%)

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Detection of Azole Resistance by PCR

- Performance of 3 commercial assays evaluated on BAL (n=103)
  - MycoGENIE® *Aspergillus fumigatus* real-time PCR kit (Adamtech)
  - Fungiplex® *Aspergillus* Azole-R IVD real time PCR kit (Bruker Daltonik)
  - AsperGenius® (PathoNostics)

Probable IPA (n=11) vs possible (n=51) /no IPA (n=41)

Kit	Sensitivity	Specificity	PPV	NPV
MycoGENIE®	80%	73.2%	26.7%	96.8%
Fungiplex®	60%	91%	42.9%	95.4%
AsperGenius®	63.6%	96.7%	70%	95.7%

- Only 1 azole-resistant isolate (TR34 mutation) detected by all three assays

Scharmann et al. *J Fungi*, 2021; Pelzer et al. *Med Mycol*, 2020

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Mucorales PCR

- Incidence of mucormycosis is increasing due to:
  - Increase in number of susceptible people
  - Change in antifungal practice
  - Improved diagnostics (PCR from culture negative tissue, BALF, serum & urine)

- Early diagnosis is key to improving survival outcomes

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Mucorales PCR for Diagnosis

- Detection in fresh or FFPE tissue resulted in increase in diagnosed cases
  - Sensitivities of 97–100% on fresh tissue & 56–91% on FFPE

- Aids diagnosis of pulmonary mucormycosis from BALF
  - PCR +ve in all 10 patients with proven/probable disease
  - PCR earliest and/or only biological evidence of disease in 4 patients
  - 6 of 24 patients (25%) co-infected with *Aspergillus*-Mucorales
  - Supports inclusion in diagnostic approach despite difficulty obtaining BALF

(Scherer et al, *JCM* 2018)

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Mucorales PCR for Screening

MODIMUCOR Prospective trial (Millon et al, *CID*, 2022)

- Assess performance of serum Mucorales qPCR for early diagnosis of mucormycosis
- 232 patients enrolled prospectively, 2x weekly screening of serum
- qPCR targets *Lichtheimia*, *Rhizomucor* & *Mucor/Rhizopus*
- Sensitivity, 85.2%; specificity, 89.8%, PLRz, 8.3; NLR, 0.17
- PCR +ve from serum 4 days before mycological/histopathological examination, 1 day before 1st imaging performed
- -ve PCR within 7 days of L-AMB associated with 85% lower 30-day mortality
- Argues for inclusion in EORTC/MSGERC definitions

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Commercial Mucorales PCR Assays

	MucorGenius® Real-Time PCR	MycoGENIE® Aspergillus Species—Mucorales Species	Fungiplex® Mucorales RUO PCR Kit
Diagnostic specimens	Bronchoalveolar lavage Biopsy samples, paraffin embedded Serum	Serum Biopsies Lower respiratory tract samples	Not specified
Species detected	*Rhizopus* spp. *Mucor* spp. *Lichtheimia* spp. *Cunninghamella* spp. *Rhizomucor* spp.	*Rh. Pusillus* *M. indicus* *M.circinelloides* *M.plombeus* *R. arrhizus* *R. stolonifera* *L. corymbifera* *L. glauca* *C. bertholletiae* *Mycotypha* sp.	*Rhizopus* spp. *Lichtheimia* spp. *Cunninghamella* spp. *Rhizomucor* spp. *Mucor* spp. *Actinomucor* spp. *Apophysomyces* spp. *Saksenaea* spp. *Syncephalastrum* spp.
Manufacturer	PathoNostics	Ademtech	Bruker

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Dannaoui, *J Fungi* 2022, 8, 457

MucorGenius® Mucorales RUO

- Detect clinically relevant Mucorales species in ~3 h
- BALF, tissue (fresh & FFPE), serum
- Lacks extensive clinical validation
- Good performance. Sensitivity, 90% (9/10); specificity, 97.9%, missed case with low fungal burden (Guegan et al. *J. Infect*, 2020)
- Sensitivity on serial blood 75%, preceded microbiological diagnosis (Mercier et al. *J Fungi*, 2019)
- Lower analytical sensitivity to in-house assay & decreased detection of *Lichtheimia corymbifera* (*J Fungi* 2022, 8, 786)

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Panfungal PCR

- Detect & identify "all" fungi from diverse specimen types
  - ITS or 28S are recommended targets
  - BLAST sequences against quality-controlled databases

- Best results from sterile specimens (not BALF)
  (Zeller et al. *J Microbiol*, 2017; Garnham et al. *Pathology*, 2020)

- Detect novel or unexpected pathogens
  - 44% (8/18) +ve with non-*Aspergillus*/*Candida* species
    (Sugaware et al. *Eur J Haematol*, 2013)

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Panfungal PCR for Diagnosis

EORTC/MSGERC recommend use of PCR & DNA sequencing for genus/species ID from fresh/FFPE tissue

- ***ONLY*** when fungal elements seen by histopathology
- ***NOT*** recommended where fungal staining is negative
- Identification ***MUST*** be consistent with histopathological features
- Rigorous quality control (+ve, -ve & internal control)
- PCR should target fungal barcoding genes (ITS or 28S)
- Every PCR product should be sequenced
- Performed ***ONLY*** in reference centres or high-volume centres
- Fulfil criteria of PROVEN fungal disease

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Donnelly et al *CID* 2020;71:1367 & Lockhart et al *CID* 2021;72:S109

Panfungal PCR on Tissue

- Diagnostic yield increases with pre-test probability
  - 71.3% patients with proven/probable IFD (variety of specimens)
  - <10% specimens with no fungal elements
  - Yield from biopsy specimens: 71.5% open resection; 50% core-needle; 0% FNA (Gomez et al, *CID*, 2017)

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Panfungal PCR on Tissue

- Limited utility on histopathology negative FFPE (n=248)
  - 28% (69/248) yielded invalid result (no HBG)
  - 18% (45/248) positive histology; 49% (22/45) positive PCR; 36% (16/45) clinically significant PCR result
  - 9% (19/203) histology negative yielded positive PCR; 3% (6/203) clinically significant PCR result
  - AU$258 histopathology positive vs AU$3105 histology negative
  - Panfungal PCR on histopathology negative FFPE tissue ***NOT*** recommended (Sparks et al, *Pathology*, 2023)

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Panfungal PCR on BALF

- Interpretation difficult – Infection, colonisation or environmental contamination?
  - *Candida, Saccharomyces*, *Rhodotorula* & "mixed" most frequently detected (Trubiano et al *Med Mycol*, 2016; Rahn et al *J Med Micro*, 2016)

- Sensitivity lower in patients receiving mould-active treatment – Negative does not exclude IFD

- Diagnostic utility & costs (Garnham et al Pathology, 2020)
  - 53% (530/1,002) yielded positive; 8.5% (45/1,002) clinically significant
  - Recommend > pre-analytical stewardship – limit to high-risk (neutropenic) patients with clinical/radiological evidence of IFD

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Pneumocystis jirovecii PCR Assays

- Increasingly used by diagnostic labs
- Detect colonisation, asymptomatic infection, sub-clinical & active infection – correlate +ve PCR with clinical, radiological & laboratory findings
  (Doyle et al, *OFID*, 2017)
  "Affords discrimination of early true disease vs rarer instances of colonisation"
- ECIL guidelines recommend real-time PCR for routine diagnosis of PCP
  (Alanio et al, *JAC*, 2016)
  - BALF best specimen (A-II)
  - Yield from BALF > induced sputum > oropharyngeal wash

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Pneumocystis jirovecii PCR Assays

- PCP qPCR incorporated into EORTC/MSGERC criteria
  - Mycological evidence of pneumocystis from BALF, IS or OW
  - Do NOT recommend threshold for positivity

- Numerous commercial assays available – excellent concordance with in-house assays (Sasso et al, *JCM*, 2016; Huh et al, *Ann Lab Med*, 2019)

- FPCRI pneumocystis working group
  - Establish consensus method
  - Assist with lab standardisation & quantification
  - Recommend SSU or mitochondrial LSU

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Candida PCR

- Fulfils many of criteria for “ideal diagnostic test for IC”

Sensitive (<5 CFU/ml)	Multiplex capability	Rapid TAT
Minimally invasive sampling	Provides speciation	+ve before culture

- Utility not clearly defined – Use differs in different clinical contexts
- Lacks standardisation & limited validation in real-life prospective settings – FPCRI developing standards

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Commercial *Candida* PCR Assays

Product	Manufacturer	Species Targeted
AusDiagnostics Sepsis	AusDiagnostics Pty Ltd	*C. albicans, glabrata, krusei, parapsilosis* & *tropicalis*
*Cand*ID & *Auris*ID	OlmDiagnostics	*C. albicans, dubliniensis, glabrata, krusei, parapsilosis* & *tropicalis; C. auris*
FungiPlex *Candida* & FungiPlex *Candida auris*	Bruker Daltonik	*C. albicans, dubliniensis, glabrata, krusei, parapsilosis* & *tropicalis; C. auris*
Magicplex Sepsis Real-time Test	Seegne	*C. albicans, glabrata, krusei, parapsilosis* & *tropicalis* (and *A. fumigatus*)
MycoReal *Candida*	Ingenetix	*C. albicans, dubliniensis, glabrata, krusei, lusitaniae, parapsilosis* & *tropicalis*
SeptiFast Real-time PCR	Roche Diagnostics	*C. albicans, glabrata, krusei, parapsilosis* & *tropicalis*
T2 *Candida* T2 *C. auris*	T2 Biosystems	*C. albicans/tropicalis*, *C. glabrata* cx/*krusei* and *C. parapsilosis* cx; *C. auris*

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Cryptococcus PCR

- Commercial assays:
  - BioFire FilmArray Meningitis/Encephalitis panel (bioMerieux)
  - AusDiagnostics Atypical Pneumonia & CSF panels
  - Limited to certain specimens (CSF & BALF)
  - Sub-optimal sensitivity due to substantial multiplexed nature
  - Cannot discriminate *C. neoformans* from *C. gattii*

- Real-time targeted PCR to detect & identify *C. neoformans* & *C. gattii* from sterile & non-sterile specimens
  (Tay et al, *J Fungi*, 2022)

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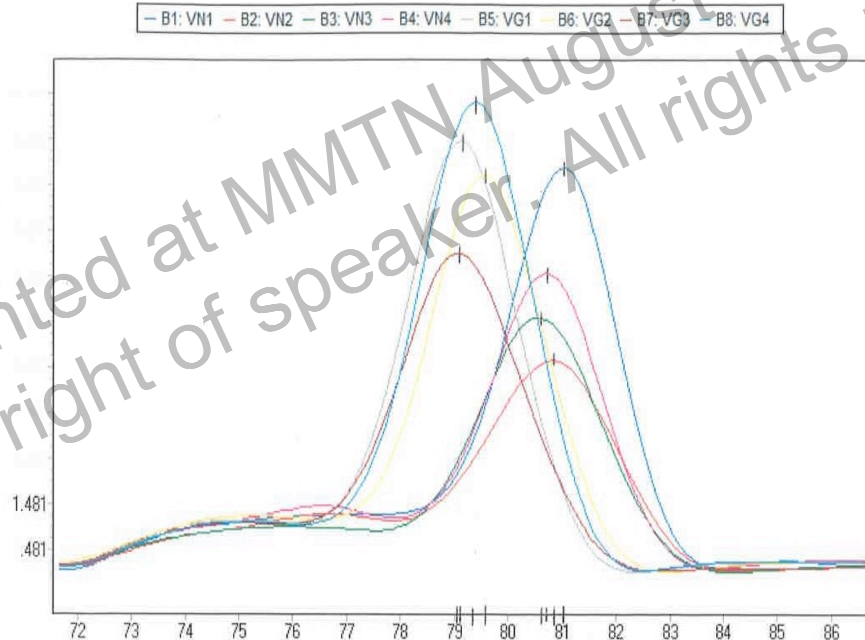
Cryptococcus PCR

- High resolution melt-curve analysis discriminates between 2 species (Tay et al *J Fungi*, 2022)

C. neoformans (81°C) & *C. gattii* (79°C)



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Conclusions

- Important advances in standardisation of PCR tests – incorporated into EORTC/MSGERC criteria
- PCR tests are NOT standalone tests – valuable "add on" tools which fulfil diagnostic gaps
  - Must optimise test algorithms
- MUST be interpreted in clinical context of patient & other findings
- Risk factor stratification – predict individuals at greatest risk

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Thank you

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