Optimising Fungal PCR

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Why we need Fungal PCR

- Gold Standard: Microscopy and Culture
  - Slow
  - Lacks sensitivity
  - Need tissue containing viable fungi
  - some fungi can't be cultured (e.g. *Pneumocystis*)
  - Fungal identification requires expertise
- Can use peripheral specimens (blood, serum, BAL fluid)
- Pre-emptive surveillance in high risk patients
  - earlier diagnosis, better management, improved outcomes ?

Fungal PCR Assays

- Specimen choice, test frequency and interpretation depends on:
  - Pre-emptive surveillance for a particular IFD in high risk patients, or
  - Definitive diagnosis where IFD is clinically evident
- Assay Design
  - Real-time PCR or qPCR (nested PCR, gel-based PCR are sub-optimal)
  - Genus or species specific, detect specific mutations
- Gene Targets
  - Ideally multi- copy to maximise sensitivity
  - Sufficiently conserved to allow amplification of target fungi
  - Adequate sequence variation to define particular genus/species/mutations

Aspergillus PCR Assays

- In use for >20 years
- Still considered experimental (until recently)
- Not included in EORTC/MSG case definitions for IA
  - Potential for false positive results (2002 criteria)
  - Lack of standardised commercial testing platform (2002 criteria)
  - No methodology Standard (2008 criteria)
  - Validation was limited clinically (2008 criteria)
- Significant efforts in last decade to overcome these issues

Standardise *Aspergillus* PCR Assays

- European *Aspergillus* PCR Initiative (EAPCRI) working group formed in 2006
- GOAL: To develop *Aspergillus* PCR Standard for incorporation into EORTC/MSG definitions (3rd edition)

EAPCRI Evaluation of PCR Protocols

- QC specimen panels distributed to 24 labs around the world
  - Whole Blood [1]
  - Serum [2]
- Labs reported detection (or not) of *Aspergillus* DNA and provided details of protocol.
- Protocol steps evaluated:
  - Volume of specimen used
  - Use of red cell lysis and/or white cell lysis steps (whole blood only)
  - Fungal lysis steps
  - DNA purification method
  - DNA elution volume
  - Use of internal controls
  - No. replicates
  - PCR format & platform
  - DNA target

Performance limited by DNA extraction method, NOT the PCR design

[1] White et al., *JCM* 2010 48:1231; [2] White et al., *JCM* 2011 49:3842

EAPCRI Recommendations - Whole Blood

- ≥3 mL blood (EDTA only)
- Red cell and white cell lysis steps required
- Bead beating essential for fungal cell lysis
- Incorporate non-human DNA internal control
- Elution volume <100 µL
- Duplicate samples
- Use real-time PCR platform, multi-copy target & genus or species specific probe
- Incorporate negative control for DNA extraction & PCR

White et al., *JCM* 2010 48:1231

EAPCRI Recommendations - Serum

- ≥0.5 mL serum
- Can use commercial DNA extraction platforms
  - Screen all kit reagents for contamination
- Incorporate non-human DNA internal control
- Elution volume <100 µL
- Duplicate samples
- Use real-time PCR platform, multi-copy target & genus or species specific probe
- Incorporate negative control for DNA extraction & PCR
- PCR positivity of 43 cycles provides best diagnostic accuracy

White et al., *JCM* 2011 49:3842

EAPCRI: Serum vs. Plasma

- Standardized *Aspergillus* PCR performed on paired plasma and serum samples from haematology patients.
- 19 proven/probable cases, 42 controls

	Serum	Plasma
Sensitivity	68.4%	94.7%
Specificity	76.2%	83.3%
Time to positivity	10.8 days before diagnosis	16.8 days before diagnosis
Earliest indicator of infection in	6 cases	13 cases

White et al., *JCM* 2015 53:2832; Springer et al., *JCM* 2016 54:705

Aspergillus PCR Assay Performance

- To assess performance of PCR assay parameters, specificity, sensitivity
- Fungal genomic DNA panels prepared, and distributed to 28 participating laboratories to test specificity of PCR methodologies.
  - *A. fumigatus, A. flavus, A. lentulus, A. niger, A. terreus, A. versicolor,*
  - *Aspergillus* spp.: genomic DNA 1-10 fg/µL and 100 fg/µL
  - *C. albicans, Lichtheimia corymbifera, Cunninghamella sp., Fusarium oxysporum, Scedosporium apiospermum, and Penicillium* sp.
  - Non *Aspergillus* spp.: genomic DNA 100-500 fg/µL

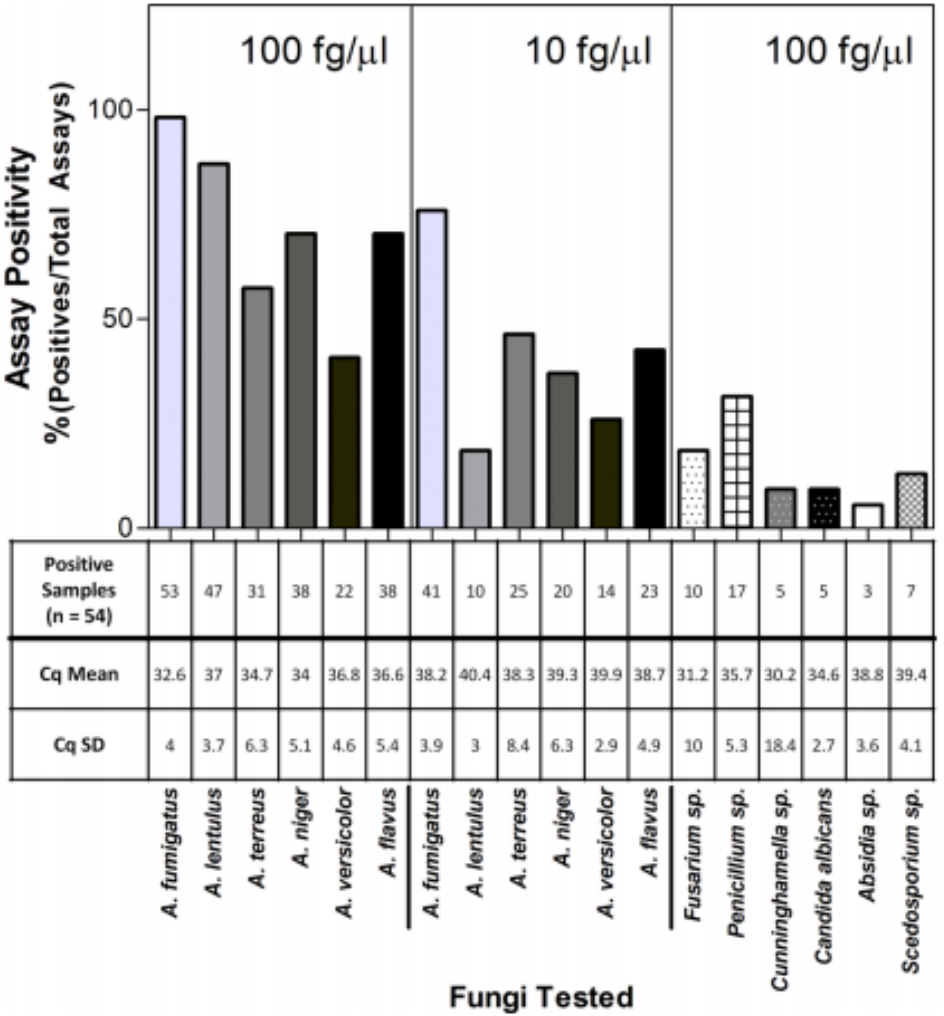
Morton et al., *Med Mycol*. Oct 7 2016.

Sample positivity

Overall (28 labs):

- *Aspergillus fumigatus* DNA tested positive significantly more than other *Aspergillus* species (P < .0001)
- False negatives: *A. lentulus* (25%) *A. versicolor* (24%), *A. terreus* (16%), *A. flavus* (15.2%), *A. niger* (13.4%), and *A. fumigatus* (6.2%).
- False positive rate 14.5%: *Penicillium* and *Fusarium* highest rate of cross-reaction.

Morton et al., *Med Mycol*. Oct 7 2016.



Influence of Technical Factors on Accuracy

- Accuracy significantly associated with:
  - PCR target: 18S/28S rRNA (71.9%) vs. ITS/other genes (56.4%)
  - Genus-specific assays (71.8%) vs. species-specific assays (47.2%)
- However, genus-specific **18S**/28S assays significantly associated with false positives
- Sensitivity significantly associated with:
  - PCR target: 18S/28S rRNA (63.8%) vs. ITS/other genes (35.3%)
  - Genus-specific assay
  - Larger reaction volumes
- No sig. assoc. between accuracy and PCR platform, DNA template volume, or final reaction volume

Morton et al., *Med Mycol*. Oct 7 2016.

MycAssay™ *Aspergillus*

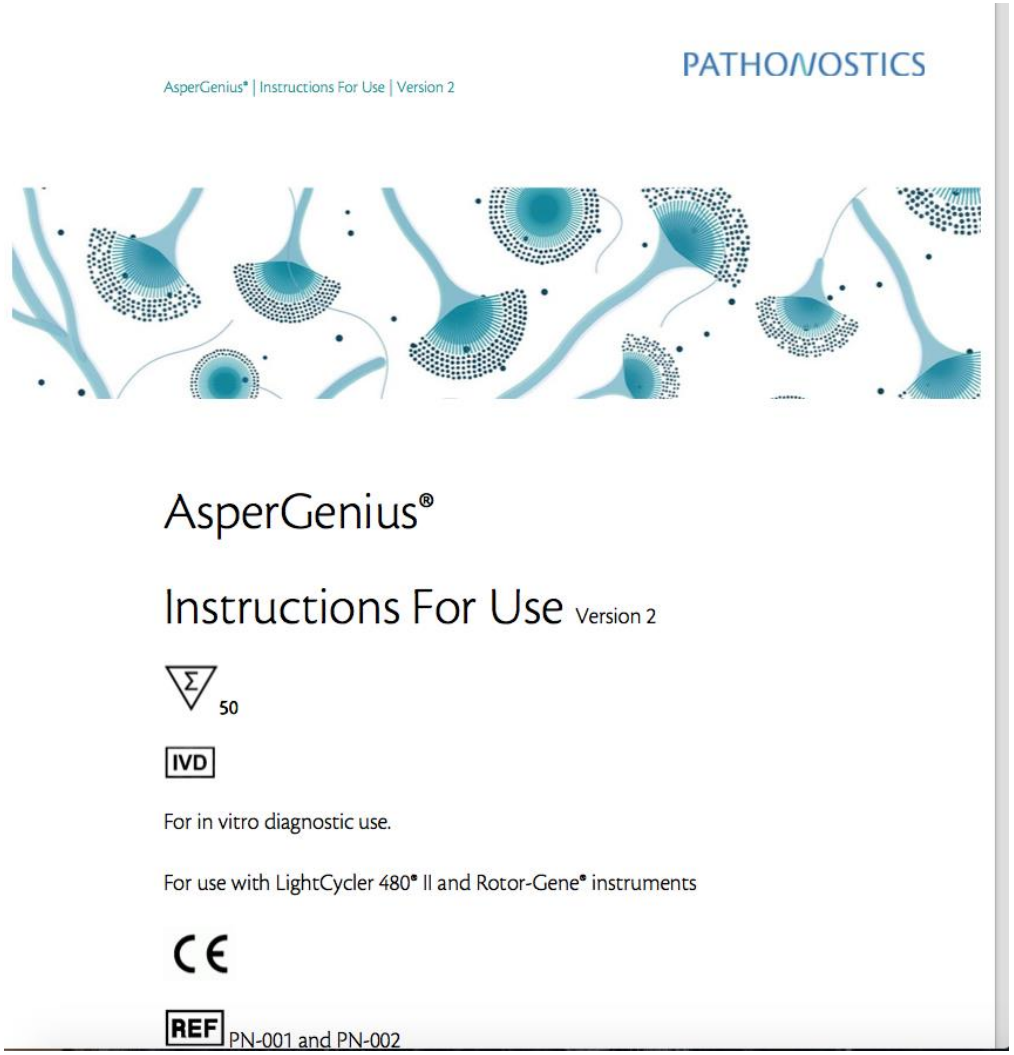
- CE marked real-time PCR assay
  http://www.myconostica.co.uk/aspergillus
- Validated for lower respiratory tract specimens & serum
- Closed tube, incorporates internal control
- Results available <3h (realistic?)
- Performance comparable to ‘in-house’ PCR assay & Galactomannan

White et al, *JCM* 2011 49:2169

AsperGenius®

http://www.pathonostics.eu/products/AsperGenius

For use with BAL specimens



AsperGenius®

- Quantitative real-time multiplex PCR assay
  - *Aspergillus fumigatus* (28S rRNA)
  - *Aspergillus terreus* (28S rRNA)
  - *Aspergillus* spp. (28S rRNA)
  - Internal control (M13 phage)
- Identifies 4 common *cyp51A* mutations conferring multi-azole resistance in *A. fumigatus*:
  - TR_{34}
  - L98H
  - T289A
  - Y121F
- Validated on BAL in Netherlands

Chong et al., *JCM* 2015 53:868

Evidence for Clinical Use of *Aspergillus* PCR

- Clinical performance comparable to Galactomannan and β-D glucan assays
- Potential to determine genus, species & azole resistance
- External QA program available (QCMD, Scotland)
- Standard for DNA extraction methodologies in place
- Commercial PCR assays available
- Standardised *Aspergillus* DNA calibrator for quantification
- Numerous publications demonstrating clinical utility
- Likely to be accepted into next revision of EORTC/MSG IA definitions

White et al., *CID* 2015 61:1293; Lyon et al., *JCM* 2013 51:2403

Candida PCR Assays

- Blood culture is gold standard for diagnosis of Invasive Candidiasis (IC)
- *Candida* PCR assays not standardised, or part of EORTC/MSG criteria
- Systematic review & meta-analysis of PCR diagnosis of IC;
  - 54 studies, 4694 patients (963 proven/probable IC)
  - Whole blood performs better than serum
  - improved performance with rRNA or P450 gene targets
  - Improved performance with detection limit ≤10 CFU/ml

Patients with proven/probable IC (n=963)	**Positivity Rates**	**Range**
PCR positive	85%	78-91%
Blood Culture positive	38%	29-46%

  - Earlier diagnosis (1 day to 4 weeks)
  - Effect on clinical outcome unknown – Need RCT

Avni et al., *JCM* 2011 49:665

Pneumocystis PCR

- BAL recommended, or if not possible use induced sputum
- Real time PCR must be used
  - qPCR (closed tube) has better sensitivity and specificity than non-qPCR
  - Express results as quantity: e.g. copies/mL, fungus/mL or Cq
  - Probes preferable to intercalating dyes
  - Use enzymatic prevention of amplicon contamination
  - Must incorporate non-human DNA internal control
- Participate in external QA program
- Commercial qPCRs ‘not superior’ to in-house assays that follow above recommendations

Alanio et al., *JAC* 2016 71:2386.

Summary

- Significant advances being made in optimization and standardization of molecular methods for diagnosis of IFD
- Choice of specimen is important – plasma for *Aspergillus* PCR
- DNA extraction method is critical to success
- Ribosomal RNA gene targets and genus-specific assays typically associated with superior performance

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Interactive discussion

Your questions are welcome

Please raise your hand for a **microphone** or submit a **question card**

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Break

Refreshments are available outside the room

Please go to Theatre 1 (next door) after the break

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5th MMTN Conference

5-6 November 2016, Bangkok, Thailand

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