

Multiplex PCRs for Fungal Diagnostics

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Invasive Fungal Infections (IFIs)

- Incidence of mycotic disease increasing
 - Immunocompromised patients
 - Immunosuppressive therapy
- Major cause of morbidity & mortality
 - Nosocomial candidiasis ↑ 10 x in 20 years
 - 66-88% mortality in HSCT recipients due to IA

Invasive Fungal Infections (IFIs)

- >90% all IFIs caused by *Aspergillus* & *Candida* spp.
- Spectrum of mycotic disease expanding
 - *Candida albicans* → *C. glabrata*, *C. tropicalis*, *C. krusei* & *C. parapsilosis*
 - *Aspergillus fumigatus* → *A. flavus*, *A. terreus* & *A. nidulans*

Diagnosis of IFIs

- Early treatment & accurate species ID essential
 - Optimal therapy
 - FLU: *C. albicans* vs *C. glabrata*
 - AMB: *A. fumigatus* vs *A. terreus*
 - Optimal duration of therapy

Diagnosis of IFIs

- Timely diagnosis limited by:
 1. Current diagnostic methods
 - ‘Gold Standard’ + histology &/or + culture
 - Slow, insensitive, rarely detect disease early
 2. Diagnostic uncertainty of disease
 - Empiric treatment
 - Emergence of resistant fungi?

Culture Independent Diagnosis

- Detection of circulating surrogate markers
 1. Serological tests – detect fungal antigens
 2. PCR based assays – detect fungal DNA
- PCR-based assays
 - Rapid
 - Sensitive
 - Specific
 - Low target number
 - Viable and non-viable cells

Culture Independent Diagnosis

- PCR-based assays
 - 20 years
 - Considered experimental
 - NOT standardised → diverging results
 - NOT included in EORTC/MSG criteria

Real-time Fungal PCR Assays

- ↓ risk of false +ve
- Fast turnaround times (<2 hrs)
- Improved specificity
 - Species specific probes & melting curve analysis
- Majority detect *Candida* or *Aspergillus* spp. or *C. albicans* or *A. fumigatus* ONLY
- Limited evaluation in relevant patient groups

Quantification of Fungal Load

- Questionable usefulness for fungi:
 - Fungaemia has low pathogen load
 - Load <100 gene copies not quantifiable
 - Fungal cells released intermittently
 - amount detected not indicative of severity of infection

Multiplex PCRs for Fungi



Multiplex PCRs for *Candida*

- Guiver *et al.*, 2001, J. Clin. Path. 54: 362-366
 - Detects & IDs 6 most clinically significant *Candida* spp.
albicans, glabrata, kefyr, krusei, parapsilosis & tropicalis
 - Targets ITS2 region of rDNA gene cluster
 - Incorporates 6 species specific primers & TaqMan probes
 - Used individually or in 2 multiplex sets
 - Spectrally distinct fluorescent dyes (FAM, TET & VIC)
 - Only evaluated on cultures

Multiplex PCRs for *Candida*

- Hsu *et al.*, 2003, J. Med. Micro. 52:1071-1076
 - Targets ITS region of rDNA gene cluster
 - Detects and IDs 7 fungal species
 - C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii* & *Cryptococcus neoformans*
 - Species specific primers, SYBR green & melt curve analysis
 - Not tested on clinical specimens

Multiplex PCRs for *Candida*

- Innings *et al.*, 2007, JCM. 45: 874-880
 - Targets RNase P RNA gene
 - Detects 8 *Candida* species
 - albicans, dubliniensis, famata, glabrata, guilliermondii, krusei, parapsilosis & tropicalis*
 - Incorporates 4 TaqMan probes
 - Candida* genus, *C. albicans*, *C. glabrata* & *C. krusei*
 - Limited clinical samples tested (20 blood)

Multiplex PCRs - *Candida* & *Aspergillus*

- Detects & IDs *A. fumigatus* & *C. albicans*
 - LightCycler – FRET & melting curve analysis
- Targets 18S rDNA gene
 - Universal primers, species-specific probes
- Clinical evaluation
 - 12 specimens, 8 patients
 - PCR -ve for *A. terreus* infection

Multiplex PCRs - *Candida* & *Aspergillus*

- Detects & IDs *Aspergillus* & *Candida* to genus level
 - MagNA Pure & LightCycler
 - 5-6hr to perform
- Targets 18S rDNA gene
- Identifies 5 *Candida* spp. with probes
 - C. albicans/dubliniensis*
 - C. glabrata*
 - C. krusei*
 - C. tropicalis*
 - C. parapsilosis*

Multiplex PCRs - *Candida* & *Aspergillus*

- In vitro sensitivity of 2 CFU/ml blood
- Specificity of species-specific probes:
 - *C. glabrata*, *C. tropicalis* & *C. krusei*, no cross-reactivity
 - *C. albicans/dubliniensis* probe cross reacted with *C. parapsilosis* DNA & vice versa
 - *C. albicans/dubliniensis* & *C. parapsilosis* differentiated by melt curve analysis

Multiplex PCRs - *Candida* & *Aspergillus*

- Clinical evaluation
 - 1650 samples (blood, other body fluids & tissue)
 - 5.3% +ve for *Candida* spp., 83% verified with conventional methods
 - 1.7% +ve for *Aspergillus* spp., 50% verified with conventional methods
- Assay useful to exclude patients at risk of IFI

Multiplex PCRs - *Candida* & *Aspergillus*

- Simultaneously detect & ID 11 medically important *Aspergillus* & *Candida* spp.
 - Aspergillus*: *fumigatus*, *flavus*, *nidulans*, *niger* & *terreus*
 - Candida*: *albicans*, *dublinsiensis*, *glabrata*, *krusei*, *parapsilosis* & *tropicalis*
- Target ITS2 region of rDNA gene cluster

Multiplex PCRs - *Candida* & *Aspergillus*

- Genus specific primers & species-specific biprobes (2 PCR mixtures)
 - Bi-probe:
Cy5-species specific sequence-biotin
- SYBR green & melt-curve analysis on LightCycler
- Probe-specific T_m guarantees high specificity

Multiplex PCRs - *Candida* & *Aspergillus*

- Species-specific T_m values

- *Aspergillus* spp.

<i>A. fum</i>	63°C	<i>A. flav</i>	59°C
<i>A. nidu</i>	66°C	<i>A. nig</i>	68°C
<i>A. terr</i>	57 or 66°C ^a		

- *Candida* spp.

<i>C. alb</i>	66 or 55°C ^{a,b}	<i>C. dubl</i>	62°C ^b
<i>C. glab</i>	65°C	<i>C. krus</i>	60°C
<i>C. para</i>	58°C	<i>C. trop</i>	63°C

^a intra-species sequence diversity @ probe binding region

^b Cross reactivity between *C. alb* & *C. dubl* strains & probes

Multiplex PCRs - *Candida* & *Aspergillus*

- High analytical sensitivity (5-10 CFU/ml)
- Preliminary clinical evaluation
 - 31 specimens (respiratory, tissue & blood)
 - Encouraging results
 - Detected mixed infections
 - Differentiation of species with T_m peak difference of 1°C difficult
 - Peak T_m can vary slightly (1°C) in clinical samples
 - $T_m = 62-66^\circ\text{C}$ requires species confirmation
- Clinical impact to be determined

Multiplex PCRs - *Candida* & *Aspergillus*

- Pan-*Aspergillus* & pan-*Candida* assay
 - Single primer pair
 - Universal TaqMan probe
- Targets 28S rDNA gene
- In vitro detection limit <10 organisms/PCR
- Limited clinical evaluation (17 specimens)
 - PCR -ve for *Fusarium* spp.
- Broad specificity → screen presence of IFI

Multiplexed Tandem-PCR (MT-PCR)

- Multiplex (≤ 72 PCR targets)
- Nested
 - 1st Amp (10-20 cycles): multiplex (≤ 72 primer pairs)
 - 2nd Amp (35 cycles): individual, target specific assays (gene disc)
- Real-time
 - High resolution melt analysis or hyb^n probes
- Rapid (< 2 hrs)

MT-PCR to Diagnose IFI

- Anna Lau – PhD project
- Identify *Candida* spp. from clinical specimens
 - +ve blood cultures & EDTA blood
 - ↓ TAT → earlier initiation of targeted therapy
- Selection of target species:
 - Frequency to cause BSI
 - Treatment
 - High likelihood of mortality

MT-PCR to Diagnose IFI

- Target species

- 1) *C. albicans*
- 2) *C. dubliniensis*
- 3) *C. glabrata*
- 4) *C. guilliermondii*
- 5) *C. krusei*
- 6) *C. parapsilosis*
- 7) *C. tropicalis*
- 8) *Cryptococcus neoformans* complex
- 9) *Fusarium* spp.
- 10) *F. solani*
- 11) *Scedosporium prolificans*

- Additional targets

Panfungal

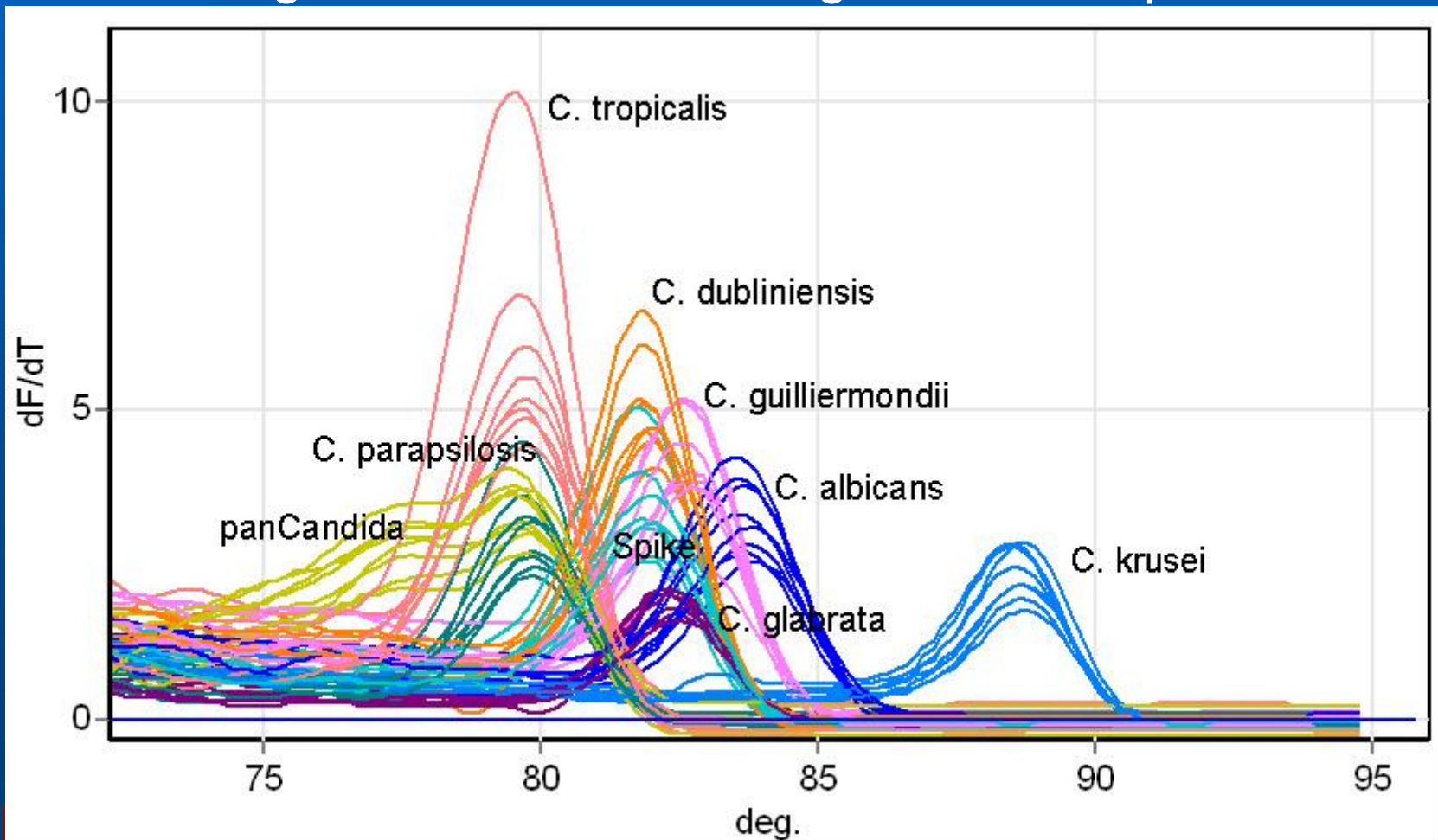
Pan-*Candida*

MT-PCR to Diagnose IFI

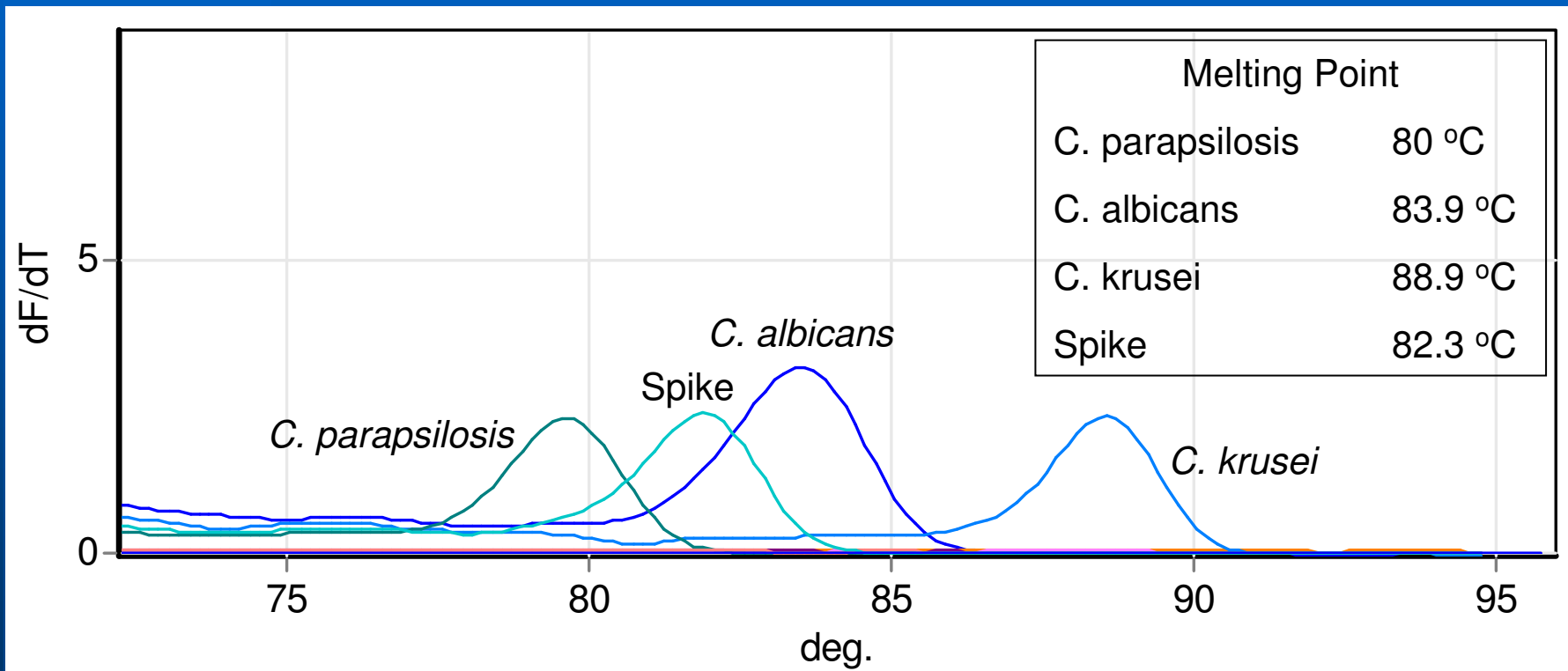
- Detection limit 10 cfu/ml
- Blood culture
 - 64 fungus +ve (60/64 MT-PCR +ve)
 - 200 bacteria +ve (All MT-PCR -ve)
 - 30 BC -ve (All MT-PCR -ve)

MT-PCR to Diagnose IFI

Melt curve generated from 44 fungal DNA samples

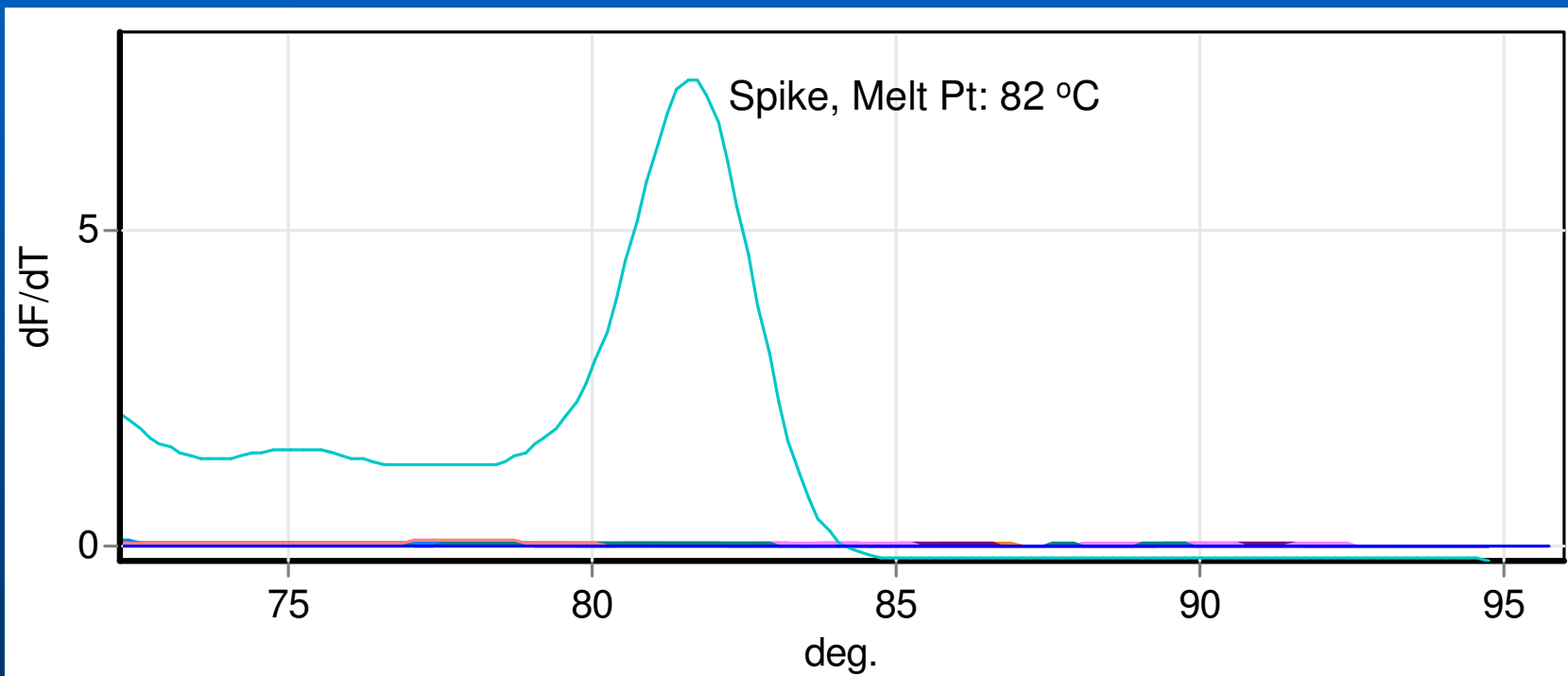


MT-PCR to Diagnose IFI



MT-PCR melt curve generated from simulated blood culture seeded with *C. albicans*, *C. krusei* & *C. parapsilosis*

MT-PCR to Diagnose IFI



MT-PCR melt curve generated from blood culture positive for *Enterococcus faecalis* & *Proteus mirabilis*

Conclusions

- Few multiplex assays to detect & ID variety of fungal species
- Role of modern clinical mycology lab:
 - Early, rapid, reliable ID
 - Improve existing methods of ID
- Real-time PCR assays
 - Promising results
 - Standardisation of DNA extraction & PCR essential
 - Need further clinical evaluation
 - EORTC/MSG criteria

Acknowledgements

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