The role of molecular diagnostics in fungal infections

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Fungal infections

Pose a diagnostic and therapeutic challenge

Invasive fungal infections (IFI)

- An overall rise in mortality due to IFI
- The absence of reliable diagnostic markers for early identification of IFI
Molecular methods in Clinical Mycology

- Polymerase chain reaction-based techniques and other molecular methods have been proven, in several cases, to be fast, cheap and reliable.
- **May provide faster and more sensitive diagnostics**
- **Can detect fungi which grew slowly (such as dermatophytes)**
- **Can detect fungi which cannot be cultured (such as Aspergillus in blood)**
- Can be automatised!
- PCR offer the potential for improved outcome in patients with IFI such as Aspergillus
Demands of molecular diagnostics in the clinical routine laboratory

- Easy to use, rapid, automated
- High sensitivity and specificity
- Low costs
- Friendly to the environment
- Faster and/or more sensitive and/or cheaper than conventional tests
The types of clinical specimens most commonly used for molecular diagnostic testing in patients at high risk of IFI include

- serum
- plasma
- whole blood
- bronchoalveolar lavage
- fresh or paraffin-embedded tissue from affected sites

- Other body fluids (such as CSF, pericard fluid, vitreous)
Molecular diagnostics

A range of different DNA extraction protocols and PCR assays:

- Conventional PCR
- Nested PCR
- Nested real-time PCR (LC)
- Real time PCR (TaqMan)
- Real time PCR (LC)
Table 1. Clinical and technical details of studies evaluating the performances of PCR in the diagnosis of IA.

<table>
<thead>
<tr>
<th>Gene targeted</th>
<th>Specimen</th>
<th>Extraction</th>
<th>Methodology</th>
<th>Micro-organism detected</th>
<th>Number of patients (samples)</th>
<th>Type of patient</th>
<th>Sensitivity/Specificity/PPV/NPV (%)</th>
<th>Detection limit</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b (mtDNA)</td>
<td>Blood, BAL, sputum</td>
<td>Chemical</td>
<td>Real-time PCR (LC)</td>
<td>A. fumigatus, A. clavatus</td>
<td>333 (1012)</td>
<td>HM, HSCT</td>
<td>100/92.6/76.5100 (BAL); 91.7/81.3/49.3/98 (Blood)</td>
<td>10 fg</td>
<td>Buchheidt et al. 2004</td>
</tr>
<tr>
<td>28S rDNA</td>
<td>Blood</td>
<td>Chemical + Mechanical disruption</td>
<td>Nested real-time PCR (LC)</td>
<td>A. fumigatus, A. flavus, A. nidulans, A. niger</td>
<td>203 (401)</td>
<td>HM, HSCT, Other</td>
<td>92.3/94.6/60/99.3</td>
<td>5 CFU.ml-1</td>
<td>White et al. 2006</td>
</tr>
<tr>
<td>18S rDNA</td>
<td>Plasma</td>
<td>Chemical</td>
<td>Real-time PCR (LC)</td>
<td>Aspergillus spp</td>
<td>96 (1251)</td>
<td>HM, HSCT</td>
<td>55/93/40/96</td>
<td>40 copies.ml-1</td>
<td>Kawazu et al. 2004</td>
</tr>
<tr>
<td>18S rDNA</td>
<td>Blood, Biopsies, BAL, CSF, sputum, others</td>
<td>Chemical + Mechanical disruption</td>
<td>Real-time PCR (LC)</td>
<td>A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus</td>
<td>379 (1650)</td>
<td>HSCT, SOT, cancer</td>
<td>ns</td>
<td>2 CFU.ml-1</td>
<td>Klingspor et al. 2006</td>
</tr>
<tr>
<td>18S rDNA and mtDNA</td>
<td>Biopsies</td>
<td>Freeze-thaw + Chemical</td>
<td>Nested PCR, Gel electrophoresis</td>
<td>A. fumigatus</td>
<td>21 (ns)</td>
<td>HM, AIDS</td>
<td>n/s</td>
<td>ns</td>
<td>Rickerts et al. 2006</td>
</tr>
</tbody>
</table>

HM, Haematological malignancies; SOT, Solid Organ Transplant; HSCT = haematopoietic stem cell transplant, CSF, Cerebrospinal fluid; BAL, Bronchoalveolar lavage; LC, lightCycler; CFU, colony forming unit; ns, not specified.
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<tr>
<td>ITS</td>
<td>Biopsies</td>
<td>Chemical</td>
<td>PCR, Gel electrophoresis</td>
<td>Panfungal</td>
<td>62 (75)</td>
<td>Proven IA</td>
<td>ns</td>
<td>ns</td>
<td>Lau et al. 2007</td>
</tr>
<tr>
<td>ITS</td>
<td>Blood, BAL, biopsies, CSF, others</td>
<td>Chemical</td>
<td>Real-time PCR (LC)</td>
<td>A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus</td>
<td>31 (31)</td>
<td>Various malignancies</td>
<td>ns</td>
<td>5-10 CFU/ml (blood), 50 CFU/ml (CSF)</td>
<td>Schabereiter et al. 2007</td>
</tr>
<tr>
<td>Cytochrome b (mtDNA)</td>
<td>CSF</td>
<td>Chemical</td>
<td>Real-time PCR (LC)</td>
<td>A. fumigatus,</td>
<td>6 (35)</td>
<td>HM</td>
<td>ns</td>
<td>15 CFU/ml</td>
<td>Hummel et al. 2006</td>
</tr>
<tr>
<td>18S rDNA</td>
<td>Blood, biopsies, BAL</td>
<td>Chemical + Mechanical disruption</td>
<td>PCR-ELISA</td>
<td>A. fumigatus, A. flavus, A. nidulans, A. terreus</td>
<td>36 (241)</td>
<td>HM, SOT</td>
<td>41.6/66.6/71.4/36.6 (blood), 87.5/58.3/80.7/70 (BAL/biopsies)</td>
<td>ns</td>
<td>Lass-Floerl et al. 2004</td>
</tr>
<tr>
<td>Alkaline Protease and mtDNA</td>
<td>BAL</td>
<td>Chemical</td>
<td>PCR, Southern blot</td>
<td>A. fumigatus, A. terreus, A. flavus, A. niger</td>
<td>249 (ns)</td>
<td>Cancer</td>
<td>80/93/38/99</td>
<td>ns</td>
<td>Raad et al. 2002</td>
</tr>
</tbody>
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HM, Haematological malignancies; SOT, Solid Organ Transplant; CSF, Cerebrospinal fluid; BAL, Bronchoalveolar lavage; LC, lightCycler; CFU, colony forming unit; n/a, ns, not specified.
PCR methods in Clinical Mycology

The implementation of this technique, has been hampered by
• a lack of standardisation of molecular targets, specimens, extraction protocols, and PCR platforms
• and is therefore urgent that a consensus is reach on the type of specimen, molecular target and technique to be used
The choice of specimen has a great influence on the extraction methodology

Easy to obtain: Serum and blood
Which blood fraction is best to test?
- Serum/plasma : Circulating DNA?
- Blood : yeast cells, hyphael elements, circulating DNA?
- EDTA- blood (Avoid citrate and heparin)

More difficult to obtain and to repeat
- BAL
- CSF
- Pleura
- Biopsi material
PCR recommendations

The current status of the technical and clinical validation of PCR for *Candida* and *Aspergillus* in blood and other fluids does not allow for a recommendation for clinical use.


*Aspergillus PCR: one step closer towards standardisation* J Clin Microbiol. 2010 Feb
EAPCRI consensus regarding Aspergillus

A consensus has been reached concerning the best blood fraction to test, blood volume, how to break the fungal cell wall and for DNA extraction

- >3 * whole-blood in EDTA tubes, in order to have access to both the free- and the cell-associated DNA
- white cell lysis buffer
- bead -beating
- either spin columns or an automated DNA extraction method can be performed
- Internal control
- DNA should be eluted < 100ul

*As the fungal load in the circulation of a patient may be less than 1 genome per ml
Which PCR assay is most suitable?

White et al. 2006 compared

- two primer sets (28S and 18S) and three machines
- Light Cycler (Roche)
- TaqMan (Applied Biosystem)
- Rotor-Gene (Corbett Research)

The sensitivity, specificity, NPV, PPV, were higher with the 28S than the 18S primer set

- The platforms influenced the assay
- Sensitivity and NPV were 100% with the Taqman machine
- Specificity and PPV were 100% with the Rotor-Gene system
Which PCR assay is most suitable?

- The sensitivity of PCR assays with blood samples from healthy donors spiked with Aspergillus conidia might not reflect the sensitivity of the assay with clinical blood samples.

- We do not know if conidia, hyphal fragments or free circulating DNA are detected.

- Genomic DNA from bacteria and virus and parasites may be present.
There are certain questions to be addressed using those assays

- The frequency of prospective sampling (2-3 times per week?)

- The number of positive results of a PCR assay required to initiate antifungal therapy is not known (2 consecutive PCR results*)

- How to interpret a single positive test? (A transient presence of fungal DNA might be possible but an infection cannot be excluded)

Florent, M., et al., J Infect Dis, 2006
Buchheidt, D., et al., Br J Haematol, 2004
Kawazu, M., et al., J Clin Microbiol, 2004
The issues of concern include:

- The validation of the techniques in clinical studies
- The correct interpretation of test results
Clinical validity also needs to be established in large scale prospective trials

- designed to determine the performance and utility of the *Aspergillus* PCR with specimens from high risk populations
MycAssay™ Aspergillus is a molecular diagnostic kit for detection of *Aspergillus* spp. genomic DNA extracted from bronchial samples

This assay offers
- a commercial, standardised assay for the molecular detection of *Aspergillus*

- fast detection of *Aspergillus* spp. and the potential for increased sensitivity for *Aspergillus* spp

- Sensitivity = 94%; Specificity = 91%;
- PPV = 97%; NPV = 83%

Bronchoalveolar lavage is positive by culture in approximately 40% of cases
Comparison of Whole Blood, Serum and Plasma for Early Detection of Candidemia by Multiplex-Tandem PCR

Candida DNA was detected in
- serum (71%)
- plasma (75%)
- whole blood (54%)

Real-time Fungal PCR assay was developed and established June 2002 at Huddinge

**DNA extraction**
Chemical + Mechanical disruption + Automatic extraction (MagNaPure LC)

**For measuring the DNA concentration**
NanoDrop ND-1000 Spectrophotometer

**Selection of target** : Multicopy gene; 18S rRNA gene

**Real-Time PCR**
LightCycler 2.0

R-T Fungal PCR assay

A method for detection of Candida and Aspergillus DNA
• in EDTA-blood samples and plasma
• body fluids such as BAL, CSF, bile, pleura, ascites
• in biopsy specimens.
R-T Fungal PCR assay with hybridisation probes

Provides rapid (6 h) and sensitive (2-10 genome) detection of

- Aspergillus and Candida to genus level

- Identification of Candida to species level
  C. albicans, C. glabrata, C. krusei, C. tropicalis,
  C. parapsilosis and C. lusitaniae.

Klingspor L, Jalal S. Clin Microbiol Infect 2006; 12:745-753
Fungal PCR results in tissue from 31 biopsies compared to culture and direct microscopy

- **Candida PCR pos 10 / 31**
  - 4 liver
  - 2 colon
  - 2 viterous
  - 1 lung
  - 1 skin

  - Sensitivity 100%
  - Specificity 100%

- **Aspergillus pos 12/31**
  - 1 cerebri
  - 2 cerebellum
  - 2 lung
  - 1 duodenum
  - 1 artery
  - 1 kidney
  - 2 pleura
  - 1 heart
  - 1 liver

  - Sensitivity 100%
  - Specificity 100%
Dermatophytes

- Diagnostic
  - Fluorescence microscopy
  - Culture 3 weeks
  - PCR 5h - 1-day
Molecular detection of dermatophyte species directly from clinical specimens

1. Multiplex PCR – Gelelectrophoresis (5h) nails
2. A PCR-ELISA method (24 h) skin scrapings & nails
3. Real-time PCR (4 h after overnight lysis) nail, skin and hair samples

The new ISHAM working group

"PCR-based diagnosis of Dermatophyte infections: on the way to a consensus"

just established!
PCR in combination with:

- Gelelectrophoresis
- ELISA
- Sequencing
  - long sequences
  - short sequences (Pyrosequencing)
- In sity hybridisation
Identification of yeasts and moulds
to species level

Conventional methods

Molecular typing of fungal isolates
- *In situ* hybridisation
- Pyrosequencing
- Sequencing
Background

- Increasing incidence of blood infections and invasive infections
- Candida species
  - *C. albicans* (35-65%)
  - Non-*Candida* albicans:
    - *C. glabrata*
    - *C. parapsilosis*
    - *C. krusei*
    - *C. tropicalis*
Procedure Overview – Simple & Easy

Prepare Smear

- Add drop from BC+
- Fix bacteria/yeast onto slide
  - Heat
  - Methanol, or
  - Flame fixation

20 min.

Hybridize

- Add PNA probe
- Probe enters cells and binds to target rRNA sequence, if present

30 min.

Wash

- Immerse slide in Wash Solution
- Unbound and excess PNA probe removed from cells and slide

30 min.

Examine

- Fluorescence microscopy using 60x or 100x oil objective
- Target bacteria/yeast fluoresce

2 min.
Yeast Traffic Light® PNA FISH®

C. albicans
C. parapsilosis

C. tropicalis
C. glabrata
C. krusei

C. albicans/C. glabrata PNA FISH®

C. albicans
C. glabrata
PNA FISH
Peptid Nucleic Acid = PNA
Fluorescence In Situ Hybridisering = Fish

[Image of a green fluorescent signal possibly representing a cell or biological sample labeled as PNA FISH]
Identification of medically important yeasts by the Pyrosequencing™ technology
**Fast** - up to 96 samples analysed in less than 6 h

**Work flow**

- Sample preparation
- PCR
- PCR product preparation
- Pyrosequencing
- Sequence analyses

**Accurate** - identification to species level by software against local database
The pyrogram™

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...peak height is proportional to the number of incorporated nucleotides

---

T GG CC GGG T C A C G A GG CCC TA ...

---

.....peak height is proportional to the number of incorporated nucleotides
Sample ID: 132
Well: B2
PSQ run: 050105 gold Kit
Entry ID: svamp20
Sequence library: andreys db pr3 (2005-01-28, 11:07)
Query sequence: CCTCAAGTAACTGTCCTGGTGTCGCCAGGAGGGCTAGCCAGAGGAAGAG

Result: Candida norvegensis (from BLAST, pr. 1)
Quality: Good

Hit 1: Candida norvegensis (from BLAST, pr. 1)
Score: 100
Identities: 53/53 (100%)
Gaps: 0/53 (0%)
E-value: 7.80e-070

Query 0: CCTCAAGTAACTGTCCTGGTGTCGCCAGGAGGGCTAGCCAGAGGAAGAG
Library 0: CCTCAAGTAACTGTCCTGGTGTCGCCAGGAGGGCTAGCCAGAGGAAGAG

Hit 2: Candida inconspicua (LOCUS AP201301)
Score: 81.3
Identities: 48/57 (84%)
Gaps: 8/57 (14%)
E-value: 6.93e-055

Query 0: CCTCAAGTAACTGTCCTGGTGTCGCCAGGAGGGCTAGCCAGAGGAAGAG
Library 0: CCTCAAGTAACTGTCCTGGTGTCGCCAGGAGGGCTAGCCAGAGGAAGAG
Zygomycosis

- Cultures from infected tissues are often negative
- The identification of a Zygomycet in tissue is difficult
- Different Zygomycetes share similar morphology according to histopathology/direct microscopy
New molecular methods for the identification of Zygomycetes in culture and tissue

Culture:
- The identification to species level of a strain isolated from culture

Some Methods: PCR+ sequencing. Real-time PCR

Tissue:
- Unfixed fresh/frozen material
- Formalin-fixed, paraffin-embedded biopsies

Some Methods: PCR+ sequencing, Real-time PCR, in situ hybridization

- Standardisation of the techniques are needed
- to improve sensitivity for identification in tissue

Dannaoui E. Clin Microbiol Infect 2009
Sequencing ABI prisma™

- A technology for long DNA sequences
- 3–4 working days
- To identify fungi from cultures (fungi that are difficult to identify by conventional methods)
Saksenea vasiformis
Molecular diagnostics in fungal infections are here to stay....
But standardisation of these techniques are needed