Diagnostic Methods for Invasive Fungal Infections in COVID-19 Patients

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Invasive fungal Infections (IFIs) present an increasing global burden in immunocompromised and other seriously ill populations.

Globally, over 300 million people are afflicted with a serious fungal infection.

Over 300 million Infected! 1.6 million killed!
IFIs complicating COVID-19

COVID-19 associated pulmonary aspergillosis (CAPA)

COVID-19 associated mucormycosis (CAM)

Invasive Candidiasis

Pneumocystis pneumonia
Oropharyngeal candidiasis in hospitalised COVID-19 patients from Iran: Species identification and antifungal susceptibility pattern

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The double-edged sword of systemic corticosteroid therapy in viral pneumonia: A case report and comparative review of influenza-associated mucormycosis versus COVID-19 associated mucormycosis

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Mucormycosis in patients with COVID-19: A cross-sectional descriptive multicentre study from Iran

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Proven Aspergillus flavus pulmonary aspergillosis in a COVID-19 patient: A case report and review of the literature

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Benefits of Early Diagnosis of IFIs in COVID-19 patients

- Antifungal selection and effectiveness
- Prevention of angioinvasion & dissemination
- Reducing healthcare costs
- Improved outcome and survival

- Prevention of angioinvasion & dissemination
Laboratory Methods for Diagnosis of IFI

Conventional Methods

- Direct examination
  - Hematoxylin and Eosin stain
  - Periodic acid Schiff stain
  - Gomori methenamine silver stain
  - KOH - Wet mount
  - Calcofluor or Blankophor

- Culture
  - Isolation, identification, and AFST
  - Conventional PCR, PCR_RFLP
  - DNA sequencing
  - Real time PCR

- Molecular methods

- Serological methods
  - B-D Glucan
  - Galactomannan
  - Immuno-histochemistry

Non-Culture based Methods
# IFI definitions

<table>
<thead>
<tr>
<th>Patient population</th>
<th>Invasive Fungal Disease*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive culture/histology</td>
<td>Proven</td>
</tr>
<tr>
<td>Prolonged neutropenia+ fever+ lung infiltrates/biomarker (GM, β-DG)</td>
<td>Probable</td>
</tr>
<tr>
<td>Prolonged neutropenia+ fever</td>
<td>Possible</td>
</tr>
</tbody>
</table>

Limitation of conventional diagnostics methods

- **Invasive specimen collection** (may be contraindicated in many neutropenic patients).
- Histopathology alone is not always complete.
- The fungal elements may not be abundant so as to be seen.
- Time-consuming
- Needs great personal expertise.
- Cultures are negative in approximately 50% of patients with documented invasive IFIs and is not possible in PJP.
Fungal species identification may take many days.

Some emerging species (such as *A. elegans* and *S. vasiformis*) fail to sporulate on routine media.

*Candida auris* (a new strain of the genus Candida) is a multidrug-resistant (MDR) yeast.
Limitation of direct examination method

- Direct examination may be confusing in case of atypical hyphae or co-infection.

- In samples from non-sterile sites, it can be difficult to distinguish real infection versus contamination, or colonization.

- Distinguish of septated hyphae of Aspergillus, Fusarium, and Scedosporium spp. are not possible.

Fungal diagnosis: how do we do it and can we do better?, Current Medical Research & Opinion. 2013
Biological infection

Clinical infection

Pathological changes

INFECTION

BDG
Aspergillus GM
PCR

Targeted prophylaxis/
Pre-emptive therapy

Empirical/targeted therapy

Conventional diagnostic methods
Non-Culture based Method
β (1,3)-D-Glucan:

A fungal cell wall component, is detectable in the blood during IFIs.

The antigen assay can be used in detecting infections caused in species of *Aspergillus*, *Candidia*, *Fusarium*, *Trichosporon*, *Saccharomyces*, *Acremonium*, *Penicillium*, *Cephalosporium*, *C. immitis*, *H. capsulatum*, and *Pneumocystis jirovecii*, except *Cryptococcus* and *Mucorales* spp.

- Sensitivity, specificity, negative and positive predictive value predictive value ranging from 64-90%, 73-100%, 83-89% and 97-100%, respectively.

- Based on excellent negative predictive value of this test (nearly 100%) lack of BDG detection is most useful for excluding invasive fungal infection.
The cause associated with false positive readings includes:

- Presence of serious bacterial Infections such as *Pseudomonas aeruginosa*.
- Cellulose membrane associated with hemodialysis.
- The surgical gauze.
- Coagulation factors
- Some antibiotics or medications such as *piperacillin tazobactam*, *ampicillin*, *amoxicillin*, *azithromycin* and *pentamidin*.
Serology

Non-Culture based Method

Galactomannan (GM)

The polysaccharide component of Aspergillus cell wall.

GM is released during hyphal growth rather than from conidia.

High assay sensitivity (76–88%) and specificity (87–100%) for IA diagnosis have been reported for GM in BAL.

Monitoring of GM during antifungal therapy allows progression of treatment to be measured.

Fungal diagnosis: how do we do it and can we do better?, Current Medical Research & Opinion. 2013
Antigen detection in bronchoalveolar lavage fluid for diagnosis of fungal pneumonia
Chadi A. Hage, Kenneth S. Knox, Thomas E. Davis and Lawrence J. Wheat

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Microscopy</th>
<th>Culture</th>
<th>Serum GM</th>
<th>BAL GM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>53%</td>
<td>50%</td>
<td>55%</td>
<td>100%</td>
<td>[3**]</td>
</tr>
<tr>
<td>Specificity</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>88%</td>
<td>CO 1.0</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>40%</td>
<td>69%</td>
<td>40%</td>
<td>100%</td>
<td>[4]</td>
</tr>
<tr>
<td>Specificity</td>
<td>NS</td>
<td>100%</td>
<td>100%</td>
<td>73/80%</td>
<td>CO 0.5/1.0</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>56%</td>
<td>69%</td>
<td>58%</td>
<td>100%</td>
<td>[5]</td>
</tr>
<tr>
<td>Specificity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>59%</td>
<td>CO 2.0</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>58%</td>
<td>[6]</td>
</tr>
<tr>
<td>Specificity</td>
<td>55%</td>
<td>69%</td>
<td>72%</td>
<td>96%</td>
<td>CO 0.5</td>
</tr>
<tr>
<td>Specificity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>96%</td>
<td>[7]</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>100%</td>
<td>[8]</td>
</tr>
<tr>
<td>Specificity</td>
<td>50%</td>
<td>40%</td>
<td>25%</td>
<td>100%</td>
<td>[9]</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>93%</td>
<td>93%</td>
<td>97%</td>
<td>84/91%</td>
<td>CO 0.5/1.0</td>
</tr>
<tr>
<td>Specificity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>54%</td>
<td>[10]</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>100%</td>
<td>CO 0.5</td>
</tr>
<tr>
<td>Specificity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>78%</td>
<td>[11]</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>73%</td>
<td>[12]</td>
</tr>
<tr>
<td>Specificity</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>92%</td>
<td>CO 0.96</td>
</tr>
</tbody>
</table>

BAL, bronchoalveolar lavage; CO, cut-off; GM, galactomannans; NS, not significant.

Galactomannan antigen
Antigen detected in plasma, serum, BAL, or CSF
Any 1 of the following:
- Single serum or plasma: ≥1.0
- BAL fluid: ≥1.0
- Single serum or plasma: ≥0.7 and BAL fluid ≥0.6

Cutoff: specificity does increase!

Cutoff: specificity does increase!
A major drawback of the GM test is a propensity for false-positive and sometimes false-negative results:

**False-positive results:**
- Use of β-lactam antibiotics
- Colonization with *Bifidobacterium*
- Presence of histoplasmosis, blastomycosis, or penicillinosis

**False negative results:**
- Antifungal (anti-mold) therapy
- Low fungal burden
- An infection that has been walled off in the tissue
Non-Culture based Method

lateral-flow assay

Results of LFA tests of BAL samples from SOT patients with IA

<table>
<thead>
<tr>
<th>Pat. No.</th>
<th>Underlying disease</th>
<th>Patient’s age (years)/sex</th>
<th>BAL GM value</th>
<th>BAL LFD result</th>
<th>Fungal growth in BAL culture</th>
<th>IPA according to EORTC 2008 criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOT patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>LTx 2012</td>
<td>43/m</td>
<td>0.52</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>HTx 2004, KTx 2011</td>
<td>48/m</td>
<td>0.42</td>
<td>+</td>
<td>Aspergillus fumigatus</td>
<td>Probable</td>
</tr>
<tr>
<td>3</td>
<td>KTx 2011</td>
<td>34/m</td>
<td>1.22</td>
<td>++</td>
<td>No</td>
<td>Probable</td>
</tr>
<tr>
<td>4</td>
<td>LTx 2008</td>
<td>67/m</td>
<td>0.7</td>
<td>+</td>
<td>Aspergillus fumigatus</td>
<td>Probable</td>
</tr>
<tr>
<td>5</td>
<td>KTx 2011</td>
<td>55/f</td>
<td>4.66</td>
<td>+++</td>
<td>Aspergillus fumigatus</td>
<td>Probable</td>
</tr>
<tr>
<td>6</td>
<td>LTx 2011</td>
<td>65/m</td>
<td>0.73</td>
<td>++</td>
<td>No</td>
<td>Possible</td>
</tr>
<tr>
<td>7</td>
<td>LTx 2012</td>
<td>58/m</td>
<td>19.86</td>
<td>+++</td>
<td>Aspergillus fumigatus</td>
<td>Probable</td>
</tr>
<tr>
<td>8</td>
<td>LTx 2007</td>
<td>63/m</td>
<td>Negative</td>
<td>–</td>
<td>Lichtheimia corymbifera</td>
<td>No</td>
</tr>
<tr>
<td>9</td>
<td>HTx 2012</td>
<td>30/m</td>
<td>Negative</td>
<td>–</td>
<td>Candida albicans</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>KTx 1999</td>
<td>67/f</td>
<td>Negative</td>
<td>–</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Comparison of LFA with the goldstandard (culture and/or India Ink) to diagnoses of cryptococcosis

<table>
<thead>
<tr>
<th>CSF</th>
<th>Culture/ India Ink</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNK</td>
<td>Positive</td>
</tr>
<tr>
<td>CrAg LFA</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Sensitivity:100% (97.91%-100%)*
Specificity:100% (96.16%-100%)*
Agreement:100% (96.72%-100%)*
*95% CI

The capsule of the fungal pathogen Cryptococcus neoformans. AdvAppl Microbiol, 2009, 68:
The serological biomarkers are not applicable in diagnosis of mucormycosis

<table>
<thead>
<tr>
<th>Method</th>
<th>Target structure</th>
<th>Clinical specimens</th>
<th>Clinical studies</th>
<th>Sensitivity/ specificity</th>
<th>Cross-reactivity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td><em>Rhizopus arrhizus</em> and <em>Rhizopus pusillus</em> antibody</td>
<td>Sera from patients infected with <em>Mucorales</em> spp. (n = 43)</td>
<td>None</td>
<td>81%/94%</td>
<td><em>Candida</em> spp., <em>Aspergillus</em> spp.</td>
<td>Minimal positivity titer dilution of 1:400</td>
</tr>
<tr>
<td>ELISA (CLISpot) or immunocytofluorimetric assays</td>
<td>Mucorales specific IFN-γ-producing T cells</td>
<td>Peripheral blood samples from patients with proven IM (n = 80)</td>
<td>None</td>
<td>None</td>
<td><em>R. pusillus</em> (6/4)</td>
<td>Positivity for 2–10 SFCs</td>
</tr>
</tbody>
</table>
DNA-based diagnostic methods are feasible for **early** and **accurate** diagnosis of IFIs.

**Direct detection of fungi in clinical specimens**
- Panfungal PCR Assays
- *Aspergillus* PCR
- Invasive Candidiasis PCR
- Mucormycosis PCR
- *Pneumocystis jirovecii* PCR

**Genome-based fungal identification**
- ITS Sequencing
- Whole Genome Sequencing

**Molecular detection of antifungal drug resistance**
- Detect azole resistance in *Aspergillus fumigatus*
- Detect azole resistance in *Candida* species
DNA extraction

The sensitivity of DNA-based diagnostic methods is strongly influenced by the amount and extractability of fungal DNA in a clinical sample, which in turn depends on the sample type.

In comparison with fresh tissue, DNA extractability from formalin-fixed paraffin-embedded tissue (FF-PET) is reduced due to the adverse effect of formalin on DNA.
Even closely related species complexes have been identified within a few minutes, but expensive.  

Conventional PCR with species-specific primers followed by gel electrophoresis is simple and cost-effective for identifying most frequently isolated fungi.
Detection and Identification of 21 clinically important yeast pathogens through 21plex-PCR

**Multiplex PCR**

- **Direct yeast colony**
  - **Tube 1**: 7 top *Candida* species
  - **Tube 2**: 7 Uncommon *Candida* species
  - **Tube 3**: 7 Common yeast species
Quantitative real-time *Pneumocystis jirovecii* PCRs have replaced microscopy and immunofluorescent stains in many diagnostic laboratories.

Although distinguishing infection may be problematic in non-HIV-infected patients.

Comparison between IFA (number of asci or trophic forms per field of vision) and PCR (copies/ml) of all tested BAL samples.

Bossart et al. BMC Res Notes. 2020
Real-Time PCR-HRM assay is highly suitable for routine clinical diagnostics.
Conclusion

Outcomes for COVID-19 patients with IFIs are best achieved when rapid and accurate diagnosis enables early treatment.

Clinicians need to better understand the newer available diagnostic tools, and combine them with each other and/or traditional diagnostics to achieve rapid, accurate diagnosis.

There is also a need for less expensive, user-friendly, and more easily implemented as point-of-care approaches to early diagnosis.
Thanks for your attention!