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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, automised**
- **High sensitivity and specificity**
- **Low costs**
- **Friendly to the enviroment**
- **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.

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

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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ITS	Biopsies	Chemical	PCR, Gel electrophoresis	Panfungal	62 (75)	Proven IA	ns	ns	Lau et al. 2007
ITS	Blood, BAL, biopsies, CSF, others	Chemical	Real-time PCR (LC)	*A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus*	31 (31)	Various malignancies	ns	5-10 CFU/ml (blood), 50 CFU/ml (CSF)	Schabereiter et al. 2007
Cytochrome b (mtDNA)	CSF	Chemical	Real-time PCR (LC)	*A. fumigatus,*	6 (35)	HM	ns	15 CFU/ml	Hummel et al. 2006
18S rDNA	Blood, biopsies, BAL	Chemical + Mechanical disruption	PCR-ELISA	*A. fumigatus, A. flavus, A. nidulans, A. terreus*	36 (241)	HM, SOT	41.6/66.6/71.4/ 36.6 (blood), 87.5/58.3/80.7/ 70 (BAL/biopsies)	ns	Lass-Floerl et al. 2004
Alkaline Protease and mtDNA	BAL	Chemical	PCR, Southern blot	*A. fumigatus, A. terreus, A. flavus, A. niger*	249 (ns)	Cancer	80/93/38/99	ns	Raad et al. 2002

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.

White PL, Bretagne S, Klingspor L, Melchers WJ, McCulloch E, Schulz B, Finnstrom N, Mengoli C, Barnes RA, Donnelly JP; Juergen Loeffler on behalf of the European Aspergillus PCR Initiative.

Aspergillus PCR: one step closer towards standardisation J Clin Microbiol. 2010 Feb

EAPCRI concensus 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 whith clinical blood samples

- we do not know if conida, 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)

* Klingspor L, Loeffler J. Med Mycol. 2009
Florent, M., et al., J Infect Dis, 2006
Buchheidt, D., et al., Br J Haematol, 2004
Kawazu, M., et al., J Clin Microbiol, 2004
Hebart, H., et al., J Infect Dis, 2000.

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**

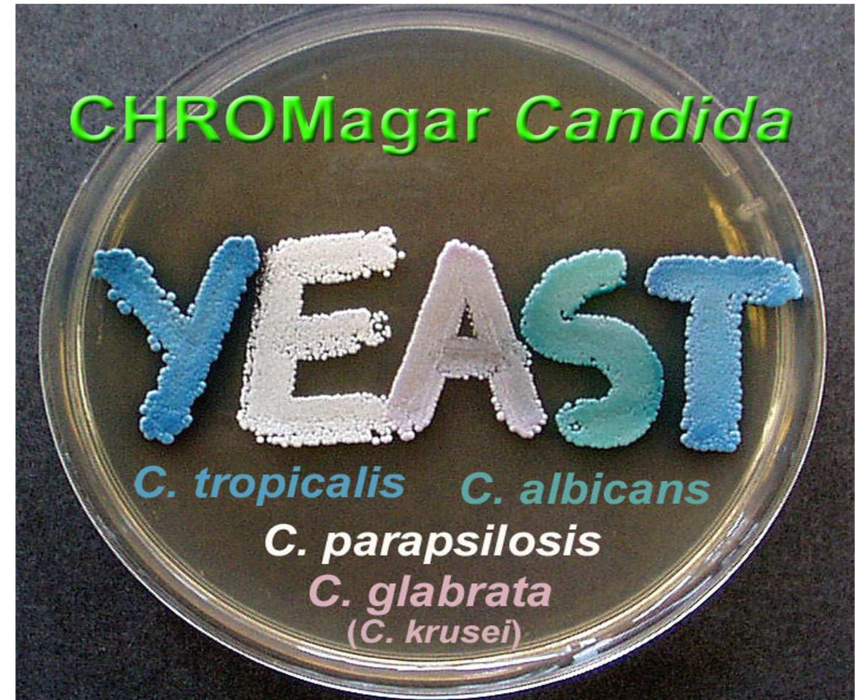
MycAssay™ Aspergillus is a molecular diagnostic kit for detection of *Aspergillus* spp. genomic DNA extracted from bronchial samples

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%)**

Lau A, et al. J Clin Microbiol. 2009

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

- **Klingspor L and Jalal S. Clin Microbiol Infect. 2006:12(8): 745-53.**

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**

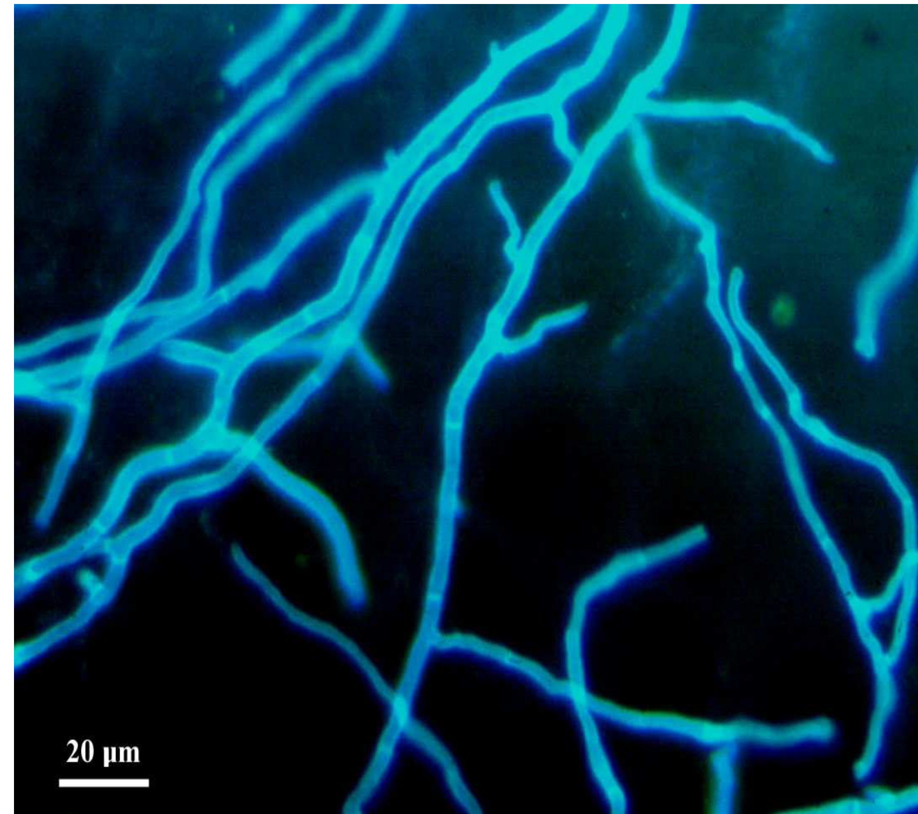
- **Sensitivity 100%**
- **Specificity 100%**

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

- **Sensitivity 100%**
- **Specificity 100%**

Dermatophytes

- **Diagnostic**
  - Fluorescens microscopi
  - Culture 3 veeks
  - 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

1. Brillowska-Dabrowska A, et Med Mycol. 2010
2. Beifuss B, et al Mycoses. 2009 Sep
3. Bergmans AM ,et al. Clin Microbiol Infect. 2009

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

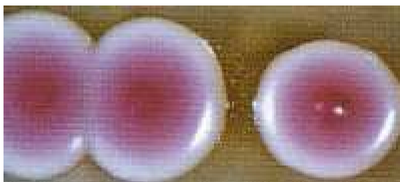
Conventional methods

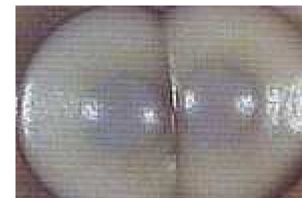
Molecular typing of fungal isolates

- ***In situ* hybridisation**
- **Pyrosequencing**
- **Sequencing**

Background

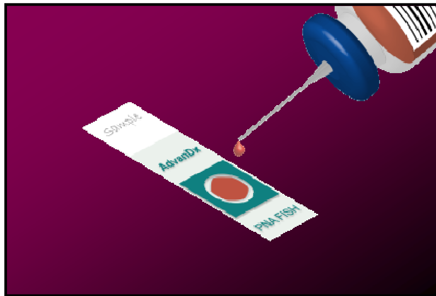
- Increasing incidence of blood infections and invasive infections
- Candida species
  → *C. albicans* (35-65%)
  → Non-Candida albicans:
    - *C. glabrata*
    - *C. parapsilosis*
    - *C. krusei*
    - *C. tropicalis*





Procedure Overview – Simple & Easy

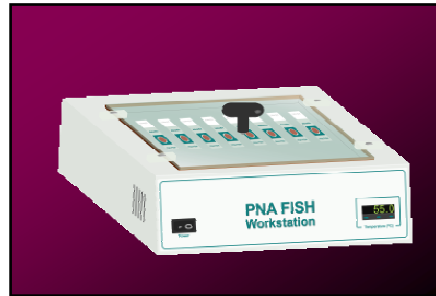
Prepare Smear



20 min.

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

Hybridize



30 min.

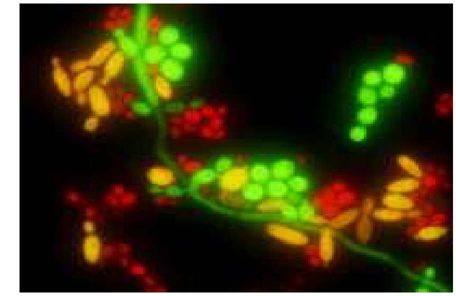
- Add PNA probe
- Probe enters cells and binds to target rRNA sequence, if present

Wash

30 min.

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

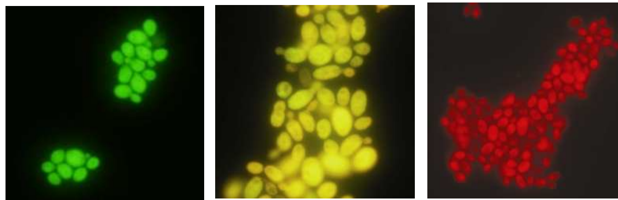
Examine



2 min.

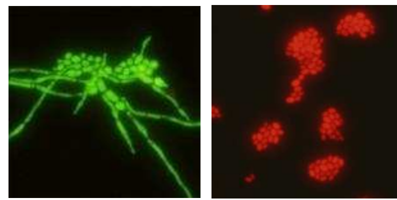
- Fluorescence microscopy using 60x or 100x oil objective
- Target bacteria/yeast fluoresce

Yeast Traffic Light® PNA FISH®



C. albicans *C. tropicalis* *C. glabrata*
C. parapsilosis *C. krusei*

***C. albicans/C.glabrata* PNA FISH®**

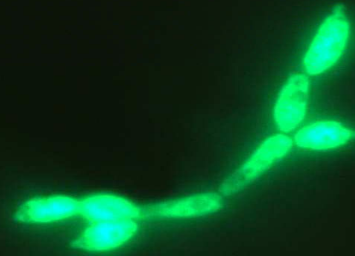


C. albicans *C. glabrata*

PNA FISH

Peptid Nucleic Acid= PNA

Fluorecence In Situ Hybridisering= Fish

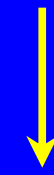


Identification of medically important yeasts by the Pyrosequencing™ technology

Fast - up to 96 samples analysed in less than 6 h

Work flow

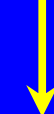
Sample preparation



PCR

PCR product preparation

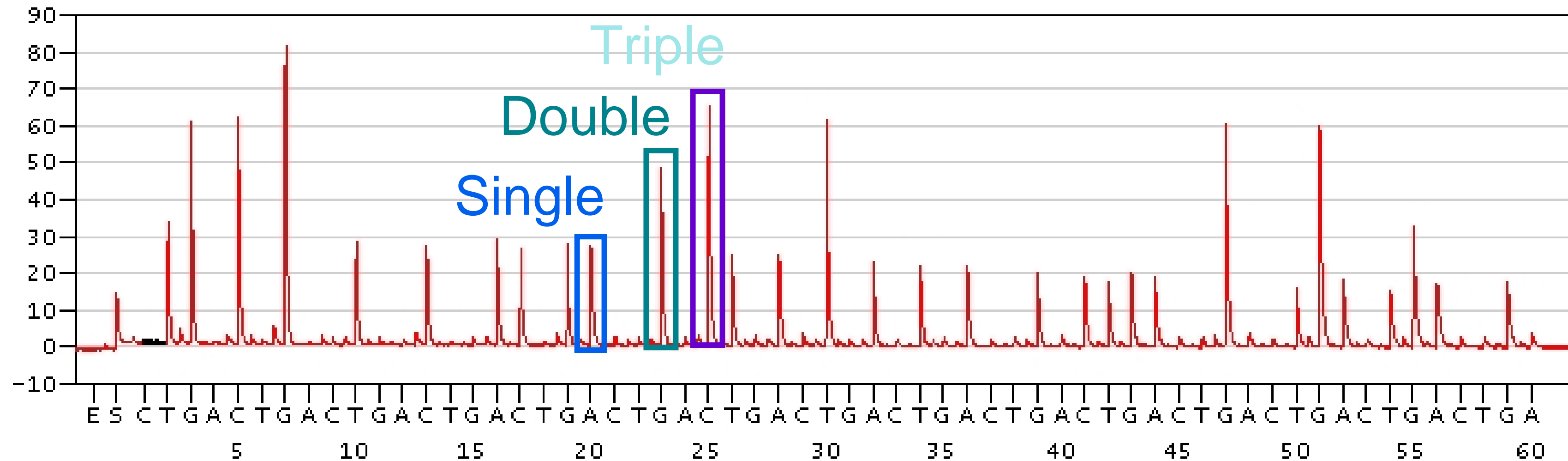
Pyrosequencing



Sequence analyses

Accurate - identification to species level by software against local database

The pyrogram™



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

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

IdentiFire Analysis report

Type

Detailed Report

Sample ID: 132

Well:	B2
PSQ run:	050105 gold kit
Entry ID:	svamp20
Sequence library:	andreys db pr1 (2005-01-28, 11:07)
Query sequence:	CCTCAAAGTAATCGTCCTGGTTCGCCGCCCACAGGGGAGGCTAGCCAGAAGGAAAAG

Result: Candida norvegensis (from BLAST, pr. 1) **Score:** 100

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  CCTCAAAGTAATCGTCCTGGTTCGCCGCCCACAGGGGAGGCTAGCCAGAAGGA 53 (57)
            |||||||||||||||||||||||||||||||||||||||||||||||||||||
Library  0  CCTCAAAGTAATCGTCCTGGTTCGCCGCCCACAGGGGAGGCTAGCCAGAAGGA 53
```

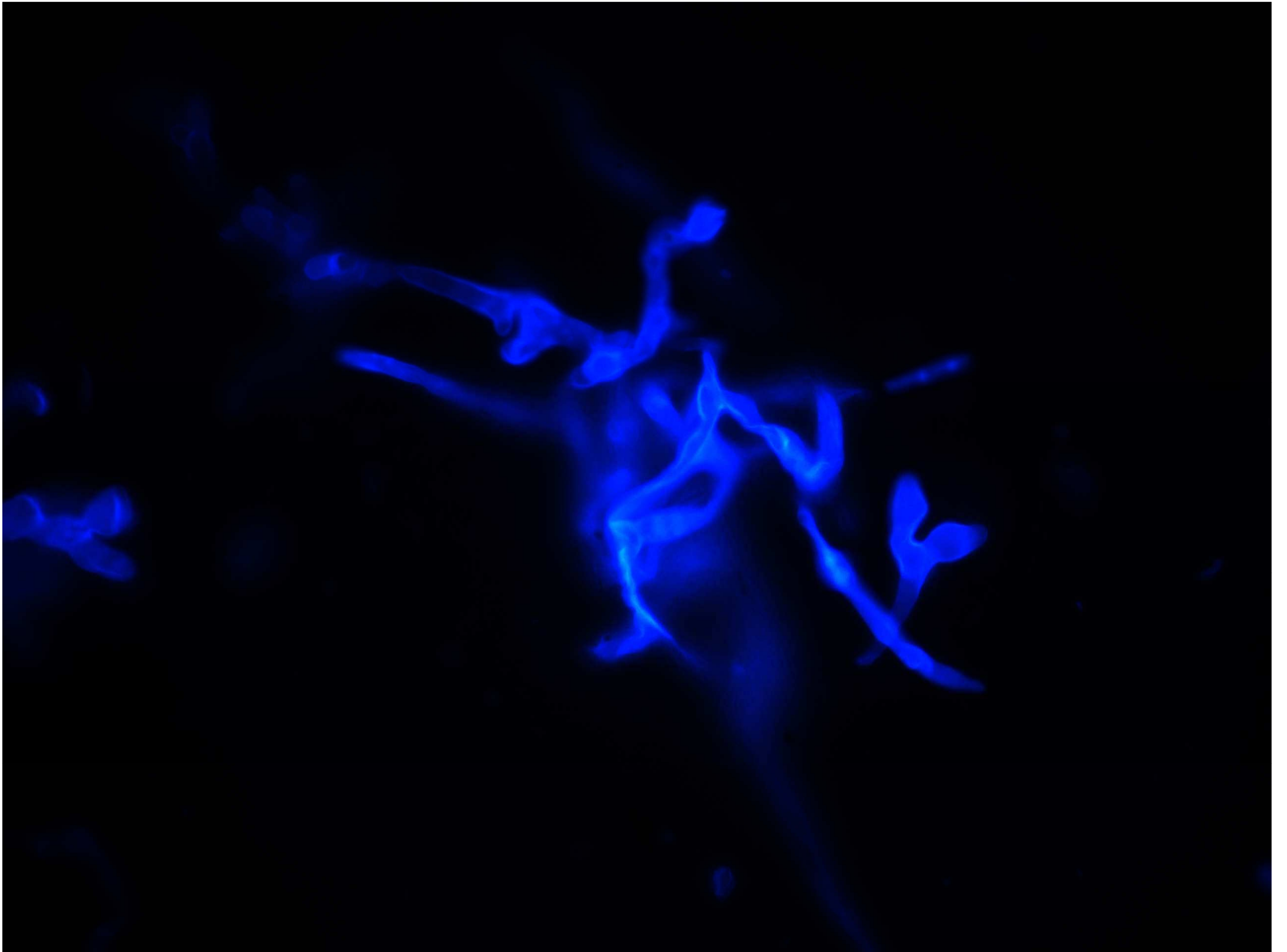
Hit 2: Candida inconspicua (LOCUS AF201301)

Score:	81.3
Identities:	48/57 (84%)
Gaps:	8/57 (14%)
E-value:	6.93e-055

```
Query    0  CCTCAAAGTAATCGTCCTGGTTCGCCGCCCACAGGGGAGGCTAGCCAGAAGGAAAAG 57
            |||||||||||||||||||||||||    ||| |||   ||||||||||||||| |
Library  0  CCTCAAAGTAATCGTCCTGGTTCGCC~~~~ACAAGGG~~~CTAGCCAGAAGGAAA~G 49
```

Zygomycosis

- Cultures from infected tissues are often negative
- The identification of a Zygomycet in tissue is difficult
- Different Zygomycetes share simular 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

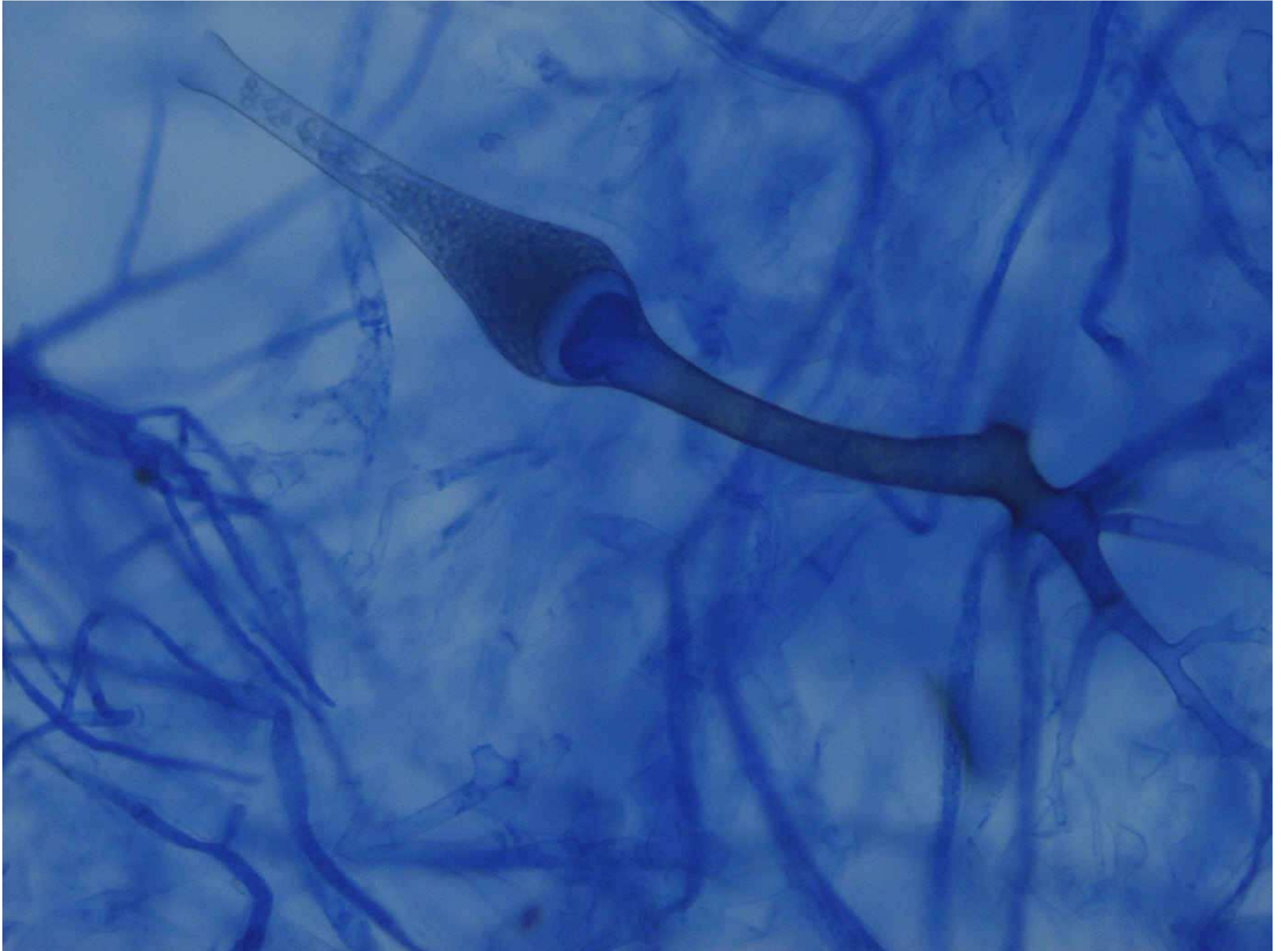
Dannaoui E. *Clin Microbiol Infect* 2009

Sequencing ABI prisma™

- A technologi for long DNA sekvenses

- 3 – 4 working days

- To identify fungi from cultures (fungi that are difficult to identify by conventionell methods)



Molecular diagnostics in fungal infections are here to stay....

BUt standardisation of these teqniques are needed