3rd Trends in Medical Mycology
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Workshop 14: Molecular Mycology

Molecular diagnostics beyond PCR

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Molecular versus "conventional" methods
Why the switch?

- In clinical microbiology, there is a constant need for alternative diagnostic methods to the existing ones
- "Traditional" diagnostic methods have well-known limitations (e. g. low sensitivity of microscopy and culture, retrospective diagnostic informations by serologic testing)

Molecular methods in the medical mycology laboratory
The main goals

- Detection of pathogenic fungi directly in the clinical specimen without prior cultivation
- Use of molecular assays to more rapidly identify fungi that are cultivated by traditional methods

Molecular methods in the medical mycology laboratory

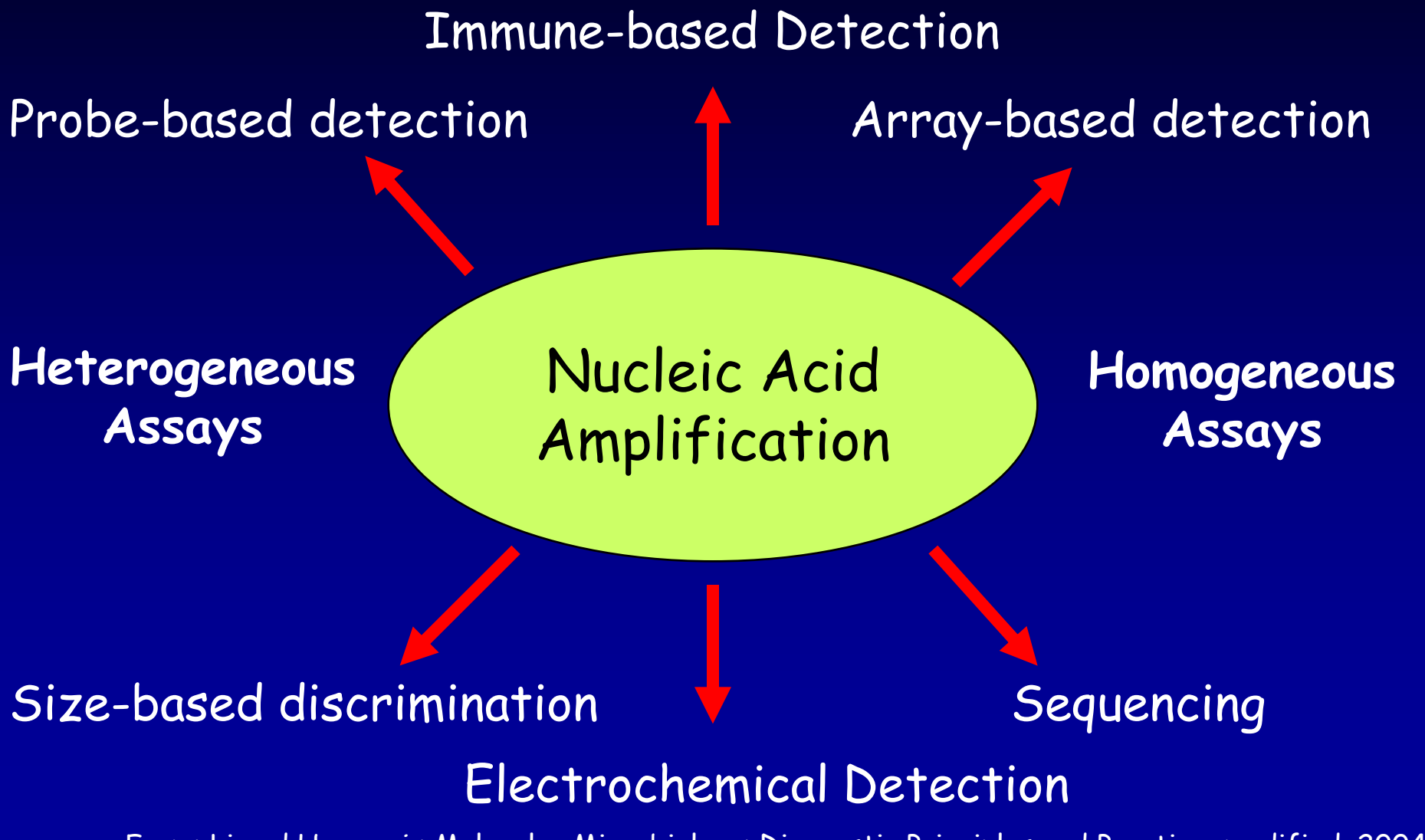
- Molecular technologies for the detection and identification of pathogenic fungi may be separated in two categories:
  - **signal amplification methods** that use hybridization probes
  - **nucleic amplification methods**

Selected commercially available DNA probes for detection of bacterial and fungal pathogens

Organism	Specimen	Sensitivity (%)	Specificity (%)	Agreement (%)
Campylobacter sp.	Stool culture	100	99.7	99.8
L. monocytogenes	Cultured isolate	100	99.7	99.8
S. pyogenes	Throat swab culture	99.0	99.7	99.3
H. influenzae	CSF or throat swab culture	97.1	100	98.7
M. tuberculosis complex	Sputum/BAL fluid culture	99.2	99.9	99.6
B. dermatitidis	Cultured isolate	98.1	99.7	99.2
C. immitis	Cultured isolate	98.8	100	99.6
H. capsulatum	Cultured isolate	100	100	100
N. gonorrhoeae	Urethral culture	95.4	99.8	99.0
C. trachomatis	Urethral swab	92.6	99.8	99.0
S. pyogenes	Throat swab	91.7	99.3	97.4

From: Li and Hanna, *in*: Molecular Microbiology: Diagnostic Principles and Practice, modified, 2004

Use of nucleic acid amplification in clinical microbiology



From: Li and Hanna, *in*: Molecular Microbiology: Diagnostic Principles and Practice, modified, 2004

Nucleic acid amplification methods

Amplification method	Enzyme used
Target amplification	
PCR	Thermophilic DNA polymerase
TAS, 3SR, NASBA, TMA	RT, RNase H, RNA polymerase
SDA	Restriction endonucleases, DNA polymerase
LCR	Thermophilic DNA ligase
Signal amplification	
bDNA	None
Hybrid capture	None
Qβ replicase	Qβ replicase
CPT	RNase H
Invader	Cleavase
RCA	DNA polymerase

Amplification methods as analogs of artificial media

Amplification method	Specimen type	Amplification result	Analogous cultivation method
Universal	Polymicrobial	Polyclonal amplification	Primary isolation medium
Universal	Normally sterile, infection with single organism	Clonal amplification	Primary isolation medium
Universal	No organism present	Negative	Primary isolation medium
Selective	Target organism present with or without other members of the flora	Positive for target organism	Selective growth medium
Selective	Target organism absent	Negative	Selective growth medium

Methods used for amplification product detection and identification

Method	Signal molecules	Amplification and detection	Clinical applications	Elapsed time (including amplification)	Comments[a]
Gel electrophoresis	Ethidium bromide staining	Separate	Detection	1 day	When used with SSCP or RFLP, this method can be used for differentiation and subtyping
Southern blot hybridization	Radioactive or chemiluminescence	Separate	Detection	1–2 days	No longer a routine method in the clinical laboratory
Microtiter plate	Enzyme	Separate	Detection and differentiation	4–8 h	No expensive equipment needed; adaptable to automation
Molecular beacons	Fluorescence	Simultaneous	Detection and differentiation	4 h	Point mutation detection and differentiation; minimal carryover contamination due to the closed system
Direct sequencing	Fluorescence	Separate	Detection, typing, and resistance determination	1–2 days	Unique ability to identify unknown and highly mutated species
Real-time PCR	Fluorescence	Simultaneous	Detection, differentiation, and quantitation	0.5–2 h	Shortest test turnaround time; minimal carryover contamination due to the closed system
Matrix hybridization (DNA chip)	Fluorescence	Separate	Detection, differentiation, and resistance determination	6–12 h	Able to resolve complex mixtures of amplicons; high capital requirement

[a] SSCP, single strand conformation polymorphism; RFLP, restriction fragment length polymorphism.

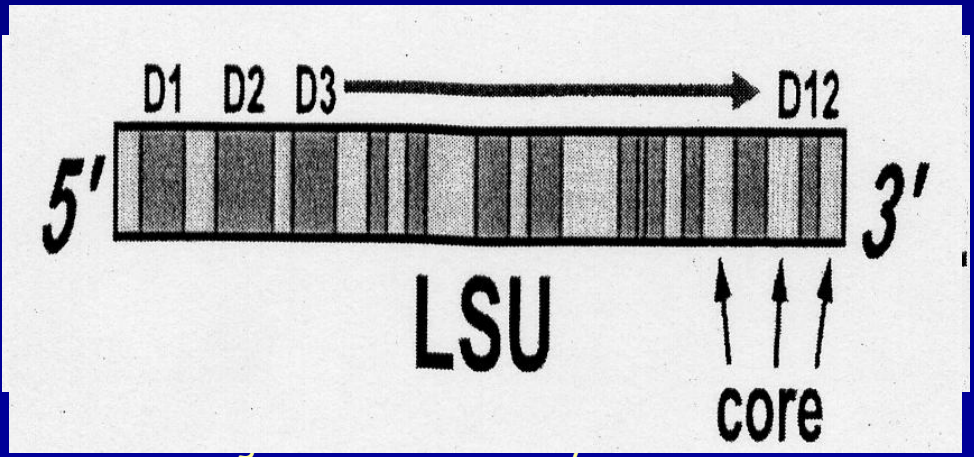
From: Tang *et al.*, *in*: Molecular Microbiology: Diagnostic Principles and Practice, modified, 2004

Sequence-based fungal identification

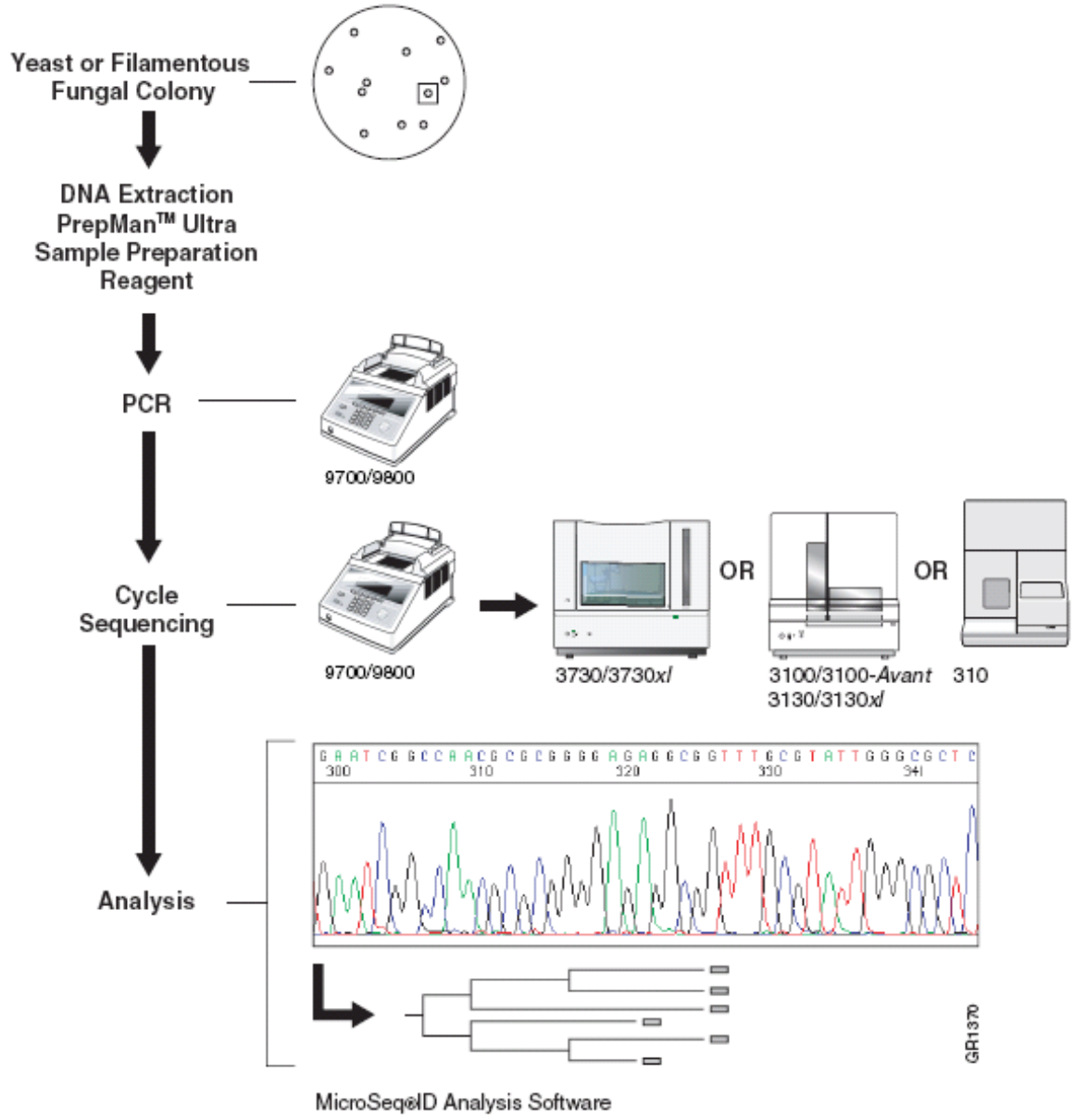
- A sequence-based molecular method consists of the following steps:
  - Nucleic acid extraction
  - Amplification of target gene fragment
  - Sequencing of the fragment
  - Comparison of the obtained sequence to other gene sequences in a database

Advantages of nuclear rDNA

- Well characterized in fungi
- Universal occurrence
- Fast- and slow-evolving regions
- Multicopy



Flowchart of the MicroSeq identification process

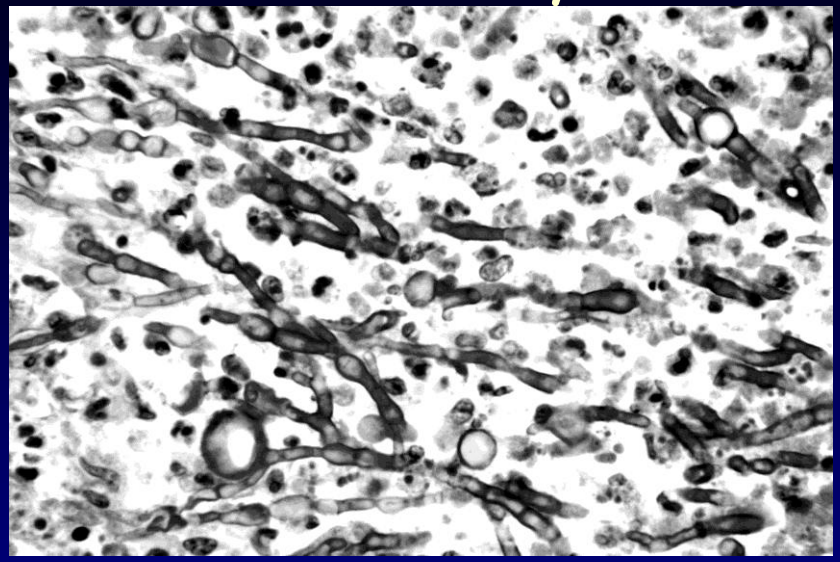


DNA is extracted by a simple procedure, then amplified by PCR and cycle sequenced. Samples can be electrophoresed on the ABI PRISM genetic analyzer. Resulting sample files are analyzed and compared to comprehensive sequence libraries for identification of the unknown sample

MicroSeq system applications

- Hall L, Doerr KA, Wohlfiel SL, Roberts GD. Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J Clin Microbiol. 2003, 41 (4): 1447-53
- Ninet B, Jan I, Bontems O, Lechenne B, Jousson O, Panizzon R, Lew D, Monod M. Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. J Clin Microbiol. 2003 41 (2): 826-30
- Woo PC, Ng KH, Lau SK, Yip KT, Fung AM, Leung KW, Tam DM, Que TL, Yuen KY. Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J Clin Microbiol. 2003 41 (5): 1996-2001
- Hall L, Wohlfiel S, Roberts GD. Experience with the MicroSeq D2 large-subunit ribosomal DNA sequencing kit for identification of filamentous fungi encountered in the clinical laboratory. J Clin Microbiol. 2004 42 (2): 622-6
- Fontana C, Favaro M, Pelliccioni M, Pistoia ES, Favalli C. Use of the MicroSeq 500 16S rRNA gene-based sequencing for identification of bacterial isolates that commercial automated systems failed to identify correctly. J Clin Microbiol. 2005 43 (2): 615-9

Early diagnosis of a case of scedosporiosis by ITS2 rDNA sequencing



Scedosporium apiospermum gene for ITS 5.8S-28S, isolate:#3004 and #7256
Length = 1486 Score = 1005 bits (507),
Expect = 0.0 Identities = 507/507 (100%)

```
Query: 1   ttagagtttgatcctggctcaggacgaacgctggcggcgtgcttaacacatgcaagtcga 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 20  ttagagtttgatcctggctcaggacgaacgctggcggcgtgcttaacacatgcaagtcga 79

Query: 61  acggaaaggccctgcttttgtggggtgctcgagtggcgaacgggtgagtaacacgtgagt 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 80  acggaaaggccctgcttttgtggggtgctcgagtggcgaacgggtgagtaacacgtgagt 139

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Sbjct: 440 gtaaaccgctttcgcctgtgacgaagcgtgagtgacggtaatgggtaaagaagcaccggc 499

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Sbjct: 500 taactacgtgccagcagccgcggtgatac 528
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[a] SSCP, single strand conformation polymorphism; RFLP, restriction fragment length polymorphism.

From: Tang *et al.*, *in*: Molecular Microbiology: Diagnostic Principles and Practice, modified, 2004

Hybridization array technology has been defined the "next wave" of technological advances to have a practical impact on the diagnosis of human disease, second only to PCR as a core technology in the clinical molecular diagnostics arena

Microarrays in microbiology

Array platform technology	Application
Microbial gene expression	Drug discovery and drug development
	Pathogenesis studies and microbial physiology
	Vaccine development
	Drug resistance detection
Host gene expression profiling	Differentiation of infectious etiologies
	Development of anti-inflammatory drugs
Diagnostic sequencing	Microbial detection, identification, and typing
	Detection of drug resistance
	Detection of host polymorphisms associated with drug metabolism or differential immune responses

From: Stover *et al.*, *in*: Molecular Microbiology: Diagnostic Principles and Practice, modified, 2004

TABLE 2. Evaluation of the array with blinded samples

No.	Isolate[b]	Isolate by conventional identification method	Array result	Sequencing result[a]
1	*C. albicans*	ST 3477-03	*C. albicans*	+
2	*C. albicans*	VA 115470-03	*C. albicans*	+
3	*C. albicans*	VA 115839-03	*C. albicans*	+
4	*C. dubliniensis*	ST 2792-03	*C. dubliniensis*	+
5	*C. dubliniensis*	VA 103469-04	*C. dubliniensis*	+
6	*C. glabrata*	VA 104009-04	*C. glabrata*	+
7	*C. guillermondii*	UR 9406-03	*C. guillermondii*	+
8	*C. krusei*	ST 3382-03	*C. krusei*	+
9	*C. lusitaniae*	ST 3324-03	*C. lusitaniae*	+
10	*C. lusitaniae*	VA 115231-03	*C. lusitaniae*	+
11	*C. parapsilosis*	VA 115230-03	*C. parapsilosis*	+
12	*C. tropicalis*	UR 9344-03	*C. tropicalis*	+
13	*Candida kefyr*	VA 116042-03	Nontarget species	*Candida kefyr*
14	*Candida norvegensis*	ST 3481-03	*C. krusei*	*Candida norvegensis*
15	*Candida pelliculosa*	ST 3352/2-03	Nontarget species	*Candida pelliculosa*
16	*A. fumigatus*	VA 104001-04	*A. fumigatus*	+
17	*A. niger*	ST 717-04	*A. niger*	+
18	*A. niger*	VA 3590-04	*A. niger*	+
19	*Aspergillus spp.*[c]	VA 103936-04	*A. flavus*	+
20	*Pseudallescheria boydii*	VA 103543-04	Nontarget species	*Pseudallescheria boydii*
21	*Saccharomyces cerevisiae*	ST 3352/1-03	Nontarget species	*Saccharomyces cerevisiae*

[a] +, concordance with the array result.
[b] Clinical isolates were identified by standard laboratory procedures.
[c] The content of this sample could not be identified to the species level by the described methods.

clinical microbiology laboratories.

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TABLE 1. Standard strains used in this study[a]

Strain no.	Species
C1a	*Candida albicans*
C2a	*Candida tropicalis*
C6a	*Candida krusei*
C4a	*Candida parapsilosis*
Y10	*Candida glabrata*
3.3440	*Mucor racemosus*
T1a	*Trichophyton rubrum*
T5a	*Trichophyton mentagrophytes*
F1d	*Epidermophyton floccosum*
M3b	*Microsporum canis*
M2b	*Microsporum gypseum*

[a] All strains listed in this table were obtained from the CMCC.

High-Thr … enic Fungi
by H … array†

maps. All 86 strains were distinguished by their hybridization maps on the microarray. The comprehensive identification results by classical methods are regarded as the final standards. All the hybridization assay results were consistent with those of the conventional methods; the consistency was 100% (86/86).

H … ogens
in a … d and
synthesized. Fur … ybridized with the
oligonucleotide r … ies were obtained.
The 122 strains … , used to test the
specificity, stabil … nting 8 genera. We
found that the r … ens to the species
level, with high s … leotide microarray
system represen … lentifying common
clinical fungal is

JCM Accepts, published online ahead of print on 22 August 2007
J. Clin. Microbiol. doi:10.1128/JCM.00942-07

DNA Microarray-based Detection and Identification of Fungal Pathogens in Clinical Samples from Neutropenic Patients

Birgit Spiess, Wolfgang Seifarth, Margit Hummel*, Oliver Frank, Alice Fabarius, Chun Zheng, Handan Mörz, Rüdiger Hehlmann, Dieter Buchheidt

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In 22/22 patients (without IFI, n=5; possible IFI, n=17), negative diagnostic results corresponded with negative microarray data. From 11 patients with proven (n=4), probable (n=2) and possible IFI (n=5) positive microarray data were validated by other diagnostic findings. In 11/11 patients with possible IFI the microarray results provided additional information. In 2 patients with proven respectively probable invasive aspergillosis, microarray results were negative.

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Features of real-time PCR

- Can be considered an "evolution" of PCR because overcome some PCR problems being able to:
  - Estimate the "microbial load" by simultaneous amplification and quantitation of nucleic acids (DNA or RNA) in clinical specimens
  - Reduce risk of carryover contamination because post-amplification manipulation is not required, so real-time PCR is described as a "closed" system

Real-time PCR methods for fungi

Genus	Specimen	Technology	Target	Status	Conventional method	Clinical sensitivity vs. conventional method	Turnaround time vs. conventional method	Reference(s)	Comments
Aspergillus	BAL fluid	iCycler TaqMan	18S rRNA	HB	Culture, histopathology	Greater	Faster	428	Compared with antigen detection
	BAL fluid, blood	LightCycler FRET HP	18S rRNA	HB	NA	NA	Faster	287	MagNA Pure vs. manual extraction
	Blood, serum	ABI 7700 TaqMan	28S rRNA	HB	Culture, histopathology	Greater	Faster	61	Compared with antigen detection
	Serum, plasma, cell pellet	ABI 7700 TaqMan	*fks*	HB	Culture, histopathology	NR	Faster	81	Evaluation of serum, white cell pellet, plasma
	Blood	LightCycler FRET HP	18S rRNA	HB	Culture, histopathology	NR	Faster	285, 385	Quantitative
	Blood, tissue	LightCycler FRET HP	18S rRNA	Expmt1	Culture, histopathology	Greater	Faster	286	Research in mice and rabbits
	Serum	LightCycler FRET HP	mito. rRNA	HB	Culture	Greater	Faster	80	Comparison with antigen detection
	Culture	ABI 7700 TaqMan	*atr*	HB	Conventional PCR	NA	Faster	445	Monitoring of efflux pump transcript levels
	Culture	ABI 7700 Molecular Beacons	*mdr*	HB	Conventional PCR	NA	Faster	331	Monitoring of expression levels
	Tissue	ABI 7700 TaqMan	18S rRNA	Expmt1	CFU quantitation	NA	Faster	41	Caspofungin efficacy monitoring in mice
Candida	Blood	ABI 5700 TaqMan	ITS2	HB	Culture	Greater	Faster	290–292	Quantitative
	Blood	LightCycler FRET HP	18S rRNA	HB	Culture	NR	Faster	285, 385	Quantitative
	Blood	LightCycler FRET HP	18S rRNA	HB	Culture	Greater	Faster	543	Detects seven species with one probe set
	Blood	LightCycler FRET HP	ITS	HB	Culture	Equal	Faster	444	Detects/differentiates six species with four probe sets
	Culture	ABI 7700 TaqMan	ITS2	HB	Culture	NA	Faster	157	Detects/differentiates six species with three probe sets
	Culture	ABI 7700 TaqMan	*erg11*	HB	Broth dilution	NA	Faster	65, 144	Fluconazole resistance genotyping
Coccidioides	Culture	LightCycler FRET HP	Ag2/PRA	HB	Culture, nucleic acid probe	NA	Faster	31	Extraction procedure safety evaluated
Conidiobolus	Tissue	LightCycler FRET HP	18S rRNA	HB	Culture, microscopy	Equal	Faster	194	1 clinical specimen
Cryptococcus	Culture	LightCycler SYBR	5.8S rRNA	HB	Culture, biochemicals	NA	Faster	188	
	Tissue	LightCycler FRET HP	18S rRNA	Expmt1	Culture	Lower	Faster	32	Research in mice; quantitative
Histoplasma	Culture, BAL fluid, tissue, bone marrow	LightCycler FRET HP	ITS	LD	Culture, nucleic acid probe	Equal except for bone marrow	Faster	303	3 clinical specimens
	Tissue	LightCycler FRET HP	18S rRNA	LD	Culture, nucleic acid probe	Equal	Faster	194	1 clinical specimen
Paracoccidioides	Culture	ABI 7700 TaqMan	20 genes	LD	Conventional PCR	NR	Faster	301	Yeast and hyphal phases differential gene expression
Pneumocystis	Oral swabs, tissue	iCycler SYBR	mt LSU	Expmt1	Stains, microscopy	NA	Equal	276	Quantitative monitoring of levels in rats
	Tissue	LightCycler FRET HP	*dhfr*	Expmt1	Stains, microscopy	NR	Equal	250	Quantitative; research in rats

From: Espy *et al.*, CMR, modified, 2006

Results of real-time PCR, conventional PCR, and GM ELISA of BAL fluid specimens from patients with IPA (1)

Patient no.	Primary disease	IPA diagnosis (EORTC criteria)	BAL fungal culture	GM ELISA	BAL PCR result	
					Real-time (copies/ml)	Conventional
1	NHL	Probable	*C. albicans*	Positive	Negative	Negative
2	NHL	Probable	*C. glabrata*	Positive	3,000	Positive
3	ML	Probable	Negative	Positive	4,500	Positive
4	AML	Probable	Negative	Positive	8,000	Positive
5	ALL	Proven	*A. fumigatus*	Positive	60,000	Positive
6	SAA	Proven	*A. flavus*	Positive	45,000	Positive
7	AML	Probable	Negative	Positive	4,200	Positive
8	SAA	Proven	*A. fumigatus*	Positive	75,000	Positive
9	ML	Probable	*C. albicans*	Positive	2,000	Positive
10	AML	Probable	Negative	Positive	3,900	Positive

From: Sanguinetti *et al.* JCM, 2003, modified

Results of real-time PCR, conventional PCR, and GM ELISA of BAL fluid specimens from patients with IPA (2)

Patient no.	Primary disease	IPA diagnosis (EORTC criteria)	BAL fungal culture	GM ELISA	BAL PCR result	
					Real-time (copies/ml)	Conventional
11	AML	Probable	Negative	Positive	9,800	Positive
12	AML	Probable	Negative	Positive	10,500	Positive
13	AREB-t	Probable	Negative	Positive	8,700	Positive
14	NHL	Proven	*A. fumigatus*	Positive	85,000	Positive
15	ALL	Probable	Negative	Positive	5,400	Positive
16	AML	Probable	*A. fumigatus*	Positive	23,000	Positive
17	NHL	Proven	*A. flavus*	Positive	100,000	Positive
18	AML	Probable	Negative	Positive	7,600	Positive
19	AML	Probable	*C. albicans*	Positive	Negative	Negative
20	ALL	Probable	*C. albicans*	Positive	3,200	Positive

From: Sanguinetti *et al.* JCM, 2003, modified

Diagnostic molecular mycology: some current and future trends

- Detection of antifungal resistance by means of real-time PCR assay or oligonucleotide microarray
- Analysis and characterization of fungal proteomes by 2D gel electophoresis and mass spectrometry
- Analysis of single nucleotide polymorphisms (SNPs) to find genes that affect disease pathology or determine drug responsiveness

JOURNAL OF CLINICAL MICROBIOLOGY, May 2004, p. 2085–2093 Vol. 42, No. 5
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Rapid Quantification of Drug Resistance Gene Expression in *Candida albicans* by Reverse Transcriptase LightCycler PCR and Fluorescent Probe Hybridization

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0066-4804/05/$08.00+0 doi:10.1128/AAC.49.2.668–679.2005

Mechanisms of Azole Resistance in Clinical Isolates of *Candida glabrata* Collected during a Hospital Survey of Antifungal Resistance

Maurizio Sanguinetti,* Brunella Posteraro, Barbara Fiori, Stefania Ranno, Riccardo Torelli, and Giovanni Fadda

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Diagnostic molecular mycology: some current and future trends

- Detection of antifungal resistance by means of real-time PCR assay or oligonucleotide microarray
- Analysis and characterization of fungal proteomes by 2D gel electophoresis and mass spectrometry
- Analysis of single nucleotide polymorphisms (SNPs) to find genes that affects disease pathology or determine drug responsiveness

Strategies for defining diagnostic candidates

- Compare the reference proteomic signatures to experimentally defined proteomic signatures
- Identify significant fungal proteins:
  - cell-associated proteins;
  - membrane-spanning proteins;
  - proteins that evoke a specific host immune response
- Combine 2D analysis with Western blotting using pateint sera

Molecular & Cellular Proteomics

Decoding Serological Response to *Candida* Cell Wall Immunome into Novel Diagnostic, Prognostic, and Therapeutic Candidates for Systemic Candidiasis by Proteomic and Bioinformatic Analyses*

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In an effort to bring novel diagnostic and prognostic biomarkers or even potential targets for vaccine design for systemic candidiasis (SC) into the open, a systematic proteomic approach coupled with bioinformatic analysis was used to decode the serological response to *Candida* wall immunome in SC patients. Serum levels of IgG antibodies against *Candida* wall-associated proteins (proteins secreted from protoplasts in active wall regeneration, separated by two-dimensional gel electrophoresis, and identified by mass spectrometry) were measured in 45 SC patients, 57 non-SC patients, and 61 healthy subjects by Western blotting. Two-way hierarchical clustering and principal component analysis of their serum anti-*Candida* wall antibody expression patterns discriminated SC patients from controls and highlighted the heterogeneity of their expression profiles. Multivariate logistic regression models demonstrated that high levels of antibodies against glucan 1,3-β-glucosidase (Bgl2p) and the anti-wall phosphoglycerate kinase antibody seropositivity were the only independent predictors of SC. Receiver operating characteristic curve analysis revealed no difference between their combined evaluation and measurement of anti-Bgl2p antibodies alone. In a logistic regression model adjusted for known prognostic factors for mortality, SC patients with high anti-Bgl2p antibody levels or a positive anti-wall enolase antibody status, which correlated with each other, had a reduced 2-month risk of death. After controlling for each other, only the seropositivity for anti-wall enolase antibodies was an independent predictor of a lower risk of fatality, supporting that these mediated the protective effect. No association between serum anti-cytoplasmic enolase antibody levels and outcomes was established, suggesting a specific mechanism of enolase processing during wall biogenesis. We conclude that serum anti-Bgl2p antibodies are a novel accurate diagnostic biomarker for SC and that, at high levels, they may provide protection by modulating the anti-wall enolase antibody response. Furthermore serum anti-wall enolase antibodies are a new prognostic indicator for SC and confer protection against it. Bgl2p and wall-associated enolase could be valuable candidates for future vaccine development. *Molecular & Cellular Proteomics 5: 79–96, 2006.*

Not only does systemic candidiasis (SC)[1] continue to be significant in incidence (1–3), but it also remains a leading infectious cause of morbidity and mortality in intensive care, post-surgical, and cancer patients (3–5) and accounts for substantial healthcare costs (6, 7). Clinical outcomes might be improved by early initiation of antifungal therapy. SC diagnosis, however, is extremely difficult because signs and symptoms of invasive disease are nonspecific. In addition, its two gold standards, blood cultures and tissue biopsies, may lack sensitivity in the first stages of infection (8) and become excessively invasive in critically ill patients, respectively. As a result, SC diagnosis is often attained after a long delay or, unfortunately, following autopsy (9, 10).

This clinical setting has prompted the search for novel prompt and accurate disease markers or, instead, for immunoprophylactic strategies. Research efforts currently focus on screening both for *Candida* cell wall polysaccharides (mannans or β-1,3-glucans), extracellular proteins (secreted aspartyl proteinase), cytoplasmic proteins (enolase or heat shock protein 90), metabolites (D-arabinitol), or nucleic acids (DNA or RNA) and for anti-*Candida* antibodies in body fluids of SC patients (11–15). The relevance of well defined *Candida* cell

Diagnostic molecular mycology: some current and future trends

- Detection of antifungal resistance by means of real-time PCR assay or oligonucleotide microarray
- Analysis and characterization of fungal proteomes by 2D gel electophoresis and mass spectrometry
- Analysis of single nucleotide polymorphisms (SNPs) to find genes that affects disease pathology or determine drug responsiveness

nature genetics

A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis

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Toll-like receptors (TLRs) and members of their signaling pathway are important in the initiation of the innate immune response to a wide variety of pathogens[1–3]. The adaptor protein Mal (also known as TIRAP), encoded by *TIRAP* (MIM 606252), mediates downstream signaling of TLR2 and TLR4 (refs. 4–6). We report a case-control study of 6,106 individuals from the UK, Vietnam and several African countries with invasive pneumococcal disease, bacteremia, malaria and tuberculosis. We genotyped 33 SNPs, including rs8177374, which encodes a leucine substitution at Ser180 of Mal. We found that heterozygous carriage of this variant associated independently with all four infectious diseases in the different study populations. Combining the study groups, we found substantial support for a protective effect of S180L heterozygosity against these infectious diseases ($N = 6{,}106$; overall $P = 9.6 \times 10^{-8}$). We found that the Mal S180L variant attenuated TLR2 signal transduction.

glycosylphosphatidylinositol (GPI) (C. Aucan *et al.*, unpublished data)[10]. The adaptor protein Mal (TIR domain–containing adaptor protein), encoded by the gene *TIRAP*, is essential for MYD88-dependent signaling downstream of TLR2 (with TLR1 and TLR6 as coreceptors) and TLR4 (refs. 1,5). After stimulation of TLR2 or TLR4, Mal triggers a signaling cascade, which culminates in the activation of the transcription factor NF-κB and the subsequent activation of proinflammatory genes. Based on the central position of Mal in the TLR2 and TLR4 pathways and our knowledge of the microbial components that associate with these TLRs, we hypothesized that genetic variation at *TIRAP* might underlie susceptibility to common infectious diseases.

TIRAP spans 14,500 bp on chromosome 11q24.2 and encodes a protein of 221 amino acids. We analyzed 33 SNPs in *TIRAP* and the surrounding region in multiple populations and found that *TIRAP* S180L consistently associated with disease (**Supplementary Note**, **Supplementary Fig. 1**, **Supplementary Table 1** and **Supplementary Table 2** online). We genotyped *TIRAP* S180L in a total of 6,106 individuals with four different diseases: two UK populations of

Parallel Genotyping of Over 10,000 SNPs Using a One-Primer Assay on a High-Density Oligonucleotide Array

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The analysis of single nucleotide polymorphisms (SNPs) is increasingly utilized to investigate the genetic causes of complex human diseases. Here we present a high-throughput genotyping platform that uses a one-primer assay to genotype over 10,000 SNPs per individual on a single oligonucleotide array. This approach uses restriction digestion to fractionate the genome, followed by amplification of a specific fractionated subset of the genome. The resulting reduction in genome complexity enables allele-specific hybridization to the array. The selection of SNPs was primarily determined by computer-predicted lengths of restriction fragments containing the SNPs, and was further driven by strict empirical measurements of accuracy, reproducibility, and average call rate, which we estimate to be >99.5%, >99.9%, and >95%, respectively. With average heterozygosity of 0.38 and genome scan resolution of 0.31 cM, the SNP array is a viable alternative to panels of microsatellites (STRs). As a demonstration of the utility of the genotyping platform in whole-genome scans, we have replicated and refined a linkage region on chromosome 2p for chronic mucocutaneous candidiasis and thyroid disease, previously identified using a panel of microsatellite (STR) markers.

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Conclusions

- Beyond sensitivity and accuracy, the **SPEED** remains a primary advantage of NAA over biological methods of amplification
  - As many factors have combined to make more practical the implementation of new molecular methods, the increasing use of microarray technology, real-time PCR, and other new technologies promises to enhance the practical utility of molecular diagnostics in clinical mycology laboratories.
  - I believe that molecular assays will find more wider application in clinical mycology laboratories as the prompt and specific diagnosis of fungal infection is shown to improve patient management and outcome.
  - In addition, as tests become adapted to automated platforms and costs decline, molecular approaches may become the tests of choice even when comparable low-technology approaches are available.