Improved diagnosis of invasive fungal infections (IFI) using DNA microarray technologyfor detection of fungal DNA in Aspergillus PCR-negative tissue biopsy samples from immunocompromised hematological patients

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Abstract

The increasing incidence of invasive fungal infections (IFI) in immunocompromised patients (pts) and the rarely achieved positive culture yields in these patients emphasize the need to improve the molecular tools for detection of fungal pathogens. We established a DNA microarray method detecting 15 fungal pathogens, which combines multiplex polymerase chain reaction (PCR) and consecutive DNA chip hybridization.
Tissue samples (n = 44) of 36 immunocompromised pts were investigated: Mostly during antifungal therapy, with proven (n = 7), probable (n = 7), possible (n = 4), and no IFI (n = 18) according to 2008 EORTC / MSG consensus criteria. DNA microarray results were compared to results from culture, histopathology, imaging, and serology.

In 14/18 pts without IFI negative conventional diagnostic results corresponded with negative microarray data. Positivity of the microarray analysis was observed for 7/7 proven, 2/7 probable, 3/4 possible and 4/18 no IFI pts yielding sensitivity and specificity values of 0.64 and 0.78, a positive likelihood ratio of 3 and a negative likelihood ratio of 0.43, respectively.

Technology

Fig. 1: Assembly and function of DNA chips. All oligonucleotides had a C12-spacer and were NH_2-modified at their 5′ ends for covalent coupling

Fig. 2: Location of primers within the fungal rRNA genes and generation of Cy3-labeled amplification products used for chip hybridization.

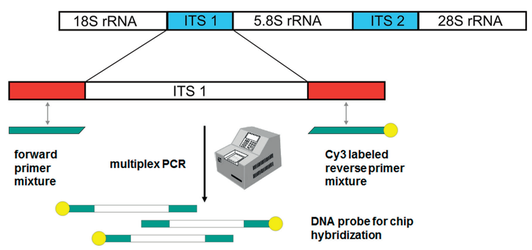


Fig.3: Grid location of fungal, human and Arabi-dopsis thaliana-specific capture probes

	1	2	3	4	5	6	7	8	9	10	11	12
A		*Candida*										
		Calb1S	Cdub1S	C.dubl2	Clusi1	Clusi2	Cglab1	Cglab2	Cglab3	Ctrop1	Ctrop2	
B	*Rhizopus*								*Fusarium*			
	Rmicr1	Rmicr2	Rmicr3	Rmicr4	Rmicr5	Rory1	Rory2		Foxys1	Foxys2	Fsola1	Fsola2
C	*Mucor*		*Scedosporium*		*Trichosporon*				*Aspergillus*			
	Mrace1	Mrace2	Sprol1	Sprol2	Tasah1	Tasah2	Aflav1	Aflav2	Aterr1	Aterr2	Afumi1	Afumi2
D		*Mucor*	Human		*Arabidopsis*		House keeping genes					
		Mrace3	Hum_ ITS1_1	Hum_ ITS1_2	Atrpl23 aA	ACT2A	RPL19 _1	RPL19 _2	G6PD-2 _1	G6PD-2 _2	G6PD-1 _1	G6PD-1 _2p

ITS1 = internal transcribed spacer 1;
Atrpl23a = A. *thaliana* ribosomal protein L23a;
ACT2 = A. *thaliana* actin 2;
RPL19 = ribosomal protein L19;
G6PD = glucose-6-phosphate dehydrogenase

Introduction

Invasive fungal infections cause high mortality rates in immunocompromised patients. The epidemiology is changing and emerging with both uncommon and resistant fungal pathogens. Concerning the outcome of patients with IFI, early initiation of antifungal treatment is crucial. Conventional microbiological diagnostic procedures are time consuming and lack sensitivity and / or specificity. In recent years molecular diagnostic tools such as PCR have been established to detect fungal pathogens, especially Aspergillus species, in clinical samples.

To facilitate and expand diagnosis of IFI, we established a DNA microarray to detect fungal genomic DNA in clinical samples.

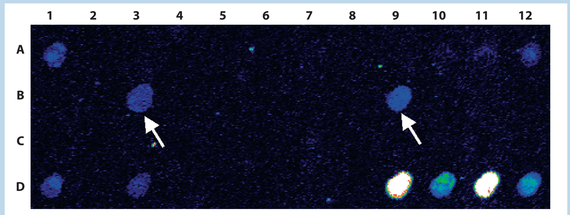
Methods

We established a DNA microarray method (Fig. 1 + 2) detecting 15 fungal pathogens (Fig. 3), which combines multiplex polymerase chain reaction (PCR) and consecutive DNA chip hybridization (Fig. 4). The array encompasses detection of Aspergillus fumigatus, A. flavus, A. terreus, Candida albicans, C. dubliniensis, C. glabrata, C. lusitaniae, C. tropicalis, Fusarium oxysporum, F. solani, Mucor racemosus, Rhizopus microsporus, R. oryzae, Scedosporium prolificans, and Trichosporon asahii. PCR primers and capture probes were generated from fungal rRNA genes. For amplification of fungal ITS1 regions and human housekeeping (G6PD, RPL19) / A. thaliana control genes, two separate PCRs were carried out. Hybridization was performed under standardized conditions (Seifarth et al., 2003). Evaluation was done using the Ima-GeneTM 4.0 tool package (BioDiscovery Inc., Los Angeles, CA, USA).

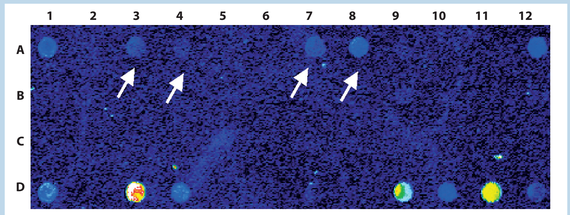
Tissue samples (n = 44) of 36 immunocompromised pts were investigated: Mostly during antifungal therapy, with proven (n = 7), probable (n = 7), possible (n = 4), and no IFI (n = 18) according to 2008 EORTC / MSG consensus definitions. DNA microarray results were compared to results from culture, histopathology, imaging, and serology.

Fig. 4: Representative hybridization patterns using DNA from clinical biopsy samples. Fungus specific positive signals are indicated by arrows.

a) Patient 1 (sinus nasalis biopsy): Fusarium oxysporium: + | Rhizopus microsporus: +



b) Patient 4 (spleen biopsy): C. glabrata: ++ | C. dubliniensis: ++



Results

Using genomic DNA of the 15 fungal species in ITS1 PCR and following hybridization, species-specific hybridization patterns were detected. PCR and hybridization showed different detection thresholds for every fungal organism (1 pg – 100 pg). Importantly there was no cross reactivity between the fungal pathogens capture probes and human or bacterial DNA.

Investigations of clinical samples from 36 patients mostly with proven or probable IFI (Tab. 1) showed positive results for C. albicans, C. dubliniensis, C. glabrata, S. prolificans, R. microsporus and R. oryzae. Detection of more than one fungal DNA points to multiple infections in the same patient.

In 14/18 pts without IFI negative conventional diagnostic results corresponded with negative microarray data. Positivity of the microarray analysis was observed for 7/7 proven, 2/7 probable, 3/4 possible and 4/18 no IFI pts yielding sensitivity and specificity values of 0.64 and 0.78, a positive likelihood ratio of 3 and a negative likelihood ratio of 0.43, respectively.

Conclusion

Using clinical samples our DNA microarray specifically detects 15 clinically relevant fungal pathogens at low detection thresholds. The benefit of a fungal microarray is the potential to detect several different fungal pathogens with one test.
First results using clinical biopsy samples show the usefulness of the microarray in the clinical context.

The evaluation in a prospective multicenter study testing clinical samples like blood, BAL, and tissue samples from immunocompromised patients, especially patients with acute leukemia, is ongoing.

References

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Tab. 1: Results of investigated tissue samples from patients with proven, probable and one exemplarily shown possible IFI. The nested PCR assay is specific for Aspergillus species. IFI = invasive fungal infection

Patients	Sample	Diagnosis	Diagnostic significance	Assumed invasive mycosis	DNA microarray results	Nested PCR (Aspergillus)
1	Sinus nasalis biopsy	AML	Proven	Culture: Mucor	R. microsporus: + F. oxysporum: +	-
2	Liver biopsy	NHL; lung cancer	Proven	Histology: Candida	C. albicans: ++ C. dubliniensis: +	-
3	Liver biopsy	Multiple myeloma	Proven	Culture: mucormycosis	S. prolificans: + R. microsporus: +	-
4	Spleen biopsy	AML	Proven	Culture: Candida tropicalis	C. dubliniensis: + C. glabrata: ++	-
5	Lung biopsy	Hematological disease; NOS	Proven	Culture: zygomycosis	C. glabrata: ++ R. oryzae: +	(+)
6	Lung biopsy	AML	Proven	Culture: Rhizopus spp.	R. microsporus: + R. oryzae: +	-
7	Cranium biopsy	AML	Proven	Culture: Mucor	Mucor racemosus: +	-
8	Spleen biopsy	AML	Probable	CT scan: atypical lung infiltrates	S. prolificans: +	-
9	Liver biopsy	Rhabdomyosarcoma	Probable	CT scan: liver infiltrates, suspected Candida infection	C. glabrata: ++	-
10	Liver biopsy	Medulloblastoma	Probable	CT scan: atypical lung infiltrates	Negative	-
11	Liver biopsy	AML	Probable	CT scan: atypical lung infiltrates	Negative	-
12	Sinus nasalis biopsy	Fungal sinusitis	Probable	Former mucormycosis with brain abscess; histology: actually no fungal hyphae	C. glabrata: ++	-
13	Lung biopsy	COPD	Probable	CT scan: atypical lung infiltrates; histology: actually no fungal hyphae	Negative	(+)
14	Lung biopsy	ALL	Probable	CT scan: suspected candidiasis; BAL: Candida spp.	Negative	-

[1] de Pauw et al.,1 2008; [2] Skladny et al., 1999.

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