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Molecular detection of invasive fungal disease in immunocompromised patients

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Abstract of the dissertation

Doctoral study programme Medical Chemistry and Biochemistry

Hradec Králové

2013

Dissertation thesis was written during combined doctoral study (PhD) study programme Medical Chemistry and Biochemistry at the Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University in Prague.

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This work has been supported by grant FWF (Austrian Science Fund) P16929 (www.fwf.ac.at).

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in doctoral study programme Medical chemistry and biochemistry

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2. Souhrn

Molekulární detekce invazivních mykotických onemocnění u imunokompromitovaných pacientů

V dizertační práci se mi podařilo vyvinout tři PCR metody pro kvantitativní detekci a identifikaci kvasinkové a plísňové DNA. Dvě metody založené na kvantifikaci v reálném čase s názvem PanAC PCR a “panfungal” PCR byly navrhnuty tak, aby detekovaly a kvantifikovaly široké spektrum plísní a kvasinek způsobujících invazivní mykotická onemocnění. Význam metod pro klinické využití byl v rámci standardizace testován retrospektivně na souborech pacientů s již dokumentovanými invazivními mykózami a dále pak prospektivně na souborech pacientů s vysokým rizikem invazivní mykózy.

Vzhledem k významu přesné identifikace původce onemocnění byla vyvinuta “semi-nested” PCR s fluorescenční detekcí pomocí kapilární elektroforézy umožňující rychlou identifikaci plísně či kvasinky v klinickém materiálu, který byl pozitivní v jedné ze širokospektrých screeningových PCR. Možnost klinického využití této metody byla taktéž testována na populaci pacientů s dokumentovanou invazivní mykózou.

3. Summary

Molecular detection of invasive fungal disease in immunocompromised patients

In my work I have been able to establish three different PCR-based assays for the quantitative detection and identification of fungal DNA. Two DNA-based detection assays termed PanAC PCR and panfungal PCR based on the real-time quantitative (RQ-PCR) technology were designed to detect and quantify the most important fungal genera currently associated with IFD including a large number of pathogenic moulds and yeasts. Upon standardization of both RQ-PCR techniques, the applicability in the clinical setting was assessed by investigating a series of clinical specimens from patients with documented fungal infection, and by prospectively studying patient cohorts at high risk of IFD.

In view of the importance of precise identification of the causative fungal pathogen, a semi-nested PCR method coupled with fluorescent capillary electrophoresis detection was established. It facilitates rapid identification of fungal species in clinical materials that test positive for IFD using one of the broad-range screening assays. This method was also tested in a population of patients with documented fungal infections to assess its clinical potential.

4. Background

Invasive fungal disease (IFD) is a leading cause of morbidity and mortality in severely immunocompromised individuals, including particularly patients with hematological malignancies, bone-marrow transplant recipients, preterm neonates, intensive care patients and individuals with acquired or innate immune deficiencies. The vast majority of IFD events are still caused by *Candida* and *Aspergillus* species, however, changes in the epidemiology have occurred over the last decades (Richardson 2008, Lass-Flörl 2009). This is partially attributable to the broad application of antifungal prophylaxis in high-risk patients resulting in the increased occurrence of resistant species or the emergence of hitherto uncommon fungal pathogens, such as *Fusarium* and *Scedosporium,* phaeohyphomycetes, *Cryptococcus*, *Trichosporon*, *Malassezia*, and, very importantly, different members of the Mucorales, namely *Rhizopus* and *Mucor*. The overall mortality associated with IFDs is high and ranges from 30 to 80 % (Lass-Flörl 2009), and even exceeding 90% with central nervous system involvement and dissemination (Walsh et al. 2008).

Early detection and identification of the fungal pathogen is crucial for appropriate antifungal therapy and thus the clinical outcome of patients with IFD. Conventional diagnostic tests mainly based on microbiological cultivation have limited sensitivity and specificity. Blood cultures were described to fail in approximately 50% of candidiases (Pemán et al. 2011). Moreover, the detection of *Aspergillus* spp. and other moulds from blood cultures is rare,

and negative findings therefore cannot readily exclude IFD (Simoneau et al. 2005). Histological analyses of CT-guided biopsies are highly sensitive and specific, but are frequently associated with bleeding complications in patients with severe thrombocytopenia (Denning, 1998). The detection of surrogate serological markers such as the fungal cell wall components galactomannan and (1,3)-β-D-glucan has become increasingly important and widely used, but the clinical interpretation of the results still remains controversial (Leeflang 2008). Therefore, efforts are ongoing to develop less invasive, yet reliable, sensitive and specific diagnostic tests for IFDs to overcome the limitations of the traditional fungus detection methods.

The introduction of molecular methods for the detection of fungal nucleic acids was an important step in this direction. Several hundred publications on the application of PCR for the detection of fungal infections have appeared and provided a new diagnostic option displaying great potential. Major advantages of these techniques include the high sensitivity and rapid availability of results, which could serve as an important prerequisite for timely onset of antifungal therapy. PCR-based methods belong to the most frequently used approaches to molecular fungus analysis either permitting species-specific, genus-specific or broad-range (panfungal) fungus detection. Several molecular formats exist for qualitative and/or quantitative detection and identification of fungi. Standard PCR (El-Mahallawy et al. 2006; Raad et al. 2002) and PCR-ELISA (Löffler et al. 1998; Florent et al. 2006) belong to the first approaches established

for clinical application, followed by nested PCR systems (Skladny et al. 1999; Holmes et al. 1994) developed to improve the detection limit of the assay. At present, a number of different variations of real-time PCR procedures are available, allowing quantification of fungal load and, depending on the setting of the assay, pathogen identification at the species level (Basková et al. 2007; Schabereiter-Gurtner et al. 2007). Moreover, special methods such as nucleic acid sequence-based amplification (NASBA) (Park et al. 2011), multiplex PCR followed by DNA microarray (Spiess et al. 2007), fragment size analysis of variable regions of the fungal genome (Landlinger et al. 2009a), DNA sequencing (Leaw et al. 2006), hybridization to specific capture probes bound to microbeads (Landlinger et al. 2009b) or pyrosequencing (Borman et al. 2010) have been developed to identify fungal pathogens in clinical samples.

The growing spectrum of fungal pathogens involved in IFDs and the requirement of highly sensitive fungus detection necessitated the selection of appropriate target genes. During recent years, the ribosomal DNA (rDNA) gene has been the most commonly targeted sequence offering the possibility to identify individual fungi (Landlinger et al. 2009a,b) or to detect a broad-range of fungal pathogens simultaneously (Löffler et al. 1998; Baskova et al. 2007; Schabereiter-Gurtner et al. 2007). The preferred gene is present in multiple copy numbers in the fungal genom, thereby increasing the sensitivity of detection.

A number of issues that arose in the studies performed could potentially affect the clinical applicability of PCR-based

testing. These include technical aspects such as the selection of appropriate clinical specimens, including tissue samples, the required sample size, the efficacy and safety of DNA extraction procedures, the availability of carefully optimized and standardized amplification assays, the validation of the techniques in large clinical trials and, very importantly, the correct interpretation of results (Preuner and Lion 2009).

5. Objectives

Study „The Pan-AC assay: a single-reaction real-time PCR test for quantitative detection of a broad range of* Aspergillus *and* Candida *species"

In the present study, we aimed at developing a rapid, sensitive and specific diagnostic test for the detection and accurate quantification of the most common pathogenic *Candida* and *Aspergillus* species. The design of the RQ-PCR assay should minimize the effect of point mutations on the detectability of the fungal species. No less importantly, the method should provide an economic approach to the screening and monitoring of invasive candidiasis and aspergillosis applicable to routine clinical diagnosis. This aspect was assessed by investigating a series of clinical specimens from patients with documented fungal infections.

Study „Diagnosis of invasive fungal diseases by a real-time panfungal PCR assay in immunocompromised patients"

IFD is a life-threatening event in immunocompromised patients, and there is urgent need for reliable screening methods facilitating rapid and reliable broad detection of pathogenic fungi. To address this task, we aimed at establishing a RQ-PCR assay for detection of a wide spectrum of fungal pathogens including fungal species

of both common and emerging fungal genera. The potential clinical utility of the assay was assessed in a combined prospective and retrospective study in severely immunocompromised pediatric patients displaying a high risk of IFD.

Study „Identification of fungal species by fragment length analysis of the internally transcribed spacer 2 region“

The rapid identification of fungal pathogens in clinical specimens is a prerequisite for timely onset of the most appropriate treatment. The aim of the present study was to develop a sensitive method facilitating the identification of a broad spectrum of fungal species with a detection limit adequate for routine clinical diagnosis of IFDs in immunosuppressed individuals. The intended clinical application of the technique is the rapid identification of fungal species in clinical specimens that have been previously shown to be positive for fungal DNA by a broad-spectrum screening technique not capable of identifying the fungal species.

6. Material and methodology

Study „The Pan-AC assay: a single-reaction real-time PCR test for quantitative detection of a broad range of Aspergillus *and* Candida *species“*

Material

Fungal strains for PCR testing were obtained from the American Type Culture Collection (ATCC, Rockville, USA) and from the German Collection of Microorganisms (DSM, Braunschweig, Germany): six *Aspergillus* species and nine *Candida* species.

The clinical specimens from patients with documented fungus infections were obtained upon informed consent. The specimens included biopsies of pulmonary infiltrations and peripheral blood from haemato-oncological patients, bronchotracheal secretions from intensive care patients, and a cornea control specimen with a culture-documented infection by *Fusarium solanii*, which is outside the detection spectrum of the Pan-AC assay. Peripheral blood specimens from healthy volunteer donors were used to test for cross-reactivity with human DNA.

Methodology

The isolation of DNA from fungal strains, as well as of fungal, human, bacterial and viral DNA from different clinical materials was

performed as described by Baskova et al. 2007, essentially using an enzymatic and mechanical pretreatment of the sample followed by DNA extraction and purification procedure based on the magnetic beads technology.

Conserved nucleotide sequences of the fungal rDNA genes of clinically relevant *Candida* and *Aspergillus* species were selected and aligned using the BLAST search software. Within the 28S large ribosomal subunit of rDNA gene, a highly conserved region was identified that was used for the design of a PanAC detection assay.

To determine the detection limit of fungal pathogens in peripheral blood, 1 ml EDTA-anticoagulated whole blood from healthy volunteer donors was spiked with tenfold serial dilutions of *A. fumigatus* conidia and *C. albicans* cells (10^5 to 1 cell).

Study „Diagnosis of invasive fungal diseases by a real-time panfungal PCR assay in immunocompromised patients"

Material

Fungal strains for PCR testing were obtained from different institutions including the ATCC (Rockville, USA), DSM (Braunschweig, Germany), and from the Institute of Hygiene and Medical Microbiology (IHMM) (Medical University of Vienna, Austria).

Clinical specimens from consecutive patients were obtained after informed consent, and were prospectively collected

as specified below. In total, 618 peripheral blood specimens from 125 pediatric hemato-oncological patients undergoing intensive chemotherapy (n=65) or allogeneic stem cell transplantation (n=60) were analyzed during 150 episodes of febrile neutropenia. On average, four peripheral blood samples were investigated during each episode (range 1-15). In selected instances, depending on availability, additional types of specimens derived from primarily sterile sites of suspected infection were collected and subjected to molecular analysis. These included cerebrospinal fluid (n=11) and lung biopsies (n=2) in patients with suspected CNS or pulmonary involvement, respectively, in order to correlate the data with PCR findings in peripheral blood samples. The specimens used as training set for initial data assessment were provided by the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands, and the specimens used as validation set by the St. Anna Children's Hospital of Vienna, Austria. Plasma or serum was usually collected from 3 ml samples of peripheral blood and all samples were stored at -80°C until further processing. Peripheral blood specimens from healthy volunteer donors were used to test for cross-reactivity with human DNA.

Methodology

The extraction of DNA from fungal strains, as well as of fungal, human, bacterial and viral DNA from different clinical materials was performed as described above (Baskova et al. 2007).

The panfungal PCR detection assay included two separate reactions, I and II, each covering different subsets of fungal pathogens, as outlined below. Both PCR reactions targeted a highly conserved region of the 28S rRNA multicopy gene. Locked nucleic acid (LNA) nucleotides were included in the primer and probe sequences to obtain high specificity despite short length (Tolstrup et al. 2003).

To determine the detection limit of the real-time PCR assays, serial logarithmic dilutions across a six-log range (1 fg to 100 pg) of genomic DNA derived from *A. fumigatus* and *C. albicans,* as representatives for moulds and yeasts, respectively, were analyzed. Standard curves were generated on the basis of the amplification profile.

For the diagnosis of fungal DNAemia, a minimum of two PCR-positive peripheral blood specimens derived at subsequent time points during close follow-up investigation were required. In the rare instances in which only one specimen from a febrile neutropenic episode was available, a single PCR-positive result was regarded as indicative of DNAemia.

Results of panfungal PCR analysis in relation to the presence or absence of possible, probable, or proven IFD by the European Organization for Research and Treatment of Cancer (EORTC) criteria were used for the calculation of sensitivity and specificity, and the corresponding negative (NPV) and positive predictive values (PPV) of the assay.

Study „Identification of fungal species by fragment length analysis of the internally transcribed spacer 2 region“

Material

The fungal reference strains tested were obtained from the ATCC (Rockville, USA), the DSM (Braunschweig, Germany), the IHMM, (Vienna, Austria), and from the Faculty of Medicine, Charles University (FMCU), (Hradec Kralove, Czech Republic).

Fungus-positive clinical specimens including biopsies of pulmonary infiltrations, bronchotracheal secretions, peripheral blood, cerebrospinal fluid, and plasma were obtained from the St. Anna Children's Hospital, Vienna, Austria and from the Division of Clinical Microbiology, IHMM, Medical University of Vienna. Ocular specimens from patients with eye infections were provided by the FMCU, Hradec Kralove, Czech Republic. All clinical specimens were obtained from the patients after informed consent. Peripheral blood specimens from healthy volunteer donors were used as negative controls.

Methodology

The extraction of DNA from fungal strains, as well as of fungal, human, bacterial and viral DNA from different clinical materials was performed as described above (Baskova et al. 2007).

Fungal DNA target sequences were amplified using a semi-nested PCR. In the first round of PCR amplification, the universal primers, ITS4 reverse primer and a newly designed forward primer, which we have termed ITS7 were used to amplify

across the entire ITS1, 5.8S, ITS2, and part of the 18S and 28S regions of the rDNA gene. The second round of PCR amplification includes the reverse primer ITS4 and two fluorescein-labeled forward primers ITS86-I and ITS86-II, which hybridize to the 5.8S region of the rRNA gene.

Fragment analysis was performed on the ABI PRISM® 3100-Avant (AB) genetic analyzer, an automated fluorescence capillary electrophoresis system. The set-up of the instrument was done according to the manufacture's instructions.

7. Results

Study „The Pan-AC assay: a single-reaction real-time PCR test for quantitative detection of a broad range of* Aspergillus *and* Candida *species“

In the present report, we describe a real-time quantitative PCR assay, developed for the detection of the most common pathogenic *Candida* and *Aspergillus* species. The single-reaction PCR assay targets a judiciously selected region of the 28S subunit of the fungal rDNA gene. The unique design of the universal primer/probe system, including a pan-*Aspergillus* and -*Candida* (Pan-AC) hydrolysis probe, facilitates the detection of numerous *Aspergillus* species (e.g. *A. fumigatus, A.flavus, A.niger, A.terreus, A.vesicolor*, and *A.nidulans*) and *Candida* species (e.g. *C.albicans, C.glabrata, C.krusei, C.tropicalis, C.parapsilosis, C.kefyr, C.guilliermondii, C.lusitaniae* and *C.dubliniensis*). The detection limit of the Pan-AC assay in spiking experiments was less than 10 organisms per PCR reaction; however, for reproducible detection and quantitative analysis a higher volume of peripheral blood providing larger overall number of fungal organisms was beneficia.

Upon standardization of the technique using cultured fungal strains, the applicability in the clinical setting was assessed by investigating a series of clinical specimen. 17 haemato-oncological or intensive care patients with fungal infections

diagnosed by other methodological approaches, including primarily culture techniques and DNA sequencing revealed positive results in the clinical specimens studied, including lung biopsies of patients with pulmonary aspergillosis or candidiasis, peripheral blood specimens from patients with different types of candidaemia, and respiratory secretions positive for different *Aspergillus* species. In one of the latter specimens, co-infection with *C. lusitaniae* was detected.

Study „Diagnosis of invasive fungal diseases by a real-time panfungal PCR assay in immunocompromised patients"

We have established a two-reaction real-time PCR-assay permitting highly sensitive detection of more than 80 different fungal pathogens. Reaction I was designed to permit detection and quantitative assessment of a variety of moulds (n≥ 29), whereas reaction II targets a broad range of yeasts and Zygomycetes (n≥ 58). The assay therefore permits the detection of co-infections by different fungal genera, which may not be uncommon in immunocompromised individuals. Owing to the exploitation of a multi-copy target gene, the detection limit of the panfungal PCR assay is in the range of 1 fg fungal DNA per reaction, which corresponds to a fraction of a single fungal genome equivalent.

To assess the clinical potential of the assay, more than 600 consecutive specimens from 125 pediatric patients carrying

a high risk of IFD were analyzed. An excellent correlation between PCR-positivity and the presence of proven, probable or possible fungal infection according to the EORTC criteria was demonstrated, as revealed by the sensitivity of the assay of 96% (95%CI:82-99%). The negative predictive value of the panfungal PCR-assay presented was 98% (95%CI:90-100%), while the specificity and the positive predictive value were 77% (95%CI:66-85%) and 62% (95%CI: 47-75%), respectively.

Study „Identification of fungal species by fragment length analysis of the internally transcribed spacer 2 region“

We employed fluorescent PCR-fragment length analysis of the highly variable internal transcribed spacer (ITS2) region to identify individual fungal species by their specific amplicon sizes. Specificity of the technique was ascertained by detailed analysis of 97 strains derived from 60 different human-pathogenic fungal species. To achieve adequate sensitivity for species identification in patients with invasive fungal infection, who often display very low pathogen loads in peripheral blood, the ITS2 region was amplified by semi-nested PCR prior to amplicon-length analysis. The reproducible detection limit of capillary electrophoresis after semi-nested PCR amplification was 1 fg for both fungal strains tested, which is equivalent to a fraction of a single fungal genome on the basis of the estimated genome mass of 32 fg for *Aspergillus* species, and 37 fg for *Candida* species.

To assess the ability of the assay to facilitate identification of specific fungal pathogens in the clinical setting, different types of specimens collected from 26 patients with documented fungal infection were investigated. All clinical specimens studied were positive for fungal DNA by PCR analysis, and the respective species were identified by comparing the ITS2 fragment sizes obtained by capillary electrophoresis to external reference standards. The cerebrospinal fluid, peripheral blood and plasma samples tested revealed *C. albicans*, *C. glabrata*, and *A. flavus*/*A. niger*, respectively. The lung biopsies showed infections by *A. fumigatus* and *C. lipolytica*. The secretion samples from the respiratory tract tested positive for *A. fumigatus* in most instances and for *Rhizopus oryzae* on a single occasion. In one of the respiratory secretion specimens, a mixed infection with *A. fumigatus* and *C. albicans* was identified. All ocular specimens investigated revealed an infection by *Fusarium oxysporum*, caused by a point source outbreak in the hospital ward from which all these specimens were derived. Since certain strains of *A. flavus* and *A. niger* showed nearly identical sizes of ITS2 PCR amplicons upon capillary electrophoresis, unambiguous identification of the pathogen present was achieved by subsequent sequencing.

8. Debate

Study „The Pan-AC assay: a single-reaction real-time PCR test for quantitative detection of a broad range of Aspergillus *and* Candida *species“*

The Pan-AC assay presented covers a broad range of clinically relevant *Aspergillus* and *Candida* species in a single reaction, using a universal detection probe and a single primer pair displaying a degenerated code.

A major problem of fungal PCR assays is the high risk of contamination, which is attributable to the ubiquitous presence of airborne fungal spores, and traces of fungal DNA in a variety of reagents and other consumables. To avoid false-positive results, it is imperative therefore to control all materials used, and to include multiple negative controls in each assay. The fungus detection assays must be performed under adequate experimental conditions, which include the preparation of reagents and processing of clinical samples under a laminar flow biohazard hood. With appropriate precautions and controls, the RQ-PCR assay presented can serve as a reliable diagnostic tool for the detection and quantitative monitoring of pathogenic fungi in clinical specimens for the presence of IFD.

In patients testing positive, antifungal agents, such as voriconazole or caspofungins, could be used as first-line treatment, because these substances can be expected to cover the entire range of fungi detected by this assay. The use of agents

with a narrower spectrum of antifungal activity, such as fluconazole or amphotericin B, would require the identification of the fungal species present, which can be performed by a number of molecular techniques.

In comparison to most earlier RQ-PCR approaches to the detection of invasive aspergillosis or candidiasis (Bu et al. 2005; Klingspor & Jalal, 2006), the Pan-AC assay covers a considerably larger spectrum of pathogenic *Aspergillus* and *Candida* species in a single reaction. Our data indicate that the Pan-AC assay can be readily implemented in routine clinical diagnosis and monitoring of the majority of IFDs.

Study „Diagnosis of invasive fungal diseases by a real-time panfungal PCR assay in immunocompromised patients"

The panfungal real-time PCR assay presented was specifically adapted to highly sensitive detection of fungal pathogens in peripheral blood or serum. In the current combined prospective and retrospective study, more than 600 clinical specimens from 150 febrile neutropenic episodes in pediatric patients with high risk of invasive fungal disease were investigated by the panfungal real-time PCR assay described. Evaluation of the molecular screening data, which had been generated in a double-blind fashion, revealed an excellent correlation with the EORTC definitions of proven, probable, and possible invasive fungal disease. The sensitivity and the NPV

in the validation cohort of patients were in the range of 100% indicating that molecular screening might be instrumental in preventing unnecessary treatment. The possible benefit of this notion is underlined by the observation that nearly half of the febrile neutropenic episodes studied were treated empirically by antifungal agents, despite the lack of evidence for IFD according to the EORTC criteria. The absence of fungemia determined by the panfungal PCR assay in about 50% of these episodes suggests that a high proportion of patients were probably over-treated.

The limited specificity and PPV of the panfungal PCR assay reflect the fact that even in severely immunocompromised patient populations, repeated or persistent detection of fungemia by sensitive, broad-spectrum PCR methods may not generally indicate an imminent risk of severe fungal disease. This observation might be interpreted as frequent occurrence of false positivity, most likely attributable to contamination. However, owing to the processing of test materials in a sterile environment, the use of internally tested reagents and the number of controls included in each assay, it may be an explanation for some, but very likely not all seemingly false-positive findings.

The limited correlation between positive panfungal PCR results and imminent fungal disease may also reflect the relatively common occurrence of fungemia caused by environmental fungi, such as *Alternaria* or *Cladosporium* species, which might display low pathogenicity even in immunosuppressed individuals (Aimanianda et al. 2009; Pastor and Guarro 2008). To assess this

notion, clinical implementation of methods facilitating reliable identification of these species at an adequate level of sensitivity would be required.

Rapid and sensitive techniques for the identification of putatively non- or low-pathogenic environmental fungal species that may cause clinically silent fungemia could improve the interpretation of results obtained by broad-spectrum PCR screening methods. This might contribute to increased specificity of molecular monitoring with regard to more reliable assessment of the expected clinical relevance of fungemia. Hence, routine clinical implementation of methods for rapid identification of fungal species could provide a basis for better assessment of the need for treatment and for selection of the most appropriate antimycotic agent, if therapy appears to be required.

Study „Identification of fungal species by fragment length analysis of the internally transcribed spacer 2 region“

We have established a pan-fungal semi-nested PCR protocol for efficient amplification of the fungal ITS2 regions using a newly designed and optimized forward primer for the first round of amplification. Our modification of the detection system based on the semi-nested amplification of the ITS2 region facilitates highly sensitive species identification, which renders the technique suitable for clinical application, especially in immunocompromised patients where conventional diagnostic methods often fail owing

to low sensitivity or specificity. The technique presented was specifically designed to permit identification of the fungal species in any clinical specimen previously shown to be positive for fungal DNA by a broad-spectrum amplification assay lacking the ability of species recognition (Klingspor and Jalal 2006; Basková et al. 2007).

The PCR fragment lengths determined by capillary electrophoresis differed from those expected on the basis of sequence analysis and from previously published data (De Baere et al. 2002; Turenne et al. 1999). This divergence can be attributed primarily to the specific properties of the capillary electrophoresis apparatus used and to migration shifts during electrophoresis caused by the fluorescent dye component. Potential sources of intrinsic variability of fragment size measurements were compensated for by implementing an internal laboratory reference panel of well defined fungal strains.

A limitation of ITS2 fragment length analysis is the potential occurrence of intra-species variations of ITS2 fragment sizes in clinical isolates which do not correspond to the ITS2 sizes of the defined fungal strains presented in the reference panel.

An essential feature of the technique presented is the ability to detect co-infections with different fungi by revealing two or more amplification signals of different size in a single electropherogram. The identification of co-infections with different fungal pathogens, which is not uncommon in immunocompromised individuals, is an important prerequisite for the most appropriate antifungaltherapy.

9. Conclusions

As a consequence of the growing number of immunocompromised patients and the expanding spectrum of newly emerging fungal species, opportunistic IFDs will continue to be a major cause of morbidity and mortality in this population. Early detection of IFD in high-risk patients is a prerequisite for rational and timely initiation of effective antifungal therapy. Conventional techniques display limited sensitivity and/or specifity in detecting systemic fungal infections, and the results are mostly available late in the course of infection. Recent advances in the development of non-invasive and culture-independent diagnostic techniques have led to significant improvement by facilitating earlier detection of IFD by incorporating biomarkers and molecular technique results into diagnostic algorithms (Ostrosky-Zeichner 2012).

The potential value of nucleic acid-based approaches to the detection and identification of fungal pathogens in immunocompromised patients is indisputable, as shown by a number of studies. To date, a large number of different molecular techniques have been published including different PCR approaches. However, the current lack of standardization and validation across a wide spectrum of diagnostic laboratories has prevented inclusion of these tests in the diagnostic criteria of IFD proposed by the EORTC/MSG Consensus Group. For clinical implementation of reliable and robust molecular methods, the laboratory procedures must be internationally standardized and

evaluated in large-scale clinical trials to permit definition of widely accepted PCR performance parameters and have to be available for routine use. In order to establish a firm basis for clinical validation of molecular testing, there is urgent need for large prospective studies combining these techniques with a variety of established diagnostic tools to determine the potential role of PCR-based methods in the clinical management of IFD. The acceptance of molecular diagnostic strategies in future consensus criteria for the diagnosis of IFD would significantly impact on clinical mycology. The inclusion of molecular testing in routine diagnostic screening would provide a basis for timely detection and identification of the causative fungal pathogen, quantification of fungal load and monitoring of the response to treatment, thereby leading to better management and control of IFD. In this respect, the techniques described in the present thesis and their implementation in an international study of pediatric cancer patients can be regarded as an important step towards the achievement of the indicated goals.

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11. Overview of own publications

1. Original papers

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2. Overview articles

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3. Lectures at professional meetings

Detection of fungal DNA using panfungal real-time quantitative PCR: options and pitfalls.
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