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A novel comprehensive set of fungal Real time PCR assays (fuPCR) for the detection of fungi in immunocompromised haematological patients—A pilot study

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ABSTRACT

Fungal infections are recognized in an increasing number of patients with immunological deficits and are associated with high rates of mortality (Brown et al., 2012a). In this pilot-study, a rapid Real time PCR (fuPCR) was designed for the detection and differentiation of fungal pathogens in clinical specimens of haematological patients. The fuPCR, targeting the internal transcribed spacer region 2 (ITS2) of rDNA region, is comprised of seven multiplex reactions, which were shown to be specific and sensitive for a comprehensive spectrum of clinically relevant fungal species. This was validated by testing respective fungal DNAs in each fuPCR reaction and 28 respiratory samples of fungal pneumonia-proven patients. Clinical sample sets of throat swab, EDTA-blood and blood sera from 50 patients with severe haematological malignancies, including haematopoietic stem cell transfer (HSCT), and samples from 30 healthy individuals were then analysed. In a first step, 198 samples of immunosuppressed patients were solely examined by fuPCR; and 50.8% (33/65) respiratory swabs, 4.8% (3/63) EDTA blood samples and 1.4% (1/70) blood serum samples were tested positive. In a second step, 56 respiratory samples of immunosuppressed patients and 30 of healthy individuals were simultaneously analysed by fuPCR and standard cultivation techniques. By both methods 30.4% (17/56) swabs of the immunocompromised patients were tested positive, 37.5% (21/56) were tested negative and 32.1% (18/56) were tested fuPCR positive and culture negative. In analysing the blood samples of the immunocompromised patients 5.4% (3/56) EDTA blood samples and 16.1% (9/56) sera samples were tested fuPCR-positive, whereas all samples of 30 healthy individuals with no signs of immunological deficits were tested negative by fuPCR.

38.9% (14/36) of the fungi detected in respiratory samples of the immunosuppressed patients, belonged to *Candida* spp., 47.2% (17/36) to *Saccharomyces* spp., 5.6% (2/36) to *Cladosporium* spp. and 8.3% (3/36) to *Alternaria* spp., whereas cultivation only identified *Candida* spp. (10/17) and *Saccharomyces* spp. (7/17).

In this pilot study a novel fuPCR assay was developed and validated for the simultaneous and comprehensive detection of fungal pathogens in clinical respiratory specimens of haematological patients. Future work will focus on the validation of the blood-stream detected fungi in pathogenicity of these patients.

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1. Introduction

Invasive fungal infections, ranging from infections of inner organs to bloodstream infections and sepsis, are increasingly recognized as severe threats for immunocompromised and critically ill patients and associated with significant morbidity and mortality (Brown et al., 2012a). Although mortality has decreased due to improved anti-fungal prophylaxis and therapy, a growing population of high risk individuals leads to an increased number of fungal infections. Patients with haematological malignancies, individuals receiving immunosuppressive therapy, after HSCT, and patients undergoing intensive care are classified as high risk population (Gullo, 2009; Richardson, 2005). The predominant fungal pathogens detected worldwide are yeasts of the genus *Candida* (Pfaller and Diekema, 2007). *C. albicans* is the major cause of candidiasis and has become the most frequently isolated pathogen

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from immunocompromised patients during the last two decades (Pfaller and Diekema, 2007). In addition, *Candida* spp. other than *C. albicans* have become more common pathogens as they were detected more frequently in clinical contexts (Pfaller et al., 2014). In the last years reports show an evident increase of trichosporonosis, which is caused by the yeast-like fungus *Trichosporon* (Caira et al., 2011; Suzuki et al., 2010). These severe invasive infections were mostly documented in patients with haematological malignancies with chemotherapy, after haematopoetic stem cell transfer (HSCT) and other medical conditions associated with immunosuppression (Colombo et al., 2011).

Mould infections caused by *Aspergillus* spp. can lead to life-threatening forms of aspergillosis (Pappas et al., 2010), with *A. fumigatus* representing the most common species. Lately, an increasing number of reports reveal the clinical importance of further emerging fungal pathogens (Kriengkauykiat et al., 2011; Low and Rotstein, 2011; Person et al., 2010). *Fusarium* causes a broad spectrum of severe infections in humans and has a wide range of resistances against antimycotic drugs. Patients with prolonged neutropenia and haematological diseases are at high risk and can be inflicted by an invasive fusariosis (Dignani and Anaissie, 2004; Nucci and Anaissie, 2007). *Scedosporium prolificans* is an emerging pathogen that is increasingly recognized as cause of infections in immunocompromised hosts (Ochi et al., 2015; Sugawara et al., 2013). Based on the challenging treatment of *Scedosporium* due to its resistance to many antifungal agents, diagnosis of the fungi is of great importance (Cortez et al., 2008). Fungal infections of the central nervous system belong to the most lethal ones and reports about an involvement of *Cryptococcus* neoformansare getting progressively noted among immunosuppressed patients (Colombo and Rodrigues, 2015). *Cryptococcus* infections are also recognized as AIDS-associated diseases (Prado et al., 2009). Zygomycetes can cause severe forms of zygomycosis which are characterized by an aggressive and rapid progression (Pagano et al., 2011). In patients at a risk fungal infections have a high mortality rate due to late and insufficient diagnosis and the difficult treatment (Kontoyiannis and Lewis, 2011). Most common fungal agents are *Rhizopus*, followed by *Mucor* and *Lichtheimia* (formerly *Absidia*) which en masse account for up to 80% of all mucormucosis cases (Chayakulkeeree et al., 2006; Skiada et al., 2013). The *Lichtheimia* family was defined by a revision of the *Absidia* and *Mycocladus* genera due to phylogenetic and morphological characteristics (Hoffmann et al., 2007, 2009). *Absidia*, *Rhizomucor* and *Cunninghamella* are other important members of the *Mucorales* that are responsible for infections in immunocompromised individuals (Alvarez et al., 2009; Gomes et al., 2011).

Well-established diagnostic methods such as histology, tissue and blood culture and microscopy are considered as gold standard for the detection of invasive fungal infections (Badiee and Hashemizadeh, 2014; Kerr, 2004; Neumeister et al., 2009; Sangoi et al., 2009), although these techniques are hampered by low sensitivities (Morrissey et al., 2011, 2013). To increase sensitivity and specificity they are often complemented by other techniques. The serological detection of genus-specific markers such as galactomannan are mainly used for *Aspergillus* spp., mannan for *Candida* spp. and (1–3‘) β-glucan assays for *Aspergillus*, *Candida* spp. and other fungi (Bernal-Martinez et al., 2016; Hachem et al., 2009; Klont et al., 2004) besides radiological imaging using X-ray and high-resolution computed tomography (Maertens et al., 2005). However, these tests do not only have low sensitivities and specificities, but cannot identify the fungal species responsible for infection.

The use of polymerase chain reaction (PCR) assays provides a powerful tool for a sensitive diagnosis of fungal infections. A variety of PCR assays have been reported during the recent years, using different technical approaches which include nested PCR (Lau et al., 2010; Taira et al., 2014), PCR in combination with FRET detection (Nabili et al., 2013) or conventional sequencing (Morjaria et al., 2015), PCR coupled with mass spectrometry (Jeng et al., 2012; Massire et al., 2013) and quantitative real-time PCR (Walsh et al., 2011). Although a variety of fungal pathogens is already known today, most PCR assays focus on detection of a single fungal genus such as *Aspergillus* (Buchheidt et al., 2004; Lass-Florl et al., 2004) or *Candida* (Lau et al., 2010; Mirhendi et al., 2006) and rarely specialize on single species detection (Maaroufi et al., 2003; Spiess et al., 2003). Some methods implement duplex assays for detection of two fungal pathogens (Klingspor and Jalal, 2006; Schabereiter-Gurtner et al., 2007) or multiplex approaches for a variety of fungal genera (Massire et al., 2013; Schabereiter-Gurtner et al., 2007). Differences between these PCRs rely on the kind of clinical specimens tested (e.g. sera, blood or BAL), methods of DNA isolation and the amplified target (Loeffler et al., 2015; Schabereiter-Gurtner et al., 2007; Taira et al., 2014). Many different genes have been suggested as target including genes coding for the lysine biosynthesis pathway (Guo and Bhattacharjee, 2006) or mitochondrial genes (Costa et al., 2001). However, PCR assays targeting ribosomal DNA (rDNA) are most frequently used. The nuclear rDNA has been widely used for fungal diagnostics and phylogenetic examinations (Begerow et al., 2010). An rDNA operon, which consists of the 18S, 5.8S and 28S rRNA genes, is separated by two internal transcribed spacer regions, ITS1 and ITS2. These ITS regions have been recommended as a universal barcode sequence for fungi (Schoch et al., 2012). Since the availability of sequenced fungal genomes has enormously increased, comprehensive PCR strategies can now be devised. Based on the presence of more or less conserved regions, sets of PCR primer can be designed which target distinct fungal families, genera or even species.

The aim of the present study was the development of a novel, comprehensive set of PCR assays (fuPCR) which enable the simultaneous detection and differentiation of medically important fungal species in respiratory samples of high risk patients. The results obtained by fuPCR were compared to those of conventional fungal culture using different respiratory samples of patient with proven (fungal) pneumonia and throat swabs as non-invasive taken specimen of immunocompromised haematological patients. In a first look, fuPCR was evaluated as a screening tool for fungal blood stream infections. Fungal detections in specimens of haematological patients were then compared to clinical data where a great discriminatory power was found by using fuPCR.

2. Materials & methods

2.1. Ethical agreement

All enrolled subjects were given detailed information and provided written agreement to participate in this study. In accordance with the Declaration of Helsinki (2014), written informed consent to take part in the study was obtained from all subjects. The study protocol for the identification, surveillance and epidemiology of fungal pathogens in immunocompromised patients was approved by the local ethics committee of the Medial Faculty of the Heinrich-Heine-University Düsseldorf (Study-No.: 4146). EDTA blood, serum and respiratory swabs of patients were collected during the course of routine treatment and examination.

2.2. Clinical characteristics of study population

In order to test the fuPCR as a screening tool a total of 50 immunocompromised adults, 31 male (62%) and 19 female (38%) were then enrolled in this study. Between May 2014 and June 2016, a sample triplet (EDTA blood, blood serum and throat swab) was taken once a week during every hospitalization. Throat swabs were

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