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Molecular detection and identification of *Candida* and *Aspergillus* spp. from clinical samples using real-time PCR

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ABSTRACT

This report describes the development of a real-time LightCycler assay for the detection and identification of *Candida* and *Aspergillus* spp., using the MagNa Pure LC Instrument for automated extraction of fungal DNA. The assay takes 5–6 h to perform. The oligonucleotide primers and probes used for species identification were derived from the DNA sequences of the 18S rRNA genes of various fungal pathogens. All samples were screened for *Aspergillus* and *Candida* to the genus level in the real-time PCR assay. If a sample was *Candida*-positive, typing to species level was performed using five species-specific probes. The assay detected and identified most of the clinically relevant *Aspergillus* and *Candida* spp. with a sensitivity of 2 CFU/mL blood. Amplification was 100% specific for all *Aspergillus* and *Candida* spp. tested. To assess clinical applicability, 1650 consecutive samples (1330 blood samples, 295 samples from other body fluids and 25 biopsy samples) from patients with suspected invasive fungal infections were analysed. In total, 114 (6.9%) samples were PCR-positive, 5.3% for *Candida* and 1.7% for *Aspergillus* spp. In patients with positive PCR results for *Candida* and *Aspergillus*, verification with conventional methods was possible in 83% and 50% of cases, respectively. In conclusion, the real-time PCR assay allows sensitive and specific detection and identification of fungal pathogens *in vitro* and *in vivo*.

Keywords *Aspergillus*, *Candida*, diagnosis, fungi, identification, real-time PCR

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INTRODUCTION

In Sweden, *Candida albicans* and other *Candida* spp., followed by *Aspergillus* spp., are the most common fungal pathogens causing invasive fungal infections (IFIs). Opportunistic IFIs are a major cause of morbidity and mortality in immunocompromised patients such as transplant recipients [1–4]. The clinical features of invasive candidiasis are non-specific, making early diagnosis of invasive candidiasis difficult [5,6]. There has been some progress in the diagnosis of invasive aspergillosis (IA) in recent years, mainly because of the use of high-resolution computerised tomography (CT) scanning and other imaging procedures, but established IA is difficult to treat, with a mortality rate of 80–90% [7].

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The incidence of nosocomial bloodstream infections caused by *Candida* spp. has increased during the last two decades. In a recent report from Sweden [8], the crude mortality rate of candidaemia was 31%. The highest mortality rates were observed in patients with haematological malignancies (41.2%), in those aged >70 years (41%), following surgery (38.5%), and in those infected with more than one *Candida* sp. (40%) or *Candida glabrata* (38%). However, blood cultures take time and have poor sensitivity [9–12]. As a consequence, the diagnosis of candidaemia and aspergillosis is generally established at a late stage, or even at autopsy, in a considerable number of cases [13].

Non-cultural techniques used previously have lacked sensitivity and specificity in immunocompromised patients [14,15]. New rapid methods that can detect IFI early in the course of disease, with high sensitivity and specificity, are thus required. More recently, PCR protocols for diagnosing fungal infections have been described [16–22]. Molecular diagnostic methods using uni-

versal fungal PCR primers and species-specific probes have been developed and evaluated for the detection of fungal DNA in clinical specimens. Since non-*albicans Candida* spp. and *Aspergillus* spp. are increasing in importance, it is necessary for the fungal PCR assay to have high sensitivity for most pathogenic *Candida* and *Aspergillus* spp.

This report describes the development of an assay that uses a fully automated laboratory robot, the MagNA Pure LC Instrument, for extraction of *Candida* and *Aspergillus* DNA, in combination with the real-time PCR LightCycler System. The whole assay takes *c*. 6 h to perform. To assess its clinical applicability, a large number of blood samples, samples from other body fluids and biopsy samples from patients with suspected IFI were analysed by the assay, and the results were compared with those obtained by conventional methods.

MATERIALS AND METHODS

Fungal cultures

Standard fungal strains used in the study were *C. albicans* ATCC 10231, *C. glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* CCUG 5570, *Candida lusitaniae* UKNEQAS 6208, *Candida guillerimondi* SMI 8302, *Candida norvergensis* SMI 115-04, *Candida dubliniensis* (sequenced), *Candida kefyr* SMI 7-01, *Cryptococcus neoformans* ATCC 24067, *Aspergillus fumigatus* UKNEQAS 5526, *Aspergillus flavus* UKNEQAS 6657, *Aspergillus niger* ATCC 1640, *Aspergillus versicolor* UKNEQAS 6406, *Aspergillus nidulans* UKNEQAS 6020, *Aspergillus clavatus* UKNEQAS 7021, *Aspergillus glaucus* UKNEQAS 5647 and *Aspergillus terreus* UKNEQAS 6323. Clinical isolates were obtained from Karolinska University Hospital Huddinge, Stockholm, Sweden.

Sample preparation

Before DNA extraction, *Candida* cultures were grown on Sabouraud–glucose agar for 48 h at 30°C, and *Aspergillus* cultures were grown for 72 h at 30°C. Fungal suspensions in saline were adjusted (0.5 × MacFarland standard) to a concentration of 1×10^6 to 5×10^6 cells/mL. Ten-fold serial dilutions (10^6–10^1 cells) were prepared to test the sensitivity and specificity of the assay.

For determination of the detection limit in blood, EDTA-anti-coagulated whole-blood samples (5 mL) from healthy volunteers were spiked with serial dilutions of *Candida* and *Aspergillus* (10^6–10^1 cells/mL). DNA was extracted and analysed according to the protocol described below.

For specificity testing of the probes, cells from the following yeasts and moulds were tested: *Candida* spp., *Aspergillus* spp., *Malazessia* spp., *Crypt. neoformans*, *Saccharomyces cerevisiae*, *Trichosporon* spp., *Fusarium* spp., *Zygomycetes* spp., *Scedosporium* spp. and *Paecilomyces* spp. (Table 1).

Preparation of total genomic DNA from clinical samples

Manual steps: blood. Blood specimens (and fluids containing red blood cells) were incubated initially with a hypotonic red cell lysis buffer (RCLB) as described previously [19]. Following lysis of the erythrocytes, the sample was centrifuged at 2500 *g* for 10 min. The pellets were transferred to 1.5-mL Eppendorf tubes

Table 1. *Candida* and *Aspergillus* spp. and additional fungal species tested by real-time PCR

	Positive by hybridisation with DNA probe specific for						
	Common *Candida*	*C. albicans*/ *C. dubliniensis*	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	*Aspergillus* spp.
Candida albicans	+	+	−	+	−	−	−
Candida glabrata	+	−	+	−	−	−	−
Candida parapsilosis	+	+	−	+	−	−	−
Candida tropicalis	+	−	−	−	+	−	−
Candida krusei	+	−	−	−	−	+	−
Candida lusitaniae	+	−	−	−	−	−	−
Candida dubliniensis	+	+	−	+	−	−	−
Candida kefyr	+	−	−	−	−	−	−
Candida norvergensis	+	−	−	−	−	−	−
Candida guillerimondi	+	−	−	−	−	−	−
Saccharomyces cerevisiae	+	−	−	−	−	−	−
Trichosporon spp.	−	−	−	−	−	−	−
Malassezia spp.	−	−	−	−	−	−	−
Cyptococcus neoformans	−	−	−	−	−	−	−
Aspergillus fumigatus	−	−	−	−	−	−	+
Aspergillus flavus	−	−	−	−	−	−	+
Aspergillus niger	−	−	−	−	−	−	+
Aspergillus versicolor	−	−	−	−	−	−	+
Aspergillus terreus	−	−	−	−	−	−	+
Aspergillus nidulans	−	−	−	−	−	−	+
Aspergillus glaucus	−	−	−	−	−	−	+
Aspergillus clavatus	−	−	−	−	−	−	+
Zygomycetes spp.	−	−	−	−	−	−	−
Fusarium spp.	−	−	−	−	−	−	−
Scedosporium spp.	−	−	−	−	−	−	−
Paecilomyces spp.	−	−	−	−	−	−	−
Penicillium spp.	−	−	−	−	−	−	−