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Regular paper

PCR and real-time PCR assays to detect fungi of *Alternaria alternata* species*

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Fungi of the *Alternaria* genus are mostly associated with allergic diseases. However, with a growing number of immunocompromised patients, these fungi, with *A. alternata* being the most prevalent one, are increasingly recognized as etiological agents of infections (phaeohyphomycoses) in humans. Nowadays, identification of *Alternaria* spp. requires their pure culture and is solely based on morphological criteria. Clinically, *Alternaria* infections may be indistinguishable from other fungal diseases. Therefore, a diagnostic result is often delayed or even not achieved at all. In this paper we present easy to perform and interpret PCR and real-time PCR assays enabling detection of *A. alternata* species. On the basis of alignment of β-tubulin gene sequences, *A. alternata*-specific primers were designed. DNA from fungal isolates, extracted in a two-step procedure, were used in PCR and real-time PCR assays followed by electrophoresis or melting temperature analysis, respectively. The assays specificity was confirmed, since positive results were obtained for all *A. alternata* isolates, and no positive results were obtained neither for other molds, dermatophytes, yeast-like fungi, nor human DNA. The assays developed here enable fast and unambiguous identification of *A. alternata* pathogens.

Key words: *Alternaria alternata*, detection, identification, PCR, real-time PCR

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INTRODUCTION

Phaeohyphomycosis is a group of opportunistic infections caused by dematiaceous molds, which are distributed worldwide as plant pathogens, usually considered as rare human pathogens. However, due to the growing population of immunocompromised patients, these fungi are increasingly recognized as etiological agents of human infections. *Alternaria* is a very large and complex genus that contains hundreds of species of dematiaceous hyphomycetes. The exact number of species is difficult to estimate because of the proliferation of nomenclature of dubious taxonomic validity (Pastor & Guarro, 2008). Many species are common saprophytes in soil and air; some are involved in organic matter decay while others are plant pathogens (de Hoog *et al.,* 2000).

Alternaria species are mostly associated with hypersensitivity pneumonitis, bronchial asthma, allergic sinusitis and rhinitis (Shugar *et al.,* 1981; Bush & Prochnau, 2004; Pant *et al.,* 2005; Stark *et al.,* 2005; Pulimood *et al.,* 2007; Chowdhary *et al.,* 2012). Less commonly, these fungi have been reported as causes of other infections including ocular infections (Laich *et al.,* 2008; Konidaris *et al.,* 2013), meningoencephalitis and arachnoiditis (Silveira *et al.,* 2013), sinusitis (Pesic *et al.,* 2015), granulomatous pulmonary disease (Lobritz *et al.,* 1979), onychomycosis (Gianni *et al.*, 1997; Romano *et al.,* 2001), cutaneous infections (Lyke *et al.,* 2001; Pereiro *et al.,* 2004; Calabrò *et al.,* 2008; Gomes *et al.,* 2011; Brás *et al.,* 2015) and subcutaneous infections (Kpodzo *et al.,* 2011; Salido-Vallejo *et al.,* 2014), and disseminated diseases (Kucan & Hall, 1985; Revankar *et al.,* 2002). Among *Alternaria* spp., *A. alternata* has been the most frequently isolated species from all types of infections. However, identification to the species level has been only performed sporadically (Pastor & Guarro, 2008).

The identification of *Alternaria* species is solely based on morphological criteria, especially size and shape of conidia and the presence (or absence) of conidial chains. Culture is regarded as mandatory for the correct identification of *Alternaria* spp., which grow on most routine laboratory media, although the clinically important species very quickly lose their ability to sporulate (Pastor & Guarro, 2008). Clinically, phaeohyphomycosis caused by *Alternaria* may be difficult to recognize. Lesions appear as non-healing ulcers, crusted lesions, erythematous macules, or subcutaneous nodules. Histopathologically, thick-walled (sub)spherical elements 10–15 μm in diameter or short chains of oblong cells may be observed. The rounded elements are mostly unpigmented, and therefore *Alternaria* infection is sometimes misdiagnosed as yeast infection or blastomycosis (de Hoog & Horré, 2002).

Moreover, the *Ulocladium* genus is morphologically very similar to *Alternaria* and has been also involved in human diseases, although remarkably less frequently (Durán *et al.,* 2003; Romano *et al.,* 2004; Badenoch *et al.,* 2006). *Ulocladium* can be distinguished morphologically from *Alternaria* only upon the observation of young conidia (Pastor & Guarro, 2008).

Current trends in diagnostics are moving towards high-throughput and simple molecular technologies that provide data regarding types of organisms present in a sample. As a result of these developments, infectious diseases will be more accurately and effectively treated. PCR is a method of *in vitro* DNA amplification that relies on knowledge of at least partial sequences of the tar-

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Abbreviations: PCR, Polymerase Chain Reaction

get DNA which are used to design oligonucleotide primers that hybridize specifically to the target sequences. A significant advancement in PCR technology is quantitative real-time PCR, in which amplification and detection of amplicons are coupled in a single reaction vessel. Real-time PCR represents a major breakthrough according to clinical applicability since it eliminates the need for post-amplification processing needed in conventional PCR (Yang & Rothman, 2004).

In this paper we present PCR and real-time PCR-based assays developed for the detection of *A. alternata* species.

MATERIALS AND METHODS

Strains and isolates. In the present study, we used a total of 213 fungal strains and isolates, representing 99 fungal species (Table 1). The strains were obtained from international culture collections (CBS-KNAW Fungal Biodiversity Centre; BCCM/IHEM Biomedical Fungi and Yeasts Collection – Belgian Coordinated Collections of Microorganisms; Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures); isolates were obtained from the Molecular Biotechnology and Microbiology Department (MBMD) collection of fungi (Gdańsk University of Technology, Gdańsk, Poland). Identification of all MBMD isolates was performed by observation of macro- and micromorphology, and then confirmed by sequencing of the ITS region (White *et al.,* 1990).

DNA extraction. Strains and isolates were cultured on Sabouraud medium (BTL, Łódź, Poland) and incubated for up to 14 days at room temperature. DNA from fungal samples (pieces of mycelium of 3–5 mm in diameter) was extracted by a 10-min incubation of the sample in 100 µl of extraction buffer (60 mM sodium bicarbonate [$NaHCO_3$], 250 mM potassium chloride [KCl] and 50 mM Tris, pH 9.5) at 95°C and subsequent addition of 100 µl of anti-inhibition buffer (2% bovine serum albumin). After 3 s vortex mixing, this DNA-containing solution was used for PCR (Brillowska-Dąbrowska *et al.,* 2010). All reagents for DNA extraction were purchased from Sigma-Aldrich (Seelze, Germany).

***Alternaria alternata*-specific PCR and real-time PCR assay.** On the basis of alignment (VectorNTI; InforMax, Inc.) of β-tubulin gene sequences deposited in the NCBI nucleotide database, *Alternaria alternata*-specific primers AaltFor (5' GTGCCTTCCCCCAAGGTCTCCG 3') and AaltRev (5' CGGAAACGAGGTGGTTCAGGTC 3') were designed. Primers were then synthesized by Genomed (Warsaw, Poland).

PCR mixtures, 20 µl each, consisted of 10 µl of 2x PCR Master Mix Plus High GC (A&A Biotechnology, Gdynia, Poland), 0.1 µl of each primer (AaltFor, AaltRev) at 100 µM, and 2 µl of DNA. PCR was performed in a 5345 Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). The time-temperature profile included initial denaturation for 3 min at 94°C followed by 35 cycles of 30 s at 94°C, and 60 s at 72°C. The presence of amplicons was examined electrophoretically on 2% agarose gels, stained with ethidium bromide.

Real-time PCR mixtures, 20 µl each, consisted of 10 µl of 2x PCR Master Mix SYBR C (A&A Biotechnology, Poland), 0.1 µl of each primer (AaltFor, AaltRev) at 100 µM, and 2 µl of DNA. Real-time PCR was performed in a LightCycler® Nano Instrument (Roche, Basel, Switzerland). The cycling conditions included an initial denaturation for 3 min at 95°C followed by 35 cycles of 15 s at 94°C, and 30 s at 72°C. The presence of amplicons was examined upon melting temperature analysis (60°C to 97°C at 0.1°C/s ramp rate), that followed cycling.

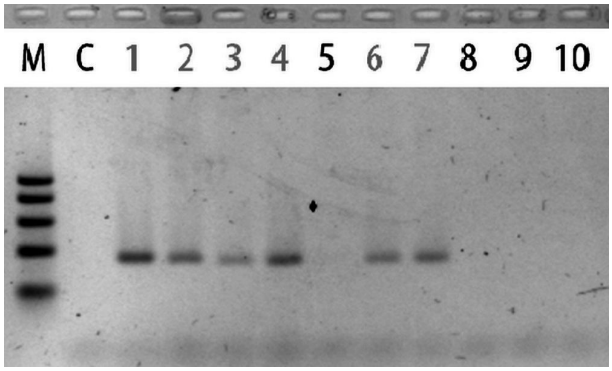

Figure 1. Example of *Alternaria alternata*-specific PCR product analysis.
M, molecular size marker (fragment sizes 500, 400, 300, 200 and 100 bp); results of *Alternaria alternata*-specific PCR performed for 1–4, *A. alternata* (environmental isolates); 5, *A. tenuissima*; 6–7, *A. alternata* (animal-derived isolates); 8–10, *A. brassicae*; C, negative control.

RESULTS

Alternaria alternata-specific PCR assay results

A 184-bp PCR product corresponding to *A. alternata* was observed for all 16 *A. alternata* DNA samples. No PCR products were detected for either 4 *Alternaria* non-*alternata* isolates, 99 other mold isolates, 65 dermatophyte isolates, 30 yeast-like isolates or human DNA (100% sensitivity and 100% specificity) (Fig. 1).

Alternaria alternata-specific real-time PCR assay results

The same results were obtained when real-time PCR was applied, as amplicon of $T_m = 88.05 \pm 0.17$°C (Ct = 26.54 ± 1.37), corresponding to *A. alternata*, was observed only for 16 *A. alternata* DNA samples and not for any other fungal or human DNA samples (Fig. 2).

DISCUSSION

At present, diagnostics of opportunistic fungal infections still relays on the evaluation of macro- and micro-

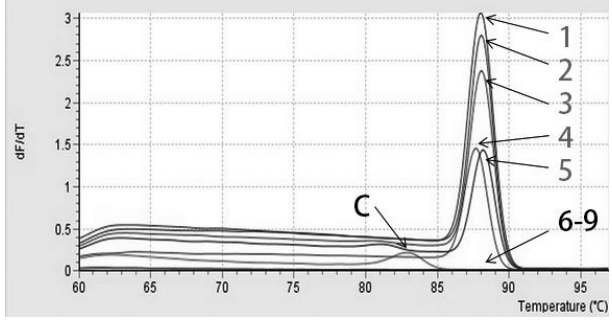

Figure 2. Example of Alternaria alternata-specific real-time PCR product melting temperature analysis performed for
1–3, *A. alternata* (environmental isolates); 4–5, *A. alternata* (animal-derived isolates); 6, *A. tenuissima*; 7–9, *A. brassicae*; C, negative control.

Table 1. DNA used in the study.

	Organism	Collection	No. of Strains/Isolates
molds	*Alternaria alternata*	MBMD (animal-derived isolates)	9
			7
	A. brassicae		3
	A. tenuissima		1
	Acremonium charticola		1
	A. kiliense		1
	A. strictum		1
	Aspergillus clavatus		2
	A. flavus		3
	A. fumigatus		8
	A. nidulans	MBMD (environmental isolates)	1
	A. niger		2
	A. versicolor		1
	Cladosporium cladosporioides		3
	C. herbarum		2
	C. macrocarpum		1
	Fusarium culmorum		1
	F. discolor		1
	F. oxysporum		2
	F. proliferatum		1
	F. solani		2
	Microascus albonigrescens	CBS	1
	M. cinereus	CBS, IHEM	2
	M. cirrosus	CBS	1
	M. longirostris		1
	M. manginii		1
	M. senegalensis		1
	M. singularis		1
	M. stoveri		1
	M. trigonosporus var. terreus		1
	M. trigonosporus var.macrosporus		1
	M. trigonosporus var. trigonosporus		1
	Mucor racemosus	MBMD (environmental isolates)	2
	M. circinelloides		1
	Ochrocladosporium elatum		1
	Penicillium chrysogenum		1
	P. carneum		1
	P. chrysogenum		2
	P. commune		2
	P. crustosum		1
	P. digitatum		1
	P. glabrum		2
	P. hirsutum		1
	P. italicum		1

	P. melinii		1
	P. paneum		1
	P. polonicum		3
	P. verrucosum		1
	Penicillium sp.		1
	Phoma herbarum		2
	Pleospora papaveracea		1
	Rhizopus oligosporus		1
	R. oryzae		2
	Scopulariopsis acremonium	DSM	1
	S. asperula	CBS, IHEM	2
	S. brevicaulis	CBS	2
		MBMD (animal-derived isolates)	2
		MBMD (human-derived isolate)	1
	S. brumptii	CBS	2
	S. canadensis		1
	S. carbonaria		1
	S. chartarum		1
	S. coprophila		1
	S. flava		1
	S. fusca	CBS, IHEM	2
	S. gracilis	CBS	1
	S. konigii		1
	S. murina		1
	S. parva		1
	Trichoderma viridae	MBMD (environmental isolates)	1
	Ulocladium chartarum		1
	U. tuberculatum		1
dermatophytes	*Epidermophyton floccosum*	MBMD (human-derived isolates)	7
	Microsporum audouinii		5
	M. canis		3
	M. gypseum		5
	M. nanum		1
	M. persicolor		3
	Trichophyton equinum		1
	T. erinacei		2
	T. interdigitale		4
	T. mentagrophytes		5
	T. rubrum		4
	T. schoenleinii		3
	T. soudanense		2
	T. terrestrae		6
	T. tonsurans		6
	T. verrucosum		5
	T. violaceum		3

yeasts	***Candida albicans***	**MBMD (human-derived isolates)**	**7**
	C. catenulata		**1**
	C. glabrata		**3**
	C. guillermondii		**1**
	C. kefyr		**2**
	C. krusei		**1**
	C. magnoliae		**1**
	C. parapsilosis		**5**
	C. tropicalis		**5**
	C. utilis		**1**
	Geotrichum sp.		**1**
	Rhodotorula mucilaginosa	**MBMD (environmental isolates)**	**1**
	Saccharomyces cerevisiae		**1**
Human		**MBMD**	1

morphology. Identification of *A. alternata* in pure culture, based on morphological criteria, remains useful since the features of conidia are quite characteristic. However, diagnosis upon histopathological examination of altered tissues is particularly challenging since phaeohyphomycosis caused by *Alternaria* fungi is difficult to distinguish from yeast infections or blastomycosis (de Hoog & Horré, 2002).

Basic Local Alignment Search Tool (BLAST) enables comparison of nucleotide sequences (mostly rDNA) of a given unidentified fungus with those deposited previously in GenBank in order to find regions of similarity among sequences. However, an important problem lies in the fact that sequences within GenBank are deposited without strict quality control of species identification, and some sequences have therefore been deposited under erroneous names. In the case of *Alternaria*, it has been estimated that about 14% of the sequences deposited in GenBank are misidentified. Moreover, analysis of internal transcribed spacer (ITS) region sequences has demonstrated that *A. longipes* and *A. tenuissima* cannot be differentiated from *A. alternata* (de Hoog & Horré, 2002). Therefore, although the method is useful, it is important to ensure that unknown sequences are compared with the sequences of reference strains that have been identified by experts.

In addition, de Hoog and Horré have developed a procedure to distinguish *Alternaria* and *Ulocladium* spp., based on a PCR-RFLP (de Hoog & Horré, 2002). However, this method is laborious and time-consuming, thus making it unlikely to be implemented in routine laboratory diagnostics.

All this underlines a need for the development of new methods that would provide simple, rapid and highly specific identification of *A. alternata*. In this study, we present PCR and real-time PCR-based assays that allow species-specific detection of *A. alternata* within few hours. The developed assays are rapid, easy to perform and interpret, and can serve as useful adjunct tools for the identification of *A. alternata* infections. An important advantage of the assays is that the applied DNA extraction procedure has a potential to extract DNA directly from clinical specimens (nails, skin scrapings etc.) thus significantly reducing the time of diagnosis (Brillowska-Dąbrowska *et al.,* 2010). However, to confirm the diagnostic accuracy of the developed assays, further studies are needed.

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