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Rapid detection of pathogenic fungi using loop-mediated isothermal amplification, exemplified by *Fonsecaea* agents of chromoblastomycosis

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ABSTRACT

Loop-mediated isothermal amplification (LAMP) was developed for rapid detection of pathogenic or allergenic fungal in the environment. Primers applied were derived from the rDNA Internal Transcribed Spacer and the 5.8S rRNA gene. The assay enabled amplification of target fungi at the level of genus or closely related species using pure cultures after 1 h reaction at 65 °C in a water bath. No cross-reactivity to related species was observed. The DNA detection limit was 0.2 fg. The method also proved to work well with fungi on non-sterile adhesive tape. Amplification products were detected by visual inspection using SYBR Green I as well as by electrophoresis on agarose gels. As a model organism we selected *Fonsecaea,* a fungal genus containing etiologic agents of chromoblastomycosis, a widely distributed tropical and subtropical skin disease in otherwise healthy patients and supposed to be acquired by environmental inoculation. It is suggested that LAMP can also be used for rapid clinical diagnosis, for environmental detection, and for retrospective studies in archived clinical samples.

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1. Introduction

Environmental detection of detrimental fungi is of major significance for understanding a wide diversity of problems related to public health. For example, fungi may contribute to allergic responses of patients due to overabundance of moulds in indoor environments (Beezhold et al., 2008). Locating the source of mycotoxin-producing *Stachybotrys* species occurring in moist homes is urgent (Kuhn and Ghannoum, 2003). Hygienic questions also arise with dermatophyte contamination of public bathing facilities such as swimming pools (Hilmarsdottir et al., 2005). Preventive measures against fungal opportunists require understanding of routes of infection, for which extensive studies on natural habitats and methods of dispersal are undertaken (Sudhadham et al., 2008). Numerous investigations have been carried out to locate the ecological niche of pathogens such as *Cryptococcus gattii* (Datta et al., 2009) and *Penicillium marneffei* (Chariyalertsak et al., 1997). Quite often, the fungi in question grow in culture only with difficulty or are unculturable (Bougoure and Cairney, 2005). In view of these problems, isolation and culturing may be far from optimal (Elliott et al., 2006), for which reason molecular detection is recommended. In the present article, we apply a recently proposed method, loop-mediated isothermal amplification (LAMP), which is rapid and allows only minute amounts of DNA, to detection of a group of pathogenic fungi.

The method is demonstrated here using potential agents of a skin disease, chromoblastomycosis, a relatively frequent disorder in (sub) tropical climate zones (Queiroz-Telles et al., 2009). The spectrum of species causing this disease is very limited: in addition to *Fonsecaea* species, mainly *Cladophialophora carrionii* (Badali et al., 2008), *Phialophora verrucosa* (Guerriero et al., 1998), *Rhinocladiella aquaspersa* (Badali et al., in press) and some scattered cases of *Exophiala* (Barba-Gomez et al., 1992; De Hoog et al., 2000, Fader and McGinnis, 1988; Padhye et al., 1996) are involved. The genus *Fonsecaea* comprises two sibling species, *F. pedrosoi* and *F. monophora* (De Hoog et al., 2004; Najafzadeh et al., 2009a), while recently a third one was proposed (Najafzadeh et al., in press-b). *Fonsecaea monophora* has repeatedly been found, in addition to causing chromoblastomycosis, as an agent of brain infection (Najafzadeh et al., in press-a,b; Surash et al., 2005).

Laboratory diagnosis of chromoblastomycosis is based on microscopy of the muriform cells in tissue and skin scrapings. Morphologic characteristics and antigen detection have also been developed for the identification of *F. pedrosoi* (Carrión and Silva-Hutner, 1971; da Silva

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et al., 2002; Iwatsu et al., 1982; Vidal et al., 2004), but these methods are time-consuming and have low specificity. Polymerase chain reaction has been developed for rapid detection of several dematiaceous fungi including members of *Fonsecaea* (Abliz et al., 2003a,b, 2004a; Caligiorne et al., 1999; De Andrade et al., 2007; Karuppayil et al., 1996). The disease is supposed to be acquired by traumatic inoculation of contaminated material into the skin (Rubin et al., 1991). Origins have been supposed to be prickly plant spines such as cactus (Fernández-Zeppenfeldt et al., 1994) or *Mimosa* (Salgado et al., 2004). However, several authors (De Hoog et al., 2007; Vicente et al., 2008) report that most fungi isolated from nature concern less virulent siblings of the pathogenic species concerned, rather than the pathogens themselves. Detection of the true agents of disease in the environment thus remains overdue.

Loop-mediated isothermal amplification (LAMP) was firstly described and initially evaluated for detection of hepatitis B virus DNA by Notomi et al. (2000). This novel nucleic acid amplification method can amplify DNA with high specificity, efficiency and rapidity under isothermal conditions. The cycling reactions result in the accumulation of 10^9 to 10^{10}-fold copies of target in less than an hour. The amplification products can be easily detected by visual assessment of turbidity, electrophoresis or by the naked eye. The sensitivity of LAMP appears not to be affected by the presence of non-target DNA in samples, neither by known PCR inhibitors such as blood, serum, plasma, or heparin (Enosawa et al., 2003; Notomi et al., 2000; Poon et al., 2005). For this reason the method is very promising as a tool for environmental detection of microorganisms. LAMP assays have been used for diagnostics of bacteria (Aoi et al., 2006; Iwamoto et al., 2003; Yoshida et al., 2005), virus (Cai et al., 2008; Hagiwara et al., 2007) and parasites (Ikadai et al., 2004; Iseki et al., 2007), but only rarely for fungi (Endo et al., 2004; Inacio et al., 2008; Ohori et al., 2006). In the present study, we developed a sensitive, specific and rapid method for the detection of members of the genus *Fonsecaea* by LAMP, in the laboratory as well as on non-sterile adhesive tape widely used for environmental screening.

2. Material and methods

2.1. Strains and culture conditions

Twelve strains of *Fonsecaea pedrosoi*, 27 of *F. monophora*, 8 of *F. nubica* and 61 reference strains of 36 related melanized fungi (Table 1) were obtained from the reference collection of the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre and grown on Malt Extract Agar (MEA) at 25 °C for 14 days.

2.2. DNA extraction and quality test

Approximately 0.5 g mycelium of 14-d-old cultures was transferred to a 2 ml Eppendorf tube containing 400 µl TEx buffer pH 9.0 (100 mM Tris, 40 mM Na-EDTA) and glass beads (Sigma G9143). The fungal material was homogenized with MoBio vortex (Bohemia, New York, U.S.A.) for 1 min. Subsequently 120 µl SDS 10% and 10 µl proteinase K (10 mg/ml) were added and incubated for 30 min at 55 °C; the mixture was vortexed for 3 min. After addition of 120 µl of 5 M NaCl and 1/10 vol CTAB 10% (cetyltrimethylammoniumbromide) solution, the material was incubated for 60 min at 55 °C. Then the mixture was vortexed for 3 min. Subsequently 700 µl SEVAG (24:1, chloroform:isoamylalcohol) was added, mixed carefully by hand and centrifuged for 5 min at 4 °C at 20,400*g* force value. The supernatant was transferred to a new Eppendorf tube with 225 µl 5 M NH_4-acetate, incubated for 30 min on ice water, and spun for 5 min at 4 °C at 20,400*g* force value. The supernatant was then transferred to another Eppendorf tube with 0.55 vol isopropanol and centrifuged for 5 min at 20,400*g* force value. Finally, the pellet was washed with 1000 µl ice cold 70% ethanol. After drying at room temperature, it was re-suspended in 48.5 µl TE buffer (Tris 0.12% w/v, Na-EDTA 0.04% w/v). The DNA concentration was detected with nano-drop DNA concentration detector at 260 nm (Thermo Scientific, U.S.A.). Identities of strains were verified by ITS sequencing as described (Najafzadeh et al., 2009a, in press-a) using the database validated by type-material, followed by BLAST analysis in GenBank (www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Seven-day-old colonies of *F. monophora* CBS 269.37, *F. pedrosoi* CBS 271.37, *F. nubica* CBS 121733 and *Exophiala dermatitidis* CBS 537.73 were used for *in situ* fungal detection. About 1 cm^2 non-sterile adhesive tape was used to collect minute amounts of fungal cells. The colony was gently touched with adhesive tape, and then the tape was transferred into a 2 ml Eppendorf tube containing 300 µl TES-buffer to extract DNA, procedures were identical to those of DNA extraction from pure cultures. To test the presence of trace DNA, the internally transcribed spacer (ITS) region of samples was amplified by nested PCR with primer V9G and LS266 in the first run, while primers ITS1 and ITS4 were used in the second run. Amplification conditions were described previously (Najafzadeh et al., 2009a).

2.3. Primer design and LAMP reaction

For primer design, the Internally Transcribed Spacer and 5.8S rDNA gene of 39 species were aligned using Bionumerics software v. 4.6 (Applied Maths, Kortrijk, Belgium). One set of LAMP primers was selected for *F. pedrosoi* CBS 271.37 using PrimerExplorer v. 4 (http://primerexplorer.jp), as follows:

forward outer primer (F3): 5′ACATTGCGCCCTTTGGTAT3′, reverse outer (B3): 5′GCACCCTTCATCCGATACG3′, forward inner primer (FIP): 5′CAACACCAAGCACAGGGGCTTTTTCGAAGGGCATGCCTGTTC3′, reverse inner primer (BIP): 5′TGGTGGAGCGAGTTCACACTTTTTTTAA-AGAAGCTCAGTGTACCGG3′.

LAMP was performed in a 25 µl reaction volume containing 0.25 µM each of F3 and B3, 1.0 µM each of FIP and BIP, 1.0 mM dNTPs, 1 M betaine (Sigma, U.S.A.), 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, U.S.A.), and 2 µl crude DNA extract as template. The reaction mixture except *Bst* DNA polymerase was denatured at 95 °C for 5 min and cooled on ice, then 1 µl *Bst* DNA polymerase was added and incubated at 65 °C for 60 min, and finally heated at 85 °C for 2 min to terminate the reaction.

2.4. Sensitivity and specificity assay

DNAs of 12 *F. pedrosoi,* 27 *F. monophora,* 8 *F. nubica* and 61 reference strains were used as templates; DNA of *F. pedrosoi* CBS 271.37 and a reaction mixture without DNA were used as controls. Sensitivity was tested by a 10-fold DNA dilution series of CBS 271.37 from 2 ng to 0.002 fg with conditions identical to those of the specificity assay. To evaluate the inhibition of non-target DNA, each of the 2 µl crude extract DNA of *F. pedrosoi* was added to the LAMP-negative samples, and tested again by LAMP.

2.5. Observation of LAMP products

The LAMP reaction products were detected by adding 2.0 µl of 10-fold diluted original SYBR Green I (Cambrex Bio Science Workingham, U.K.) to the reaction tube separately. Change of the solution color was observed directly by naked eye or using a UV transilluminator. Amplified products were also analyzed by electrophoresis on 1% agarose gels, followed by ethidium bromide staining and photography. Smart DNA ladder (Eurogentec, Seraing, Belgium) was used as a molecular weight standard.

Table 1
Isolates used in this study with results of ITS amplification and LAMP test.

Species	CBS number	GenBank number	ITS-PCR	LAMP
Fonsecaea pedrosoi	CBS 122741	EU938589	+	+
	CBS 122740	EU938590	+	+
	CBS 671.66	EU938586	+	+
	CBS 274.66	EU938587	+	+
	CBS 273.66	AY366916	+	+
	CBS 253.49	AY366921	+	+
	CBS 212.77	AY366912	+	+
	CBS 102247	AY366919	+	+
	CBS 102245	AY366918	+	+
	CBS 201.31	AY366913	+	+
	CBS 670.66	EU938588	+	+
	CBS 117910		+	+
	CBS 271.37(T)	AY366914	+	+
F. monophora	CBS 102238	AY366927	+	+
	CBS 102242	EU938583	+	+
	CBS 102246	AY366928	+	+
	CBS 102248	AY366926	+	+
	CBS 117238	AB240949	+	+
	CBS 269.37 (T)	AY366906	+	+
	CBS 289.93	AY366925	+	+
	CBS 397.48	EU938585	+	+
	CBS 117542	EU938584	+	+
	CBS 117237	EU938580	+	+
	CBS 122742	EU285266	+	+
	CBS 122845	EF513737	+	+
	CBS 123849		+	+
	CBS 121721	EF513768	+	+
	CBS 121722	EF513769	+	+
	CBS 121723	EF513767	+	+
	CBS 121724	EF513766	+	+
	CBS 121725	EF513770	+	+
	CBS 121726	EF513765	+	+
	CBS 121727	EF513764	+	+
	CBS 121728	EF513763	+	+
	CBS 121729	EF513762	+	+
	CBS 121730	EF513761	+	+
	CBS 121731	EF513760	+	+
	CBS 121732	EF513759	+	+
	CBS 117236	AB240948	+	+
	CBS 102229	EU938581	+	+
F. nubica	CBS 121733 (T)	EF513756	+	+
	CBS 121734	EF513758	+	+
	CBS 121720	EF513757	+	+
	CBS 444.62	AY366931	+	+
	CBS 269.64	EU938592	+	+
	CBS 277.29	EU938594	+	+
	CBS 271.33	AB114227	+	+
Cladophialophora carrionii	CBS 114392	EU137267	+	−
	CBS 160.54 (T)	EU137266	+	−
	CBS 260.83	EU137292	+	−
	CBS 101252	EU140590	+	−
C. bantiana	CBS 678.79	EU103992	+	−
	CBS 648.96	EU103993	+	−
	CBS 444.96	EU103994	+	−
	CBS 173.52 (T)	EU103989	+	−
C. emmonsii	CBS 640.96	EU103995	+	−
	CBS 979.96 (T)	EU103996	+	−
C. minourae	CBS 556.83 (T)	AY251087	+	−
C. mycetomatis	CBS 454.82	EU137293	+	−
	CBS 122637 (T)	FJ385276	+	−
C. yegresii	CBS 114405	EU137322	+	−
	CBS 114406	EU137323	+	−
C. satumica	CBS 114326	AY857507	+	−
	CBS 109628	EU103983	+	−
C. immunda	CBS 834.96 (T)	EU137318	+	−
	CBS 110551	FJ385274	+	−
	CBS 102227	FJ385269	+	−
Exophiala pisciphila	CBS 661.76		+	−
	CBS 537.73	AF050272	+	−
	CBS 464.81	AF050273	+	−
E. spinifera	CBS 899.68	AY156976	+	−
	CBS 668.76	AY156973	+	−
	CBS 101537	AY156970	+	−
E. jeanselmei	CBS 507.90 (T)	FJ358310	+	−
E. heteromorpha	CBS 579.76	AY857533	+	−
E. castellanii	CBS 525.76	X78481	+	−
	CBS 526.76	AB025831	+	−
E. nigra	CBS 546.82	EF551550	+	−
E. oligosperma	CBS 725.88 (T)	AY163551	+	−
E. salmonis	CBS 157.67	AF050274	+	−
E. dopicola	CBS 537.94		+	−
E. dermatitidis	CBS 207.35	FJ974060	+	−
	CBS 115663	AY663828	+	−
	CBS 292.49	AY554286	+	−
	CBS 149.90	AF050248	+	−
	CBS 748.88	AF050270	+	−
E. moniliae	CBS 520.76	AB100668	+	−
E. alcalophila	CBS 520.82	AF361051	+	−
E. lecanii-comi	CBS 232.39	FJ974061	+	−
E. prototropha	CBS 534.94	X91898	+	−
E. phaeomuriformis	CBS 131.88	AJ244259	+	−
Capronia moravica	CBS 602.96	AF050254	+	−
	CBS 552.79	AF050265	+	−
Cap. coronata	CBS 617.96	AJ232939	+	−
Cap. epimyces	CBS 606.96	AJ232938	+	−
Cap. munkii	CBS 615.96	AF050250	+	−
Cap. acutiseta	CBS 618.96	AJ232942	+	−
Cap. villosa	CBS 616.96		+	−
Cap. fungicola	CBS 614.96	AJ232941	+	−
Cap. pulcherrima	CBS 609.96	AJ232944	+	−
Cap. dactylotricha	CBS 604.96	AF050243	+	−
Rhinocladiella mackenziei	CBS 650.93 (T)	AF050288	+	−
Phaeococcomyces catenatus	CBS 650.76	AF050277	+	−
Phialophora americana	CBS 840.69 (T)	AF050283	+	−
	CBS 273.37	AF050281	+	−
	CBS 102233		+	−
P. verrucosa	CBS 839.69	DQ404353	+	−
	CBS 102234		+	−

Positive ITS-PCR was used as criterion for DNA quality of the sample. Abbreviations used: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; T, ex-type strain.

3. Results

The LAMP assay could be performed within 1 h at 65 °C in a water bath. LAMP products were visualized directly by the naked eye or under UV trans-illumination after adding SYBR Green I dye, positive reactions showing bright green fluorescence, while a negative reaction remained light orange (Fig. 1). Prior to the addition of SYBR Green I, white turbidity of the reaction mixture by magnesium pyrophosphate (by-product of LAMP) was also observed (data not shown). The products of LAMP reaction could also be detected by electrophoresis on 1% agarose gels, and showed ladder-like patterns (Fig. 2). Positive results were obtained with *F. pedrosoi* (lane 1), *F. monophora* (lane 2) and *F. nubica* (lane 3), while all reactions with fungi included for comparison (Table 1) remained negative, with a response identical to the negative control. The specificity assay thus showed that positive amplification is obtained with the genus *Fonsecaea* but not in any other

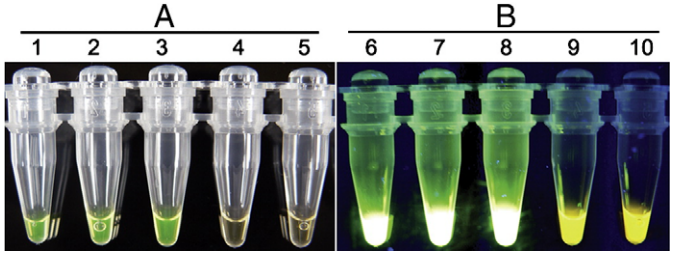

Fig. 1. Visual appearance of LAMP reactions from isolates after addition of SYBR Green I. Panel A: *Fonsecaea pedrosoi* (tube 1), *F. monophora* (tube 2), *F. nubica* (tube 3), negative control (tube 4) and tube without DNA templates (tube 5). Panel B: under UV trans-illumination, *F. pedrosoi* (tube 6), *F. monophora* tube 7), *F. nubica* (tube 8), negative control (tube 9) and tube without DNA templates (tube 10).

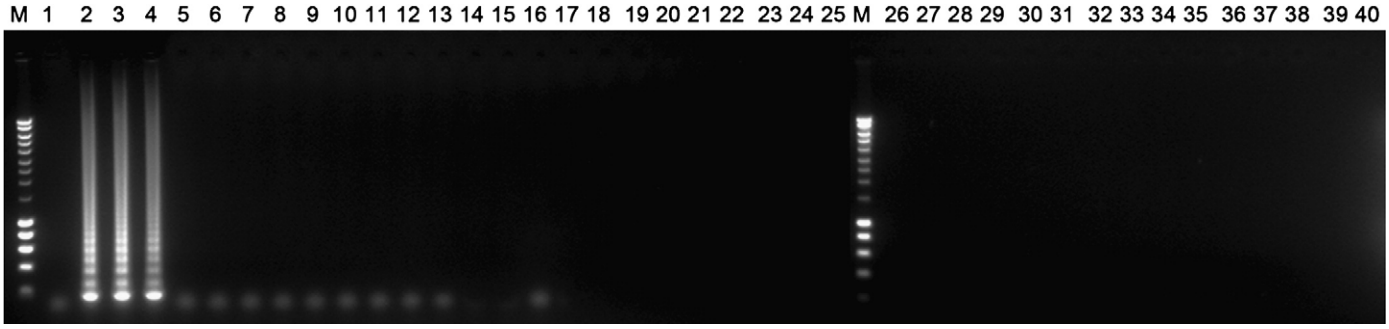

Fig. 2. Agarose gel electrophoresis of LAMP products from tested strains obtained after application of the primer set designed in this work. Left to right: lane "M" Smart DNA marker; lane 1, negative control without DNA; lane 2, *F. pedrosoi*; lane 3, *F. monophora*; lane 4, *F. nubica*; lanes 5–40, *Cladophialophora carrionii, C. bantiana, C. emmonsii, C. minourae, C. mycetomatis, C. yegresii, C. satumica, C. immuda, Exophiala pisciphila, E. spinifera, E. jeanselmei, E. heteromorpha, E. castellanii, E. nigra, E. oligosperma, E. salmonis, E. dopicola, E. dermatitidis, E. moniliae, E. alcalophila, E. lecanii-corni, E. prototropha, Capronia moravica, Cap. coronata, Cap. epimyces, Cap. munkii, Cap. acutisela, Cap. villosa, Cap. fungicola, Cap. pulcherrima, Cap. dactylotricha, Rhinocladiella mackenziei, Phaeococcomyces catenatus, Phialophora americana, P. verrucosa, Exophiala phaeomuriformis.*

member of *Chaetothyriales* (black yeasts and relatives) (Table 1). The detection limit was found to be 0.2 fg (Fig. 3). The inhibition test showed that all LAMP-negative samples turned positive after the addition of 2 µl crude DNA solution of *F. pedrosoi* or *F. monophora*, LAMP reaction was not inhibited by other fungal DNA.

To evaluate the potential application of this LAMP assay in screening possible pathogens in environmental samples, minute amounts of fungal cells were adhered to non-sterile adhesive tape by gently pressing a fungal colony; subsequently the DNA was extracted successfully. This direct trace DNA test on *in situ* environmental samples showed that the fungal DNA from members of the genus *Fonsecaea* could be detected by nested PCR but not by direct PCR (Table 2). The LAMP *in situ Fonsecaea* assay remained negative for *Exophiala dermatitis*.

4. Discussion

LAMP is a powerful innovative gene amplification technique emerging as a simple and rapid diagnostic tool for early detection and identification of microbial agents of disease. The technique was firstly described and initially evaluated for detection of hepatitis B virus DNA by Notomi et al. (2000) and depends on the *Bst* DNA polymerase performing autocycling strand displacement DNA synthesis. A set of four or six specific primers recognize six or eight distinct sequences of the target DNA. The cycling reactions result in billions of copies of target gene within an hour at isothermal conditions. With recent improvements (Boehme et al., 2007) and combination with hybridization (Nagamine et al., 2002), ELISA (Lee et al., 2009) and rolling circle amplification (Marciniak et al., 2008) the value of the method as a potential technique for the detection minute quantities of DNA and protein has increased.

We used the fungal genus *Fonsecaea* as a set of model organisms, because it is relatively homogeneous in its ecological and clinical preferences and taxonomically well-demarcated containing three species only. Cladistic analysis of the genes coding for small-subunit rRNA (SSU rDNA) supported a close relationship of *Fonsecaea* with other dematiaceous hyphomycetes of the order *Chaetothyriales* in the form-genera *Exophiala, Phialophora*, and *Rhinocladiella* (Spatafora et al., 1995). Sequence analysis of total-SSU and of the D1/D2 domains of LSU ribosomal DNA showed that between some of the close relatives of black yeast-like fungi the diversity may be extremely small: as few as 0 changes were detected between species of *Fonsecaea,* some *Exophiala* species, and *Cladophialophora bantiana*, while two mutations were observed with *P. verrucosa* (Abliz et al., 2004b). SSU and LSU obviously are insufficiently variable for the identification of pathogenic melanized fungi and relevant species in sequencing analysis. Zeng and De Hoog (2008) demonstrated that in melanized pathogens the rDNA ITS region is much more useful as a specific marker. This domain has been used to identify anamorphs of *Chaetothyriales* including the genus *Fonsecaea* (Abliz et al., 2003b; Chen et al., 2001; da Silva et al., 2002; de Andrade et al., 2007; Iwen et al., 2002; Najafzadeh et al., 2009a). In this study, we proved that the LAMP technique successfully amplified DNA of the genus *Fonsecaea* but not of related fungi, such as *C. carrionii, C. bantiana* and *P. verrucosa*. In addition, it was not inhibited by the presence of other fungal DNA.

The LAMP assay can be performed within 1 h at 65 °C in a water bath, heating block or PCR machine, and the reaction products can easily be observed by electrophoresis or directly by the naked eye. The detection limit was found to be 0.2 fg. Moreover, our results proved that it could be used to detect minute amounts of fungi adhering to adhesive tapes applied to non-sterile surfaces with fungal colonization. Given its specificity, sensitivity, easy handing and cost-efficiency, the LAMP assay is judged to be suitable for a wide range of applications. In this paper it has been used for the detection of *Fonsecaea*, a pathogenic fungus that due to its simple morphology is difficult to identify by microscopic methods (De Hoog et al., 2004, Xi et al., 2009). In addition, in retrospective studies LAMP provides an accurate and specific procedure for the detection of the pathogen in archived

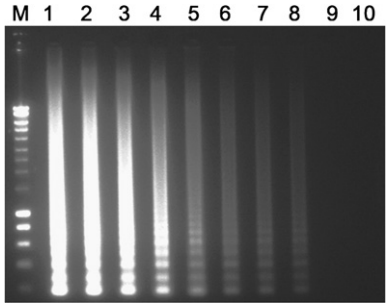

Fig. 3. Analytical sensitivity of LAMP for detection of the ITS rDNA gene. Left to right: Lane "M", Smart DNA ladder; lanes 1–10, 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg, 0.2 fg, 0.02 fg, 0.002 fg/tube, respectively.

Table 2
Results of detection of minute amounts of fungal DNA in non-sterile adhesive tape samples by nested PCR and LAMP.

Species	Strain	Nested PCR		LAMP
		First run	Second run	
F. monophora	CBS 269.37 (T)	−	+	+
F. pedrosoi	CBS 271.37 (T)	−	+	+
F. nubica	CBS 121733 (T)	−	+	+
E. dermatitidis	CBS 537.73	−	+	−

Abbreviation used: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; T, ex-type strain.

samples. The limited need for equipment and techniques makes the LAMP assay suitable for diagnosis in a routine clinical laboratory.

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