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MINI-REVIEW

Current state and future perspectives of loop-mediated isothermal amplification (LAMP)-based diagnosis of filamentous fungi and yeasts

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Abstract Loop-mediated isothermal amplification is a rather novel method of enzymatic deoxyribonucleic acid amplification which can be applied for the diagnosis of viruses, bacteria, and fungi. Although firmly established in viral and bacterial diagnosis, the technology has only recently been applied to a noteworthy number of species in the filamentous fungi and yeasts. The current review gives an overview of the literature so far published on the topic by discussing the different groups of fungal organisms to which the method has been applied. Moreover, the method is described in detail as well as the different possibilities available for signal detection and quantification and sample preparation. Future perspective of loop-mediated isothermal amplification-based assays is discussed in the light of applicability for fungal diagnostics.

Keywords Fungi · Yeast · Detection · Identification · Loop-mediated isothermal amplification · Primer design

Introduction

Information about the microbiological status of persons, materials, and the environment is a crucial factor in modern concepts such as human and animal healthcare, quarantine, food safety, environmental monitoring, or criminal forensics. All these areas have developed their arsenal of specialized methods for detection, enumeration, and identification of microorganisms, among which fungi may play a minor role when compared to the importance of bacteria. Nonetheless, since fungi do have an impact on human and animal health and nutrition as pathogens, toxicants, and spoilers, it would be a culpable mistake to ignore this group of organisms. The invention of deoxyribonucleic acid (DNA)-based technologies led to a general switch in diagnostic targets from multiplication of living cells to specific multiplication of nucleic acid. Hence, specificity of a diagnostic assay was no more depending on several different environmental factors but was reduced to only one factor, the nucleotide sequence of a DNA or ribonucleic acid (RNA) target. The first system for in vitro amplification of DNA was the polymerase chain reaction (PCR) in which heat denaturation of DNA double strands was used to permit exponential multiplication of the target sequence in discontinuous mode. Shortly after the invention of PCR, a multitude of alternative amplification techniques were published which used non-thermostable DNA or RNA polymerases under isothermal conditions for continuous amplification of a target nucleic acid (see reviews by Gill and Ghaemi 2008 and Yan et al. 2014). It can be assumed that much of the effort given to develop those systems was due to the fact that the PCR technology was rigidly covered by patents hampering general access to the fast growing market of nucleic acid-based diagnostics. Although meanwhile established as the Gold Standard in many areas of diagnosis and research, PCR turns out to be more suitable in situations in which accuracy is of importance rather than time to result. In many diagnostic situations, however, time to result is the most crucial factor since an information-based short-term decision has to be made, often with far-reaching consequences in terms of health, public safety, or economic considerations. Under such circumstances, some of the isothermal in vitro DNA amplification techniques may have an advantage over PCR since they can be operated at one continuous temperature obliviating thermal cycling equipment. Sample preparation,

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which is an important issue in PCR, can be kept to a minimum with some of the available isothermal methods and also signal detection is possible using simple equipment or the naked eye. Loop-mediated isothermal amplification (LAMP) of target DNA is an amplification technology with high diagnostic potential which is currently well on its way to replace PCR as a diagnostic tool in many fields such as clinical point of care diagnostics, phytoquarantine, and food safety. Although fungal organisms are rarely as acutely live threatening as some bacteria or viruses are, this group of microorganisms has a big impact on the health of humans, animals, and plants as well as on the safety of our food supply due to their production of mycotoxins. The following sections will, therefore, elucidate how LAMP can be used to improve fungal diagnosis in different fields of application.

The classical way of detecting and enumerating filamentous fungi and yeasts involves several steps of sample preparation, dilution, plating, incubation, enumeration, isolation, and identification. Various different media can be applied depending on the source to be analyzed and the group of fungi to which the analysis is aiming. Detailed descriptions of methods and media for the culture-based analysis of sources such like food (Pitt and Hocking 2009), soil (Alef and Nannipieri 1995), water (Mesaphy 2013), and air (O'Loughlin et al. 2013) have been published in the literature. Various different culture media are used to grow fungal isolates under different environmental conditions in order to identify them to species level based on culture characteristics, micro morphological features, and biochemical properties. Specificity of growth media can be improved by inhibition or even exclusion of unwanted groups of organisms or by selective enhancement of the organisms to be detected. Tsao (1970) published a comprehensive review about the general principles of developing selective fungal growth media. Development of automated detection and identification systems is strongly driven by clinical microbiologists due to their demand for rapid analysis and high throughput systems. To meet this demand, media were developed to selectively grow and identify fungal and yeast colonies of interest in one step by addition of chromogenic substrates (Odds and Bernaerts 1994; Baumgartner et al. 1996; Sendid et al. 2007; Ghelardi et al. 2008). Although more rapid culture-based identification systems exist for some groups of fungi, the routinely use of such methods for fungal diagnosis in clinical and industrial settings is generally hampered by their high requirement for time and specialization. In addition to culture based systems, immunochemical assays have been developed for identification of some filamentous fungi and yeasts as well as for diagnostic detection of specific antigens present in the blood stream of patients as an indicator for invasive fungal infections (Buckley et al. 1992). However, direct detection of filamentous fungi in formats such as enzyme-linked immunosorbent assay (ELISA) or lateral flow device (LFD) is limited by the fact that the mycelial surface does not carry antigenic structures which would enable sufficiently specific antigenic determination as present in other groups of organisms such as the Gram negative bacteria, in which the composition of pepticoglucan layers of the cell walls follows the taxonomical grouping of species (Schleifer and Kandler 1972). As for other groups of microorganisms, DNA sequence-based techniques have gained increasing importance in the diagnosis of fungal plant pathogens (McCartney et al. 2003; Atkins and Clark 2004), human pathogens (Yeo and Wong 2004; White et al. 2009), or fungi relevant to food safety and hygiene (Niessen 2008). Until recently, assays were mainly based on the concept of PCR. During recent years, amplification of DNA using isothermal conditions gained increasing attention due to the fact that they can be run with less effort and expense as compared to PCR. Loop-mediated isothermal amplification is a technique which has become especially important as an alternative technology for the detection of all groups of microorganisms, including viruses.

The LAMP reaction

LAMP is a novel approach to nucleic acid amplification. It relies on auto cycling strand displacement DNA synthesis performed by *Bst* DNA polymerase or similar thermophilic DNA polymerases under isothermal conditions with a set of four specifically designed primers hybridizing to six different parts of the target DNA sequence (Notomi et al. 2000). Figure 1 gives a schematic representation of the basic reaction steps involved. The method makes use of the large fragment of the *Bst* DNA polymerase from *Geobacillus stearothermophilus*. The large fragment of the enzyme contains the 5′–>3′ polymerase activity but lacks 5′–>3′ exonuclease activity. The enzyme is produced by an *Escherichia coli* strain containing a genetic fusion of the *Bst* DNA polymerase large fragment and the maltose binding protein (MBP) of *E. coli*. MBP is used for affinity purification and removed by cleavage of the fused proteins (Kong et al. New England Biolabs, unpublished data). *Bst* DNA polymerase large fragment displaces third strand DNA with high efficiency during primer-initiated polymerization of new DNA leaving a double stranded product and a single stranded DNA strand which can act as the matrix for further primer annealing and DNA polymerization. Since *Bst* DNA polymerase has a very high activity, vast amounts of high molecular weight DNA are produced within short time. The exceptionally high specificity of LAMP is due to the fact that a set of four primers with six binding sites must hybridize correctly to their target sequence before DNA biosynthesis occurs (see Fig. 1a). A third pair of primers (loop primers) can be added optionally to the reaction in order to further amplify the amount of DNA produced during LAMP (Nagamine et al. 2002). One of the primer pairs is constructed in such a way that the reverse complement of a

binding site downstream of the F2c/B2c binding site (F1c/B1c) is attached to the 5′-end of a primer binding to that site. These composite primers are essential for the specificity of the amplification reaction and have, thus, to be chosen very carefully. Both parts of each FIP/BIP primer should be checked for cross reactivity by in silico analysis prior to application in LAMP reactions. A pair of outer primers (F3/B3) anneals upstream of the F2c/B2c binding site to displace the initial LAMP product strand from the DNA matrix. Specificity of outer primers can be regarded as being of lower importance since they are not involved in any of the following amplification reactions and a low number of base mismatches will not prevent amplification. The process is initiated by attachment of primers to the DNA target (see Fig. 1b). Primers are elongated and the second matrix strand is displaced from the target DNA. The newly synthesized product itself is displaced from the matrix strand by the F3/B3 product strand. Primers F3 and B3 have no further function once the amplification process has been initiated. As the final product of the amplification initiation step, a dumbbell structured single-stranded DNA is formed by hybridization of both ends of the molecule to complementary downstream sequences forming two loops. Starting from this structure, primers FIP and BIP continuously hybridize to newly formed binding sites and are elongated, displaced, and refolded while forming ever longer multimers of the basic dumbbell structure (see Fig. 1c). Loop primers are designed to hybridize to the single-stranded loop structures present in the dumbbell structures as well as in the multimeric DNA formed during autocycling DNA amplification. They prime the production of novel template DNA to which FIP/BIP primers can bind to initiate synthesis of even higher concentrations of DNA. Addition of loop primers, therefore, does not increase the speed of amplification but rather enables earlier detection of a LAMP signal as compared to a reaction run without loop primers.

LAMP-based assays for the detection of fungal organisms

The number of publications involving loop-mediated isothermal amplification as an alternative method in molecular

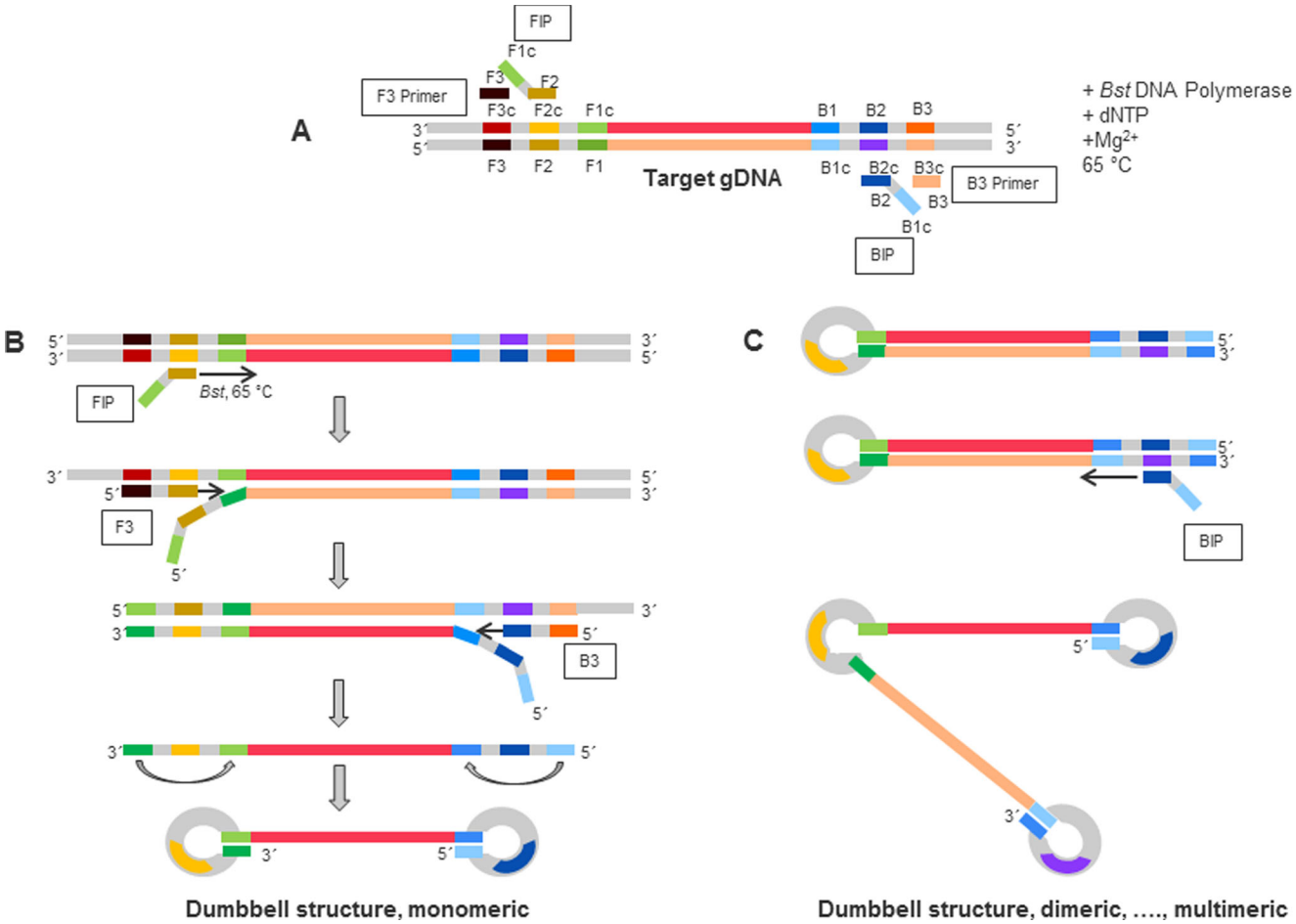

Fig. 1 Schematic representation of the LAMP reaction. **a** Primers, binding sites, and conditions. **b** Initiation of the LAMP reaction resulting in production of a double-loop stem structure (dumbbell structure). **c** Autocycling enzymatic DNA amplification during LAMP resulting in multimers of different size of the monomeric double-loop stem structure

diagnosis of microorganisms is steadily increasing (Niessen et al. 2013). However, publications dealing with LAMP-based detection and identification of fungal organisms (55 as of 2 September 2014) represent only a relatively small proportion in relation to the total number of publications involving LAMP (1,252 as of 2 September 2014, Science Citation Index). Table S1 (supplementary materials) gives an overview of published assays with species names, sequence source used to design primer sets, and several other details characterizing individual assays, including sample preparation. No reaction details such as incubation temperature were included in Table 1 since they are subject to variation and depend widely on the hardware used in individual labs. It will therefore be necessary to optimize incubation temperatures if assays are to be reproduced. Column 1 in Table 1 provides all species detected by a given primer set, including species which were found to cross react with specific assays. Table S1 (supplemental materials) provides sequences of all primers used in individual LAMP assays. All FIP/BIP primers were checked for correctness of sequences by comparing sequences with the corresponding gene as given in GenBank. The two parts of each primer are indicated by hyphenation in order to make *in silico* analysis of primer specificity easier for the reader.

LAMP assays for the detection of clinically relevant yeasts and filamentous fungi

Target organisms of LAMP assays can be grouped into three different categories according to their relevance, e.g., yeasts and fungi with clinical relevance in humans and animals, plant pathogenic fungi, and fungi relevant to the quality and safety of food and feed. As with other fields of applications of LAMP-based assays, also in the field of fungi the development started with the development of assays targeting clinically relevant filamentous fungi and yeasts. The first assay published on LAMP-based detection of a fungal organism was by Endo et al. (2004, see also Tatibana et al. 2009) who set up an assay for *Paracoccidioides brasiliensis* based on the sequence of the *gp43* gene (GenBank accession no. HQ878437) which codes for a 43-kDa secreted glycoprotein involved in the attachment of the yeast form of the fungus to macrophages (Mendes-Giannini et al. 2000). A review of the pathology and ecology of the pathogen can be found in Restrepo (1985). A LAMP-based assay for diagnosis of *Pneumocystis jirovecii* was published by Uemura et al. (2008). The yeast-like fungus is an opportunistic pathogen in immunocompromised patients showing symptoms of *Pneumocystis carinii* pneumonia. LAMP analysis of sputum and bronchoalveolar lavage fluid samples showed that the sensitivity of the LAMP assay was considerably higher as compared to a nested-PCR assay for the fungus. Lucas et al. (2010) used primer sets based on the *cap59* gene which harbors serotype associated alleles on which they based specific differentiation of several *Cryptococcus* species (Enache-Angoulvant et al. 2007). Recently, Ishikawa et al. (2014) described a LAMP assay for detection of *Filobasidiella neoformans*, the theleomorph of *C. neoformans*, which they applied to the detection of the mycelial form of the pathogen in a probiotic dairy product. *Candida* species, i.e., *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* group, and *Candida tropicalis* are dimorphic yeasts known as causative agents of superficial or systemic candidal infections in humans (Fidel et al. 1999). Jensen Søe et al. (2013) developed isoPCR as a two-stage nested-like amplification technique that showed exceptionally high sensitivity and specificity combined with low operating time as compared to PCR-based assays of *Candida* yeasts. *Talaromyces marneffei* (syn. *Penicillium marneffei*) is the only (hypothermal) dimorphic species known among fungi with a *Penicillium* anamorphic state. The fungus causes systemic infections mostly in immunocompromised patients and has been found to be widely distributed as an AIDS related illness. Sun et al. (2010a) set up a LAMP assay which was based on the DNA sequence of the ITS region of the rRNA gene of *T. marneffei* and applied it to the analysis of skin, lung, and lymph node tissues of *T. marneffei* culture positive patients.

Beside yeast targeted assays, LAMP was also used for the diagnosis of filamentous fungi of clinical relevance. The dematiaceous mycelial fungus *Ochroconis gallopava* is an opportunistic zoonotic pathogen of concern mainly in immunocompromised or immunosuppressed patients with a mortality rate of 20 % after 6 month (Qureshi et al. 2012); hence, early diagnosis is of eminent importance. Ohori et al. (2006) developed a set of LAMP primers for the fungus which was based on the sequence of the D1/D2 domain within the 28S ribosomal RNA gene. The assay was applied successfully to the detection of *O. gallopava* in brain and spleen tissues of infected mice. Primers also picked up *Ochroconis calidifuminalis*, a sibling species of *O. gallopava*. Aspergillosis is a collective name for invasive human lung diseases caused by *Aspergillus* species (Patterson et al. 2000). Beside *Aspergillus fumigates*, the closely related *Aspergillus nidulans* and *Aspergillus quadrilineata* are mainly responsible for high mortality rates of the disease. Matsuzawa et al. (2010) developed sets of specific LAMP primers for either of the latter two species to enable correct diagnosis which is important for the proper treatment of patients since *A. nidulans* and *A. quadrilineata* display different susceptibility against antifungal drugs. Chromoblastomycosis is a collective designation for chronic, localized and slowly progressing subcutaneous fungal infections characterized by the development of brown-pigmented (demaciaceous) rounded sclerotic bodies in affected tissues (López Martínez and Mendéz Tovar 2007). *Fonsecaea pedrosoi*, *Fonsecaea monomorpha*, and *Fonsecaea nubica* (Najafzadeh et al. 2010) were found to be

Table 1 Overview of LAMP assays for the detection of fungal organisms

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
Amanita phaloides	Quant.[a] real-time LAMP	SYBR Green1 fluorescence in fluorimeter	*rRNA*-ITS[d]	Mushroom caps	Spin column purification	64 pg gDNA/rxn[f]	Vaagt et al. (2013)
	LAMP[b]	LFD[c] visual detection				2 % (*w*/*w*)[g] death cap in mushroom mix	
Aspergillus caelatus	LAMP	calcein fluorescence visual detection under UV[h] light	*acl1*	Conidial suspension Lavage fluid Brazil nuts gDNA[e] Brazil nuts	Direct addition of conidia Addition of supernatant from conidial suspension after mechanical disruption and boiling	n.d.[i]	Luo et al. (2014a)
A. carbonarius	LAMP	HNB[j] visual detection	*Acpks*	PCM[k]	Direct addition of mycelia	100 pg gDNA/rxn	Storari et al. (2013)
A. flavus *A. minisclerotigenes* *A. oryzae* *A. parasiticus* *A. parvisclerotigenus* *A. sojae* *A. toxicarius*	LAMP	Calcein fluorescence visual detection under UV light	*acl1*	Conidial suspension PCM Lavage fluid Brazil nuts and green coffee beans gDNA of Brazil nuts and green coffee beans	Direct addition of pure culture conidia Addition of supernatant from conidial suspensions after mechanical disruption and boiling	2.4 pg gDNA/rxn 1×10^{4} spores/rxn after direct addition 1×10^{2} spores/rxn after spore disruption and boiling	Luo et al. (2012) Luo et al. (2014a) Luo et al. (2014b)
A. niger	LAMP	HNB visual inspection	*Anpks*	PCM	Direct addition of mycelia	100 pg gDNA/rxn	Storari et al. (2013)
A. nomius	LAMP	calcein fluorescence visual detection under UV light	*amy1*	Conidial suspension PCM Lavage fluid Brazil nuts and green coffee beans gDNA of Brazil nuts and green coffee beans	Direct addition of pure culture conidia Addition of supernatant from conidial suspensions after mechanical disruption and boiling	7.6 pg gDNA/rxn 1×10^{5} spores/rxn after direct addition 1×10^{1} spores/rxn after spore disruption and boiling	Luo et al. (2012) Luo et al. (2014a) Luo et al. (2014b)
A. parasiticus *A. sojae*	LAMP	calcein fluorescence visual detection under UV light	*amy1*	Conidial suspension PCM	Direct addition of pure culture conidia	20 pg gDNA/rxn 1×10^{5} spores/rxn after direct addition	Luo et al. (2012) Luo et al. (2014a) Luo et al. (2014b)
A. toxicarius				Lavage fluid ground nuts, corn, and green coffee beans gDNA of ground nuts corn and green coffee beans	Addition of supernatant from conidial suspensions after mechanical disruption and boiling	1×10^{2} spores/rxn after spore disruption and boiling	

Table 1 (continued)

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
Botrytis cinerea	Real-time LAMP[l]	nondescript intercalating dye from OptiGene in fluorimeter	*rRNA*-ITS	PCM Rose petals *Pelargonium* leafs	Spin column purification Isopropanol precipitation of gDNA after phenol-chloroform extraction	65 pg/rxn	Tomlinson et al. (2010a)
Brettanomyces naardenensis	Quant. real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA-ITS*	Pure culture cell suspension	Direct addition of cell supernatant	63 cfu[m]/ml culture	Hayashi et al. (2007)
Candida albicans	isoPCR[n]	SYBR Green 1 fluorescence	*rRNA*-26S	Pure culture cell suspension	Spin column purification	gDNA equivalent to 10 genome copies/rxn	Jensen Søe et al. (2013)
C. albicans	Real-time LAMP Multiplex real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA*-ITS	Pure culture cell suspension dairy products	Isopropanol precipitation of gDNA after mechanical treatment	32 cells/ml 160 cells/ml in multiplex assay	Kasahara et al. (2014)
C. glabrata	Real-time LAMP Multiplex real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA*-ITS	Pure culture cell suspension dairy products	Isopropanol precipitation of gDNA after mechanical treatment	32 cells/ ml 160 cells/ml in multiplex assay	Kasahara et al. (2014)
C. glabrata	isoPCR (SYBR green 1)	SYBR Green 1 fluorescence	*rRNA-26S*	Pure culture cell suspension	Spin column purification	gDNA equivalent to 1 genome copy/rxn	Jensen Søe et al. (2013)
C. parapsilosis group	Real-time LAMP Multiplex real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA*-ITS	Pure culture cell suspension dairy products	Isopropanol precipitation of gDNA after mechanical treatment	*C. metaplisosis* (160 cells/ml) *C. orthopsilosis* (6.4 cells/ml) *C. parapsilosis* (160 cells/ml)	Kasahara et al. (2014)
C. tropicalis	Real-time LAMP Multiplex real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA*-ITS	Pure culture cell suspension dairy products	Isopropanol precipitation of gDNA after mechanical treatment	800 cells/ml culture 4,000 cells/ml culture in multiplex assay	Kasahara et al. (2014)
Cryptococcus neoformans var. *grubii* (= serotype A)	LAMP	AGE[o] visual detection after ethidium bromide staining	*CAP59*	Pure culture cell suspension	Direct addition of cell supernatant	0.1–0.5 pg gDNA/μl extract	Lucas et al. (2010)
Cry. neoformans var. *neoformans* (= serotype D)	LAMP	AGE visual detection after ethidium bromide staining	*CAP59*	Pure culture cell suspension	Direct addition of cell supernatant	0.1–0.5 pg gDNA/μl extract	Lucas et al. (2010)
Cry. gattii (= serotypes B/C)	LAMP	AGE visual detection after ethidium bromide staining	*CAP59*	Pure culture cell suspension	Direct addition of cell supernatant	0.1–0.5 pg gDNA/μl extract	Lucas et al. (2010)

Table 1 (continued)

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
Dekkera anomala	Quant. real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA-ITS*	Pure culture cell suspension	Direct addition of cell supernatant	9.8 cfu/ml culture	Hayashi et al. (2007)
D. bruxellensis	Quant. real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA-ITS*	Pure culture cell suspension beer wine	Direct addition of cell supernatant	78 cfu/ml culture	Hayashi et al. (2007)
D. custersiana	Quant. real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA-ITS*	Pure culture cell suspension	Direct addition of cell supernatant	9.3 cfu/ml culture	Hayashi et al. (2007)
Emericella nidulans	Real-time LAMP Nested real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rodA*	PCM	Ethanol precipitation after chemical cell lysis	10 pg gDNA/µl extract (LAMP) 0.1 pg gDNA/µl extract (nested LAMP)	Matsuzawa et al. (2010)
E. quadrilineata	Real-time LAMP Nested real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rodA*	PCM	Ethanol precipitation after chemical cell lysis	10 pg gDNA/µl extract (LAMP) 0.1 pg gDNA/µl extract (nested LAMP)	Matsuzawa et al. (2010)
Filobasidiella neoformans	LAMP	Mg-pyrophosphate precipitation visual inspection	*rRNA-ITS*	Pure culture cell suspension dairy product	Isopropanol precipitation of gDNA after mechanical treatment Direct addition of sample supernatant	100 cfu/ml culture	Ishikawa et al. (2014)
Fonsecaea pedrosoi *F. monophora* *F. nubica*	LAMP	SYBR green 1 fluorescence visual inspection under UV light	*rRNA*-ITS	PCM	Isopropanol precipitation of gDNA after mechanical treatment	0.2 fg gDNA/rxn	Sun et al. (2010b)
Fusarium graminearum	LAMP	Calcein fluorescence visual inspection under UV light	*gaoA*	PCM Wheat seeds	Isopropanol precipitation of gDNA after mechanical treatment Direct addition of culture surface scrapings Spin column purification	2 pg gDNA/rxn	Niessen and Vogel (2010); Abd-Elsalam et al. (2011); Almoammar et al. (2013); Niessen (2013)
F. mangiferae	Quant. real-time LAMP	SYTO9 fluorescence in fluorimeter SYBR Green 1 visual inspection at day light	RAPD[p] marker	PCM Shoots of mango	96 well extraction plate-based purification Isopropanol precipitation of gDNA after mechanical treatment	2.26×10^{-4} ng plasmid DNA/µl extract	Pu et al. (2014)
F. oxysporum f. sp. *cubense* tropical race 4	LAMP	SYBR Green 1 fluorescence visual detection under UV light	SCAR[q] marker	PCM Banana tissue soil	Ethanol precipitation of gDNA from after phenol-chloroform extraction Direct addition of diluted tissue extract	0.01 pg gDNA/rxn	Li et al. (2013a)

Table 1 (continued)

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
					Spin column purification		
F. oxysporum f. sp. *cubense* tropical race 4	quant. real-time LAMP	SYTO 9 fluorescence in fluorimeter SYBR Green 1 fluorescence in day light	*rRNA-IGS*[r]	PCM soil	96 well extraction plate-based purification after mechanical treatment Isopropanol precipitation of gDNA Spin column purification	10^3 spores/g soil	Zhang et al. (2013)
F. oxysporum f. sp. *lycopersici*	LAMP real-time LAMP	Mg-pyrophosphate precipitation visual detection HNB visual detection GeneFinder visual detection SYBR Green 1 visual detection Ethidium bromide visual detection under UV light	*rRNA*-28S	PCM	Isopropanol precipitation of gDNA after mechanical treatment Direct addition of supernatant from aqueous mycelial solution	gDNA equivalent to 2×10^3 cfu/ml culture	Almasi et al. (2013)
F. oxysporum f. sp. *niveum*	Quant. real-time LAMP	SYTO 9 fluorescence in fluorimeter	RAPD fragment (GenBankaccession no. EU603504)	PCM	96 well extraction plate-based purification of gDNA after mechanical treatment	1.2 pg dDNA/rxn	Peng et al. (2013)
		SYBR Green 1 fluorescence visual detection in day light		Soil	Isopropanol precipitation of gDNA Spin column purification	10^3 spores/g soil	
F. tricinctum species complex	LAMP	Calcein fluorescence visual detection under UV light	*acl1*	PCM Barley seeds	Isopropanol precipitation after mechanical treatment Direct addition of culture surface scrapings and single barley seeds Direct addition of surface lavage from bulk grain sample	0.95 pg gDNA/rxn	Niessen et al. (2012)
F. solani	LAMP	SYBR Green 1 fluorescence detection under UV light	*tef1*	PCM human blood serum	Isopropanol precipitation of after mechanical treatment	gDNA equivalent to 1 genome copy/rxn	Ferdousi et al. (2014)
Fusarium spp. group specific for gushing inducing species	LAMP	Calcein fluorescence detection under UV light	*fchyd5*	PCM	Isopropanol precipitation of gDNA after mechanical treatment	0.74 pg gDNA/rxn	Denschlag et al. (2012); Denschlag et al. (2013)
	Quant. real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter V13 fluorescence in fluorimeter		Cereal grain Brewing malt	DNA extraction kit after mechanical treatment Direct addition of culture surface scrapings Direct addition of surface lavage		

Table 1 (continued)

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
					Spin column purification		
Fusarium spp. group specific for trichothecene producing species	Multiplex real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*tri5/tri6*	PCM	Isopropanol precipitation of gDNA after mechanical treatment	4 pg–15.72 ng gDNA/rxn depending on species	Denschlag et al. (2014)
				Cereal grain	DNA extraction kit after mechanical treatment		
				Brewing malt	Direct addition of culture surface scrapings		
					Direct addition of surface lavage		
					Spin column purification		
Guignardia citricarpa	Real-time LAMP	Nondescript intercalating dye from OptiGene in fluorimeter	*rRNA*-ITS	PCM *Citrus* tissue	Isopropanol precipitation of gDNA after mechanical treatment Affinity purification of gDNA	0.6 pg gDNA/rxn	Tomlinson et al. (2013)
Glomus intraradices *G. proliferum* *G. lamellosum* *G. cerebriforme* *Gigaspora rosea*	LAMP	Mg-pyrophosphate precipitation visual inspection	*btub*	Fungal spores Carrot roots	Direct addition of supernatant Spin column purification	10 pg gDNA/rxn	Gadkar and Rillig (2008)
Metarhizium anisopliae var. *anisolpliae*	LAMP	SYBR Green 1 fluorescence visual inspection under UV light	*rRNA*-IGS	PCM soil	Spin column purification	0.01 pg per rxn 50 conidia per rxn in soil	Li and Cai (2011)
Ochroconis gallopava *O. calidifuminalis*	LAMP	AGE visual inspection after ethidium bromide staining	*rRNA*-28S D1/D2 domain	PCM brain and spleen tissue	Direct addition of gDNA	100 fg gDNA/rxn	Ohori et al. (2006) Yarita et al. (2007) Yarita et al. (2010)
Ophiostoma bruneo-ciliatum	Real-time LAMP	Nondescript intercalating dye from OptiGene in fluorimeter	*btub*	PCM Wood tissue of Scots pine	Isopropanol precipitation of gDNA after mechanical treatment	20 pg gDNA/rxn	Villari et al. (2013)
O. clavatum[1]	Real-time LAMP	Nondescript intercalating dye from OptiGene in fluorimeter	*Btub*	PCM Wood tissue of Scots pine	Isopropanol precipitation of gDNA after mechanical treatment	40 pg gDNA/rxn	Villari et al. (2013)
Paracoccidioides brasiliensis	Real-time LAMP	Nondescript intercalating dye from Eiken LoopAmp kit in fluorimeter	*gp43*	Pure culture cell suspension paraffin embedded tissue sputa	Ethanol precipitation of gDNA after enzymatic treatment Direct addition of gDNA containing supernatant	100 fg gDNA/rxn	Endo et al. (2004) Tatibana et al. (2009)
Phytophthora kernoviae	Real-time LAMP	PicoGreen fluorescence in fluorimeter visual detection on LFD	*rRNA*-ITS	PCM Rhododendron tissue	Spin column purification Isopropanol precipitation of gDNA after mechanical treatment	17 pg gDNA/rxn	Tomlinson et al. (2010b)
P. melonis	LAMP	SYBR Green1 fluorescence visual	*Ypt1*	PCM Cucumber soil	Isopropanol precipitation of gDNA DNA preparation from cucumber and soil not described	10 fg gDNA/rxn	Chen et al. (2013)

Table 1 (continued)

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
		inspection under UV light					
P. ramorum	Real-time LAMP	PicoGreen fluorescence in fluorimeter visual detection on LFD	*rRNA*-ITS	PCM Rhododendron tissue	Spin Column purification Isopropanol precipitation of gDNA after mechanical treatment Direct addition of LFD membrane	50 pg gDNA/rxn	Tomlinson et al. (2007); Tomlinson et al. (2010b)
P. sojae	LAMP	HNB visual inspection	*A3aPro*	PCM Soybean tissue	Spin column purification of gDNA.	10 pg gDNA/rxn	Dai et al. (2012)
Pneumocystis jirovecii	LAMP real-time LAMP	Mg-pyrophosphate precipitation visual inspection YO-PRO1 fluorescence in fluorimeter	*rRNA*-18S	PCM Sputum BALF[s] from PCP[t] patients	Isopropanol precipitation of gDNA after mechanical treatment MiniMAG purification from sputum and BALF	50 plasmid copies/rxn	Uemura et al. (2008)
Pseudallescheria boydii	LAMP	SYBR Green 1 fluorescence visual inspection under UV light	*btub*	PCM Sputum	Addition of gDNA after immobilization on Whatman FTA filter paper	20 pg gDNA/rxn 5×10^2 cells/μl culture	Lu et al. (2011)
P. minutispora	LAMP	SYBR Green 1 fluorescence visual inspection under UV light	*btub*	PCM Sputum	Addition of gDNA after immobilization on Whatman FTA filter paper	20 pg gDNA/rxn 5×10^2 cells/μl culture	Lu et al. (2011)
Puccinia striiformis f. sp. *tritici*	LAMP	SYBR Green 1 fluorescence visual inspection under day light	*btub*	PCM Wheat leaves	Isopropanol precipitation of gDNA after mechanical treatment	2 pg gDNA/rxn	Huang et al. (2011)
Pythium aphanidermatum	LAMP	Mg-pyrophosphate precipitation visual inspection	*rRNA*-ITS	PCM Hydroponic solution Tomato roots	Ethanol precipitation of gDNA after mechanical treatment Direct addition of membrane bound gDNA Direct addition of sample supernatant	10 fg gDNA/rxn	Fukuta et al. (2013)
P. helicoides	Real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter		PCM roots of *Poinsettia*	Ethanol precipitation of gDNA after mechanical treatment Direct addition of supernatant from root washings	100 fg gDNA/rxn	Takahashi et al. (2014)
P. myriotylum	Real-time LAMP	Nondescript intercalating dye from Nippon Gene in fluorimeter	*rRNA*-ITS	PCM Hydroponic solution	Ethanol precipitation of gDNA after mechanical treatment	100 fg gDNA/rxn	Fukuta et al. (2014)
Saccharomyces bayanus	Real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*RAD18*	Pure culture cell suspension Red wine beer	Direct addition of cell supernatant Direct addition of cell lysate from wine and beer	1.9×10^2 cfu/ml culture 3.2×10^2 cfu/ml beer 3.9×10^2 cfu/ml wine	Hayashi et al. (2009)
S. cerevisiae	Real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*Sc-MET16*	Pure culture cell suspension	Direct addition of cell supernatant.	4.4×10^3 cfu/ml culture	Hayashi et al. (2009)

Table 1 (continued)

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
Amyloytic *S. cerevisiae* strains (= *S. diastaticus*)	Real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*STA1*	Pure culture cell suspension	Direct addition of cell supernatant	4.4×10^3 cfu/ml culture	Hayashi et al. (2009)
S. cerevisiae *S. pastorianus*	Real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*Sc-CYS3*	Pure culture cell suspension	Direct addition of cell supernatant	n.d.	Hayashi et al. (2009)
S. paradoxus	Real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*TLC1*	Pure culture cell suspension	Direct addition of cell supernatant	2.6×10^3 cfu/ml culture	Hayashi et al. (2009)
S. pastorianus	Real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*Lg-MET16*	Pure culture cell suspension	Direct addition of cell supernatant	5.2×10^2 cfu/ml culture	Hayashi et al. (2009)
Scedosporium aurantiacum	LAMP	SYBR Green 1 fluorescence visual observation under UV light	*btub*	PCM Sputum	Addition of gDNA after immobilization on Whatman FTA filter paper	20 pg gDNA /rxn 5×10^2 cells/μl culture	Lu et al. (2011)
S. apiospermum	LAMP	SYBR Green 1 fluorescence visual observation under UV light	*btub*	PCM Sputum	Addition of gDNA after immobilization on Whatman FTA filter paper	20 pg gDNA/rxn 5×10^2 cells/μl culture	Lu et al. (2011)
S. dehogii	LAMP	SYBR Green 1 fluorescence visual observation under UV light	*btub*	PCM Sputum	Addition of gDNA after immobilization on Whatman FTA filter paper.	20 pg gDNA/rxn 5×10^2 cells/μl culture	Lu et al. (2011)
S. prolificans	LAMP	SYBR Green 1 fluorescence visual observation under UV light	*btub*	PCM Sputum	Addition of gDNA after immobilization on Whatman FTA filter paper	20 pg gDNA/rxn 5×10^2 cells/μl culture	Lu et al. (2011)
Sclerotinia sclerotiorum	LAMP	HNB visual observation	hypothetical protein (XP_001584860.1)	PCM rapeseed tissue	Spin column purification	0.1 fg gDNA/rxn	Duan et al. (2014)
Talaromyces marneffei	LAMP	SYBR Green 1 fluorescence visual observation under UV light and at day light	*rRNA*-ITS	PCM paraffin embedded samples of human and bamboo rat tissue	Addition of gDNA after extraction kit	gDNA equivalent to 2 genome copies/rxn	Sun et al. (2010a)
Trichosporon asahii	Multiplex real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA*-IGS	Pure culture cell suspension dairy products	Isopropanol precipitation of gDNA after mechanical treatment	1.3 cells/ml culture 6.4 cells/ml culture in multiplex assay	Kasahara et al. (2014)
Trichosporon mucoides	Multiplex real-time LAMP	Mg-pyrophosphate precipitation in turbidimeter	*rRNA*-IGS	Pure culture cell suspension dairy products	Isopropanol precipitation of gDNA after mechanical treatment	160 cells/ml culture 160 cells/ml culture in multiplex assay	Kasahara et al. (2014)
Verticillium dahliae	LAMP	Ca- and Mg-pyrophosphate precipitation visual inspection Ethidium bromide fluorescence visual inspection under UV light SYBR Green 1 visual inspection under day light HNB visual inspection GeneFinder visual inspection at day light	RAPD marker (GenBank accession no. AJ302674)	PCM soil	Spin column purification Direct addition of aqueous soil supernatant	0.5 pg gDNA/rxn (type ND) 0.05 pg gDNA/rxn (type D)	Moradi et al. (2014)

Table 1 (continued)

Species/group detected	Assay type	Signal detection	Target gene	Source of DNA	Sample preparation	Sensitivity	Reference
Yeast species	Hybridization LAMP	Hybridization of DIG-labeled yeast specific LAMP product to membrane bound species specific oligonucleotide probes Visual observation of anti-DIG antibody bound enzyme activity after substrate addition	*rRNA*-26S	Pure culture cell suspension	Direct addition of gDNA from diluted culture supernatant	0.05 pg gDNA/rxn	Inacio et al. (2008)

[a] *quant. real-time LAMP* quantitative real-time loop-mediated isothermal amplification

[b] *LAMP* LAMP with endpoint signal measurement

[c] *LFD* lateral flow device

[d] *ITS* internal transcribed spacer region in ribosomal RNA gene

[e] *gDNA* genomic DNA

[f] *pg gDNA/rxn* picogram purified genomic DNA per reaction

[g] *%* (*w*/*w*) percent weight by weight

[h] *UV* ultraviolet

[i] *n.d.* not determined

[j] *HNB* hydroxynaphtol blue indicator for metal titration

[k] *PCM* pure culture mycelium

[l] *real-time LAMP* LAMP reaction with real-time signal monitoring, e.g., turbidity or fluorescence

[m] *cfu* colony forming units

[n] *IsoPCR* A LAMP reaction in which the sequence between FIP and BIP primers is used as template after pre-amplification on a PCR reaction

[o] *AGE* agarose gel electrophoresis

[p] *RAPD* random amplified polymorphic DNA

[q] *SCAR* sequence characterized amplified region

[r] *IGS* intergenic spacer separating ribosomal RNA gene repeats

[s] *BALF* bronchoaveolar lavage fluid

[t] *PCP* pneumocystis carinii pneumonia

the most important ethiological agents worldwide. Therapy of the disease is strongly supported when taxonomical identification of the ethiological agent is available before treatment. Aimed at rapid identification of *Fonsecaea* agents of chromoblastomycosis, Sun et al. (2010b) set up a highly sensitive LAMP-based assay which gave a positive signal with all three species. Several species in the anamorphic genus *Scedosporium* and their teleomorphs in the genus *Pseudoallesheria* are known as causative agents of traumatically inoculated subcutaneous mycoses (Cortez et al. 2008; Pihet et al. 2009). Due to the fact that the relevant species differ widely in virulence and susceptibility to antifungal drugs, accurate identification and detection of the most relevant species are necessary. Lu et al. (2011) developed separate species specific LAMP assays for each of the six medically relevant pathogens within the *Pseudoallesheria*/*Petriella* complex of species.

LAMP-based assays for detection of plant pathogenic fungi

Botrytis cinerea (tel. *Botryotinia fuckelina*) is a necrotrophic fungus that affects the fruits of many plant species (Williamson et al. 2007), although wine grapes are supposed to be the most notable host leading to high economical losses (Elmer and Michailides 2007). Tomlinson et al. (2010a) developed a LAMP-based assay using primers designed from the nucleotide sequence of the ITS region of the rRNA gene of *B. cinerea* and applied it to the detection of the fungus in infected plants with high sensitivity. Several species within the genus *Fusarium* are well known plant pathogens. *Fusarium oxysporum* is one of the most abundant and widespread fungal species of the global soil mycobiota (Gordon and Martyn 1997). Genetically, the species represents a highly heterogeneous polytypic morphospecies (Waalwijk et al. 1996) containing formae speciales adapted to about 100 different host plants, among them ecomically important crops such as melon, tomato, cotton, and banana (Michielse and Rep 2009). LAMP assays have been set up for *F. oxysporum* f. sp. *cubense* as a wilt pathogen of banana plants (Panama disease). Using different target genes, Li et al. (2013a) and Zhang et al. (2013) independently published LAMP assays for the detection of tropical race 4 of the pathogen as the economically most important race and detected it in banana plantation soils with high sensitivity. LAMP-based assays for two other economically important formae speciales of *F. oxysporum* were published by Almasi et al. (2013) for f. sp. *lycopersici* (tomatoes) and by Peng et al. (2013) for f. sp. *niveum* (water melon). *Fusarium mangiferae* represents one of three lineages in the Gibberella fujikuroi complex of *Fusarium* species causing mango malformation (Lima et al. 2009). Pu et al. (2014) recently developed a LAMP-based assay for the sensitive detection of the pathogen in mango shoots. *Guignardia citricarpa* is pathogenic to a variety of *Citrus* species causing Citrus Black Spot on citreous fruits (EPPO 2009) and some other host. In order to replace time consuming microbiological analysis of the quarantine fungus, Tomlinson et al. (2013) set up a LAMP assay which they applied to the detection of the pathogen from citrus fruit lesions. Several closely related *Glomus* species are of pervasive importance to terrestrial ecosystems producing arbuscular mycorrhizas in plant roots (Rillig 2004). In order to detect four of the more important *Glomus* spp. together with *Gigaspora rosea* in one reaction, Gadkar and Rillig (2008) developed a group specific LAMP assay to detect presence of the fungi in carrot roots. Several species within the oomycetous genus *Phytophthora* are well known plant pathogens (Kroon et al. 2012). LAMP-based detection assays were developed for detection of quarantine species such as *Phytophthora kernoviae* and *Phytophthora ramorum* (Tomlinson et al. 2007, 2010b) in tissues of *Rhododendron* trees, *Phytophthora melonis* in cucumbers and potting soils (Chen et al. 2013) or *Phytophthora sojae* in tissues of soybean plants (Dai et al. 2012). *Puccinia striiformis* f. sp. *tritici* (PCT) is the causal agent of wheat stripe rust. The obligate biotrophic parasite is difficult to cultivate on artificial media. In order to facilitate management of the pest through early and sensitive detection in early growth stages, Huang et al. (2011) developed a LAMP-assay using primers specific for the ß-tubulin gene of the fungus and applied it to the detection of PCT in wheat leaves. Many species in the oomycetous genus, *Pythium* cause symptoms similar to those caused by *Phytophthora* spp., i.e., root rot and wilting. Fukuta et al. (2013, 2014) used LAMP-based assays for the detection of *Phytophthora aphanidermatum* and *Phytophthora myriotylium* in hydroponic fluids as well as in infected roots of tomato plants, respectively. Recently, Takahashi et al. (2014) published a set of LAMP primers which they applied in the detection of *Phytophthora helicoides* in roots of *Poinsettia*, an economical important ornamental plant. The ascomycete *Sclerotinia sclerotiorum* infects rapeseed and cotton plants. The LAMP-based assay published by Duan et al. (2014) was designed to analyse presence of the fungus in rapeseed plants and seeds with a detection limit in sub-femtogram range. Verticillium wilt is a disease known from more than 300 cultivated plant species. *Verticillium dahliae* and *Verticillium albo-atrum* are the most important pathogenic species within the genus. In order to test soil samples for the presence of *V. dahliae*, Moradi et al. (2014) recently set up a LAMP assay which detected both pathotypes of the fungus (D, ND) at the level of femtograms of DNA per reaction.

LAMP-based assays for detection of food relevant fungi and yeasts

The role of fungi and yeasts in the production of food and feed may be either positive, e.g., as starter cultures for food production or as biotechnological producers of food ingredients, or negative, e.g., as organisms inducing food poisoning or spoilage. The motivation behind all developments of LAMP-based assays

published so far for the detection of filamentous fungi in food sources was the ability of the species to produce mycotoxins. The only exception so far is the assay developed by Denschlag et al. (2012, 2013) who detected the *hyd5* gene in a group of closely related *Fusarium* species. The gene codes for a protein which is suspected to induce gushing (over foaming) of bottled beer (Sarlin et al. 2005). A variety of *Aspergillus* species are able to produce potent carcinogenic mycotoxins of the aflatoxin type (Bennett and Klich 2003). Luo et al. (2012) set up primer sets based on housekeeping genes to detect major aflatoxinogenic species (*Aspergillus flavus*, *Aspergillus nomius*, and *Aspergillus parasiticus*) on food commodities such as corn, groundnuts, Brazil nuts and green coffee beans. Specificity testing revealed cross reactions with closely related species especially with the *A. flavus* specific primer set. Comparison of LAMP results with aflatoxin contents showed that LAMP can be used to predict the presence or absence of aftlatoxins in Brazil nuts (Luo et al. 2014a). Storari et al. (2013) applied the nucleotide sequences of the *pks* genes within the ochtratoxin A biosynthetic pathways of Aspergillus carbonarius and Aspergillus niger to design species specific primer sets for both species and to analyze pure cultures obtained from vineyards for the presence of the gene. In order to detect and quantify producers of trichothecene mycotoxins among *Fusarium* spp. in cereal grain and malt, Denschlag et al. (2014) designed two sets of LAMP primers each of which was specific to a subset of species representing two chemotypes producing either type A or B trichothecenes. Multiplexing of both primer sets in one reaction enabled detection of the six most abundant species among trichothecene producers. Real-time LAMP results and concentrations of the mycotoxin deoxynivlenol (DON) found in corresponding samples were well correlated. *Saccharomyces cerevisiae* sensu stricto includes yeast strains used in a variety of artisan and industrial food and beverage fermentation processes. Aimed at discriminating the four economically important sibling species of *S. cerevisiae*, i.e., *S. cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, and *Saccharomyces paradoxus* (Guillamon et al. 1994) as well as *Saccharomyces diastaticus*, Hayashi et al. (2009) set up species specific primer sets for each of them an applied the LAMP assays for the discrimination of pure cultures of the yeasts and for the detection of *S. bayanus* as contaminant in wine and beer. Aimed at differentiating the most abundant species within *Brettanomyces*/*Dekkera*, Hayashi et al. (2007) designed primers based on sequence differences within the ITS region of the rRNA genes and set up LAMP-based assays to detect *Dekkera bruxellensis* in samples of beer and wine spiked with low numbers of yeast cells.

Selection of target sequences and LAMP primer design

The majority of LAMP-based assays for fungi and yeasts have been targeted to standard sequences which are readily available from public data bases. Housekeeping genes, such as *acl1* (Niessen et al. 2012; Luo et al. 2014a), *amy1* (Luo et al. 2012), *tef1* (Ferdousi et al. 2014), and *btub* (Gadkar and Rillig 2008; Lu et al. 2011; Villari et al. 2013), or other genes of ubiquitous function in fungi, such as *rodA* (Matsuzawa et al. 2010) or *ypt1* (Chen et al. 2013), are universal sources of sequence information available for a greater number of species and strains. Sequence information from genes coding for ribosomal RNA (rRBA) are even more universally available and have been used in nearly half of the total number of published LAMP assays. Different parts of the gene operon have been applied as sequence source. The ITS region as the universal fungal barcoding marker (Schoch et al. 2012) has been used particularly often (for example, Hayashi et al. 2007; Tomlinson et al. 2007; Sun et al. 2010b; Ishikawa et al. 2014; Kasahara et al. 2014). Other parts of the operon have been used less frequently such as the part coding for the 28S (26S) ribosomal subunit (Ohori et al. 2006; Inacio et al. 2008; Almasi et al. 2013; Jensen Søe et al. 2013) or the intergenic spacer region (Li and Cai 2011; Zhang et al. 2013; Kasahara et al. 2014). Beside their universal availability, rRNA sequences can be used to the advantage of higher assay sensitivity since the ribosomal genes are organized in multicopy clusters in eukaryotes ranging from a few to several hundred copies per genome (Prokopowich et al. 2003). Some of the LAMP assays developed so far have been targeted to certain properties of the detected fungi. In such cases, the selection of a sequence source is rather guided by considerations such like involvement of the target sequence in a particular biosynthetic pathway leading to the production of certain toxins (Storari et al. 2013; Denschlag et al. 2014) or other unwanted compounds (Denschlag et al. 2012). Lucas et al. (2010) selected the *cap59* gene as LAMP target since it is involved in the extracellular trafficking of capsular glucuronoxylomannan in *Cryptococcus* spp. where its sequence variations correlate with the different serotypes of that medically important yeast. A similar approach was taken by Endo et al. (2004) who designed their LAMP primers from the sequence of *gp43*, a gene coding for a 43-kDa protein which is the main exocellular antigen recognized by sera from patients with paracoccidioidomycosis (Travassos et al. 1995). Niessen and Vogel (2010) used the gene coding for galactose oxidase (*gaoA*) as target for the detection of *Fusarium graminearum* since the enzyme is a typical feature characterizing this fungal species (Ögel et al. 1994). A few authors used genomic marker sequences of unknown function and localization as sequence source for primer design. Pu et al. (2014) and Moradi et al. (2014) used sequence characterized amplified regions (SCAR) which resulted from random amplification of genomic DNA of target organisms with random sequence PCR primers as the basis for primer design in LAMP assays detecting *Fusarium mangiferae* and *Verticillium dahliae*, respectively. A similar approach was taken by Li et al. (2013a)

for LAMP-based detection of *F. oxysporum* f. sp. *cubense* TR4 in banana tissues and by Peng et al. (2013) for *F. oxysporum* f sp. *niveum* in cucumber plantation soil. Dai et al. (2012) applied sequences of the *A3aPro* deletion element of known localization but unknown function present in the genome of certain *P. sojae* races to detect the pathogen in soybean tissues in a LAMP assay and Duan et al. (2014) applied the sequence of a hypothetical protein of unknown function which was found by incident during annotation of the *S. sclerotiorum* genome.

Signal detection in fungal LAMP assays

Detection of amplified product in LAMP has originally been accomplished by agarose gel electrophoresis which typically reveals a ladder like pattern of DNA fragments as shown in Fig. 2b. The smallest (monomer) fragment spans from the 5′ end of the Fc1 part of the forward inner primer (FIP) to the 5′ end of the B1c part of the backward inner primer (BIP). Multimers and polymers of that monomeric structure are produced with sizes of a few hundred bp up to the formation of a smear of high molecular weight DNA of several kilobases in size. However, due to massive production of DNA during LAMP, the risk of cross contamination of samples by aerosolized product is very high and may compromise analytical results. This may be the reason why only few authors used this method to analyze the results of LAMP reactions (Ohori et al. 2006; Yarita et al. 2007; Lucas et al. 2010). A different approach to ex-tube detection of LAMP product was applied by Tomlinson et al. (2010b) and also by Vaagt et al. (2013). Both authors used LFD to detect LAMP product which was specifically double-labeled by using a pair of loop primers with either biotin or DIG/FITC in a commercially available standard format. However, also the use of LFD for detection necessitates opening of LAMP reaction vessels.

In order to prevent cross contamination by aerosolized LAMP product, various different methods for in-tube detection of DNA amplification have been developed and were used also in LAMP assays targeted at fungal DNA. In-tube detection of amplified product does not involve opening of LAMP reaction vessels. Direct staining of double stranded DNA using fluorogenic intercalating dyes is one possible way to achieve this goal. Substances such as SYBR green 1 (Sun et al. 2010b; Huang et al. 2011; Lu et al. 2011; Jensen Søe et al. 2013; Vaagt et al. 2013), SYTO9 (Peng et al. 2013; Zhang et al. 2013; Pu et al. 2014), YOPRO1 (Uemura et al. 2008), PicoGreen (Tomlinson et al. 2007; Tomlinson et al. 2010b), or ethidium bromide (Almasi et al. 2013; Moradi et al. 2014) have been applied for the detection of LAMP product in fungal and yeast diagnostic assays with the first three fluorochromes applied mostly in real-time LAMP assays (see Fig. 2c). Since most of these substances substantially reduce productivity of the *Bst* DNA polymerase during LAMP reactions, they must be added after the reaction has run taking into account the risk of cross contamination due to opening of reaction vessels as previously described. In order to prevent inhibition of SYBR Green 1 in LAMP assays, Pu et al. (2014) placed the dye as a droplet in the lid of reaction vessels and mixed it by centrifugation after the reaction was finished. Intercalating fluorescent dyes which can be added before the reaction starts are the V13 01184 dye from Dyomics (Denschlag et al. 2012), the undisclosed fluorescent dyes present in ready-to-use master mixes provided with LAMP kits from OptiGene for applications running on real-time LAMP scanners of the Genie® series and in the Isothermal Master Mix from Nippon Gene (Tomlinson et al. 2010a; Tomlinson et al. 2013; Villari et al. 2013) or in LoopAmp kits from Eiken Chemicals (Tatibana et al. 2009). Also Gene Finder has been described as an intercalating fluorescent dye which has been used by Moradi et al. (2014) in an assay detecting *Verticillium dahliae* in soil samples. It is interesting to note, however, that the authors applied the fluorophore for visual detection of positive LAMP reactions since the compound results in a color change from orange to dark green when binding to double stranded DNA. Precipitation of the DNA produced during LAMP with a fluorescently labeled cationic polymer or the use of a specific fluorescently labeled probe have been shown to be an alternative for direct in-tube detection of the LAMP product (Mori et al. 2006). However, neither of the two methods has yet been used in LAMP assays involving fungal DNA.

Indirect in-tube detection of LAMP product is an alternative to the use of intercalating fluorescent dyes and labeled polymers or probes. It is based on the fact that pyrophosphate is a typical by-product of enzymatic DNA synthesis. The molecule forms an insoluble complex with bivalent cations such as Ca^{2+}, Mg^{2+}, or Mn^{2+}. The turbidity which occurs upon precipitation of the complex was found to be proportional to the concentration of template DNA initially present in a LAMP reaction (Mori et al. 2001). As a consequence, monitoring of turbidity over time enables real-time monitoring of LAMP reactions (see Fig. 2c) and has been applied to the quantification of LAMP results (Mori et al. 2004). Turbidity assays have been used in several LAMP applications for the detection of fungi, either as end point analysis (Gadkar and Rillig 2008; Almasi et al. 2013; Fukuta et al. 2013; Moradi et al. 2014) or by real-time analysis using a dedicated turbidimeter (Hayashi et al. 2007; Denschlag et al. 2014; Ishikawa et al. 2014; Kasahara et al. 2014; Takahashi et al. 2014). In addition to haze formation, the ability of pyrophosphate to form a complex with bivalent cations can be applied during LAMP reactions to revert quenching of a complexometric fluorophore such as calcein (Diehl and Ellingboe 1956). Its fluorescence is initially quenched by the addition of manganese and subsequently restored by complexation of the metal

ion to the pyrophosphate produced during DNA synthesis (Tomita et al. 2008). Fluorescence intensity was found to be proportional to the initial concentration of target DNA in LAMP reactions. Several assays involving calcein fluorescence to detect fungal DNA have been published (Niessen and Vogel 2010; Luo et al. 2012; Niessen et al. 2012; Luo et al. 2014a). Calcein fluorescence can also be used for real-time monitoring of LAMP reactions. However, the technology has not yet been applied for the analysis of fungal DNA. Apart from calcein, other complexometric dyes have been used which change color in the visible part of the spectrum. Goto et al. (2009) demonstrated the usefulness of hydroxynaphtol blue for indirect detection of DNA biosynthesis during LAMP with no need for UV light. The dye was applied for endpoint LAMP analysis of fungal DNA only in recent publications (Almasi et al. 2013; Dai et al. 2012; Storari et al. 2013; Duan et al. 2014; Moradi et al. 2014).

Quantification of fungal DNA using real-time LAMP

In contrast to PCR where DNA amplification is a function of the number of melting–amplification cycles (C_t value), biosynthesis of DNA is a continuous process in LAMP reactions and is therefore a function of incubation time rather than cycle number. Because of its discrete mode of dependence, the concentration of target molecules used in a PCR can be calculated rather precisely from the concentration of amplification product observed after a known number of amplification cycles. The equation used to calculate copy numbers or DNA concentrations in qPCR is biased only by factors which influence amplification efficiency. Because of the dependence of DNA amplification on reaction time in LAMP, a theoretical calculation of target concentrations from a given concentration of amplification product as in qPCR is impossible. Quantitative estimation of target concentrations in a given LAMP reaction therefore has to be done by comparison of the time needed for the signal to reach a certain threshold (T_t value) or, in analogy, to reach a positive signal (T_p value). Few attempts were made so far to find quantitative correlations between LAMP signals and either cell counts (Hayashi et al. 2007), concentrations of purified template DNA (Denschlag et al. 2013; Peng et al. 2013; Vaagt et al. 2013), or concentrations of a plasmid standard containing the target DNA sequence (Zhang et al. 2013; Pu et al. 2014). Authors reported coefficients of correlation of <0.98 but standard deviations between repetitions were high in most cases. A reason for that fact could be that instruments used for real-time LAMP work with quite long data acquisition intervals (6 to 20 s) which might be generally inadequate in the case of a continuous process running at the speed of a usual LAMP reaction, where 2–5 min suffice from zero to maximum signal intensity.

Sample preparation

DNA polymerases used in any kind of amplification-based assay can interact with a variety of components introduced into the reaction together with sample of DNA to be analyzed. Inhibitors include constituents of body fluids such as hemoglobin, urea or heparin, food constituents such as phenolic compounds, glycogen, polysaccharides, enzymes, or fat as well as environmental materials such as humic acids, calcium, and heavy metals. Inhibitive interactions have been specifically well analyzed for *taq* DNA polymerase and other heat resistant DNA polymerases used in PCR (see reviews by Wilson 1997; Alaeddini 2012; Schrader et al. 2012). However, the mechanisms involved have been elucidated only for a limited number of compounds. In order to circumvent limitations imposed by inhibitive interactions, samples have to be highly processed in order to exclude inhibitors from the sample DNA used in PCR analysis (Schrader et al. 2012). Moreover, proper standards must be included in order to control the influence of a DNA sample on the outcome of the analysis since inhibitive potential of different compounds may widely vary between different PCR assays (Huggett et al. 2008). One of the features characterizing the LAMP reaction is its comparably low susceptibility to typical inhibitory compounds occurring in samples such as full bood, stool, soil or food materials (Boehme et al. 2006; Mori and Notomi 2008). Its robustness against variation of reaction conditions such as pH and temperature have been established (Francois et al. 2011).

Table 1 provides information about the DNA extraction procedures applied for LAMP analysis. With a few exceptions, authors apply highly purified genomic DNA (gDNA) for testing specificity and sensitivity of their LAMP reactions. For isolation and purification of gDNA from pure culture mycelia or yeast pure cultures, most authors basically use the method described by Raeder and Broda (1985) with several modifications. Genomic fungal DNA is precipitated by isopropanol or ethanol addition to a cell lysate commonly produced by mechanical maceration of mycelia or yeast cells to provide better accessibility of DNA. Maceration is mostly done by grinding of fresh, frozen or freeze dried mycelia or by application of sand, glass beads or steel ball bearings in a bead beating device. Alternatively, lysis can be accomplished by several cycles of freeze–thawing and/or boiling of cells (Lu et al. 2011). Extraction buffers are most commonly based on Tris–HCl as buffering agent with addition of NaCl to regulate ion strength and ethylenediaminetetraacetic acid as heavy metal chelator. Sodium dodecyl sulfate or benzyl chloride is added in order to facilitate cell wall and membrane disintegration (Fukuta et al. 2013). CTAB can be added to precipite sugars and polysaccharides from the extracts (Vaagt et al. 2013). Addition of polyvinylpolypyrrolidone (PVPP) is often necessary in cases where extracts contain polyphenols

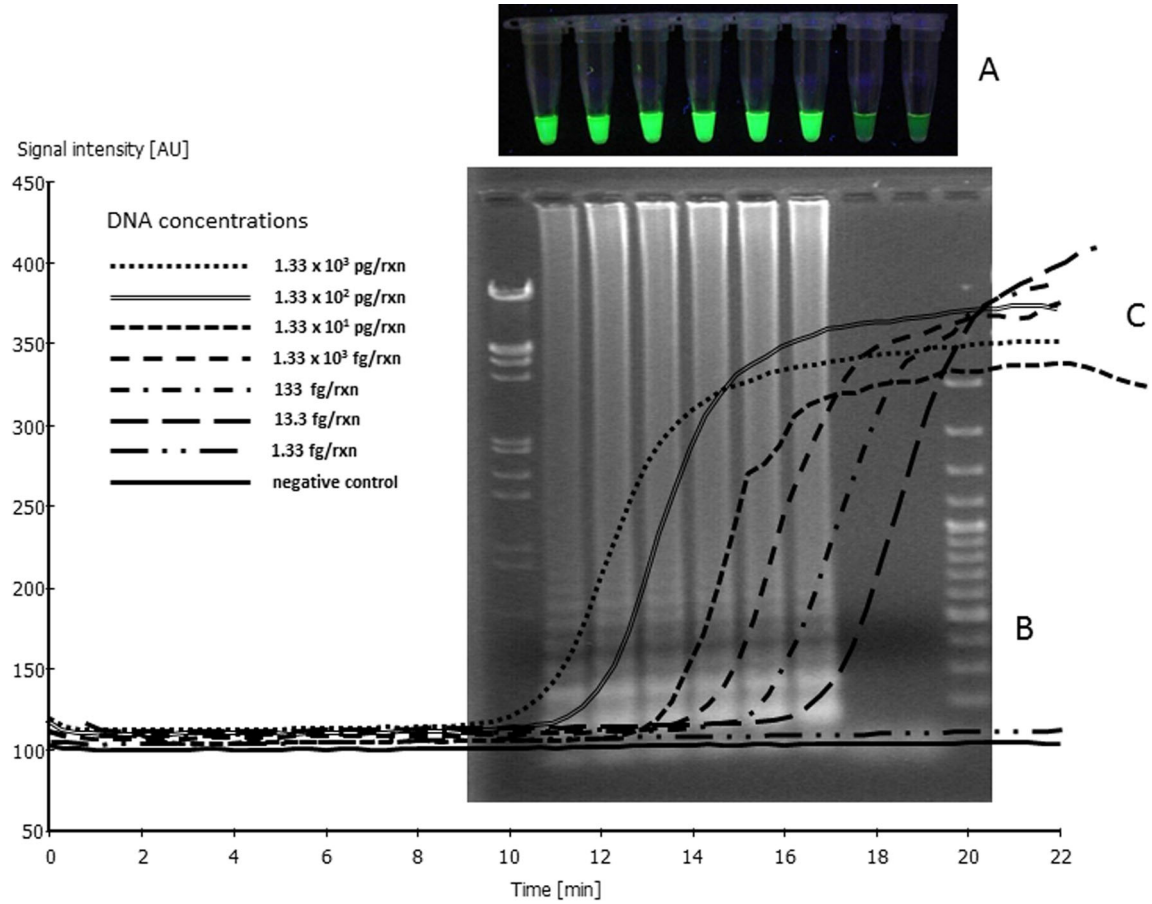

Fig. 2 Different techniques frequently used for signal detection in LAMP. Detection of signals in LAMP reactions performed with a tenfold serial dilution of target fungal DNA. **a** Calcein fluorescence for end point determination of positive LAMP reactions, inspection under UV light. **b** Reactions shown in **a** after electrophoretic separation on agarose gel and staining with ethidium bromide. **c** Real-time monitoring of V13 fluorescence in the same serial DNA dilution, arbitrary fluorescence units plotted against time. Time to threshold (Tt) can be defined according to absolute signal values or derivative signal value, e.g., signal slope

(Tomlinson et al. 2010a). Also, the combination of PVPP with Sephadex has been described for that purpose (Zhang et al. 2013). A lysis step at 55–65 °C is often integrated in order to intensify extraction procedures and improve DNA yield. To provide higher purity of precipitated DNA, organic compounds can be extracted from lysates with chloroform or mixtures of phenol and chloroform for additional protein precipitation (Sun et al. 2010b). Final DNA precipitation is by addition of half a volume of isopropanol or one volume of ethanol to the extract supernatant after maintaining high salt concentrations. Precipitated DNA is directly used in LAMP reactions or undergoes a further purification step using silica based spin column systems available from several commercial providers.

Preparation of highly purified DNA from pure cultures or from sample materials is a cumbersome business which takes a few hours if not days to be completed. Many authors have benefited from the fact that LAMP can be run with DNA of low purity with the effect that time needed for sample preparation can dramatically be reduced. To reach this goal, many authors use spin column based kit systems to extract, concentrate and purify LAMP ready target DNA from pure cultures or from contaminated sample materials (Niessen et al. 2012; Jensen Søe et al. 2013; Li et al. 2013a; Denschlag et al. 2014). An even simpler method of providing purified target DNA for LAMP reactions is the use of a FTA membrane (Whatman) to specifically bind gDNA and enable removal of non-DNA materials by a simple washing step. DNA bound to FTA membrane material can be added directly to a LAMP master mix to amplify target DNA (Lu et al. 2011). Similarly, Tomlinson et al. (2010b) applied a nitrocellulose membrane commonly used in LFD to bind and purify target DNA. LAMP was performed using a small segment of the membrane to provide the target for amplification. Other authors used raw cell or sample extracts after boiling in an extraction buffer or water with or without previous mechanical treatment from which an aliquot was directly added into the LAMP reaction (Hayashi et al. 2007; Lucas et al. 2010; Storari et al. 2013; Ishikawa et al. 2014). The highest degree of simplification in sample preparation is direct addition of materials into

the LAMP master mix with no further handling necessary. This was demonstrated for pure culture materials (Luo et al. 2014a; Niessen and Vogel 2010; 2012; Denschlag et al. 2012) or for infected barley grains which were immersed directly into the master mix prior to the start of the LAMP reaction (Niessen et al. 2012).

Future perspectives of fungal LAMP

Review of the literature as given in the previous sections has demonstrated that the potential of LAMP is based on its sensitivity, rapidness and specificity, as well as convenience in practical implementations. Similar to other nucleic acid based methods, LAMP provides detection and identification in one reaction. Based on DNA amplification, the LAMP technique cannot distinguish whether or not fungi and yeasts detected are alive and multiplying. However, this fact may have serious implications in some areas such as medicine and food safety where treatment of patients or food and food raw materials is unnecessary if pathogens or spoiling organisms are not viable. However, no attempts have so far been made in that direction by application to fungal organisms. Only recently, Minenko et al. (2014) published the first paper describing the use of reverse transcription LAMP for the selective detection of a fungal mRNA template in a one-step reaction by directly adding fungal mycelia to the LAMP reaction or after mRNA isolation. Attempts to use sample treatment with ethidium monoazide or propidium monoazide prior to RNA isolation for the selection of live target cells as previously reported for living bacteria (Wang and Huo 2012; Wang et al. 2012) have not yet been used for fungi.

Within the near future LAMP-based diagnostics will neither replace sequencing-based identification nor the classical microbiological analysis of fungi and yeasts in the clinical, food, or environmental laboratory. However, it will provide a valuable tool in all such applications in which rapid and sensitive identification of organisms is a priority in order to facilitate decision making about actions to be taken, e.g., in medical point-of-care situations, sanitation and quarantine testing, and food quality control applications. Application of LAMP has been described now for major fungal pathogens in humans, animals, and plants. Only a few LAMP assays have yet been focused on organisms which compromise the quality of food and beverages and it can be anticipated that more such assays will be developed in the near future because the method is ideally suited for application in HACCP concepts in various industries. Ready to use kit systems have been patented mainly for bacteria and viruses but also systems for fungi or yeast will presumably become available in the future, especially for diagnosis of medically important organisms. Also the development and application for LAMP-based systems for diagnosis of quarantine organisms, especially plant pathogens, will become a growing field in the future since it will considerably speed up the release of plants and animals during import. Some such systems have already been developed (Tomlinson et al. 2010b; Li et al. 2013a; Tomlinson et al. 2013; Zhang et al. 2013; Moradi et al. 2014), but the list of organisms recommended for regulation as quarantine pests as approved by EPPO Council is long enough to keep scientists working late hours during the coming years.

As regards to speed of analysis, the recent release of the newly developed thermophilic *Bst* 2.0 DNA polymerases for LAMP applications has improved reaction speed a lot and similar types of enzymes have been released now from market competitors. Enzymes of that type can reduce reaction times by about 50 % according to the authors' experience (Denschlag et al. 2013). In combination with modern portable devices for incubation and signal readout now available on the market, testing with a simple yes/no result will be possible within 10–15 min or even less with sensitivity similar to what has been shown in lab experiments. Other recently detected thermophilic DNA polymerases such as OmniAmp Pol expressed from the genome of PhyroPhage 3173 and modified for LAMP application may further improve the preparation of dried ready-to-use master mixes (Chander et al. 2014).

Another future trend in the application of LAMP for on-site testing is the use of micro fluidic instrumentation. Developments in that area are strongly driven by virological and bacteriological research. None of the developments has yet been applied or adapted in the field of mycological diagnostics. However, the field will certainly benefit a lot from the developments described below in applicactions such as medical diagnosis or diagnosis of quarantine plant panthogens. Recently, Ahmad and Hashsham (2012) reviewed the literature available on miniaturized nucleic acid amplification systems and their possible application for point-of-care analysis and described a variety of different systems already available or under development. At the current point of development, LAMP-based assays do not yet fulfill the WHO recommendation according to which point-of-care diagnostic devices developed for low-resource settings should follow the ASSURED criteria; affordable, sensitive, specific, user-friendly, rapid and robust, electricity free, and deliverable to end users (Peeling et al. 2006). Recent research towards the development of low equipment microLAMP shows progress towards this goal for future applications since electricity-free LAMP (tube based) was recently demonstrated by LaBarre et al. (2011), who used calcium oxide as heat source in combination with a phase change material in a NINA (non-instrumented nucleic acid amplification) process to create a constant temperature of 65 °C over 1 h without electricity and demonstrated the feasibility of the method to perform a LAMP reaction. Also such systems would be highly desirable for fungal diagnosis in clinical settings or in the field.

Besides making LAMP more easily operated, using the method for the analysis of multiple parameters in one run will be a future trend. Preliminary concepts have been demonstrated and prototype devices were published recently. It appears that two different approaches to multiplexing the LAMP reaction do currently develop in parallel. One approach is parallel multiplexing using microfluidic devices in which a sample is split into several subsamples which are than analyzed with multiple primer sets in separate LAMP reactions resulting in a positive/negative pattern readout (Fang et al. 2012; Zhu et al. 2012). Another approach is the use of simultaneous multiplexing using multiple primer sets in one reaction as demonstrated by Tanner et al. (2012) who applied the FRET-based assimilating probes technology previously described by Kubota et al. (2011). Separate detection of different amplification products obtained with different primer sets was achieved by integration of specific probes each of which was coupled to a different fluorescent marker corresponding to different channels of a fluorescence reader, e.g., real-time thermal cycler.

Some very recent developments show that LAMP has the potential to be a useful technology not only for the diagnosis of microorganisms. Combination of antibodies or aptameric probes for proteins or even low molecular weight compounds such as mycotoxins with a LAMP-based detection system can provide ultrasensitive systems for the analysis and quantification as an alternative to ELISA-based detection (immunoLAMP). Pourhassan-Moghaddam et al. (2013) combined antibody-based detection of proteins with LAMP-based amplification of a DNA marker sequence either attached to an antibody or specific aptamer or packed in attached liposomes which open upon binding and set the LAMP target free for subsequent amplification and detection. A similar system for specific quantification of ochratoxin A (OTA) was developed by Yuan et al. (2014) who used an OTA specific aptamer to react specifically with sensor bound toxin. The binding event was detected and quantified using a polynucleotide probe that was amplified in a subsequent LAMP reaction with $Ru(phen)_3^{2+}$ based electrochemiluminiscent signal readout. The assay had a detection limit of 10 f. of OTA in a model solution. Xie et al. (2014) showed that the method is also applicable for the detection of the toxin in red wine samples. Other recently developed concepts have shown that LAMP can be combined with enzyme free DNA nanotechnologies such as catalytic hairpin assembly (Li et al. 2012) or enthropy driven DNA assembly such as strand displacement driven hybridization chain reaction (Li et al. 2013b). Implication of such newly developed technologies into the concept of LAMP-based detection systems as well as further completion of the portfolio of detectable species and groups will certainly cause a considerable improvement of the diagnostic tools for fungi and yeasts within the near future.

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