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Efficient Identification of Clinically Relevant *Candida* Yeast Species by Use of an Assay Combining Panfungal Loop-Mediated Isothermal DNA Amplification with Hybridization to Species-Specific Oligonucleotide Probes▽

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The occurrence of invasive mycoses has progressively increased in recent years. Yeasts of the genus *Candida* remain the leading etiologic agents of those infections. Early identification of opportunistic yeasts may contribute significantly to improved disease management and the selection of appropriate antifungal therapy. We developed a rapid and reliable molecular identification system for clinically relevant yeasts that makes use of nonspecific primers to amplify a region of the 26S rRNA gene, followed by reverse hybridization of the digoxigenin-labeled products to a panel of species-specific oligonucleotide probes arranged on a nylon membrane macroarray format. DNA amplification was achieved by the recently developed loop-mediated isothermal DNA amplification technology, a promising option for the development of improved laboratory diagnostic kits. The newly developed method was successful in distinguishing among the major clinically relevant yeasts associated with bloodstream infections by using simple, rapid, and cost-effective procedures and equipment.

The occurrence of nosocomial invasive mycoses in immunocompromised patients has increased over the last decade (24). Yeasts of the genus *Candida* are the main etiologic agents of those infections, with a high prevalence of *C. albicans*. However, other species (e.g., *C. krusei* and *C. glabrata*) have emerged as opportunistic pathogens associated with systemic infections (14, 21), posing difficulties due to the different susceptibilities of these yeasts to antifungal therapy. Sensitive, reliable, and rapid identification of these pathogenic yeasts is of paramount importance to improve disease management and enable the selection of adequate treatment.

Currently, yeast identification in clinical laboratories usually involves the analysis of phenotypic properties, a time-consuming and expensive procedure that often fails to provide clear-cut results. PCR-based methods and other successful molecular diagnostic techniques, such as the peptide nucleic acid-fluorescent in situ hybridization method (1, 39, 47), evaluating the hybridization of specific fluorescent probes to RNA target sites, have been developed, but their implementation for the identification of medically important yeasts in the clinical laboratory has not yet been routinely established, possibly because they are not so easy to perform and require more or less sophisticated equipment.

In order to bypass the PCR step, which until recently was patent protected, several groups have engaged in developing alternative nucleic acid amplification technologies (see, e.g., references 6 and 25). Of particular interest in this context are isothermal amplification processes, which could facilitate their integration in bench molecular diagnostic kits. One such technology is loop-mediated isothermal DNA amplification (LAMP), first described by Notomi et al. (33) and subsequently refined (26–28, 30, 31). This elegant, robust, and very promising isothermal DNA amplification technique relies on autocycling strand displacement DNA synthesis (Fig. 1), using specially designed primer sets that recognize at least six distinct sequences on the target DNA and a DNA polymerase with strand displacement activity. The reaction runs very rapidly in the presence of template DNA and deoxynucleoside triphosphates, usually in less than 90 min at a constant temperature (e.g., 60 to 65°C). The final amplification products present stem-loop DNA structures, encompassing alternate inverted repeats of the target sequence with multiple loops, and appear with a ladder-like pattern in agarose gel electrophoresis. LAMP provides high amplification efficiency, with DNA being amplified 10^9- to 10^{10}-fold, and shows a detection limit and a specificity comparable to those of standard PCR. Moreover, the sensitivity of LAMP appears not to be affected by the presence of nontarget DNA in samples (33), and the method is also more tolerant of the presence of known PCR inhibitors such as blood, serum, plasma, or heparin (8, 36).

The high potential of LAMP for the development of improved DNA-based diagnostic kits, its simplicity, and the fact that it does not require specific equipment fully justify the increasing number of recent reports on the utilization of this technique for the detection and identification of organisms of clinical and biotechnological importance. There has been a clear emphasis on the diagnosis of viral and bacterial infections (8, 11, 13, 16, 17, 35, 37, 42), but parasites such as *Plasmodium falciparum* (36) and *Trypanosoma* spp. (19, 44) and pathogenic fungi such as *Paracoccidioides brasiliensis* (7) and *Ochroconis gallopava* (34) have also been addressed. LAMP-based approaches have been applied to a wide range of samples, such as paraffin-embedded tissues (7), whole blood (36), nasopharyn-

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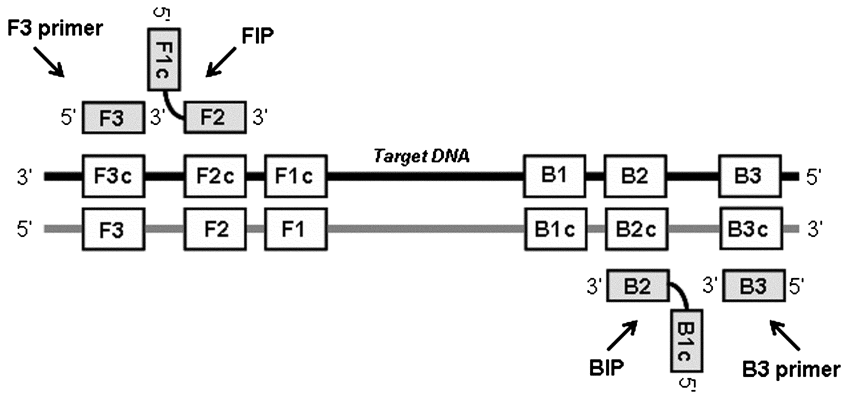

FIG. 1. General location of the LAMP primer set in relation to previously defined regions of the target DNA. Forward (F3) and backward (B3) outer primers and forward (FIP) and backward (BIP) inner primers are indicated. The specially designed inner primers, FIP and BIP, contain two distinct sequences (F1c plus F2 and B1c plus B2, respectively) corresponding to sense and antisense segments of the target DNA, one for priming in the first stage and the other for self-priming in a subsequent amplification reaction stage (33).

geal swabs (17, 40), dental plaques (23), eggs (13), and potato leaf samples (32).

Previous reports on the application of "isothermal" nucleic acid amplification techniques to yeast identification (3, 4, 22, 46) are all based on nucleic acid sequence-based amplification (6), but this method is rather unspecific due to the need to use a relatively low temperature (40°C) for amplification (33). We are interested in the development of a simple and user-friendly bench DNA-based diagnostic kit for the identification of clinically relevant yeasts. To the best of our knowledge, this is the first report on the utilization of LAMP to amplify digoxigenin (DIG)-labeled yeast DNA amplicons. Our concept is different from that used in all LAMP-based methods published so far in that they involve the utilization of species-specific LAMP primer sets for the detection and identification of a single organism. In contrast, our system progresses in two steps. The first involves the utilization of a relatively conserved panfungal LAMP primer set that leads to the amplification of a common DIG-labeled DNA fragment from a broad range of yeast species. A specific species, either alone or in a mixed yeast population, can be identified subsequently by reverse hybridization to an array of membrane-bound species-specific oligonucleotide probes.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study are listed in Table 1 and are maintained at the Portuguese Yeast Culture Collection (PYCC), Caparica, Portugal. Eight yeast species were selected on the basis of their clinical importance in relation to invasive mycosis: *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, *C. krusei*, *Pichia anomala*, and *Saccharomyces cerevisiae*. All the other species were used as negative controls for the reverse hybridization assays. We took the option to conserve common yeast names recognized in the clinical set, despite changes in nomenclature for some of the species (see Table 1 footnotes).

DNA extraction. For DNA extraction, two loopfuls of cultures grown on MYP agar (0.05% [wt/vol] yeast extract, 0.7% malt extract, 0.25% Soytone, and 1.5% agar) for 2 to 5 days at 25°C were suspended in 500 μl lysing buffer (50 mM Tris, 250 mM NaCl, 50 mM EDTA, 0.3% sodium dodecyl sulfate [SDS] [pH 8]) plus the equivalent of a 200-μl volume of 425- to 600-μm-diameter glass beads (Sigma). After being vortexed for 2 min, the tubes were incubated for 1 h at 65°C and vortexed again for another 2 min. The suspensions were centrifuged for 15 min at 14,000 rpm, and the supernatant (DNA concentration, 10 to 30 ng/μl) was diluted in sterilized double-distilled water (1:750) and used directly for DNA amplification. The concentration of genomic DNA was estimated by visual comparison to serial dilutions of reference standards (GeneRuler DNA ladder mix; Fermentas) in an ethidium bromide-stained agarose gel. DNA solutions could be kept for several months at −20°C without noticeable degradation.

LAMP primers. A set of LAMP primers targeting relatively conserved sequences within the D1/D2 domains of the fungal 26S ribosomal DNA (rDNA) was designed in order to amplify a 190-bp DNA fragment from a variety of yeast species. The primers were as follows: F3, forward outer primer (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′); B3, backward outer primer (5′-CCT TCC CTT TCA ACA ATT TCA C-3′); FIP, forward inner primer (5′-CTG CAT TCC CAA ACA ACT CGA CTC ACA GAG GGT GAG AAT CCC G-3′); BIP, backward inner primer (5′-TAT TGG CGA GAG ACC GAT AGC GTT TCA CTC TCT TTT CAA AGT TC-3′). The primer set is fully complementary to segments inside the 26S rDNAs of three of the species under study, *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. For the other species, primer FIP had the maximum number of nucleotide substitutions: five nucleotide substitutions in comparison to the sequences of *C. krusei* and *C. lusitaniae*. Primers were designed according to the instructions of Notomi et al. (33) and those found at the LoopAmp Eiken Genome website (http://loopamp.eiken.co.jp/e/lamp/index.html). All 26S rDNA sequences used for primer design were retrieved from GenBank, with special emphasis on the sequences made available earlier by comprehensive yeast systematics studies (9, 20).

Species-specific oligonucleotide probes. A universal probe for fungi, U210, was designed. Species-specific oligonucleotide probes were designed for selected clinically relevant yeasts based on the comparative analysis of 26S rDNA sequences retrieved from GenBank (Table 2). The targets for the specific probes are located inside the FIP/BIP LAMP-amplified fragment (Fig. 1). All probes were synthesized with an additional 3′ tail of six thymine bases to ensure efficient binding to nylon membranes and capture of the target amplicons (5). All primers and probes were synthesized by STAB Vida Lda. (Oeiras, Portugal).

LAMP reaction. The LAMP reaction mixture was optimized for our identification system and contained 1.6 μM (each) FIP and BIP, 0.2 μM (each) F3 and B3, 900 μM each deoxynucleoside triphosphate, 1.4 μl of the template DNA solution, 0.8 M betaine (Sigma), 3 mM $MgCl_2$, 3.2 U *Bst* polymerase, and the respective 1× buffer from New England Biolabs, for a final volume of 10 μl. When the amplicons were labeled, 1/40 of the dTTP was in the form of DIG-labeled dUTP (Roche Diagnostics). The template DNA was denatured (at 94°C for 4 min; then it was kept on ice) prior to the amplification reaction. The LAMP mixture was incubated at 64°C for 90 min in a heater block, followed by a final step of 80°C for 5 min to inactivate the enzyme. Amplicons were separated by subjecting the amplification mixture to electrophoresis in a 1.4% agarose gel and were detected with ethidium bromide. LAMP reactions were also performed by the addition of whole-yeast-cell suspensions directly to the reaction mixture (cells grown for 2 to 5 days on MYP agar at 25°C were suspended in water [at a McFarland standard of 5] and heated to 99°C for 5 min before amplification).

Reverse hybridization. DIG-labeled LAMP amplicons were hybridized to a panel of species-specific oligonucleotide probes in a nylon membrane macroarray format. The 11 oligonucleotide probes (Table 2) were first immobilized on nylon strips (1 by 2 cm; Hybond-N; Amersham Pharmacia Biotech): 0.3 μl of each 50 pM probe aqueous solution was spotted onto a specific location on the

TABLE 1. List of yeasts used in this study

Yeast	Strain	Origin
Candida albicans	PYCC 3436^T	Skin of man with interdigital mycosis
	PYCC 2411	Sputum (Portugal)
	PYCC 2746	Sputum of asthma patient (Norway)
	PYCC 4079	Unknown
Candida glabrata	PYCC 2418^T	Human feces
	PYCC 3109	Digestive tract of domestic animal (Germany)
	PYCC 2716	Unknown
Candida krusei[a]	PYCC 3341^T	Sputum of bronchitic patient (Sri Lanka)
	PYCC 2631	Digestive tract of a horse (Portugal)
	PYCC 4740	Seawater (Portugal)
Candida lusitaniae[b]	PYCC 2705^T	Cecum of pig (Portugal)
	PYCC 4093[c]	*Citrus* essence (Israel)
	PYCC 4175	Unknown
Candida maltosa	PYCC 3860^T	Neutralizing tank for monosodium glutamate (Japan)
Candida oleophila	PYCC 4296	Unknown origin
Candida parapsilosis	PYCC 2545^T	Case of sprue (Puerto Rico)
	PYCC 5124	Unknown
Candida tropicalis	PYCC 3097^T	Bronchitic patient
	PYCC 4672	Soil near a polluted river (Portugal)
	PYCC 2508	Liver of an elephant (Portugal)
Candida viswanathii	PYCC 2811	*Penaeus braziliensis* (shrimp) (Gulf of Mexico)
Kluyveromyces polysporus[d]	PYCC 3887^T	Soil (South Africa)
Lodderomyces elongisporus	PYCC 4136^T	Concentrated orange juice (United States)
Pichia anomala	PYCC 4121^T	Unknown
	PYCC 3294	Pus from lung of a dead tuberculosis patient (Italy)
	PYCC 5618	Unknown
Saccharomyces bayanus	PYCC 4456^T	Turbid beer
Saccharomyces cerevisiae	PYCC 4455^T	Brewer's top yeast (The Netherlands)
Saccharomyces exiguus[e]	PYCC 2543^T	Japan
Saccharomyces paradoxus	PYCC 4570^T	Tree exudate
Stephanoascus ciferrii	PYCC 3818	Neck of a cow (The Netherlands)

[a] Anamorph of *Issatchenkia orientalis*.
[b] Anamorph of *Clavispora lusitaniae*.
[c] Type strain of *Clavispora lusitaniae*.
[d] Current synonym of *Vanderwaltozyma polyspora*.
[e] Current synonym of *Kazachstania exigua*.

nylon membrane, followed by irradiation with short-wave UV light for 2.5 min to cross-link the oligonucleotides to the membranes. Membranes were washed once in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 2 min at 37°C to remove any unbound probes. The strips were dried and stored at room temperature. For the hybridization, each strip was transferred to a 2-ml microcentrifuge tube containing 1 ml of prewarmed hybridization buffer (Dig Easy Hyb; Roche Diagnostics) and incubated with gentle agitation for 10 min at 55°C. After denaturation of the DIG-labeled LAMP amplicons (at 95°C for 5 min), after which they were kept on ice, 4 μl of the reaction mixture was added to the microcentrifuge tube containing the strip and hybridization buffer. Hybridization was performed for 3 h at 55°C, with inversion of the tubes. The strip was then removed from the tube and washed once in 0.25× SSC–0.1% SDS (40 ml for each batch of 30 strips, in a Falcon tube) at 55°C for 10 min. Positive hybridization was detected by using an alkaline phosphatase-labeled anti-DIG antibody and a color substrate detection system according to the manufacturer's instructions (Dig labeling and detection kit; Roche Diagnostics). Color developed 5 to 30 min after the start of the reaction. The universal probe U210 was used as a positive control on each strip, and water was used as a negative control.

RESULTS

LAMP primers. The first and perhaps the most important step in LAMP optimization is the primer set design. We cor-

TABLE 2. DNA probes developed in this study

Probe	Sequence (5′→3′)	% GC[a]	T_m (°C)[a]	Target species
U210	TCG AGT TGT TTG GGA ATG CAG CTC	50.0	65	Panfungal
Ca170	TGA GAT GAC CCG GGT CTG TGT AAA	50.0	65	*Candida albicans*
Ca176	GAC CCG GGT CTG TGT AAA GTT CC	56.5	66	*C. albicans*
Cg175	GTG TCA GTT CTT TGT AAA GGG TGC TCG	48.0	68	*C. glabrata*
Cd176	GGC CCG GGT CTA TGT AAA GTT CC	56.5	66	*C. dubliniensis*
Cl180	GAC TCT TTG CAC CGC GGC TCC	66.7	67	*C. lusitaniae*
Ct171	GAG ATG ATC CAG GCC TAT GTA AAG T	44.0	64	*C. tropicalis*
Cp171	GAG ATG TCC CAG ACC TAT GTA AAG TTC	44.4	67	*C. parapsilosis*
Ck175	GGC GGA AGC AGT GAG GCC CTT CT	65.2	70	*C. krusei*
Pa176	GCC CAT TCC TAT GTA AGG TGC TAT C	48.0	66	*Pichia anomala*
Sc176	GTG CGG TTC TTT GTA AAG TGC CTT CG	50.0	68	*Saccharomyces cerevisiae*

[a] Data obtained with Oligo Analyzer, version 1.0.2 (Teemu Kuulasmaa, University of Kuopio, Finland).

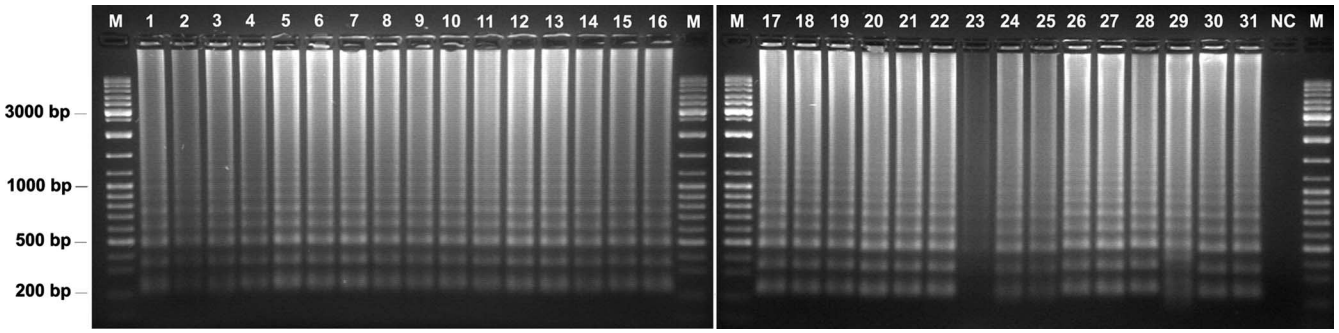

FIG. 2. Agarose gel electrophoresis of LAMP products from clinically relevant yeasts obtained by using the primer set designed in this work. Lanes: 1 to 3, *Candida lusitaniae* PYCC 2705^T, PYCC 4093, and PYCC 4175; 4, *Saccharomyces cerevisiae* PYCC 4455^T; 5, *S. bayanus* PYCC 4456^T; 6, *S. paradoxus* PYCC 4570^T; 7, *Saccharomyces exiguus* PYCC 2543^T; 8 to 10, *C. glabrata* PYCC 2418^T, PYCC 3109, and PYCC 2716; 11 to 14, *C. albicans* PYCC 3436^T, PYCC 2411, PYCC 2746, and PYCC 4079; 15 to 17, *C. tropicalis* PYCC 3097^T, PYCC 4672, and PYCC 2508; 18 and 19, *C. parapsilosis* PYCC 2545^T and PYCC 5124; 20 to 22, *Pichia anomala* PYCC 4121^T, PYCC 3294, and PYCC 5618; 23 to 25, *C. krusei* PYCC 3341^T, PYCC 2631, and PYCC 4740; 26, *C. viswanathii* PYCC 2811; 27, *C. maltosa* PYCC 3860^T; 28, *C. oleophila* PYCC 4296; 29, *Lodderomyces elongisporus* PYCC 4136^T; 30, *Kluyveromyces polysporus* PYCC 3887^T; 31, *Stephanoascus ciferrii* PYCC 3818; NC, negative control; M, molecular weight marker (GeneRuler DNA ladder mix; Fermentas).

roborate the experience of others (2, 8) in that it may be necessary to design several primer sets before finding one that works efficiently in the LAMP reaction. The LAMP primers developed in this investigation targeted conserved sequences inside the fungal D1/D2 domains of the 26S rDNA. This rDNA region was chosen not only because it is much used in yeast systematics and identification studies but also because of the availability of its sequences in public databases and its earlier utilization for designing fungal LAMP primers (34). Unfortunately, some of the available sequences were of low quality and/or were incorrectly labeled. Regions of the rDNA unit have been used before (34, 36) to develop LAMP primers, but the majority of LAMP-based identification systems targeted species- or group-specific genes, such as the *gp43* gene of *Paracoccidioides brasiliensis* (7), which encodes the major glycoprotein antigen of this fungus, the pertussis toxin gene promoter region of *Bordetella pertussis* (17), or the *amoA* gene in ammonia-oxidizing bacteria (2).

Species-specific oligonucleotide probes. The probes we designed are listed in Table 2. The probes designed for *C. albicans*, Ca170 and Ca176, have identical sequences in the closely related and recently described species *Candida africana* (45). The *C. tropicalis* probe, Ct171, has an identical sequence in *C. sojae* and one mismatch in *C. maltosa*. The *C. parapsilosis* probe, Cp171, has an identical sequence in *C. orthopsilosis* and one mismatch in *C. metapsilosis*, both recently described (43). Probe Pa176, for *Pichia anomala*, has an identical sequence in *Pichia subpelliculosa*, and probe Sc176, for *S. cerevisiae*, has one mismatch relative to *Saccharomyces bayanus* and *Saccharomyces paradoxus*.

LAMP amplification. The LAMP primer set designed in this work successfully amplified genomic DNA from all yeasts tested, producing the expected ladder-like patterns on the agarose gel electrophoregram (Fig. 2). Occasionally, with a very low frequency (10^{-2}), LAMP produced a minor amount of amplified DNA (e.g., *C. krusei* PYCC 3341^T [Fig. 2, lane 23]). To determine the sensitivity of the LAMP reaction, the extracted genomic DNAs from *C. albicans* PYCC 3436^T and *C. krusei* PYCC 3341^T were quantified, serially diluted, denatured, and used as templates in LAMP reactions (Fig. 3A). The detection limit assessed with denatured genomic DNA of *C. albicans* PYCC 3436^T, which shows a 26S rDNA segment fully complementary to the designed primer set, was around 50 fg (Fig. 3A). This sensitivity is comparable to that of standard PCR and similar to values (10 to 100 fg) mentioned by other authors for their LAMP-based systems (7, 17, 34). However, the LAMP detection limit was only 1 pg when *C. krusei* PYCC 3341^T genomic DNA was used (Fig. 3A), which may be ascribed to the five mismatches between the FIP primer and the respective *C. krusei* target site. An alternative to circumvent this lower sensitivity could be to utilize degenerated primers or a mixture of primers, which has proved successful in other LAMP assays (10, 38). We confirmed that the LAMP reaction proceeds without a previous thermal denaturation of the template DNA (30), making this technique really isothermal (Fig. 3B). However, as other authors found (17), the detection limit was 5 to 10 times less sensitive when a nondenatured DNA template was used (Fig. 3B). There was no difference in band intensity over the genomic DNA concentration range tested, which has also been observed by other authors (17, 19, 41). It is possible that the incubation time used (90 min) was sufficient to complete the reaction, even when the lowest amount of template DNA was used.

To shorten the identification response time by avoiding the DNA extraction step, the direct utilization of heat-treated whole yeast cells in the LAMP assay was tested. Heat-treated *C. albicans* cell suspensions prepared directly from growth on plates showed a detection limit for the LAMP reaction consistently below 10 cells in the reaction mixture (Fig. 3C), in good agreement with the results of other authors who followed the same experimental approach using whole bacterial cells (13, 15). A different electrophoretic banding pattern was occasionally (<1%) detected in LAMP assays (Fig. 2, lane 29, and Fig. 3C, lane 8). This unusual banding pattern was not observed consistently for a given species or strain. Since it occurred with a negative control, with the *Lodderomyces elongisporus* DNA template (Fig. 2, lane 29), and in a sensitivity assay of the isothermal amplification step (Fig. 3C, lane 8), we were not

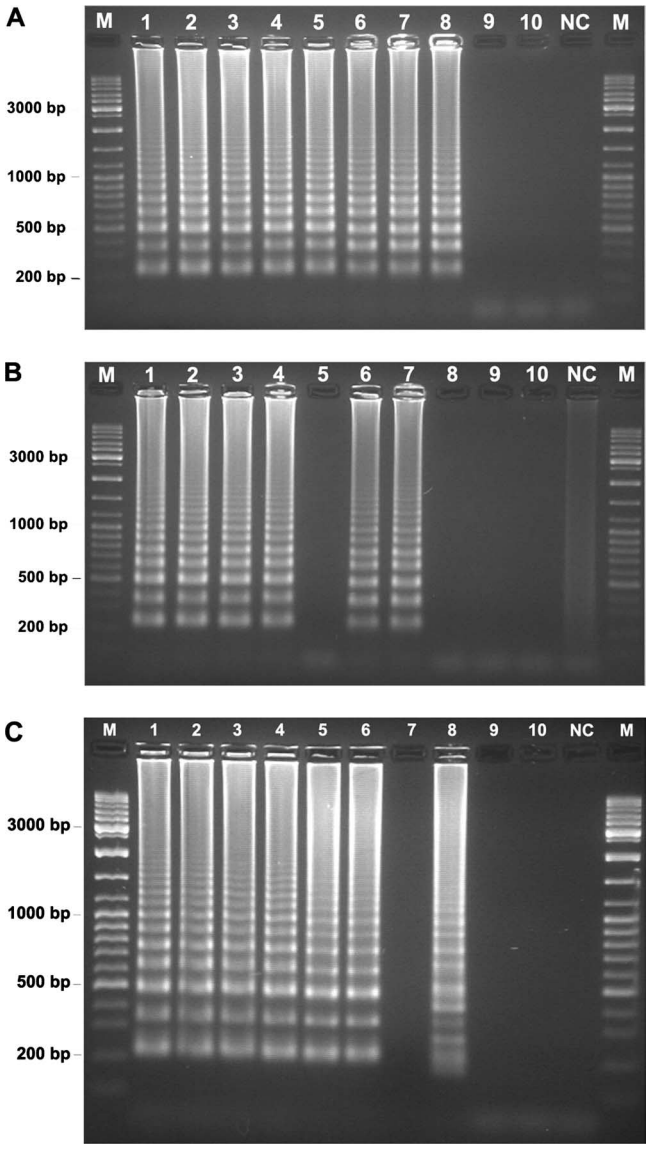

FIG. 3. LAMP sensitivity. (A and B) Different amounts of genomic DNA from *C. albicans* PYCC 3436^T (lanes 1 to 5) and *C. krusei* PYCC 3341^T (lanes 6 to 10), subjected (A) or not (B) to a previous thermal denaturation step, were used in the reaction mixture: 500 pg (lanes 1 and 6), 5 pg (lanes 2 and 7), 1 pg (lanes 3 and 8), 0.5 pg (lanes 4 and 9). and 0.05 pg (lanes 5 and 10). (C) LAMP sensitivity determined with heat-treated whole cells of *C. albicans* PYCC 3436^T placed directly in the reaction mixture. Estimated numbers of cells in 10 μl of the reaction mixture are as follows: lane 1, 7×10^3; lane 2, 3.5×10^3; lane 3, 10^3; lane 4, 700; lane 5, 70; lane 6, 7; lane 7, 1; lanes 8 to 10, <1. Lane NC, negative control; lane M, molecular weight marker (GeneRuler DNA ladder mix; Fermentas).

able to determine whether the respective LAMP products can hybridize with the species-specific probes. This banding pattern was observed only once in reactions using DIG-modified nucleotides, with *L. elongisporus*. In this case, the LAMP product hybridized only with the panfungal U210 probe, as expected. We have no clear explanation for the formation of these odd banding patterns, but it appears to be the result of specific linear target isothermal multimerization and amplification of the template DNA (19), a property of the *Bst* DNA polymerase that has been demonstrated by Hafner et al. (12).

Reverse hybridization. The reverse hybridization of the DIG-labeled LAMP amplicons to a panel of species-specific oligonucleotide probes (Table 2) on nylon membrane strips correctly identified the respective yeast species. Each amplicon yielded a clearly visible hybridization signal with the respective species-specific probe and the U210 universal probe for fungi, albeit with different intensities depending on the probe (Fig. 4). In a future improvement of the method, it should be possible to standardize signal intensities for all probes, e.g., by modifying their concentrations and/or the size of the 3′ thymine tail (5). Control yeast species, e.g., *Candida viswanathii* PYCC 2811 and *Kluyveromyces polysporus* PYCC 3887^T, for which no specific probe was available, hybridized intensely only to the universal probe. Weak, cross-reacting hybridization signals were observed for *S. bayanus* PYCC 4456^T and *S. paradoxus* PYCC 4570^T DNA with the *S. cerevisiae* probe, Sc176 (Fig. 4, strips 5 and 6, respectively). This is most likely the result of the fact that the *S. bayanus* and *S. paradoxus* sequences have only one internal mismatch with the Sc176 probe sequence. This weak cross-reactivity contrasts with the strong, distinct signal observed for *S. cerevisiae* PYCC 4455^T DNA with the Sc176 probe (Fig. 4, strip 4). Similar weak cross-reactivity between *C. maltosa* PYCC 3860^T DNA and the Ct171 and Cp171 probes was observed; *C. maltosa* DNA has only one and two internal mismatches with the Ct171 and Cp171 probe sequences, respectively. The signal produced can be well differentiated from those obtained with the same probes and DNAs from *C. tropicalis* (Fig. 4, strips 15 to 17) and *C. parapsilosis* (Fig. 4, strips 18 and 19), respectively. When DNA mixtures from two yeast species were used for the LAMP reaction (Fig. 4, strips 32 to 35), hybridization signals were obtained only with the corresponding species-specific probes. The reverse hybridization system also allowed the detection of LAMP amplicons that could hardly be visualized after gel electrophoresis, as in the case of *C. krusei* PYCC 3341^T (Fig. 2, lane 23, versus Fig. 4, strip 23), which demonstrates the high sensitivity of the method developed.

Our LAMP amplicons obtained with the panfungal probe produced clearly more-intense specific hybridization signals than standard PCR for 26S rDNA-based amplification products under similar experimental conditions (data not shown). The production of higher-molecular-weight DIG-labeled amplicons containing several inverted repeats of the target DNA certainly contributes to that better performance. The oligonucleotide probe panel can easily be extended to accommodate additional species and/or variants.

DISCUSSION

This report describes the development of a DNA-based identification system for clinically relevant yeasts that provides accurate identification of an isolate in less than 6 h. Our concept comprises the amplification of a 26S rDNA fragment relatively conserved in a wide range of yeast species, followed by reverse hybridization to a panel of species-specific oligonucleotide probes for identification at the species level. The present work provides the proof of a principle that ultimately may be applied to the development of an improved qualitative

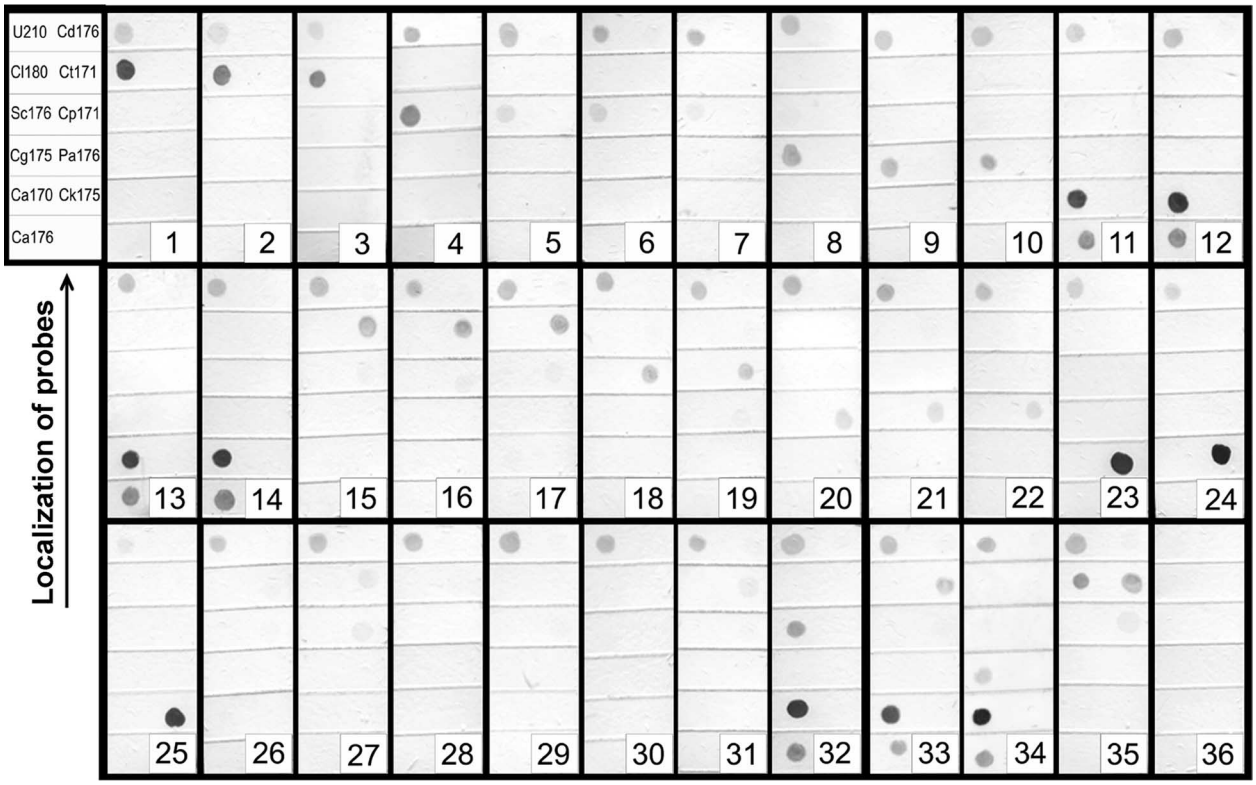

FIG. 4. Hybridization of DIG-labeled LAMP amplification products to species-specific probes. (Upper left panel) Spatial distribution of the DNA probes immobilized onto each nylon membrane strip (U210, universal panfungal probe; Cl180, *C. lusitaniae* probe; Sc176, *S. cerevisiae* probe; Cg175, *C. glabrata* probe; Ca170 and Ca176, *C. albicans* probes; Cd176, *C. dubliniensis* probe; Ct171, *C. tropicalis* probe; Cp171, *C. parapsilosis* probe; Pa176, *P. anomala* probe; Ck175, *C. krusei* probe). The species in strips 1 to 31 correspond to those in Fig. 2, lanes 1 to 31; strips 32 to 35, DNA mixtures from *C. albicans* PYCC 3436^T plus *S. cerevisiae* PYCC 4455^T, *C. albicans* PYCC 3436^T plus *C. tropicalis* PYCC 3097^T, *C. albicans* PYCC 3436^T plus *C. glabrata* PYCC 2418^T, and *C. lusitaniae* PYCC 2705 plus *C. tropicalis* PYCC 3097^T, respectively; strip 36, negative control (DNA replaced with water).

yeast identification molecular diagnostic kit. Validation with clinical samples will be required before application to a clinical setting. Routine methods in clinical diagnostics must be reliable, sensitive, simple to execute, and cost-effective. PCR-based diagnostics combine some of these characteristics but involve the use of expensive equipment and/or reagents. Simple diagnostic kits are in high demand, because they can be used wherever a shortage of resources exists. In order to overcome the limitations, we adapted and optimized a recently described isothermal DNA amplification technology, known as LAMP, for use with yeasts. Compared to a standard PCR protocol, LAMP requires a single bath at a constant temperature instead of a thermal cycler.

The performances of LAMP- and PCR-based diagnostic systems, including real-time technologies (23, 40), have been extensively compared. In general, LAMP was found to be either similar or superior to PCR, and more specific (e.g., 8, 13, 15), but a few studies proved otherwise, such as those reported by Kato et al. (18), who showed that although LAMP was 10-fold more sensitive than standard PCR for the detection of the *Clostridium difficile* toxin B gene (*tcdB*), an optimized nested-PCR assay performed much better than LAMP. In our experience, an optimization step can be critical for improving the sensitivity of both LAMP- and PCR-based methods. For instance, we obtained better results in the LAMP reaction with a 90-min incubation at 64°C than with incubation for a standard period of 60 min or less. Some authors would corroborate this result (7, 8, 36), while others would disagree (29). Another possibility for increasing LAMP sensitivity and accelerating the response time would be the additional utilization of loop primers in the reaction mixture (31). These hybridize to the stem-loops in amplified template DNA and initiate new strand displacement DNA synthesis.

Occasionally, we observed false-positive LAMP reactions in negative controls. Kuboki et al. (19) also mentioned the occurrence of false positives in work with *Trypanosoma* spp., probably due to cross-contamination. To avoid this, they recommended a few precautions and careful manipulation in preparing the samples and reaction mixtures. We stress the need to guarantee a clean environment by sterilizing all the labware utilized in the LAMP reaction and using a UV-sterilized laminar flow chamber. The utilization of a lower $MgCl_2$ concentration in the reaction mixtures (see Materials and Methods) also helped to eliminate the occurrence of false-positive results in negative controls. A rough estimate of costs involved in a single identification by the LAMP-based system reported here provides a value of around 3.8 euros, approximately half the amount spent in clinical mycology laboratories for current identification systems (e.g., API 20 C AUX and API *Candida* galleries).

Overall, our results indicate that robust and simple "PCR-free" isothermal DNA amplification methodologies could greatly contribute to the development of rapid and reliable molecular diagnostic kits to be used in clinical laboratories worldwide for the identification of pathogens in general and infectious yeasts in particular.

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