Three Isothermal Amplification Techniques for Rapid Identification of Cladophialophora carrionii, an Agent of Human Chromoblastomycosis

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In this study, we developed rapid and sensitive assays for the detection of Cladophialophora carrionii, a common agent of human chromoblastomycosis. The isothermal techniques evaluated were rolling-circle amplification (RCA), multiplex ligation-dependent probe amplification (MLPA), and loop-mediated isothermal amplification (LAMP). The probes for RCA and MLPA were designed with target sequences in the rDNA internal transcribed spacer gene (ITS) and elongation factor 1α (EF1α) region, and LAMP primers were designed using the elongation factor 1α gene (EF1α); these probes and primers specifically amplified DNA of isolates of the species. The three techniques were sufficiently specific and sensitive for discriminating target DNA of C. carrionii from related Cladophialophora species and other agents of chromoblastomycosis. RCA, MLPA, and LAMP are advantageous in their reliability and ease of operation compared to standard PCR and conventional methods.

Chromoblastomycosis is an endemic, mutilating skin disease caused by melanized fungi. Several species have been reported as etiologic agents, but in the great majority of proven cases, Fonsecaea pedrosoi, Fonsecaea monophora, Fonsecaea nubica, or Cladophialophora carrionii were concerned. C. carrionii is prevalent in arid and semiarid climates (e.g., South and Central America and Australia [1, 2]). Lesions associated with the disease are polymorphic or hyperkeratotic and, in view of therapy, must be differentiated from those associated with other infectious or autoimmune disorders. Conventional diagnosis of chromoblastomycosis is made by identification of the fungus in clinical specimens by microscopy, culture, and stained histopathological sections. These procedures may be time-consuming (3); hence, there is need for experimental diagnostic methods. Serological diagnosis (4) is tedious because it requires paired acute- and convalescent-phase sera, and the results may be influenced by contamination or cross-reaction.

Innovations in molecular diagnostics are rapidly reaching routine laboratories. Most molecular methods are PCR based, but during the past 2 decades, several isothermal amplification techniques, which amplify DNA by strand replacement without using a thermocycler, have become available. These non-PCR-based techniques have the advantages of simple application in the clinical lab and suitability for processing large numbers of specimens. In earlier studies on pathogenic fungi, Sun et al. (5, 6) developed loop-mediated isothermal amplification (LAMP) assays for the rapid diagnosis of Talaromyces marneffei and for Fonsecaea species. LAMP proved to be a fast and sensitive method for the routine diagnosis of T. marneffei in the clinical lab, whereas the specificity of the method was insufficient to distinguish between the different Fonsecaea species causing chromoblastomycosis. Najafzadeh et al. (7) introduced rolling-circle amplification (RCA) for use with Fonsecaea species, and this assay successfully distinguished the three species involved. Thus far, the multiplex ligation-dependent probe amplification (MLPA) method has rarely been applied in medical mycology. Zhang et al. (8) successfully adapted MLPA to identify and characterize T. marneffei infections in cultured cells and paraffin-embedded tissue sections. The sensitivity and specificity of MLPA proved to be high, and the assay was therefore found to be a useful tool for the prompt and accurate diagnosis of chromoblastomycosis and for use in epidemiological studies.

Here, we introduce three isothermal amplification methods (rolling-circle amplification [RCA], multiplex ligation-dependent probe amplification [MLPA], and loop-mediated isothermal amplification [LAMP]) for the detection of Cladophialophora carrionii. These techniques differ in their requirements for sample volumes, specimen preparation, and methods of amplification and detection. There are strengths and weaknesses of each amplification system. In this study, we aimed to develop a highly specific and technically attractive method to discriminate C. carrionii from closely related species without the need for sequencing.

MATERIALS AND METHODS

Strains and culture conditions. Strains and sequences (internal transcribed spacer gene [ITS] and elongation factor 1α gene [EF1α]) used in

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TABLE 1 Primers and probes used in this study

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCA1</td>
<td>ATGGGCAAGCAAGCAAGCA</td>
</tr>
<tr>
<td>RCA2</td>
<td>CGGCAAGCAAGCAAGCA</td>
</tr>
<tr>
<td>COC</td>
<td>p-TGGCAGGAGGCGCCAGAGCATGTCCTGAGTTCGACAT</td>
</tr>
<tr>
<td></td>
<td>tactivagtagagtagtagtcGCGGAGACAGCAGATGtagctAGAAGTGGTGGCTG</td>
</tr>
<tr>
<td>MLPA probes and primers</td>
<td></td>
</tr>
<tr>
<td>MLPA-AW</td>
<td>TCG GGG GAC AGC GGC CCA GAT CTC GGG CAA TTC GAT GGC CCT ATA GTC AGG TCT TCT CTA TTT CCA CCG TAT GCA CAT CTC GGA ACT AAG CTG</td>
</tr>
<tr>
<td>MLPA-FW</td>
<td>ACT GGA TTC AGG TTC AGG AAC ATG CAT TAT GCA TGA CTA CCA GCA GTG TAG TAC AGC GCC GGT GAA ATT ATC GCC ACA GGC CCT TGA GGT GTG CTG</td>
</tr>
<tr>
<td>Primer 1</td>
<td>CAG CTT AGT TCC GAG ATG T</td>
</tr>
<tr>
<td>Primer 2</td>
<td>ACT GGA TTC AGG TTC AGG A</td>
</tr>
<tr>
<td>LAMP primer set</td>
<td></td>
</tr>
<tr>
<td>FIP (F1c to F2)</td>
<td>GAGCCCTTGGCGAGTTCAGCTTTTCCCGAGCTGATCAACT</td>
</tr>
<tr>
<td>F3</td>
<td>CCGTACAGTAGTTACACAT</td>
</tr>
<tr>
<td>BIP (B1c to B2)</td>
<td>ATGCCCTGAGTTCGAGCAGCTGCTCGAAGTCCAGAGCG</td>
</tr>
<tr>
<td>B3</td>
<td>CATCGATAGGGTGACAGAAG</td>
</tr>
</tbody>
</table>

* COC, Cladophialophora carrionii molecular inversion probe-specific primers; FIP, forward inner primer; F3, forward outer primer; BIP, back inner primer; B3, back primer.

At the 5’ end of the probe, p indicates 5’ phosphorylation. The underlined, lowercase sequences are the binding arms of the molecular inversion probes, which are backbone of the probe that includes the nonspecific linker region.

this study are listed in Table S1 in the supplemental material. The set included 81 Cladophialophora carrionii isolates in total. Reference strains of species which are taxonomically close to C. carrionii within the order Chaetothyriales and five species outside the Chaetothyriales order were applied to evaluate the specificity of the three methods. The strains were obtained from the reference collection of the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS) and grown on malt extract agar (MEA) at 25°C for 1 week.

Isolation of genomic DNA. DNA was extracted and ITS amplicons were generated as described previously (9). PCR amplification of elongation factor 1 (EF1) gene regions was performed with the primers EF1-728F (5’-CATCGAGAAGTITCGGAGAAG-3’) and EF1-1567R (5’-ACH GTRCCRATACCACCRATCTT-3’) (see http://www.atloff.org/pdfs/EF1 primer.pdf). The EF1 region amplification was conducted with one cycle of denaturation for 5 min at 96°C, followed by 40 cycles at 95°C for 60 s, 45°C for 60 s, and 72°C for 60 s, with a final extension at 72°C for 7 min. The ITS amplicons were used as targets for the RCA and MLPA reactions. Genomic DNA was used directly for the LAMP reaction.

RCA. We used ITS amplicons for the RCA reactions. Sequences of the two primers used for RCA and the molecular inversion probes (MIP) (original term, oligonucleotide padlock probes) based on the ITS region are listed in Table 1. One microlter of ITS amplicon (approximately 300 ng) was mixed with 2 U PFU DNA ligase (Epicentre Biotechnologies, Madison, WI, USA) and 0.1 μmol molecular inversion probes in 20 mmol Tris-HCl (pH 7.5), 20 mmol KC1, 10 mmol MgCl2, 0.1% Igepal, 0.01 mmol rATP, and 1 mmol dithiothreitol (DTT) for a total reaction volume of 10 μl. Molecular inversion probe ligation was conducted with one cycle of denaturation for 5 min at 95°C, followed by five cycles at 95°C for 30 s and 4 min of ligation at 61.5°C. Two microliters of the ligation product was used as the template for RCA. The total volume of the RCA reaction was 50 μl and contained 8 U Bst DNA polymerase (New England Biolabs, Hitchin, United Kingdom), 400 mmol·1−1 deoxynucleoside triphosphate (dNTP) mix, and 10 pmol of each RCA primer in distilled water. The reaction was performed at 65°C for 60 min, and the accumulation of double-stranded DNA products was visualized on a 1% agarose gel. Positive reactions showed a ladder-like pattern, whereas no signal was detected in the negative reactions.

MLPA. For this technique, a standard protocol (10) was used with minor modifications. Approximately 500 pg DNA was used as the template. Probes and primer sequences based on the ITS region for C. carrionii are listed in Table 1. Total reaction volumes of 10 μl contained 1 μl of ITS amplicon, 1 U PFU DNA ligase (Epicentre Biotechnologies), 0.1 mol MLPA probe mix (MLPA-AW and MLPA-FW), and 1 μl 10× amplification DNA ligase BsaI buffer. Probe ligation was conducted with one cycle of denaturation for 5 min at 95°C, followed by five cycles at 95°C for 30 s and 15 min of ligation at 63.5°C. Five microliters of ligation product was used as the template for MLPA. The total volume was 25 μl and contained 0.5 U Taq polymerase, 1 μl primer 1 and primer 2 (10 pmol), and 1 μl dNTP (5 mM). The program for MLPA included one cycle of denaturation for 5 min at 95°C, followed by 25 cycles at 95°C for 35 s, 55°C for 30 s, and 72°C for 1 min. The amplified products were analyzed by electrophoresis on 2% agarose gels; a BenchTop 100-bp DNA ladder (0.1 to 15 kb; Promega) was used as the DNA size standard. One band with a size of 195 bp after MLPA was detected in positive reactions but not in the negative reactions.

LAMP. Briefly, the LAMP method used in the present study detects C. carrionii with a combination of the F3, B3, FIP, and BIP primers designed from the partial sequence of the EF1 region of C. carrionii; the primer set based on the ITS region did not distinguish C. carrionii specifically from closely related species within the genus Cladophialophora (data not shown). The species-specific LAMP primer set was designed based on the EF1 region (GenBank accession no. KJ609515) of C. carrionii using PrimerExplorer V4 software (http://primerexplorer.jp). Primer sequences for C. carrionii are listed in Table 1. DNA (approximately 500 pg) was used as the template. All LAMP reagents were bought from New England BioLabs. LAMP was carried out in a 25-μl reaction mixture that contained 4 μl each FIP and BIP (10 pmol), 0.5 μl MgSO4 (80 mM), 2 μl dNTP (5 mM), 4 μl of 5 M betaine (Sigma, Zwijndrecht, the Netherlands), 2.5 μl 10× Thermo buffer, 1 μl Bst DNA polymerase (New England BioLabs), 5 μl double-distilled water (ddH2O), and 500 pg template DNA (1 μl), followed by incubation of the mixture at 65°C for 70 min. Additionally, to evaluate the detection limit of the LAMP assay, 1 μl of each 10-fold serial dilution was used in each LAMP reaction. Genomic DNA of C. carrionii CBS 114393 (concentration range, 3.2×102 to 3.2×105 copies per DNA) was used. Positive reactions showed a ladder-like pattern, whereas negative reactions showed a clean background. The amplified products were analyzed by electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed. A DNA SmartLadder (0.2 to 10 kb; Eurogentec) was used as the DNA size standard.
RESULTS

RCA. Tests using the species-specific molecular inversion probes (Table 1) yielded positive results in all 81 C. carrionii strains (data not shown), while results remained negative with the 20 isolates used for reference, including those belonging to closely related Cladophialophora species. No cross-reactions were observed between the C. carrionii and reference strains (Fig. 1). Thus, the specificity was 100%.

MLPA. Probe pairs targeting the ITS1 domain of C. carrionii (Table 1) yielded amplicons of 195 bp. Amplification products were detected by electrophoresis on agarose gel. Positive responses showed the expected band (195 bp) after MLPA in all of the C. carrionii strains tested (data not shown), whereas negative results were obtained with the 20 isolates used for reference, including strains of closely related Cladophialophora species; the backgrounds remained clean. No cross-reactions were observed between C. carrionii and any of the reference species (Fig. 2). Therefore, the specificity was 100%.

LAMP. One set of primers was designed to amplify their target genes for C. carrionii (Table 1). Amplification products were detected by electrophoresis on agarose gels. Positive reaction products appeared as a ladder of multiple bands. LAMP with the primer set successfully amplified all 81 C. carrionii strains (data not shown). Positive reactions included all haplotypes known in

FIG 1 Gel representation of the specificity of C. carrionii-specific RCA probe compared to that for related species. Amplification of probe signals was seen only with matched template-probe mixtures. Empty lanes denote absence of signals with unmatched template-probe mixtures; lanes C, C. carrionii; lane M, DNA SmartLadder (0.2 to 10 kb); lanes 1 to 5, five Cladophialophora species (CBS 147.84, C. devriesii; CBS 556.83, C. minouae; CBS 114405, C. yegrezi; CBS 126.86, C. boppii; and CBS 173.52, C. bantiana); lanes 6 to 15, other relatives within the Chaetothyriales order (CBS 207.35, Exophiala dermatitidis; CBS 507.90, Exophiala jeanesienei; CBS 899.68, Exophiala spinifera; CBS 269.37, Fonsecaea monophora; CBS 271.37, Fonsecaea pedrosoi; CBS 269.64, Fonsecaea rubra; CBS 273.37, Phialophora verrucosa; CBS 117642, Exophiala xenobiota; CBS 101.67, Capronia mansonii; and CBS 650.93, Rhinocladiella mackenziei); lanes 16 to 20, remotely related species of Aspergillus, Candida, and dermatophytes (CBS 116044, Aspergillus fumigates; DTO 112 C4, Aspergillus niger; CBS 8758, Candida albicans; CBS 118534, Trichophyton violaceum; and CBS 118547, Microsporum ferrugineum); CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; DTO, identification collection of the CBS-KNAW Fungal Biodiversity Center, Utrecht, the Netherlands.

FIG 2 Gel representation of the specificity of C. carrionii-specific MLPA probe compared to that for related species. A single band with a size of 195 bp is present with matched template-probe mixtures. Lanes C, Cladophialophora carrionii; lane M, BenchTop 100-bp DNA ladder (0.1 to 15 kb); lanes 1 to 20, see Fig. 1 legend.
the species (S. Deng and G. S. de Hoog, 2014, unpublished data). An evaluation of specificity of the LAMP assay was carried out by comparison with five Cladophialophora species and other relatives within the Chaetothyriales order. Positive reactions showed a ladder-like pattern, whereas negative reactions showed a clean background. The LAMP assay was also tested on remotely related species of Aspergillus and Candida and on dermatophytes, which all gave a negative response (Fig. 3). The specificity was 100%. Sensitivity testing showed that LAMP yields positive results in wide ranges of genomic DNA concentration, down to $3.2 \times 10^2$ copies of DNA (Fig. 4).

**DISCUSSION**

**RCA.** Rolling-circle amplification using molecular inversion probes (10) is a robust and technically simple isothermal in vitro DNA amplification technique that is emerging as a new tool for the rapid detection of pathogenic fungi. The method is based on the replication of a short single-stranded DNA circle by Bst DNA polymerases at constant temperature. In the present study, we developed and evaluated a molecular inversion probe (MIP) assay for the identification of Cladophialophora carrionii using pure cultures. The method showed 100% specificity for this agent of human chromoblastomycosis. Our data suggest that the method is highly suitable for this aim.

**MLPA.** MLPA is multiplex PCR that uses several primer pairs to simultaneously generate a product band for each primer pair. The method, first described by Schouten et al. (11), is now widely used to identify point mutations, indels, and duplications. In this study, we adapted the MLPA assay to identify C. carrionii infections. As with RCA, MLPA showed 100% specificity.

**ITS** sequencing is the gold standard for identifying C. carrionii, but sequencing is expensive, time-consuming, and impractical for the analysis of large numbers of isolates. Furthermore, profes-

**FIG 3** Gel representation of the specificity of C. carrionii-specific LAMP primer set compared to that for related species. Amplification of the primer set signals was seen only in lanes C (C. carrionii); lane M, DNA SmartLadder (0.2 to 10 kb); lanes 1 to 20, see Fig. 1 legend.

**FIG 4** Gel representation of the sensitivity in detecting genomic DNA of the EF1 region of C. carrionii with LAMP primer set: analytical sensitivity of LAMP in detecting the artificial template of the EF1 region. DNA of C. carrionii CBS 114393 was used as a sample. Lane M, DNA SmartLadder (0.2 to 10 kb); lanes 1 to 12, $3.2 \times 10^3, 3.2 \times 10^4, 3.2 \times 10^5, 3.2 \times 10^6, 3.2 \times 10^7, 3.2 \times 10^8, 3.2 \times 10^{-2}, 3.2 \times 10^{-3}, 3.2 \times 10^{-4}$, and $3.2 \times 10^{-5}$ copies of DNA, respectively; H2O, Milli-Q water. Sensitivity testing showed that LAMP yields positive signals in wide ranges of genomic DNA concentration, down to $3.2 \times 10^2$ copies of DNA.
sional databases for comparison are needed. The RCA and MLPA reactions are relatively free from requirements for laboratory equipment and would be suitable for routine application because they are easy to handle; amplicons are identified by isothermal incubation at 62°C to 65°C in a water bath within 2 to 4 h. However, the detection limit enables quantification of DNA copies (300 ng is equal to 10^8 DNA copies). Thus, sensitivity requires further optimization to capture the low copy numbers of templates in clinical samples. Thus, these two methods are advantageous in terms of ease of operation compared with standard PCR methods.

**LAMP.** Loop-mediated isothermal amplification of DNA (LAMP), a recently developed detection technique (12), may offer a cheaper alternative without compromising sensitivity and speed. LAMP relies on autocycling amplification of DNA using the strand displacement property of Bst DNA polymerase. LAMP requires two specially designed inner primers and two outer primers which amplify the target DNA with high efficiency. Since the LAMP reaction is done under isothermal conditions (60°C to 65°C), simple incubators are sufficient for DNA amplification. LAMP is based on direct amplification of fungal DNA, whereas for RCA and MLPA, amplicons are needed. Although a 362-bp region which is included in the ITS gene can be used to specifically identify *C. carrionii* using routine PCR amplification (13), the LAMP assay based on the ITS region did not distinguish *C. carrionii* specifically from the related species in our study. We changed the targeted region for the LAMP assay for a primer set in the *EF1* gene. This region appeared to provide a good target for the detection of *C. carrionii* with LAMP. The selection of an appropriate target region is crucial for the specificity of LAMP technology. With the new primer set, we found a specificity of 100% and high sensitivity. In principle, LAMP is a simple isothermal DNA amplification technique that allows for the rapid detection of specific nucleic acid sequences from genomic DNA directly, without requiring sequencing. Once set up, the tests can be performed within 2 h, and they are particularly useful for rapidly screening for agents of human chromoblastomycosis in areas of hyperendemicity.

In conclusion, the degrees of *ITS* nucleotide polymorphisms that were sufficient to allow for successful RCA and MLPA diagnostics were inadequate for LAMP for *C. carrionii*. The more variable *EF1* gene proved to be appropriate for LAMP. Thus, these three isothermal amplification techniques, RCA, MLPA, and LAMP, can be recommended as suitable lab tools for the rapid identification of *C. carrionii*, an agent of human chromoblastomycosis, and LAMP may be used for the quantification of gene copy numbers.

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