Biodiversity, pathogenicity, antifungal susceptibility and rapid identification of Fonsecaea and relatives
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Citation for published version (APA):

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Rapid detection and identification of fungal pathogens by rolling circle amplification (RCA) using Fonsecaea as a model

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In press in: Mycoses
Abstract

We aimed to describe a rapid and sensitive assay for identification of pathogenic fungi without sequencing. The method of rolling circle amplification (RCA) is presented with species of *Fonsecaea*, agents of human chromoblastomycosis, as a model. The internal transcribed spacer (ITS) rDNA region of 103 *Fonsecaea* strains was sequenced and aligned in view of designing three specific padlock probes to be used for the detection of single nucleotide polymorphisms in three *Fonsecaea* species. The 38 strains included for testing the specificity of RCA comprised 17 isolates of *Fonsecaea pedrosoi*, 13 of *Fonsecaea monophora* and eight of *Fonsecaea nubica*. The assay successfully amplified DNA of the target fungi at the level of species, while no cross reactivity was observed. The amplification product was visualised on a 1% agarose gel to verify the specificity of probe–template binding. Amounts of reagents were minimised to avoid the generation of false-positive results. The simplicity, sensitivity, robustness and low costs provide RCA a distinct position among isothermal techniques for DNA diagnostics as a very practical identification method.

Key words: *Fonsecaea*, chromoblastomycosis, RCA, rapid diagnosis

Introduction

Fungal infections that cause life-threatening infections in critically ill and immunocompromised patients have increased significantly over the last decades. In addition, the burden of disease of mutilating infections in healthy individuals, such as chromoblastomycosis, is increasingly recognised. Rapid and specific identification of fungal pathogens in early stages of the infection is important for timely and appropriate treatment with antifungal agents. Conventional methods for fungal identification in the clinical laboratory rely on morphological and physiological tests and need several days or weeks and are frequently unspecific. Molecular identification mostly implies sequencing, which is relatively expensive and time-consuming.

Rolling circle amplification (RCA) was introduced as a rapid and specific tool for molecular diagnostics of microbial agents including fungi. The RCA method is discussed in this article using the genus *Fonsecaea*. This genus of black yeast-like fungi comprises potential agents of chromoblastomycosis, a chronic, cutaneous and subcutaneous infection with high morbidity and characterised by the presence of muriform fungal cells in tissue. Chromoblastomycosis is found worldwide, but most reports of the disease are from tropical and subtropical climates. The genus comprises two sibling species, *Fonsecaea pedrosoi* and *Fonsecaea monophora*, while a third one, *Fonsecaea nubica*, was described recently. The latter taxon was reported from Brazil and southern China. Patients with chromoblastomycosis are supposed to have acquired their infection after traumatic inoculation of contaminated material such as spines of cactus or of *mimosa* plants. However, fungi isolated directly from the environment often concern less virulent siblings of the species rather than the pathogens themselves. With environmental search for the culprit pathogen yielding many avirulent
counterparts, precise identification at the level of molecular species is therefore essential. Laboratory diagnosis of chromoblastomycosis relies on presence of the muriform cells in tissue and on microscopic morphology of the agent, which both have low specificity. Rolling circle amplification is a sensitive, specific and reproducible isothermal DNA amplification technique for rapid molecular identification of microorganisms. RCA-based diagnostics are characterised by good reproducibility, with less amplification errors as compared with PCR. The method uses a padlock probe, a circularisable oligonucleotide consisting of two segments complementary to the 3’ and 5’ ends of the target and a linker sequence. When the 3’ and 5’ terminal regions of the oligonucleotide probes are juxtaposed to the sequence of interest, the probe ends can be joined by a DNA ligase to form a circular DNA molecule that can be amplified by RCA. Recently the power of padlock probes to accurately identify target nucleic acid sequences, with high specificity down to the single nucleotide polymorphism level, has been demonstrated. RCA assays have been used for rapid identification of bacteria, viruses and fungi. In this study, we develop a specific, sensitive and rapid method for identification of clinically important Fonsecaea species by RCA.

Material and methods

Fungal strains
Thirty-eight Fonsecaea isolates were studied, including 17 F. pedrosoi, 13 F. monophora and eight F. nubica. Thirty isolates originated from patients with chromoblastomycosis, two from brain infection, one from an animal ear and five from the environment. Twenty-five strains came from the South and Central America, five from Southern China and eight isolates came from other countries. Strain data and GenBank accession numbers have been published elsewhere. To evaluate the specificity of three Fonsecaea-specific padlock probes, we tested five closely related species: Cladophialophora bantiana (CBS 678.79, Gen Bank EU103992), Cladophialophora minourae (CBS 556.83, GenBank AY251087), Cladophialophora modesta (CBS 985.96), Cladophialophora saturnica (CBS 109630, GenBank FJ385270) and a hitherto unidentified Cladophialophora species (dH 12336). The affinity of Fonsecaea to these species was established using a phylogenetic tree constructed with Small SubUnit (SSU) rDNA sequences. Strains were maintained on slants of 2% malt extract agar (MEA) and oatmeal agar (OA) at 24°C. Species identity was confirmed by sequencing rDNA internal transcribed spacer (ITS), partial β-tubulin (BT2), actin (ACT1), and cell division control protein (cdc42) genes, supplemented with Amplified Fragment Length Polymorphism profiles.

DNA extraction and ITS PCR amplification
DNA extraction and PCR amplification of ITS regions were performed as described previously. We used ITS amplicons for RCA reactions.

Tree building
A consensus tree of 38 members of Fonsecaea species based on ITS constructed with the Tree
Finder algorithm (v. June 2007, by Gangolf Jobb) with 100 bootstrap replicates and edited with MEGA4 software. Based on SSU data strain CBS 306.94 (*Cladophialophora arxii*) was off among the species closest to *Fonsecaea* species and was taken as outgroup.

### Padlock probe design

For the selection of padlock probes, sequences of ITS regions of 103 *Fonsecaea* strains (*F. monophora*, *F. pedrosoi* and *F. nubica*) from the CBS reference collection were aligned and adjusted manually using BioNumerics v. 4.61 (Applied Maths, Kortrijk, Belgium) to identify informative nucleotide polymorphisms. Three padlock probes targeting the ITS region were designed and were ordered from Invitrogen Inc (Breda, The Netherlands). In order to optimise binding efficiency to target DNAs, the padlock probes were designed with minimum secondary structure and with Tm of the 5´ end probe binding arm close to or above ligation temperature (63°C, see below). To increase its discriminative specificity, the 3´ end binding arm was designed with a Tm 10–15°C below ligation temperature. The linker regions of each *Fonsecaea* species-specific probe were taken from Zhou *et al.* and the 5´ and the 3´ binding arms were designed in this article (Table 1). Sequences of the two primers used for RCA and the oligonucleotide padlock probes are listed in Table 1. The oligonucleotide probes used were c. 92–99 nt in length and consisted of two adjacent target complementary sequences (14–20 nt) with a spacer region (63 nt) to facilitate loop formation and provide a template for RCA primer binding.

### Ligation of padlock probe

One microlitre of ITS amplicon was mixed with 2 U pfu DNA ligase (Epicentre Biotechnologies, Madison, WI, USA) and 0.1 lmol l⁻¹ padlock probe in 20 mmol l⁻¹ Tris-HCl (pH 7.5), 20 mmol l⁻¹ Cl, 10 mmol l⁻¹ MgCl₂, 0.1% Igepal, 0.01 mmol l⁻¹ rATP, 1 mmol l⁻¹ DTT with a total reaction volume of 10μl. Padlock probe ligation was conducted with one cycle of denaturation for 5 min at 94°C, followed by five cycles of 94°C for 30 s and 4 min ligation at 63°C.

### Exonucleolysis

Exonucleolysis is required to remove unligated padlock probe and template PCR product and thus reduce subsequent ligation-independent amplification events. It was performed in a 20 μl vol by addition of 10 U each of exonuclease I and III (New England Biolabs, Hitchin, UK) to the ligation mixture and incubation at 37°C for 30 min, followed by 94°C for 3 min to inactive the exonuclease reaction.

### Rolling circle amplification (RCA) reaction

Two microlitre of ligation product was used as template for RCA. The total volume was 50 μl containing 8 U Bst DNA polymerase (New England Biolabs), 400 μmol l⁻¹ deoxynucleoside triphosphate mix, 10 pmol of each RCA primer in distilled water. Probe signals were amplified by incubation at 65°C for 60 min, and accumulation of double stranded DNA products was visualised on a 1% agarose gel to verify the specificity of probe–template
Results

Fonsecaea species were easily distinguished from each other and from other members of the order Chaetothyriales (black yeast and relatives) by ITS sequence analysis (Fig. 1). Each of the three recently recognized clinically relevant species had several unique nucleotide positions. The duration of the RCA assay was 2 h. Positive responses proved to be highly specific for all strains; individual species-specific probes being correctly identified in all three Fonsecaea species analysed (F. pedrosoi, F. monophora and F. nubica). No cross reaction was observed between the three Fonsecaea species (Fig. 2a, b). The products of the RCA reaction were visualised by electrophoresis on 1% agarose gels. Positive responses showed ladder-like patterns after RCA, whereas with negative results the background remained clean. When the exonuclease step was omitted, a single weak band was visible on the gel representing a non-specific band that did not interfere with the RCA reaction (data not shown). The concordance of RCA results and identification with ITS sequencing was 100%. The five closely related relevant species used for comparison, i.e. C. bantiana (CBS 678.79), C. minourae (CBS 556.83), C. modesta (CBS 985.96), C. saturnica (CBS 109630) and a hitherto unidentified Cladophialophora species (dH 12336), yielded negative results with the Fonsecaea-specific padlock probes (data not shown).

Discussion

Rolling circle amplification is a robust and simple, isothermal in vitro DNA amplification technique emerging as a tool for rapid detection of specific nucleic-acid sequences in DNA samples. The use of a padlock probe to circularise oligonucleotides was discovered by Nilsson et al. The method is based on the replication of a short, single stranded DNA circle binding. Positive reactions showed a ladder-like pattern, whereas negative reactions showed a clean background.

Table 1. Rolling circle amplification padlock probes and padlock probe-specific primers used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>RCA1 [4]</td>
<td>5'-ATGGCCACCGAAGAAGCA-3'</td>
</tr>
<tr>
<td>RCA2 [4]</td>
<td>5'-CGCGCAGACACGATA-3'</td>
</tr>
<tr>
<td>FOP</td>
<td>5'Pa-497AAAGAGCTGACGTATCCGGG477</td>
</tr>
<tr>
<td></td>
<td>gatcaTGCTTCCTCCGGTGCCCACAAgctagctaccGCAGACACGATAgcta</td>
</tr>
<tr>
<td></td>
<td>S14CGGATACGTGTCACATA498 3'</td>
</tr>
<tr>
<td>FOM</td>
<td>5'Pa-111AGCCTTCTCCAGCG96</td>
</tr>
<tr>
<td></td>
<td>gatcaTGCTTCCTCCGGTGCCCACAAgctagctaccGCAGACACGATAgcta</td>
</tr>
<tr>
<td></td>
<td>S25CAAGCCGCCATTG112 3'</td>
</tr>
<tr>
<td>FON</td>
<td>5'Pa-397CAGGGGCTTAGGGG479GTAT376</td>
</tr>
<tr>
<td></td>
<td>gatcaTGCTTCCTCCGGTGCCCACAAgctagctaccGCAGACACGATAgcta</td>
</tr>
<tr>
<td></td>
<td>S422CGTCAAACCAAGCGG398 3'</td>
</tr>
</tbody>
</table>

FOM, Fonsecaea monophora, FOP, Fonsecaea pedrosoi; FON, Fonsecaea nubica; RCA, rolling circle amplification. At the 5’ end of probe, “P” indicates 5’-phosphorilation. The underlined sequences are the binding arms of the padlock probes, which are joined by the backbone of the probe including the non-specific linker region, which is the same for all three padlock probes.
Fig. 1. Consensus tree of *Fonsecaea* based on Internal Transcribed Spacer (ITS) ribosomal DNA of 38 strains, constructed with the Tree Finder algorithm (v. June 2007) with 100 bootstrap and edited with Mega 4 software. CBS 306.94 was taken as outgroup (bootstrap values >70 are shown at branches in bold).
by BstDNA polymerases at constant temperature. The technique was first applied by Fire et al. \cite{29} and Liu et al. \cite{29,30} in the mid 1990s. The sensitivity of RCA enables accurate and reliable detection and quantification of gene copy numbers, discrete antigen–antibody complexes, and mRNA expression levels in individual cells.\cite{5} Its technical simplicity makes the method also applicable for rapid and specific routine diagnostics of pathogenic fungal agents. In this study, the RCA assay was developed for the identification of the three currently recognised pathogenic Fonsecaea species. ITS sequencing is the gold standard for species identification of black yeast and relatives\cite{31} but sequencing is expensive, time-consuming and impractical for analysis of large numbers of isolates.\cite{8} In addition, validated databases for comparison are required, as GenBank data are polluted within correctly identified sequences. The RCA reaction is relatively free from requirement for expensive laboratory equipment and can be performed within 2 h isothermally at 65ºC in a water bath, thermocycler, heating block or microwave.\cite{32} However, a positive signal was usually evident 15 min after commencement of the RCA reaction when detected by real time PCR.\cite{8,9} In this study, we used the fungal genus Fonsecaea as a model organism, as it contains three species which potentially cause the same disease, chromoblastomycosis, and are morphologically indistinguishable from each other. Rapid identification of the agents is significant because virulence differs between species, F. monophora also being an agent of primary encephalitis.\cite{33} RCA amplicons were detected here with gel electrophoresis, but other researchers used fluorescence, \cite{34} radiolabelling\cite{35} or UV absorbance.\cite{36} Recently also loop-mediated isothermal amplification (LAMP) has been proposed for rapid diagnosis of Fonsecaea.\cite{27} LAMP proved to be a fast and sensitive method based on direct amplification of fungal DNA, whereas for RCA, ITS amplicons are needed. However, with the LAMP assay, we could not distinguish between the different species of the genus Fonsecaea, because they differ in relatively few nucleotide polymorphisms that were sufficient to allow successful RCA diagnostics. Thus, RCA is more specific than LAMP, but LAMP is more sensitive than RCA.\cite{27} Thus far, the RCA method has only rarely been

![Fig. 2. Gel representation of specificity of RCA probes. Amplification of probe signals was seen only with matched template-probe mixtures (empty lanes denote absence of signals with unmatched template-probe mixtures). The species-specific probes are labelled as shown at the top of the figure (FOM, Fonsecaea monophora; FOP, Fonsecaea pedrosoi and FON, Fonsecaea nubica). (A) Lane “M” is DNA smart ladder; lane 1, 4, and 7, F. monophora (CBS 269.37); lane 2, 5 and 8, F. pedrosoi (CBS 271.37); lane 3, 6 and 9, F. nubica (CBS 269.64). (B) Lane “M” is DNA smart ladder; lane 1- 4 are F. monophora (CBS 117238, CBS 102225, CBS 121727 and CBS 121724 respectively); lane 5- 8 are F. pedrosoi (CBS 253.49, CBS 122741, CBS 122849 and CBS 274.66 respectively); lane 9- 12 are F. nubica (CBS 444.62, CBS 272.29, CBS 270.37 and CBS 121734 respectively).]
applied in medical mycology. Papers were limited to Candida, Aspergillus and Scedosporium, Cryptococcus and Trichophyton. In this study, we have demonstrated that RCA is a very efficient method for specific and sensitive identification of fungal pathogens and opportunistic agents, with species-specific detection of Fonsecaea taxa and excluding the closely related species of the genus Cladophialophora. Judging from these results and given the simplicity of the method, it is our impression that RCA deserves a place in routine testing in laboratories for fungal diagnostics where large numbers of samples are to be screened. The establishment of the test is relatively expensive, but with high throughput applications, the performance of testing will be rapid and inexpensive. For identification of very low volume of cases, the method is less suitable.

**Acknowledgements**

This study was financially supported by the Ministry of Health and Medical Education of Iran, Mashhad University of medical sciences, Mashhad, Iran and Brazilian Government fellowship from Coordenação de Pessoal de Nível Superior (CAPES).

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