Chapter 11
Molecular Techniques (PCR)

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Key Features

- The polymerase chain reaction (PCR) is a highly sensitive and specific molecular technique for onychomycosis diagnosis.
- The commercial kits allow an ideal DNA extraction from clinical samples of onychomycosis and fungal cultures.
- The genomic regions mostly used for onychomycosis diagnosis are the ITS1 and ITS2 region. Other useful genes are the chitin synthase 1 and β-tubulin.
- The panfungal primers are the principal ones for fungal species identification.
- The real-time PCR helps identify fungal presence and its species in clinical samples in a fast and specific way.
- The sequencing of the PCR-amplified DNA is highly sensitive and specific for fungal species identification.

Introduction

The conventional method for the identification of the etiological agents of onychomycosis is the fungal culture, considered the gold standard; however, it has several disadvantages such as a high rate of false-negative results and when it does grow,

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the utilized time may take up to 4 weeks to do so. These disadvantages are being tried to decrease with the development of molecular techniques that allow species identification in a time span of 24–48 h, which is a considerable reduction when compared to 4 weeks. These techniques also permit the detection of difficult-to-grow fungi and generate reproducible results with high specificity and sensibility.

Although molecular techniques are still not used as routine tests for these purposes, its applications in investigative protocols have been constantly increasing during the last years and, thus, have allowed a cost reduction in its supplies making it more accessible. The downside of molecular biology is that it requires qualified personnel and the specific infrastructure to develop the procedures.

State of the Art

DNA Extraction

DNA extraction is a crucial step for any molecular technique either for basic or clinical research with any microorganism or cell type. The extraction methodology must allow the DNA obtained to be of the highest quality, purity, and integrity. The different methods vary according to the origin of the DNA sample, from a clinical sample or from a pure culture. When working with fungi, specialized techniques are required because of the characteristic components of the fungal cell wall; they can be mechanic, physic, or enzymatic [1–3]. The majority of DNA isolation methods involve three steps: cellular lysis, inactivation of nucleases, and purification.

For DNA extraction by enzymatic digestion (cellular lysis), Proteinase K, lyticase, zymolyase, or other cell wall-degrading enzymes are used [4, 5]. The mechanic procedure includes freezing with liquid nitrogen and crushing of the sample with mortar and pestle, sand, or glass beads. The physical methods may break the cellular wall with microwaves or sonication [6–8]. To obtain a sufficient amount of DNA, these methods require a large quantity of clinical sample and DNA-purifying substances such as phenol-chloroform and RNAse treatment. Something that we must keep in mind is that the physical and mechanical methods may cause DNA rupture or degradation [1–3, 9].

For DNA extraction, the noncommercial methods are very efficient when using a large amount of fungal culture; however, they are not recommended for working with large amounts of clinical samples or paraffin-embedded tissues. The use of commercial methods is very helpful for molecular diagnosis in all sample types and avoids cross contamination, they can handle large number of samples, and the methodological standardization and reagents are simple and faster to use and sometimes even more cost-effective [6, 10–12]. The process includes cellular lysis, RNA removal, protein and polysaccharide elimination, and the union by centrifugation of

DNA to the column. The centrifugation is usually done under refrigeration and requires several wash cycles with tube change in every extraction. The purity, integrity, and amount of DNA obtained are higher than the obtained via noncommercial methods [13–15].

For the reasons mentioned above, the use of commercial kits has increased when fungal DNA extraction is required. Some of the most popular are Fast DNA Kit (Qbiogene, Irvine, CA, USA), ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA), DNeasy Plant Mini Kit or DNeasy Blood and Tissue DNA Extraction Kits (Qiagen, Valencia, CA, USA), UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), MasterPure Yeast DNA Purification Kit (Epicenter, Madison, WI), and High Pure PCR Template Kit (Roche, Basel, Switzerland) among others. All the commercial extraction systems share the use of columns.

Molecular Techniques

The development and application of molecular techniques have increased during the last decades with the purpose to improve the sensitivity and/or specificity of diagnosis, as well as to decrease the periods of time normally required for detection and identification of etiological agents.

In clinical samples from patients with onychomycosis, several strategies are used based on the DNA detection for dermatophytes and non-dermatophytes. The classic technique is the polymerase chain reaction (PCR).

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a molecular technique widely used to make multiple copies of a specific gene or gene fragments.

This molecular tool is very precise and can be used to amplify or copy a specific DNA target from a mixture of DNA molecules in vitro, where the DNA polymerase enzyme synthetizes a complementary sequence of DNA using two short DNA sequences called primers, free nucleotides, MgCl2, and nucleotides (A, T, C, G); the mixture is placed in a PCR machine, called thermocycler. The chain reaction involves a process of exponential amplification: one DNA molecule is used to produce two copies of specific fragment and then four, then eight, and then millions of copies [16]. Despite that one of the most important characteristics of PCR is its high sensitivity, it can produce false-positive results due to exogenous contamination. To avoid this, some strategies must prevail such as physical separation of work areas: DNA extraction area, PCR area, and electrophoresis area.

A classic PCR is called end point PCR, which may be uniplex or simple, multiplex, and nested. Also, this can be combined with other techniques such as restriction fragment length polymorphism analysis (PCR-RFLP), where restriction enzymes that recognize specific sites in the material obtained by PCR generate fragments with different lengths which conform patterns unique to each species of dermatophyte or non-dermatophyte involved. Direct sequencing to the product obtained by PCR has proven to be a great technique for species or subspecies identification. PCR may be used in a real-time protocol (PCR real time) with the intention to quantify the relative levels of the transcription of the genetic marker of choice and to estimate the viability of the sample. Although PCR has the advantage of consuming much less time than fungal culture, it has the disadvantage of being a more expensive technique, particularly the PCR sequencing or PCR real time [17–28].

Gene Target

For gene target options, the internal transcribed spacer regions ITS1 and ITS2 are the gene region most commonly used for sequencing and identifying a large number of fungi. They are variable regions located between the conserved genes that codify for the ribosomal subunits 18S, 5.8S, and 28S.

Ribosomal DNA (rDNA) is a tandem region of 50–100 copies in the fungal haploid genome. It is composed of the gene of the small subunit (SSU) rDNA (18S), the gene from subunit 5.8S, and the gene from the large subunit (LSU) rDNA (28S). When separating the subunits 18S and 5.8S, and subunits 5.8S and 28S, we find the intergenic transcribed spacers (ITS), ITS1 and ITS2, respectively (Fig. 11.1). Besides this cluster, we find a second repeated unit of the gene of the subunit 5S rDNA flanked by a region of the non-transcribed spacer (NTS). Either cluster can be used as a marker for PCR. The genes in the subunits of rDNA are highly conserved and the ITS regions are highly variable among fungi [29].

The region ITS1 can be amplified from a wide variety of fungus by using the primers ITS1 (5-TCCGTAGGTGAACCTTGCGG-3) and ITS2 (5-GCTGCGTTCTTCATCGATGC-3). A second variable zoned among the cluster

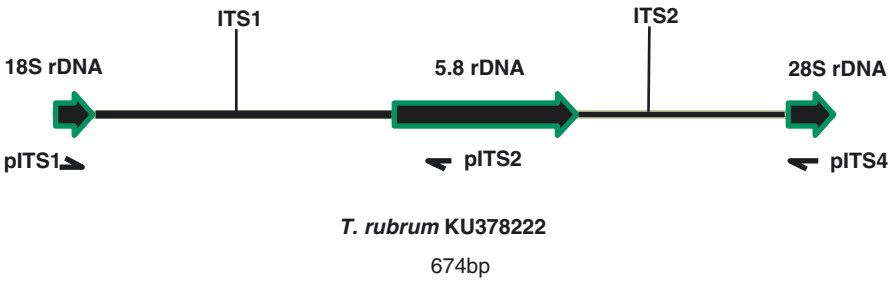

Fig. 11.1 Map of rDNA locus from *Trichophyton rubrum*

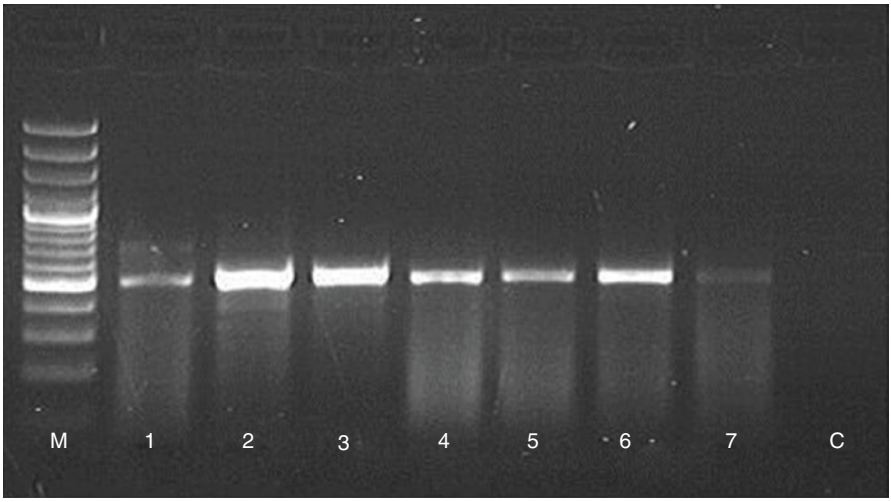

Fig. 11.2 The 18S-ITS1-5.8S-ITS2-28S rDNA amplification using the ITS1 and ITS4 primers of *Trichophyton rubrum* from nail samples. M, 100 bp DNA ladder; line 1, *T. rubrum* positive control; lines 2–7, DNA from nails with onychomycosis; C, negative control

of ribosomal DNA has an area named domain D1/D2, located in the rDNA 28S subunit. This region can be amplified with ITS1 primers (5-TCCGTAGGT GAACCTTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (Fig. 11.2). They are highly conserved and informative and much less variable than the ITS. The conserved region of the domains D1/D2 is the anchorage point for the primers that, along with the variable nature of the ITS regions, provides a specific combination for each species. One of the main advantages of the ribosomal locus in fungi is the high number of copies from the target gene, 10–100 times, compared to genes with only one copy, what in the end translates in higher sensitivity [11, 19, 22, 30–33].

A gene frequently used is the chitin synthase 1 (CHS1), and in cases where the ribosomal target is not sensitive enough for discriminating between closely related species, additional loci may be sequenced, such as the β-tubulin [11, 19, 22, 30–33].

More recently, real-time PCR (quantitative PCR [qPCR]) has been used to identify onychomycosis, using molecular beacons which are small single-chain probes of hairpin type that fluoresce when linked to the target site. The same way as with conventional PCR (end point PCR), both variable and conserved regions (ITS1-2/ITS1-4) can be used to design the probe. This will allow the universal identification of the fungal presence in the sample or the involved species [34].

On the other hand, in the same qPCR platform, TaqMan technology or hybrid probes can be used, such as the carboxyfluorescein (FAM)-labeled probe with a Black Hole Quencher (BHQ1) [35, 36]. This technique can detect and quantify quickly the nucleic acids directly from human and animal tissue samples. It has high sensitivity with a detection threshold of one single molecule and depends from one efficient DNA extraction and purification to avoid PCR inhibitors as well as an adequate fungal cell wall lysis [11, 12].

Panfungal Amplification Technique

The gene regions in the SSU and the LSU rDNA have highly conserved regions and variable regions [31, 37]. These permit the development of panfungal primers based on the conserved regions of the rRNA cluster which are capable of identifying a great number of fungal species and can even be species specific [27, 29, 31]. These characteristics have made this the preferred PCR mode. After the amplification of the mentioned region, different genders and species can be identified using the same amplification product by RFLP, hybridization with specific probes marked with radioactivity or digoxigenin, and, with highly specific tool, the direct sequencing of the amplification product [38–41].

A general problem with PCR as detection method for the causative agents of onychomycosis is the lack of worldwide standardization, as well as the deficit of availability of commercial systems in some countries. Several studies report excellent results with "in-house PCR"; however, the majority of these don't make adequate comparisons mainly in clinical practice [22, 42]. The DNA extraction methods, genetic markers, as well as the different types of clinical samples (blood, DNA from fungal culture, nails, the hair, the skin, or fluids) are factors that influence the comparison between different PCR protocols.

Outlook: Future Developments

Molecular biology is a tool under continuous development and improvement for onychomycosis diagnosis.

The challenges ahead are:

- Availability of molecular tests to reduce the time consumed in some techniques such as PCR sequencing, corroborate sensitivity, and specificity of the currently available tests and improve them if possible with the aim to reduce the clinical sample required that at present must be large
- Improvement of keratin extraction and fungal cell wall rupture techniques
- Reduction of costs
- Readily accessible kits for fast etiological identification in onychomycosis, particularly when caused by non-dermatophyte molds or yeasts, which enables the indication of adequate treatment options
- Improvement of subspecies identification
- More than one fungal determination in the same clinical sample
- Differentiation of saprophytes from etiological agents
- Performance of epidemiological studies to redefine onychomycosis frequency and its etiological agents
- Implementation of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) in clinical samples

- Identification of the species of causative agents with PCR-terminal restriction fragment length polymorphism (PCR-TRFLP)
- Implementation of the use of other operons as genes that codify for non-ribosomal proteins and determine if relapses are by the same causative agent

Summary for the Clinician

Molecular techniques are particularly useful for fast identification of onychomycosis in atypical or mycological negative cases, gender and species identification in 24–48 h allowing a prompt diagnosis, and thus optimal management.

Clinical Pearls

Consider molecular biology for diagnosis in difficult onychomycosis cases:

- Identify fungal genus and species in onychomycosis if traditional techniques have failed to do so.
- Send enough of the clinical sample for adequate processing.
- Contact a reference center that has access to molecular biology for difficult onychomycosis cases.

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