ITS-RFLP optimization for dermatophyte identification from clinical sources in Alagoas (Brazil) versus phenotypic methods

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Abstract

Introduction: Dermatophytoses are superficial mycoses, and the identification of their etiological agents is routinely performed by culture and microscopic features, which is time-consuming and relies on personnel expertise. Molecular approaches have been developed to provide faster and reliable results; therefore, this study aimed to identify dermatophytes isolated from Alagoas state patients, employing phenotypical and molecular methods.

Methodology: Clinical samples for morphological identification were collected from private and public laboratories and cultivated on Sabouraud dextrose agar. DNA extraction was followed by ITS amplicon analysis after restriction enzyme digestion DdeI (ITS-RFLP).

Results: Out of fourteen representative strains, ITS-RFLP with DdeI efficiently identified Microsporum canis, Nannizzia gypsea, and Trichophyton rubrum, while species of the complex T. tonsurans/T. mentagrophytes presented the same restriction pattern. After genotyping, 2 T. tonsurans and 1 Microsporum sp. strain were reclassified as T. rubrum.

Conclusions: RFLP of ITS-region followed by DdeI digestion produced faster and relatively reliable results than classic methods; however, this method has not been as efficient for closely related dermatophytes cryptic species.

Key words: Dermatophytes; Trichophyton; ITS-RFLP; PCR.

J Infect Dev Ctries 2022; 16(11):1773-1777. doi:10.3855/jidc.17077

(Received 14 July 2022 – Accepted 12 October 2022)

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Introduction

Dermatophytes are superficial infections diagnosed frequently in the dermatological clinical routine, caused by filamentous fungi (dermatophytes) which attack keratinized tissues in humans and animals [1]. Advances in phylogenetic taxonomy culminated in the adoption of the polyphasic nomenclature, with the recognition of nine holomorphic genera of dermatophytes, of which seven had species that could cause infections in humans, including the anamorphic genera (Trichophyton, Microsporum, and Epidermophyton), Nannizzia, Paraphyton, Lophophyton, and Arthroderma [2–4]. Dermatophytes thrive under humid conditions and temperatures ranging from 25-28 °C, making skin and its annexes excellent environments for their growth and spread [5].

According to their ecological habitat, dermatophytes are classified as anthropophilic, geophilic, or zoophilic, and can cause dermatophytosis in humans, as well as in small and large animals, configuring an important zoonotic and public health problems due to the potential risk of transmission from animals to humans [6,7], which can be amplified with the transmission between domestic animals. Etiological agent identification is essential for epidemiological purposes and accurate infection management since there is clinical significance: anthropophilic species cause more chronic and non-inflammatory dermatophytosis, while zoo-geophilic species cause acute and inflammatory mycoses [2]. The clinical routine of dermatophyte identification is based on phenotypical observation of culture and direct microscopic examination, low-cost methods that rely on technical expertise and diagnosis, often time-consuming and inconclusive at the species level [8].

The main fungal etiological agents on the American continent belong to the Trichophyton genus [9–13], as found in studies from northeastern Brazil, confirming the high prevalence of the genus [6,14]. Although they affect approximately one-quarter of the population worldwide [15], dermatophytoses are often neglected due to their non-invasive nature, with limited or no
medical care whatsoever, increasing the pathogen spread and chronic mycoses cases [16,17].

Restriction enzyme analysis (REA) and restriction fragment length polymorphism (RFLP) have shown satisfactory results in dermatophyte species identification for faster and reproducible diagnosis [18]. Molecular approaches based on ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequence have been employed as the best method to close the gap in dermatophyte identification [18–20]. Once PCR-based methods increase the accuracy of the identification, the present study aimed to track patients diagnosed with dermatophytosis by phenotypic methods in a public hospital and private laboratories for molecular identification of etiological agents, comparing the phenotypic with molecular results to elucidate the most common species in Alagoas.

Methodology

Clinical strains

This research was approved by the Research Ethics Committee (No. 23065.017665/2011-64) (SisGen No. A5B1165), according to resolution 466/2012 (Brazil). Patients who underwent medical evaluation due to lesions with suspected dermatophyte infection were referred for laboratory diagnosis in a public hospital and 3 private laboratories in Maceió (Alagoas/Brazil), and clinical specimens were collected and processed using phenotypic methods for fungal structure identification via direct microscopic examination with lactophenol cotton blue and cultivated on Sabouraud dextrose agar (SDA; 14 days, 37 ºC). Among positive samples, representative strains were cultured in our laboratory, and stored in distilled water for reactivation on potato dextrose agar or SDA with chloramphenicol (0.05 g/L) (Sigma-Aldrich, Burlington, USA) at 37 ºC; 7 days), followed by centrifugation (2 minutes; 14,000 × g) and DNA extraction by the Wizard® Genomic DNA Purification Kit (Promega®, Madison, USA), 0.1 mM dNTP set, 25pmol of each primer, 2U GoTaq® DNA Polymerase, 1.5 µL of DNA and ultrapure water (q.s.p. 25 µL). Samples were amplified with an initial denaturation at 94 ºC (6 minutes), 35 cycles of denaturation (94 ºC; 30 seconds), annealing (58 ºC; 1 minute), extension (72 ºC; 1 minute), and final extension at 72 ºC (10 minutes). Each amplicon (~700 bp) was evaluated by electrophoresis on a 1.2% agarose gel, and 2 µL were digested (37 ºC for 2 hours) with 5U of the restriction endonuclease DdeI (Promega®, Madison, USA) and the products were separated by agarose gel electrophoresis (1.5%). Reference strains of anamorphic forms (positive controls) were provided by Dr. Regina Celia Cândido (FCFRP-USP, Brazil): Trichophyton rubrum ATCC 28188, T. interdigitale ATCC 9533 Microsporum gypseum ATCC 24102 and M. canis ATCC 36299. Finally, the results of molecular and conventional methods were compared.

Results

A total of 402 patients with dermatophytosis (214 female/182 male/6 without a gender identity) were diagnosed with clinical and laboratory tests (phenotypical methods) between 2009 and 2015, aged 1 to 83 years. Of these, 14 dermatophytes were recovered and identified as T. tonsurans (8), T. mentagrophytes (2), T. rubrum (1), Trichophyton sp. (1), Microsporum canis (1), and Microsporum sp. (1), all directed to ITS-RFLP identification (DdeI digestion).

We identified 4 different digestion patterns for the reference strains (Table 1): T. rubrum (320bp, 300bp), T. interdigitale (450, 290bp), N. gypse (420, 210bp), and M. canis (260, 210bp). Twelve out of 14 isolates were confirmed with a reference strain, and 5 of these turned out to be misidentified earlier by conventional methods (MF04, MF07, MF29, MF31, MF33) and re-identified after ITS-RFLP/DdeI (Table 1 and Figure 1). Two clinical isolates did not match with any restriction pattern found for reference strains in this study (MF15 and MF07) and were misidentified as T. rubrum and T.

Table 1. Fragment size patterns observed for the reference strains after DdeI digestion of ITS amplicons.

| Reference strain                  | Restriction pattern after DdeI digestion |
|-----------------------------------|----------------------------------------|
| Microsporum canis ATCC 36299      | 260, 210 bp                            |
| Microsporum gypseum / Nanniza gypsea ATCC 24102 | 420, 210 bp                            |
| Trichophyton interdigitale ATCC 9533 | 450, 290 bp                            |
| Trichophyton rubrum ATCC 28188    | 320, 300 bp                            |
tonsurans by direct mycological examinations, respectively (Table 2).

**Discussion**

Superficial mycotic infections, including dermatophytosis, are considered the most common fungal infections worldwide [16,21]. Classic laboratory diagnosis relies upon morphological features under culture on solid media and microscopy (macro and micromorphology), which can vary according to outside factors such as temperature incubation, medium, and chemotherapy [6,22]. Since dermatophytes can be similar or frequently vary into the same species, phenotypical methods are subject to misidentifications between species and even genera [6] and molecular methods have been frequently applied for proper identification.

Here we identified 12 isolates among 14 by ITS-RFLP as belonging to the *Trichophyton* genus, an expected finding since it has been reported as the most prevalent in Latin America, Brazil, and in Alagoas State [6,9,11,13]. It is important to note that the reference strain ATCC 9533, previously *T. mentagrophytes* var. *interdigitale*, has recently been reclassified as *T. interdigitale* and based on the knowledge that *T. tonsurans*, *T. mentagrophytes*, and *T. interdigitale* have similar ITS sequences and share a taxonomic complex [23], strains phenotypically identified as the first two but presenting the same restriction pattern as *T. interdigitale* was considered as a match. Isolate MF07, phenotypically identified as *T. tonsurans*, did not match

![Table 2. Comparison of dermatophyte identification with phenotypic methods and molecular profile after PCR-restriction enzyme analysis with DdeI.](image)

| Sample | Anatomical site | Phenotypical identification | Restriction pattern after DdeI | ITS-RFLP identification |
|--------|-----------------|----------------------------|-------------------------------|-------------------------|
| MF33   |                 | * M. canis                | 260, 210 bp                   | * M. canis              |
| MF39   | Toenail         | Microsporum sp.           | 320, 300 bp                   | * T. rubrum             |
| MF26   | Foot            | * T. mentagrophytes       | 450, 290 bp                   | * T. interdigitale †     |
| MF27B  |                 | * T. mentagrophytes       | 450, 290 bp                   | * T. interdigitale †     |
| MF15   |                 | * T. rubrum               | 230, 190 bp                   | -                       |
| MF04   | * T. tonsurans  | 320, 300 bp               | * T. rubrum                   |                         |
| MF05   | * T. tonsurans  | 450, 290 bp               | * T. interdigitale †          |                         |
| MF07   | * T. tonsurans  | 380, 160, 110 bp          | * T. schoenleinii / *M. canis*|                         |
| MF09   | Skin scrapings  | * T. tonsurans            | 450, 290 bp                   | * T. interdigitale †     |
| MF29   | Foot            | * T. tonsurans            | 320, 300 bp                   | * T. rubrum             |
| MF30   | Foot            | * T. tonsurans            | 450, 290 bp                   | * T. interdigitale †     |
| MF31   | * T. tonsurans  | 320, 300 bp               | * T. rubrum                   | * T. rubrum             |
| MF32   | Scalp           | * T. tonsurans            | 450, 290 bp                   | * T. interdigitale †     |
| MF23A  | Foot            | * T. tonsurans            | 450, 290 bp                   | * T. interdigitale †     |
| MF04, MF29, MF31 and MF39: *Trichophyton rubrum*; MF05, MF09, MF23A, MF26, MF27B, MF30 and MF32: *T. interdigitale*; MF33: *Microsporum canis*. The last four lanes, respectively: ATCC strains of *T. rubrum*, *T. interdigitale*, *M. canis* and *N. gypseae*. Without molecular identification: MF07 and MF15.

![Figure 1. Electrophoretic profile of ITS-PCR products digested with DdeI.](image)
any of the available ATCC strains, but it was considered
as a mismatch since it lacks the > 400 bp fragment after
Ddel digestion reported in other works for this species
[24]. ITS-RFLP was shown to be a reproducible, fast,
and reliable method to identify important
dermatophytes species from clinical samples [19,25];
however, for some strains this technique was not
sufficiently precise for the identification of species
level [26,27].

Regarding enzyme choice, many enzymes were
tested for the digestion of dermatophyte ITS amplicon,
such as Mval, HindI, Ddel, and HaeIII, with Mval being
the most widely tested and chosen by researchers as the
best-performing enzyme [26,28]. Ddel restriction
patterns for T. interdigitale and T. rubrum found in our
study were confirmed by another report [28], as well as
the M. canis and N. gypsea digestion patterns [26].
Although Ddel is a suitable enzyme for the
differentiation of some dermatophyte species, the
 distinction between closely related species such as T.
rubrum/T. raubitschenkii or M. canis/M. audouini
using Ddel has proven to be difficult [26]. When we
compared the digestion patterns of the strains by ITS-
RFLP/Ddel with the results of phenotypic methods, 4
isolates previously misidentified were reclassified, as
reported in other studies concerning molecular
identification of dermatophytes [19,25,29].

Ghojoghi et al. [30] emphasized the importance of
accurate identification of dermatophytes for appropriate
treatment and control of potential environmental
sources of infection after misidentifying 58 samples by
phenotypic methods, re-identifying as T. interdigitale
and T. rubrum after ITS-RFLP. PCR-based methods
have brought advances in the distinction between
species and strains but are not yet applied in the routine
clinical diagnosis of dermatophyosis due to the high
cost of equipment, reagents, and specialized
professionals [28].

Conclusions
This was the first molecular approach to
dermatophytes in our state. ITS-RFLP analysis of the
ITS-rDNA followed by Ddel treatment produced faster
and more reproducible results than classical culture-
based methods. However, the definitive molecular
identification for less frequent dermatophyte species
only can be achieved through DNA sequencing.

Acknowledgements
We would like to thank the Brazilian research agency CNPq
and the Alagoas State agency FAPEAL for the financial
support (20110829-011-0025-0009).

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Conflict of interests: No conflict of interests is declared.