JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Brief Original Article

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

Luisa Andrea Villanueva da Fonseca[1], Maria Anilda Santos Araújo[2], Denise Maria Wanderlei Silva[3], Fernanda Cristina de Albuquerque Maranhão[3]

[1] *Microbiology Postgraduate Program, University of São Paulo, São Paulo, Brazil*
[2] *Tiradentes University Center, Alagoas, Brazil*
[3] *Federal University of Alagoas, Institute of Biological and Health Sciences, Alagoas, Brazil*

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 *Dde*I (ITS-RFLP).

Results: Out of fourteen representative strains, ITS-RFLP with *Dde*I 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 *Dde*I 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)

Copyright © 2022 Fonseca *et al*. This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

Dermatophytoses 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 ºC, 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.

Molecular analyzes

A small amount of mycelium was transferred into 1 mL potato dextrose broth medium for incubation (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). The amplification of ITS1-5.8S-ITS2 rDNA was performed using panfungal primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) (Sigma-Aldrich®, Burlington, USA) in a reaction mix: 2.5 µL of 5X Green GoTaq® Flexi Buffer and 2.5 µL of $MgCl_2$ (25 mM) (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 *Dde*I (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 (*Dde*I digestion).

We identified 4 different digestion patterns for the reference strains (Table 1): *T. rubrum* (320bp, 300bp), *T. interdigitale* (450, 290bp), *N. gypsea* (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/*Dde*I (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 *Dde*I digestion of ITS amplicons.

Reference strain	Restriction pattern after *Dde*I digestion
Microsporum canis ATCC 36299	260, 210 bp
Microsporum gypseum / Nannizia gypsea ATCC 24102	420, 210 bp
Trichophyton interdigitale ATCC 9533	450, 290 bp
Trichophyton rubrum ATCC 28188	320, 300 bp

Table 2. Comparison of dermatophyte identification with phenotypic methods and molecular profile after PCR-restriction enzyme analysis with *Dde*I.

Sample	Anatomical site	Phenotypical identification	Restriction pattern after *Dde*I	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 / T. mentagrophytes*
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*
MF32	Scalp	*T. tonsurans*	450, 290 bp	*T. interdigitale* †
MF23A	Foot	*Trichophyton* sp.	450, 290 bp	*T. interdigitale* †

* Unavailable information; - Did not correspond to any of the available ATCC strains; † Due to taxonomic and genetic similarities, strains previously identified as *Trichophyton mentagrophytes* and *T. tonsurans* that presented *T. interdigitale* restriction patterns were not considered as a mismatch.

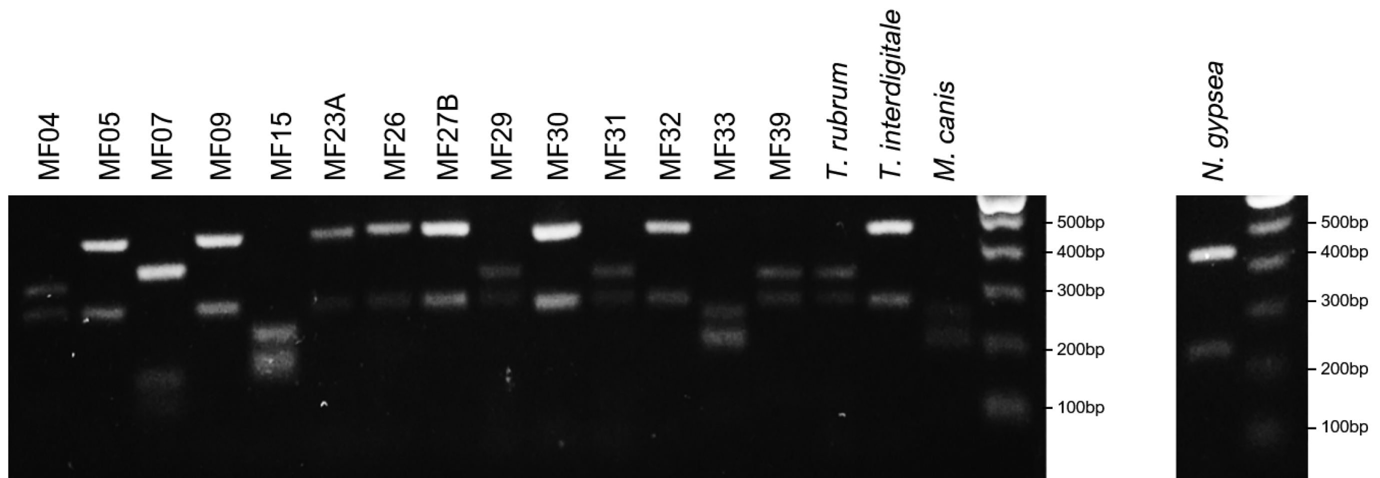
tonsurans by direct mycological examinations, respectively (Table 2).

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

Figure 1. Electrophoretic profile of ITS-PCR products digested with DdeI.



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. gypsea*. Without molecular identification: MF07 and MF15.

any of the available ATCC strains, but it was considered as a mismatch since it lacks the > 400 bp fragment after *Dde*I 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 *Mva*I, *Hinf*I, *Dde*I, and *Hae*III, with *Mva*I being the most widely tested and chosen by researchers as the best-performing enzyme [26,28]. *Dde*I 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 *Dde*I 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. audouiini* using *Dde*I has proven to be difficult [26]. When we compared the digestion patterns of the strains by ITS-RFLP/*Dde*I 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 dermatophytosis 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 *Dde*I treatment produced faster and more reproducible results than classical culture-based methods. However, the definitive molecular identification for less frequent dermatophyte species can only 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).

References

1. Heidrich D, Garcia MR, Stopiglia CDO, Magagnin CM, Daboit TC, Vetoratto G, Schwartz J, Amaro TG, Scroferneker ML (2015) Dermatophytosis: A 16-year retrospective study in a metropolitan area in southern Brazil. J Infect Dev Ctries 9: 865–871. doi: 10.3855/jidc.5479.
2. de Hoog GS, Dukik K, Monod M, Packeu A, Stubbe D, Hendrickx M, Kupsch C, Stielow JB, Freeke J, Göker M, Rezaei-Matehkolaei A, Mirhendi H, Gräser Y (2017) Toward a novel multilocus phylogenetic taxonomy for the dermatophytes. Mycopathologia 182: 5–31
3. de Macedo PM, Freitas DFS (2021) Superficial infections of the skin and nails. In Zaragoza Ó, Casadevall A, editors. Encyclopedia of Mycology. Oxford: Elsevier. 707–718.
4. Baert F, Stubbe D, D'hooge E, Packeu A, Hendrickx M (2020) Updating the taxonomy of dermatophytes of the BCCM/IHEM collection according to the new standard: A phylogenetic approach. Mycopathologia 185: 161–168.
5. Cafarchia C, Iatta R, Latrofa MS, Gräser Y, Otranto D (2013) Molecular epidemiology, phylogeny and evolution of dermatophytes. Infect Genet Evol 20: 336–351.
6. Maranhão FC de A, Oliveira-Júnior JB, dos Santos Araújo MA, Silva DMW (2019) Mycoses in northeastern Brazil: Epidemiology and prevalence of fungal species in 8 years of retrospective analysis in Alagoas. Braz J Microbiol 50: 969–978.
7. Chang CC, Wechtaisong W, Chen SY, Cheng MC, Chung CS, Lin LS, Lien YY, Tsai YL (2022) Prevalence and risk factors of zoonotic dermatophyte infection in pet rabbits in Northern Taiwan. JoF 8: 627.
8. Kanbe T (2008) Molecular approaches in the diagnosis of dermatophytosis. Mycopathologia 166: 307–317.
9. de Oliveira Pereira F, Gomes SM, Lima da Silva S, Paula de Castro Teixeira A, Lima IO (2021) The prevalence of dermatophytoses in Brazil: A systematic review. J Med Microbiol 70.
10. Foster KW, Ghannoum MA, Elewski BE (2004) Epidemiologic surveillance of cutaneous fungal infection in the United States from 1999 to 2002. J Am Acad Dermatol 50: 748–752.
11. Mazza M, Refojo N, Davel G, Lima N, Dias N, Passos da Silva CMF, Canteros CE, Mycology Network of the Province of Buenos Aires (MNPBA) (2018) Epidemiology of dermatophytoses in 31 municipalities of the province of Buenos Aires, Argentina: A 6-year study. Rev Iberoam Micol 35: 97–102.
12. Welsh O, Welsh E, Ocampo-Candiani J, Gomez M, Vera-Cabrera L (2006) Dermatophytoses in Monterrey, México. Mycoses 49: 119–123.
13. Béjar V, Villanueva F, Guevara JM, González S, Vergaray G, Abanto E, Napám K, Velasque L, Vergaray S (2014) Epidemiology of dermatomycoses in 30 years of study at the Daniel A Carrión Institute of Tropical Medicine, Universidad Nacional Mayor de San Marcos, Lima, Peru. AnFac med 75: 167–172 [Article in Spanish].
14. Brilhante RSN, Cordeiro RA, Medrano DJA, Rocha MFG, Monteiro AJ, Cavalcante CSP, Meireles TEF, Sidrim JJC

(2005) Onychomycosis in Ceará (Northeast Brazil): epidemiological and laboratory aspects. Mem Inst Oswaldo Cruz 100: 131–135.
15. Zhan P, Liu W (2017) The changing face of dermatophytic infections worldwide. Mycopathologia 182: 77–86.
16. Havlickova B, Czaika VA, Friedrich M (2008) Epidemiological trends in skin mycoses worldwide. Mycoses 51: 2–15.
17. Qadim HH, Golforoushan F, Azimi H, Goldust M (2013) Factors leading to dermatophytosis. Ann Parasitol 59: 99–102.
18. Begum J, Mir NA, Lingaraju MC, Buyamayum B, Dev K (2020) Recent advances in the diagnosis of dermatophytosis. J Basic Microbiol 60: 293–303.
19. Ahmadi B, Mirhendi H, Shidfar MR, Nouripour-Sisakht S, Jalalizand N, Geramishoar M, Shokoohi GR (2015) A comparative study on morphological versus molecular identification of dermatophyte isolates. J Mycol Med 25: 29–35.
20. Mohammadi R, Abastabar M, Mirhendi H, Badali H, Shadzi S, Chadeganipour M, Pourfathi P, Jalalizand N, Haghani I (2015) Use of restriction fragment length polymorphism to rapidly identify dermatophyte species related to dermatophytosis. Jundishapur J Microbiol 8: e17296.
21. White TC, Findley K, Dawson TL, Scheynius A, Boekhout T, Cuomo CA, Xu J, Saunders CW (2014) Fungi on the skin: Dermatophytes and *Malassezia*. Cold Spring Harb Perspect Med 4: a019802–a019802.
22. Brilhante RSN, Rocha MFG, Cordeiro RA, Rabenhorst SHB, Granjeiro TB, Monteiro AJ, Sidrim JJC (2005) Phenotypical and molecular characterization of *Microsporum canis* strains in north-east Brazil. J Appl Microbiol 99: 776–782.
23. Gräser Y, Kuijpers AFA, Presber W, De Hoog GS (1999) Molecular taxonomy of *Trichophyton mentagrophytes* and *T. tonsurans*. Med Mycol 37: 315–330.
24. Brilhante RSN, Cordeiro RA, Gomes JMF, Sidrim JJC, Rocha MFG (2006) Canine dermatophytosis caused by an anthropophilic species: Molecular and phenotypical characterization of *Trichophyton tonsurans*. J Med Microbiol 55: 1583–1586.
25. Didehdar M, Shokohi T, Khansarinejad B, Ali Asghar Sefidgar S, Abastabar M, Haghani I, Amirrajab N, Mondanizadeh M (2016) Characterization of clinically important dermatophytes in North of Iran using PCR-RFLP on ITS region. J Mycol Med 26: 345–350.
26. Shin JH, Sung JH, Park SJ, Kim JA, Lee JH, Lee DY, Lee ES, Yang JM (2003) Species identification and strain differentiation of dermatophyte fungi using polymerase chain reaction amplification and restriction enzyme analysis. J Am Acad Dermatol 48: 857–865.
27. Rezaei-Matehkolaei A, Makimura K, Shidfar MR, Zaini F, Eshraghian MR, Jalalizand N, Nouripour-Sisakht S, Hosseinpour L, Mirhendi H (2012) Use of single-enzyme PCR-restriction digestion barcode targeting the internal transcribed spacers (ITS rDNA) to identify dermatophyte species. Iran J Public Health 41: 82–94.
28. Elavarashi E, Kindo AJ, Kalyani J (2013) Optimization of PCR-RFLP directly from the skin and nails in cases of dermatophytosis, targeting the ITS and the 18S ribosomal DNA regions. J Clin Diagn Res 7: 646–651.
29. Heidemann S, Monod M, Gräser Y (2010) Signature polymorphisms in the internal transcribed spacer region relevant for the differentiation of zoophilic and anthropophilic strains of *Trichophyton interdigitale* and other species of *T. mentagrophytes sensu lato*. Br J Dermatol 162: 282–295.
30. Ghojoghi A, Falahati M, Pagheh AS, Abastabar M, Ghasemi Z, Ansari S, Farahyar S, Roudbary M (2015) Molecular identification and epidemiological aspects of dermatophytosis in Tehran, Iran. Res Mol Med 3: 11-16.

Corresponding author

Professor Fernanda C. de A. Maranhão, PhD
Laboratory of Clinical Microbiology
Institute of Biological and Health Sciences, Federal University of Alagoas
(57072-900) - Maceió-AL, Brazil.
Tel: + 55-82-3214-1065
Email: fcam@icbs.ufal.br

Conflict of interests: No conflict of interests is declared.