MOLECULAR IDENTIFICATION OF FUSARIUM spp.

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ABSTRACT

Fusarium spp. have been widely studied because the mycotoxins they produce were a big threat to plants, animal and human health. This genus have species as known human opportunistic pathogens, mainly *F. solani* species complex (FSSC) and *F. oxysporum* species complex (FOSC). These species are able to cause a broad spectrum of infections in humans, ranging from skin infections such as keratitis and onychomycosis, up to disseminated infections. Classical methods are important for fungal identification, being the most used in the laboratory routine. However, molecular techniques are necessary for rapid diagnostic. In addition, for *Fusarium* spp. molecular approaches allow us to distinguish between species complexes. Seven clinical *Fusarium* isolates from patients with suspected onychomycosis were included to this study. Six samples from toenails (85.8%). By classical techniques, the clinical isolates were identified as *Fusarium solani* (71.5%) and *Fusarium oxysporum* (28.5%). The ITS region sequencing also identified these two *Fusarium* species. However, for two isolates was observed discrepant results between classical and molecular identification. Therefore, development of rapid and safe diagnostic methodology is essential for the correct management of patients. Molecular techniques are promising in this proposal. However, *Fusarium* species identification is still a major challenge.

KEY WORDS: *Fusarium* spp.; clinical isolates; PCR; DNA sequencing.

1 INTRODUCTION

Fusarium genus contemplates filamentous fungi widely distributed in soils and organic substrates of tropical regions. Many species have been described as phytopathogens, generating high costs for the agriculture sectors. Otherwise, *Fusarium* is able to cause a broad spectrum of infections in humans. In the immunocompetent host can generally cause a local infection such as onychomycosis, cellulitis, sinusitis and keratitis (AL-HATMI; et. al, 2018). This fungus has the ability to form a biofilm community, provides the survival of this fungus in hostile environments, as well as its high resistance to external stresses such as heat, cold and UV light (PEIQIAN; et. al, 2014).

Currently, among the species capable of causing infection in humans, two are the most prevalent: *F. solani* species complex (FSSC) and *F. oxysporum* species complex (FOSC) (VAN DIEPENINGEN; et. al, 2015). Due to the wide variety of species, accurate diagnosis, economically accessible and early, have still been a great challenge. The classical identification techniques, as such morphological analysis have been used. However, molecular identification techniques have been developed, providing greater speed, high sensitivity and specificity (VAN DIEPENINGEN; et. al, 2014). Thus, the present study aimed to obtain and sequence the internal transcribed spacer (*ITS*) region from seven clinical *Fusarium* isolates, focusing on molecular identification.

2 MATERIALS AND METHODS

Seven clinical *Fusarium* isolates were provided by *Laboratorio de Ensino e Pesquisa em Análises Clínicas* (LEPAC), Division of Medical Mycology, *Universidade Estadual de Maringa* (UEM). This project was approved by the Research Ethics Committee of UEM (number 2.748.843). Samples were identified, by classic methods (De HOOG), between 2015 and 2018 from patients with suspected onychomycosis.

The fungal DNA were extracted as described by BONFIM-MENDONÇA; et. al, 2013, with modifications to filamentous fungi. The DNA quality was verified by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized on a UV transilluminator. The PCR amplification and sequencing were performed in an ABI3730 automated sequencer (Applied Biosystems). Two regions were selected: the ITS1-5.8S rDNA-ITS2 region, using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (ZARRIN; GANJ; FARAMARZI, 2016). Comparisons of regions were performed using GenBank and a BLAST analysis. Phylogenetic study based on ITS sequence constructed with maximum likelihood, based on the Tamura-Nei model and Gamma distribution (T92+G), performed in MEGA v.7. Bootstrap support was calculated from 1000 replicates.

3 RESULTS AND DISCUSSION

Identification of filamentous fungi at the species level using classical techniques, such as morphological methods, is time-consuming. In addition, the classical identification techniques are not sufficient to distinguish members within the species complex. In table 1, the frequency of onychomycosis caused by *Fusarium* spp. in the toenails was 85.8%, affecting 71.5% females, in patients range 39 - 72 age. From seven clinical isolates, 71.5% (5/7) were identified to *F. solani* and 28.5% (2/7) *F. oxysporum*.

Table 1. Morphological identification of clinical isolates provided by LEPAC.

Clinical isolates	Age	Sex	Specimen	*Fusarium* spp.
LMMC 30	53	Female	toe nail	*F. oxysporum*
LMMC 33	68	Male	fingernail	*F. solani*
LMMC 40	72	Female	toe nail	*F. solani*
LMMC 44	54	Female	toe nail	*F. oxysporum*
LMMC 45	55	Female	toe nail	*F. solani*
LMMC 47	39	Male	toe nail	*F. solani*
LMMC 100	45	Female	toe nail	*F. solani*

Classical methods are important for fungal screening, phenotypic identification was effective 85.8% in this study. However, molecular techniques are necessary, to validate morphological identification between species-specific, promoving high specificity, similarity and reliability. The recognition of internal transcribed sequence (ITS) region is frequently used to the molecular identification of fungi (VAN DIEPENINGEN, et. al, 2015). In order to confirm the identification performed by classical methods, the ITS region was amplified and analyzed for the seven clinical isolates. The molecular identification shown that the clinical isolates LMM33, LMM47 and LMM100 clustering with *F. solani* 140079 TYPE, belong to FSSC. The clinical isolates LMM30, LMM40, LMM44 and LMM45 clustering with *F. oxysporum* CBS 127149, belong FOSC. Two clinical isolates showed discrepant results between classical and molecular identification (LMM40 and LMM45). However, Thomas and colleagues (2019) have proposed that the fungal identification from a culture is more accurate when performed with EF1α. The ITS region, would be more appropriate for a primary sample, as such biopsy material (THOMAS, et. al, 2019). In addition, for *Fusarium* spp. this region is too conserved and may not even distinguish between species complexes, let alone down to the species level (VAN DIEPENINGEN; et. al, 2014). In order to confirm

the species identification, a new morphological evaluation and complementary tests will be performed.

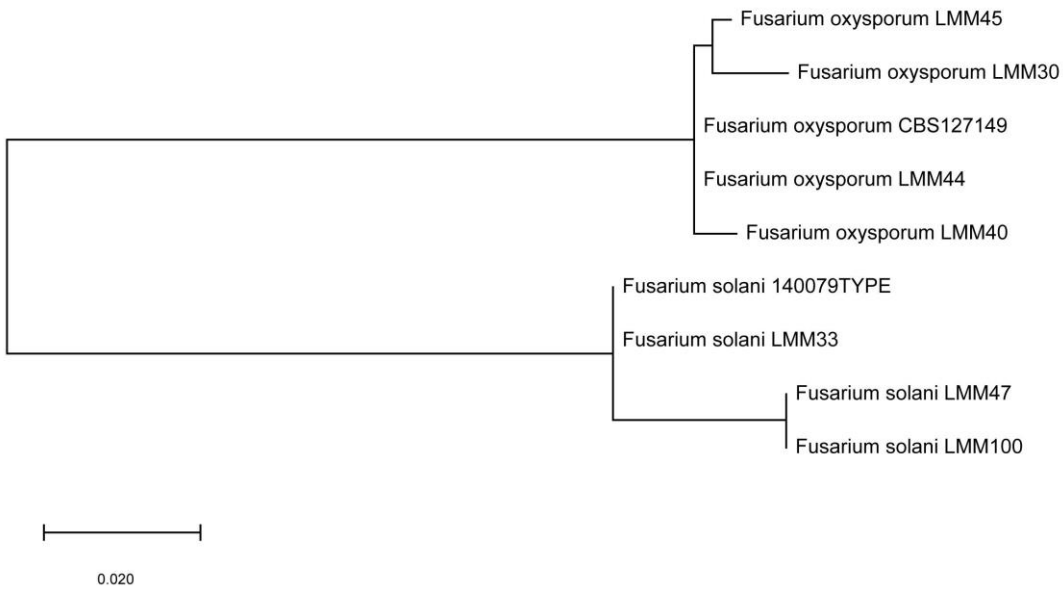

Fig 1. Phylogenetic analysis of clinical isolates from *Fusarium* spp. Phylogenetic study based on ITS sequence constructed with maximum likelihood, based on the Tamura-Nei model and Gamma distribution (T92+G), performed in MEGA v.7. Bootstrap support was calculated from 1000 replicates.

The next step will be the EF1α gene sequencing, as proposed by Thomas (THOMAS, et. al, 2019). The smaller size of EF1α allows one-piece sequencing for laboratories with modest equipment, with a high power of discrepancy among the members within the species complex.

4 FINAL CONSIDERATIONS

Techniques fast and safe are required in order identification fungus, mainly to particularly for management of patients. The molecular approaches, such as PCR and DNA sequencing, have contributed with the rapid and correct identification of pathogens. However, for *Fusarium* spp. this ideal identification is still a big challenge, considering the particularities of this opportunistic pathogen.

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