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INVASÃO DE *Fusarium oxysporum* EM UNHA CARACTERIZADA POR ESPECTROSCOPIA VIBRACIONAL USANDO MODELO *ex vivo* DE ONICOMICOSE

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências da Saúde.
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Orientador: Profª Drª Terezinha Inez Estivalet Svidzinski

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à minha querida avó Li.

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Invasão de *Fusarium oxysporum* em unha caracterizada por espectroscopia vibracional usando modelo *ex vivo* de onicomicose

RESUMO

Infecções oportunistas por fungos causam diversas patologias em humanos e acometem principalmente pacientes com imunocomprometimento. Entre os fungos filamentosos causadores dessas infecções, o gênero *Fusarium* é a segunda causa mais comum e a fungemia está presente em 60% dos casos de disseminação da fusariose. Embora a via respiratória seja a principal forma de infecção por este fungo, a onicomicose fusarial é uma possível porta de entrada para a infecção invasiva, uma vez que *Fusarium* spp. podem migrar da unha e posteriormente causar uma infecção sistêmica. Contudo ainda não é bem esclarecida essa etiopatogenia e a relação fungo-hospedeiro. Para entender melhor o processo de patogênese da onicomicose fusarial, é importante ressaltar que a unha humana é formada por diferentes camadas, com propriedades e funções distintas, as quais oferecem padrões distintos à permeação de agentes externos. Dentre as técnicas de espectroscopia vibracional, a espectroscopia no infravermelho via transformada de Fourier por reflexão total atenuada (FTIR-ATR) tem sido utilizada no estudo de fungos. É uma técnica óptica que permite obter os modos vibracionais da amostra de interesse, os quais são atribuídos a grupos químicos. Assim, o objetivo deste estudo foi comprovar a capacidade de invasão de *Fusarium oxysporum* em modelo de unha humana *ex vivo*, caracterizando por meio da histopatologia e por espectroscopia FTIR-ATR. Para a realização deste estudo foi utilizado o isolado *F. oxysporum* (CMRP 2915) obtido de um caso clínico de onicomicose. Inóculo aferido deste isolado foi pipetado sobre fragmentos de unha humana saudável em uma concentração $1{,}2\times10^7$ conídios/mL em solução salina esterilizada (0,85%). O inóculo foi incubado por sete dias a 35 ºC. Após este período foram realizadas as análises por histopatologia e espectroscopia FTIR-ATR. Este estudo permitiu identificar a presença de *F. oxysporum* em diferentes camadas de unhas e também diferenças no grau de invasão, de acordo com o lado da unha que foi inoculado, revelando que a camada inferior da unha é mais permeável ao fungo, provavelmente devido à sua formação estrutural.

Palavras-chave: FTIR-ATR, espectroscopia, onicomicose, unha, *Fusarium*

Invasion of *Fusarium oxysporum* on nail characterized by vibrational spectroscopy using *ex vivo* model of onychomycosis

ABSTRACT

Fungal opportunistic infections cause several pathological conditions in humans, affecting primarily immunocompromised patients. Among the filamentous fungi that cause these infections, the *Fusarium* genus is the second most common cause and fungemia is present in 60% of fusariosis dissemination cases. Although the respiratory tract is the main point of entry for infection by this fungus, fusarial onychomycosis is a possible way for the invasive infection, since *Fusarium* spp. are able to migrate from the nail and cause a systemic infection later. However, it is still not well understood this etiopathogenesis and the fungus-host relationship. To better understand the pathogenesis process of the fusarial onychomycosis, it's important to emphasize that the human nail is composed by different layers, with distinct properties and functions, which offer distinct patterns to the permeation of external agents. Among the vibrational spectroscopy techniques, the Fourier-transform infrared spectroscopy by attenuated total reflection (FTIR-ATR) has been used in the study of fungi. It is an optical technique that allows to obtain the vibrational modes of the sample of interest, which are attributed to chemical groups. Thus, the objective of this study was to verify the penetration capacity of *Fusarium oxysporum* in an ex vivo human nail model, characterized by histopathology complemented by FTIR-ATR spectroscopy. This study was carried out utilizing a *F. oxysporum* isolate (CMRP 2915) obtained from a onychomycosis clinical case. Inoculum of this isolate was pipetted onto healthy human nail fragments in a concentration of $1,2\times10^7$ conidia/mL in sterile saline solution (0,85%). The inoculum was incubated for seven days at 35ºC. After this period, the analyzes were performed by histopathology and FTIR-ATR spectroscopy. This study made it possible to identify the presence of *F. oxysporum* in different layers of nails and also differences in the degree of invasion, according to the side of the nail that was inoculated, revealing that the bottom layer of the nail is more permeable to the fungus, probably due to its structural conformation.

Key-words: FTIR-ATR, spectroscopy, onychomycosis, nail, *Fusarium.*

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SUMÁRIO

CAPÍTULO I

INFECÇÃO SISTÊMICA POR FUNGOS OPORTUNISTAS

Infecções oportunistas por fungos causam diversas patologias em humanos, particularmente em pacientes com imunocomprometimento, como transplantados, portadores de tumores malignos ou de doenças hematológicas. Com a introdução do fluconazol como padrão na profilaxia e tratamento de infecções por *Candida* spp. houve uma ascensão dos casos por outros tipos de fungos os quais essa classe de antifúngico não tem ação. Entre esses pode-se destacar: *Aspergillus* spp., *Fusarium* spp., *Scedosporium, Rhizopus* spp., *Mucor* spp., *Rhizomucor* spp. e *Absidia* spp. (Galimberti *et al.*, 2012). Atualmente entre as infecções por fungos filamentosos, a fusariose causada por *Fusarium* spp. é a segunda causa mais comum, precedida apenas pela aspergilose (Lanternier *et al.*, 2012; Kollipara *et al.*, 2016).

O gênero *Fusarium* agrupa fungos filamentosos amplamente distribuídos na natureza, são encontrados no solo, nas plantas e no ar. Algumas espécies são importantes patógenos das plantas e ocasionalmente podem causar infecção em animais (Nelson et al., 1994; Evans et al., 2004).

Existem mais 50 espécies de *Fusarium* identificadas, incluindo patógenos vegetais e animais. Doze dessas espécies foram mais freqüentemente associadas a doenças em seres humanos, sendo que 20% dessas infecções e a maioria dos casos de onicomicose fusarial causadas por *F. oxysporum* (Nucci *et al.*, 2013).

FUSARIOSE HUMANA

A fusariose humana inclui várias manifestações clínicas desde infecções cutâneas localizadas como onicomicose e queratite até infecção sistêmica, de grande gravidade, porém a etiopatogenia da fusariose ainda é pouco conhecida, por isso estudos nesse sentido são necessários. A apresentação clínica dessa doença, quando disseminada geralmente inclui febre, lesões cutâneas e infecções sinopulmonar.

Esta infecção pode afetar o fígado, baço, rim, coração e olhos, entre outros órgãos (Nucci *et al.*, 2013). A fungemia está presente em 60% dos casos de disseminação da fusariose e muitas vezes é detectável após o início de lesões na pele.

As infecções sistêmicas são desencadeadas pela disseminação do fungo no organismo. Na maioria das vezes, a causa da inoculação é desconhecida, entretanto a inalação, ingestão e entrada do fungo através de trauma da pele e/ou unha estão entre os mecanismos mais conhecidos. Além disso, a contaminação pode acontecer após cirurgia ou queimadura

(Galimberti *et al.*, 2012; Muhammed *et al.*, 2013). Lesões de pele podem evidenciar o local de introdução do fungo, porém em algumas vezes aparecem somente após disseminação do fungo no organismo hospedeiro. Comumente as lesões cutâneas evoluem de um a cinco dias da disseminação e geralmente ocorrem no tronco e nas extremidades, estas lesões têm geralmente aspectos necróticos e podem se tornar úlceras e desenvolvem uma borda escura (Kollipara *et al.*, 2016) conforme ilustra a Figura 1.

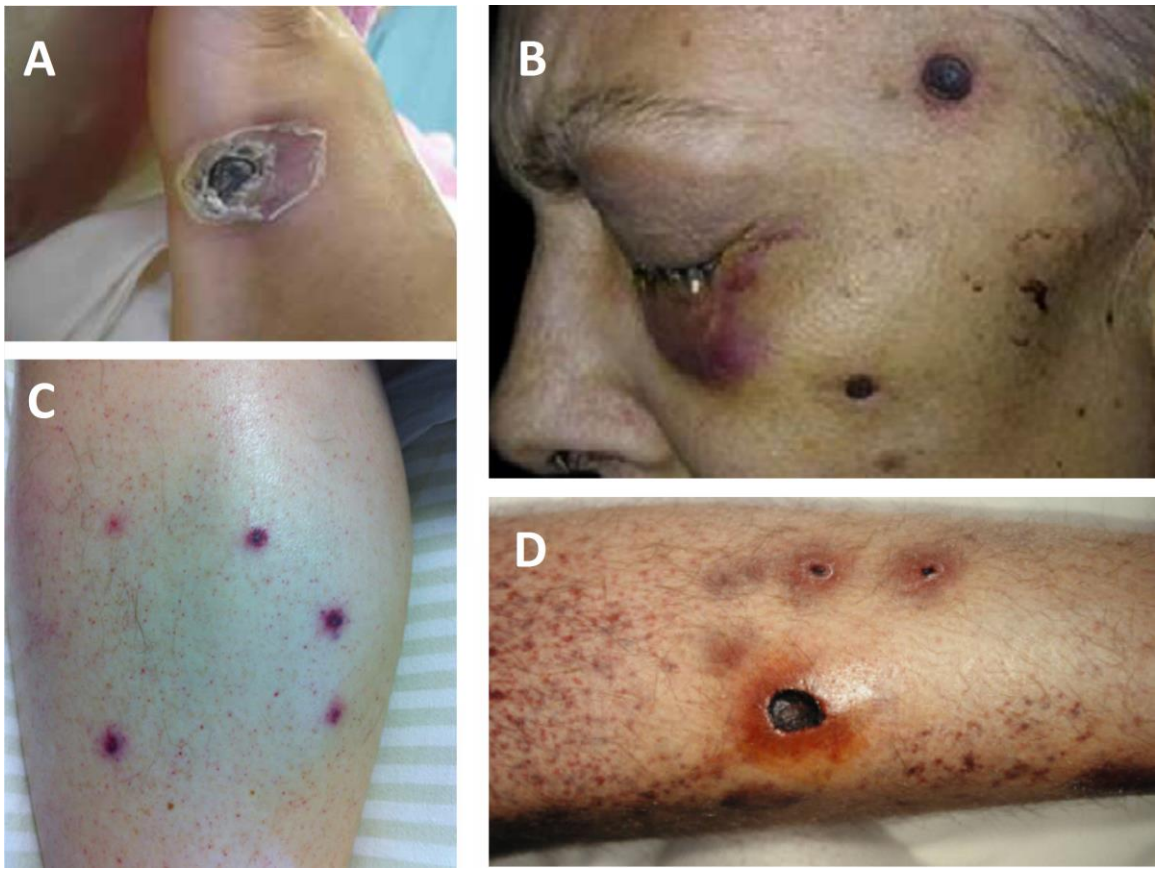

Figura 1. Lesões necróticas e inflamatórias causadas por *Fusarium* spp. reportados em diferentes trabalhos da literatura. Fonte: A) Nucci *et al.*, 2013; B) Galimbert*et al.*, 2012; C) Kollipara*et al.*, 2015; D) Galimbert *et al.*, 2012.

A forma clínica da fusariose é dependente do sistema imune do hospedeiro e da porta de entrada da infecção. Entre os hospedeiros imunocompetentes, a queratite e a onicomicose são as infecções mais comuns (Garnica e Nucci, 2013).

Varon *et al.*, 2014, avaliaram a relação entre infecções de pele e/ou onicomicose como reservatório de *Fusarium* spp. e o desenvolvimento de fusariose invasiva em pacientes humanos imunossuprimidos. Concluíram que os pacientes que desenvolveram a fusariose sistêmica no

hospital já portavam o fungo em lesões cutâneas e/ou onicomicoses quando foram admitidos no hospital.

A fusariose sistêmica é uma das doenças fúngicas mais devastadoras e com altíssimas taxas de mortalidade. Em virtude dessa importância, elucidar a capacidade de invasão *Fusarium* spp. em tecidos humanos como unha é uma prioridade nos dias atuais (Nucci *et al.*, 2013).

ONICOMICOSE FUSARIAL

A palavra onicomicose é derivado do vocabulário grego "onyx" que significa unha e "mico" que significa fungo, ou seja, esse termo é usado para descrever infecção por fungos em uma ou mais unhas. Onicomicose é responsável por aproximadamente 50% das onicopatias (Ghannoum *et al.*, 2000; Baswan *et al.*, 2017) e pode ser causada por dermatófitos, leveduras ou fungos filamentosos não dermatófitos (Faergemann e Baran, 2003).

Dermatófitos são os principais fungos causadores dessa infecção nas unhas, entretanto, casos de onicomicoses por fungos não dermatófitos estão emergindo, entre esses fungos, *Fusarium* spp. é o fungo mais frequentemente isolado nas infecções das unhas por não dermatófitos no Brasil (Guilhermetti *et al.*, 2007; Godoy-Martinez *et al.*, 2009). Além disso, estudos recentes relatam aumento súbito de *Fusarium* spp. em onicomicose em pacientes saudáveis além dos casos em imunocomprometidos, sendo que nestes já seria esperado por ser um fungo oportunista (Galletti *et al.*, 2015; Baswan *et al.*, 2017).

A onicomicose fusarial, diferentemente da onicomicose por dermatófitos, está associada à queixa clínica de inflamação, edema, dor e com acometimento dos tecidos moles (Figura 2). Porém pouco se sabe como é o comportamento do fungo durante o processo de colonização e infecção da unha. Além disso, a onicomicose fusarial apresenta resistência aos antifúngicos e recidivas a tratamentos (Garnica e Nucci, 2013; Galletti *et al.*, 2016).

É importante ressaltar que em pacientes com imunocomprometimento, a onicomicose fusarial é uma possível porta de entrada, pois é confirmado que o fungo pode migrar da unha e posteriormente causar uma disseminação sistêmica (Muhammed *et al.*, 2013; Nucci *et al.*, 2013; Varon *et al.*, 2014).

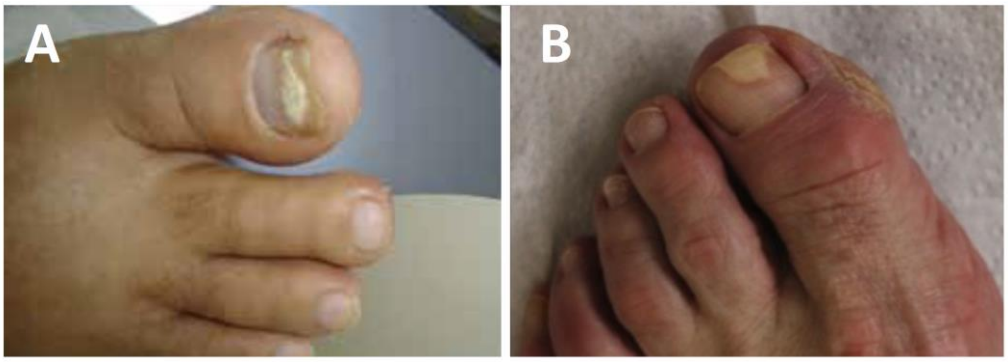

Figura 2. Onicomicose fusarial. Fonte: A) Nucci, 2013 B) Próprios autores.

UNHA

Em seres humanos, a placa de unha é completamente composta por células queratinizadas achatadas e fundidas na superfície superior da ponta de cada dedo (Walters e Flynn, 1983; Baswan *et al.*, 2017)

O tecido ungueal é composto por três camadas, dorsal, intermediária e ventral (Figura 3), sendo constituído, em sua grande maioria, por uma proteína fibrosa chamada alfa-queratina (Walters e Flynn, 1983; Baswan *et al.*, 2017). A camada dorsal é a mais externa e rígida, a camada intermediária é mais espessa, porém flexível e a camada ventral é composta por poucas camadas de células, sendo a camada mais fina. Por essas características as camadas apresentam diferentes resistências à penetração de agentes externos (Sowa *et al.*, 1995), sejam drogas ou micro-organismos.

Estudos mostram que a face ou camada ventral é mais susceptível ao crescimento de fungos do que a dorsal. Há uma grande quantidade de pesquisas que demonstram a capacidade de permeação de drogas nas unhas, contudo até este estudo não havia sido discutido a forma de penetração de fungos no tecido ungueal (Farren *et al*., 2004; Geyer *et al*., 2004)

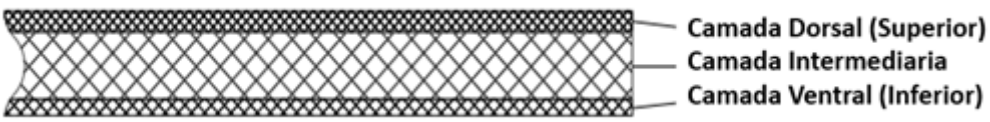

Figura 3. Representação esquemática das camadas das unhas humanas: Dorsal, intermediária e ventral.

FTIR-ATR

Entre as técnicas de espectroscopia vibracional, a espectroscopia no infravermelho via transformada de Fourier por reflexão total atenuada (FTIR-ATR) é amplamente utilizada no estudo de fungos (Salman *et al.*, 2011). É uma técnica óptica que permite obter os modos vibracionais da amostra de interesse, os quais são atribuídos a grupos químicos.

Existe grande quantidade de informações sobre as bandas obtidas a partir de espectros de amostras de fungos na medição *in vivo* (Salman *et al.*, 2011). Neste sentido, a FTIR-ATR é uma técnica com elevada, o que a torna uma ferramenta promissora e valiosa para a detecção de marcadores que identificam agentes patogênicos, podendo ser também empregado como diagnóstico (Salman *et al.*, 2011).

FTIR-ATR é amplamente utilizada em estudos análiticos, qualitativos e quantitativos por meio da vibração molecular e, consequentemente, estruturas das moléculas que compõem o material são obtidas (Sowa *et al.*, 1995; Salman *et al.*, 2012). Deste modo, é utilizada como ferramenta analitica na caracterização bioquímica de compostos bióticos devido às vantagens que apresenta, como sua sensibilidade para detectar pequenas mudanças estruturais das moléculas (Salman *et al.*, 2012).

JUSTIFICATIVA

A onicomicose, infecção fúngica das unhas, é uma onicopatia comum em humanos, atingindo aproximadamente 50% de todos os problemas das unhas e sua prevalência em relação a população mundial varia entre 2 e 18% (Baswan *et al.*, 2017). Esta micose é principalmente ocasionada por fungos dermatófitos, contudo o número dos fungos não dermatófitos está emergindo, entre esses *Fusarium* spp. é o fungo mais frequentemente isolado nas onicomicoses no Brasil, com maior incidência de *F. solani* e *F. oxysporum* (Galletti *et al.*, 2015). Sabe-se que a onicomicose fusarial é uma possível porta de entrada para a fusariose invasiva, pois o fungo pode migrar da unha e posteriormente causar uma infecção sistêmica (Muhammed *et al.*, 2013). Contudo ainda não é bem esclarecido a etiopatogenia fungo-hospedeiro. Sendo assim a caracterização dessa micose no tecido ungueal é de fundamental importância para compreensão da infecção, tratamento mais adequados e profilaxia da fusariose disseminada. Nesse contexto, é de grande interesse conhecer a forma de penetração de *F. oxysporum* na unha e sua permeação.

OBJETIVOS

GERAL

Avaliar a capacidade de invasão de *Fusarium oxysporum* em modelo *ex vivo*, simulando uma infecção de unha humana saudável.

ESPECÍFICOS

1. Padronizar um modelo de estudo *ex vivo* utilizando fragmentos de unha humana saudável para infecção com *F. oxysporum;*
2. Avaliar a capacidade de *F. oxysporum* de infectar unhas humanas *in vitro* utilizando a técnica de espectroscopia FTIR-ATR e histopatologia;
3. Estudar os efeitos da permeação de *F. oxysporum* em unha *ex vivo* utilizando a técnica de espectroscopia FTIR-ATR e histopatologia;
4. Verificar as diferenças quanto à capacidade de invasão *de F. oxysporum n*as diferentes camadas da unha saudável utilizando a técnica de espectroscopia FTIR-ATR e histopatologia;

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CAPÍTULO II

Artigo 1: FTIR-ATR spectroscopy for detection of *Fusarium oxysporum* invasion in *ex vivo* onychomycosis model

FTIR-ATR spectroscopy for detection of *Fusarium oxysporum* invasion in *ex vivo* onychomycosis model

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HIGHLIGHTS

1. *Fusarium oxysporum* has the capacity to invade healthy nail layers;
2. The infection by *Fusarium oxysporum* on onychomycosis is successful by permeating between the nail layers;
3. Bottom layers of nail are more prone to infection than upper layers.

ABSTRACT

The present study proposes to evaluate the ability of *Fusarium oxysporum* to permeate the sterile human nail, using them as its only nutritional resource. FTIR-ATR spectroscopy is considered an appropriate method of detection for molecular changes in cells. This study enabled to identify the presence of *F. oxysporum* in different nail layers and differences in the degree of invasion according to the side of infection, revealing that the bottom layer is more permeable due to its structural formation.

Keywords: FTIR-ATR, spectroscopy, onychomycosis, nail, *Fusarium.*

1 Introduction

Onychomycosis is the most frequent onychopathy in the worldwide (Baswan *et al.*, 2017). It is a common superficial or cutaneous fungal infection of the nails that accounts for approximately 50% of all onychopathies. Among the main clinical signs and symptoms are discoloration, nail layer thickening, and onycholysis. Three groups of fungi are responsible by

onychomycosis: filamentous fungi dermatophytes, non-dermatophyte molds (NDMs) and yeasts (Galletti *et al.*, 2015; Baswan *et al.*, 2017).

Dermatophytes are the most frequent etiological agent of onychomycosis, nevertheless cases of onychomycosis due to NDM are emerging, among which *Fusarium* spp. seems to be the most frequently isolated NDM in nail infections (Galletti *et al.*, 2015). Recently studies reported an important increases in onychomycosis by *Fusarium* spp. and it has been observed frequently in health patients, beyond the immunocompromised. Clinically a fusarial onychomycosis can cause pain, discomfort, an elevated sensation and pruritus (Guilhermetti *et al.*, 2007; Tardivo *et al.*, 2015; Baswan *et al.*, 2017). *Fusarium* spp. affects the great nail after trauma or dystrophic abnormalities and can be associated to others predisposing conditions of immunosuppression. Many *Fusarium* species have an invasive potential and shown lack of responsiveness to antifungal agents (Muhammed *et al.*, 2013; Varon *et al.*, 2014; Shah *et al.*, 2016). We have previously demonstrated the effects of compounds produced by *Fusarium* on tissue. It was proved metabolites from this fungus have ability to permeate the skin and cause undesirable toxic effects, such as inflammatory responses, necrosis, apoptosis and disorganization of the extracellular matrix even in the fungal absence (Marangon *et al.*, 2009; Melo *et al.*, 2014) however, the fungus effect on nail we not have evaluated yet.

In immunocompetent patients, fusarial onychomycosis as cutaneous lesions. These lesions besides of an aesthetic problem, they are clinically relevant due painful and inflammatory aspects (Guilhermetti *et al.*, 2007), and they require long-term treatment. On the other hand, in immunocompromised patients *Fusarium* spp. can cause severe disseminated infections, with poor options of antifungals and onychomycosis to be a portal of entry of fungus (Nucci *et al.*, 2013). Invasive fusariosis is one of the most devastating fungal diseases, this kind of infection is thought to be acquired by inhalation of conidia, with subsequent systemic dissemination. Despite nail and skin may be a gateway, since the fungi is able to migrate by tissue breakdown or onychomycosis to others sites (Muhammed *et al.*, 2013; Nucci *et al.*, 2013)

Nevertheless, despite its importance, fusarial nail infections are not well explicit, considering *Fusarium* spp are originally regarded as ambiental contaminants, is not clear its invasiveness into epithelial cells ability as well as if this genus is a true onychomycosis agent or if is just a secondary agent like others contaminant fungi.

The Fourier Transform Infrared (FTIR) spectroscopy with Attenuated Total Reflection (ATR) is a method that offers a large information about the spectral bands obtained from living cells combined with infrared spectroscopy features like sensitivity, speed, low expense and simplicity. It is a promising method and valuable tool for detection of pathogens, including

fungi (Salman *et al.*, 2011; Salman *et al.*, 2012). Here we intend the use of FTIR-ATR, for the first time, allied to histopathology to elucidate the pathogenesis of fusariosis. Thus, the present study proposes to evaluate the invasion ability of *F. oxysporum* into sterilized human nail, using it as its only source of nutritional resource.

2 Materials and Methods

2.1 Fungi

This study was conducted with a NDM strain isolated from a patient suffering of onychomycosis which was preliminarly identified according its macromorphology: colony color and texture, border type, and radial growth rate, as well as its micromorphology: features of hyphae, conidiophorus and conidia (Larone, 1993). Following preliminary further molecular studies were performed for to confirm the identification as *Fusarium oxysporum* This isolated was deposited at Microbial Collections of Paraná Network- TAX online at Federal University of Paraná under number (CMRP 2915).

2.2 Ex vivo nail infection

This analysis was developed based on the technique described by Galletti *et al.*, 2015, with some modifications. One serie of sterilized nail fragments that were collected from the a same healthy female volunteer, approved by ethical committee under number no.615.643/2014 were placed on glass slides and 3 μL of *Fusarium* conidial suspension in sterile saline (0.85%) containing 1.2×10^7 conidia/mL in a Neubauer chamber and were pipetted over them. In a half of nail fragments fungi were deposited externally on dorsal (upper) nail face and another half on ventral (bottom) of nail layers. This set was incubated for seven days at 35°C, in a humid chamber. All experiments were carried out in triplicate and they were repeated six times in different days.

2.3 FTIR-ATR measurements

The measurements were performed using a Fourier Transform Infrared Spectrometer (BRUKER, Vertex 70V) coupled to equipped with diamond crystal ATR accessory (BRUKER, Platinum). The spectral range was 400-4000 cm^{-1} with 128 scans and a resolution of 4 cm^{-1}. A computer connected to the spectrometer makes the data acquisition via software (OPUS7.2), with ATR and background correction.

To evaluate the permeation of *F. oxysporum* in the dorsal and ventral layers of the infected nails, readings were made starting from the superficial layer opposite to the application

of the fungus, sequentially that layer was manually sanded with gentle movements every ~50µm and, next, this procedure was done until the last layer of the nail.

2.4 Histopathology

The contaminated nails and negative control (equal volume of saline, without fungus) were made histological sections, which were analysed, using the histopathology processing methods. The nails fragments were embedded in paraffin and continuous 4 mm sections were stained with Periodic Acid–Schiff (PAS) method, using standard procedures. The infection was monitored by light microscopy (Motic®, Hong Kong).

3 Results and discussion

Fusarium spp. is an opportunistic pathogen causing a high spectra of human infections from superficial to disseminated and severe forms. Onychomycosis are one of the most common cutaneous infections. The incidence of onychomycosis by *Fusarium* species is seen to be increasing (Galletti *et al.*, 2015; Varon *et al.*, 2014), but despite the clinical and epidemiological relevance, its physiopathology is speculative, since little is known about. According to literature, fusarial onychomycosis is usually associated to trauma or dystrophic abnormalities and some immunodeficiencies, however, increasing involvement of immunocompetent hosts, without traumatic action, has been documented (Guilhermetti *et al.*, 2007). In addition this fungus has a high potential for virulence, since lesions in soft tissues are usually inflammatories and necrotic (Nucci *et al.*, 2013) suggesting high invasive capacity. However, to our knowledge there are few research that show etiopathogeny of *Fusarium* on human nail (Galletti *et al.*, 2015; Baswan *et al.*, 2017).

To better understand this process, it is important highlight the human nail is comprised of four main structures: the proximal nail fold, the nail matrix, the nail bed and the hyponychium (Farren *et al*., 2004). These structures together form the nail layer, which are flat, rectangular, translucent and keratinised and the epithelial cells into the nail layer are closely packed, adherent. Moreover, the nail is organized in three tightly bound layers: dorsal, intermediate and ventral (Baswan *et al.*, 2017).

PAS staining revealed the ability of *F. oxysporum* to permeate the human nail causing disorganization, as shown on Figure 1B. Furthermore, it was possible to observe that *F. oxysporum* penetration was different for the two nail layers (dorsal and ventral), invading more the ventral than dorsal layer, Figure 1 (C and D), respectively. These nail layers present different

morphological and biochemical compositions, and such characteristics allow differentiated permeations. The dorsal layer is considered the barrier limiting the rate of permeation when compared with other layers. Analysis of the biochemistry of nail layers suggests the interpretation of dorsal layers being the primary barrier for nail permeation (Baswan *et al.*, 2017). The greater fusarial permeability by ventral layer, observed in Figure 1C, should be attributed to composition of this layer, since it is made of soft keratin, and is laid down towards the middle of the nail bed, our results are entirely in agreement with the literature (Baswan *et al.*, 2017; Farren *et al.*, 2004). Cells of this side of layer emerge from the nail bed and are an easy target for invasion and consequently for the disease.

In order to amplify the histopathology informations the FTIR-ATR spectroscopy technique was successful to complementing the permeation analyzes. This technique has already been described aiming to detect the presence of fungus in the different samples (Salman *et al.*, 2011). Salman *et al.*, 2011 have proved the potential of FTIR-ATR spectroscopy for supply specific spectra of *F. oxysporum* samples, enabling good classification on the level of fungal isolates. In current study, this fungus has been detected into the health human nail fragment. For this, all the FTIR-ATR spectra were normalized by the total area of itself spectrum already with baseline connection for the best analyzes the spectra were divided in two regions (3000-2800 cm^{-1}) and (1780-850 cm^{-1}). Peaks were obtained from the acquired the spectra separately in the readings of *F. oxysporum* and from the healthy nail identified and are described in Table 1.

Comparing with Salman, *et al.* (2011) finding that identified *F. oxysporum* by FTIR-ATR spectroscopy, our results showed regions of higher frequency of spectrum, which is composed primarily by lipids resulting from the stretching of asymmetric and symmetric of CH_2. The region of lower frequency is mainly composed by amide I due to C-O and C-C stretching, besides carbohydrates and nucleic acids (Figure 2A), characterizing *F. oxysporum* by FTIR-ATR spectroscopy.

Figure 2B shows the spectra of the human nail layers, used as control before the infection by *F. oxysporum*. The numbers indicate the main identified peaks and are described in Table 1. The spectrum of the healthy human nail has a range of higher frequency, just as the fungus, for the spectra referring to the lipids, amide I and amide II, being distributed in a non homogeneous way over the nail layers (Sowa *et al.*, 1995). Highlighting the heterogeneity of human neil wherein the dorsal is the more rigid and outermost layer, the intermediate is the thickest and more flexible one while the ventral is made up of few layers of cells, being the thinnest layer (Sowa *et al.*, 1995). Due to the proteinaceous nature of nails, the region of lower

frequency of the spectrum is composed mainly by keratin, a protein found in the 1800–400 cm^{-1} spectral band (Shin *et al*., 2017). It is noted that the human nail presents low intensity in the region between 1140-950 cm^{-1}, region of high intensity in *F. oxysporum* spectrum, which is mainly attributed to glycogen, carbohydrate and nucleic acid vibrations, components of its metabolismo Salman, *et al*. (2011).

To evaluate the permeation of *F. oxysporum* in nail by FTIR-ATR, it was manually sanded every ~50μm on the side opposite to the infection and, next, this procedure was done until the last layer of the nail (Figure 3). Figure 4 shows the spectra of *F. oxysporum* in human nail and it is possible to note the presence of characteristic band for *F. oxysporum* in 1028 cm^{-1}, in the ventral (Figure 4C) and dorsal layer (Figure 4D) before being sanded, indicated by arrows. Interestingly, regardless of which side the nail was infected, the fungus was able to grow around the nail. This fact is observed when analyzing in Figure 4 where the spectrum (between 1140 - 950 cm^{-1}) of the fungus is evidenced by arrows at L_0.

Comparing the spectra *F. oxysporum* control with the fungus spectra with the nail, we notice spectral changes that are not characteristic of the infected nail or pure fungus, especially a band appearing at ~ 1750 cm^{-1}. This band (~ 1750 cm^{-1}) observed in Figure 4 (C and D), in both dorsal and ventral infection, may be related to the presence of -C = O in the initial layer of infection (Salman, *et al*., 2011). Recent studies show that fungi are able of causing infection structured in the form of biofilm, and according to Gupta *et al*., (2016) onychomycosis is related to on biofilm form. In this sense, is razonable think this band evidenced ~ 1750 cm^{-1} may be related the presence of biofilm on infected nail, confirming the suggestion of Gupta *et al.* (2016). Since this band only appears in this situation, not being observed when analyzing the fungus alone, fungus in culture medium, only culture medium, saline and healthy nail (date in the show). This idea is reinforced by statement that in the presence of biofilm, the extracellular matrix of filamentous fungi showed diverse composition consisting of proteins, polysaccharides and lipids (Ząbek *et al.*, 2017).

In relation to *F. oxysporum* spectrum on the infected nail, the presence of fungus was differentiated among layers L_2 to L_5, since the fungus graphic was only evident in layer L_5 when it has been inoculated by ventral layer, fact that this does not occur when the inoculum was put in contact to nail by the dorsal layer. Thus, the results show that *F. oxysporum* permeated more easily through the ventral layer of the nail. These data corroborating with histopathological analysis.

Thus, our data support that *F. oxysporum* use the nail as the only source of nutrient, and the fungus is able to use the physiological characteristics of nail to penetrate and infect the

human nail preferably by the ventral layer. This nail region consists of a few layers of cells, reinforcing the opportunistic profile of this genus. This fact is in agreement and explicates the symptoms observed in onychomycosis by *Fusarium* spp., besides it may justified the nail as a portal of entry this fungus to invasive fusariosis, which is a fatal infection.

4 Conclusion

In current study, using FTIR-ATR spectroscopy in tandem with histopathology we were able to prove, with confidence, the invasiveness of *F. oxysporum* into health human nail *ex vivo*. It was possible to confirm our previous finding that this fungus is able to use nail as a single source of nutrients, in addition that it can invade the healthy nail by itself. Besides, we showed differences in the degree of invasion in the nail by the fungus according to the side of infection start, revealing that the bottom layer is more permeable, probably due to its structural formation.

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Conflict of interest

The authors declare that they have no conflict of interests.

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Table 1. Peak assignment of the FTIR-ATR spectra of *F. oxysporum* and human nail (Sowa et al., 1995; Salman et al., 2011; Salman et al., 2012; Shin et al., 2017)

Band assignment	Wavenumber (cm^{-1})			
	F. oxysporum	Human nail		
		Dorsal	Intermediate	Ventral
$\nu_{as}(CH_2)$: Lipids	2920	2920	2920	2920
$\nu_{s}(CH_2)$: Lipids	2850	2850	2850	2850
ν(C=O): Amide I	1642	1642	1642	1642
Amide II	1553	1537	1513	1537
$\delta(CH_2)$ $\delta(CH_3)$	-	1456	1456	1456
ν(C-N): Cytosine guanine	1375	-	-	-
Amide III	1230	1230	1230	1230
ν(C-O)ν(C-C): Glycogen	1151	-	-	-
Skeletal (CC) of DNA	1074	1072	1072	1072
δ (CH)	-	1030	1030	1030
ν(C-O): Glycogen	1028	-	-	-

ν, stretching mode; ν_s, symmetric streching; ν_{as}, asymmetric streching; δ, bending.

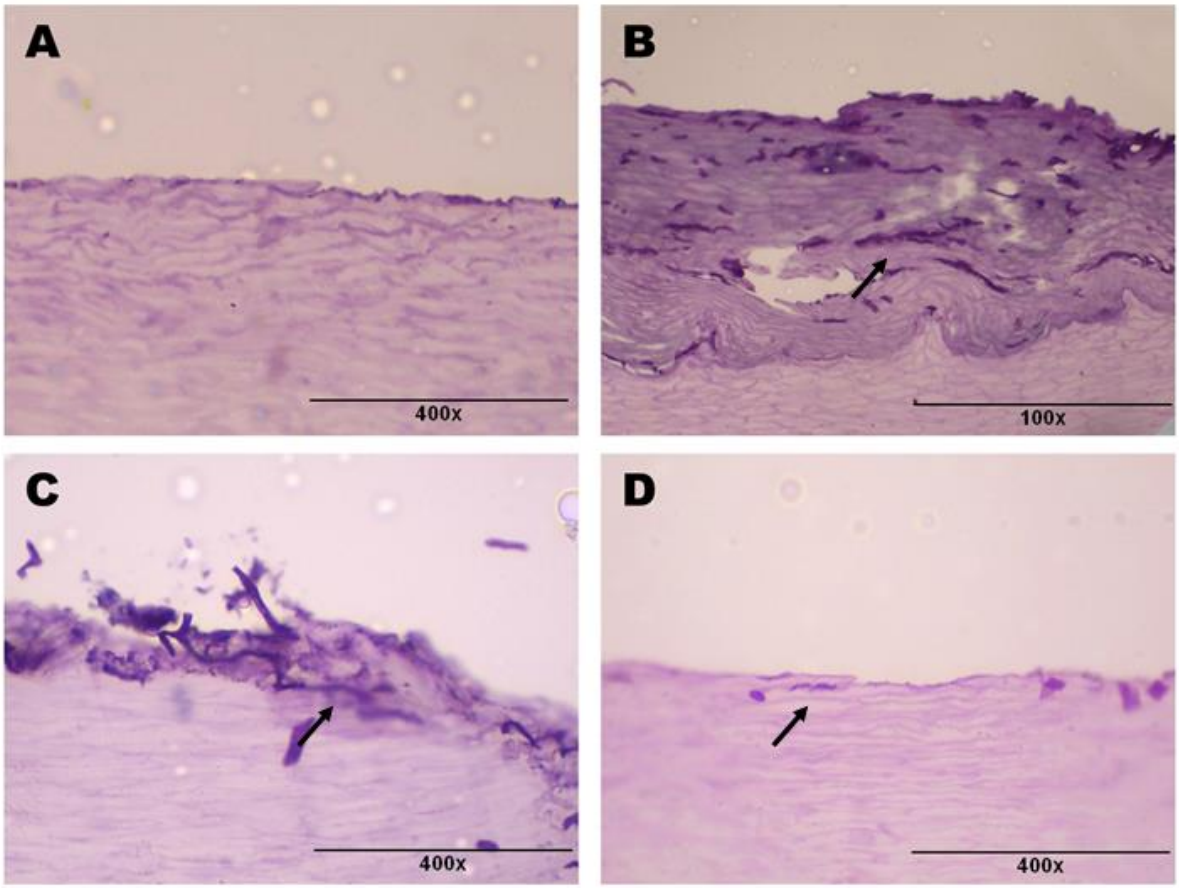

Figure 1: Histological sections of nail infected with 1.2×10^7 conidia/mL of *F. oxysporum*. A) Nail, ventral layer, without fungal (negative control); B) Disorganization caused by the fungus in the nail in ventral layer; C) Nail with fungal hifas reaching deeply (ventral) the corneal layer; D) Nail with fungal hyphae into superficial nail layer (dorsal). Images were performed seven days after incubation. Light microscopy.

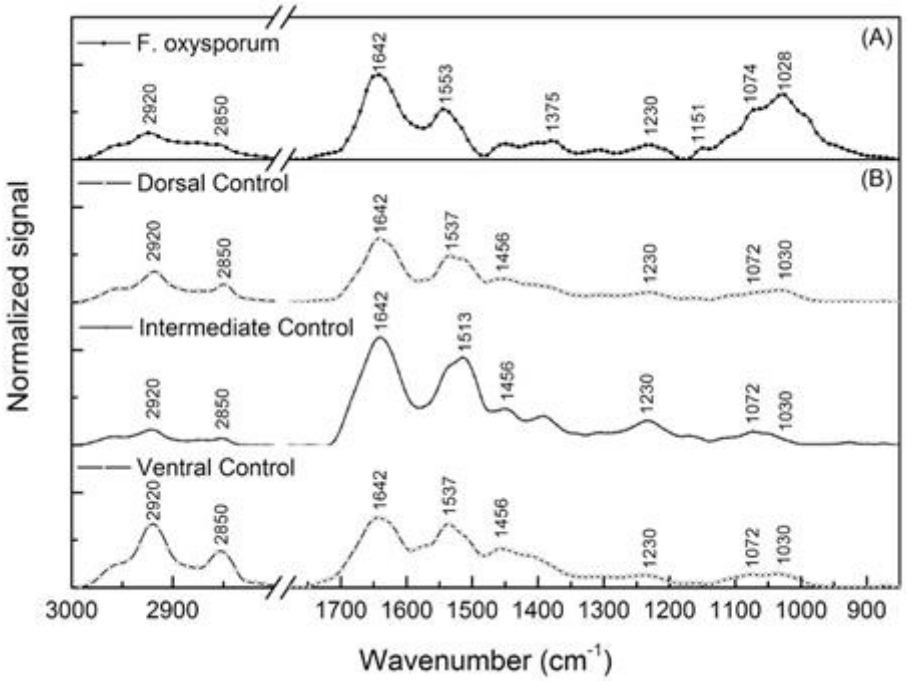

Figure 2. Obtained through FTIR-ATR spectra. (A) *F. oxysporum*; (B) Layers of the human nail.

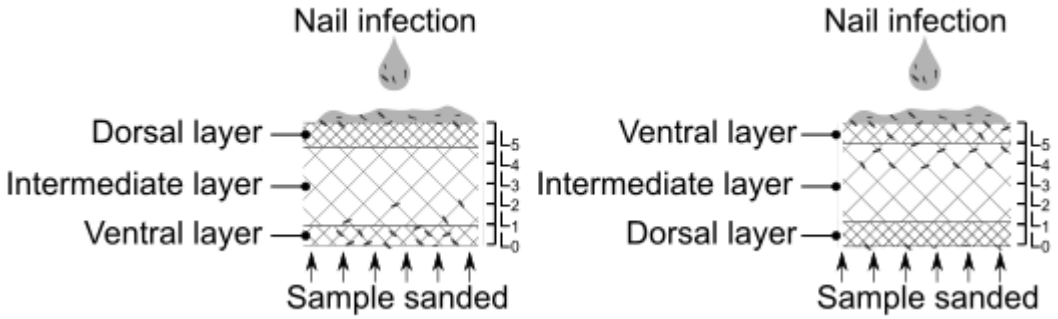

Figure 3. Design of procedure to obtain the FTIR-ATR spectrum. L_0 is the initial sample thickness (μm) and from L_1 to L_5 are the depths after the following removal of ~50μm from the side opposite to the infection.

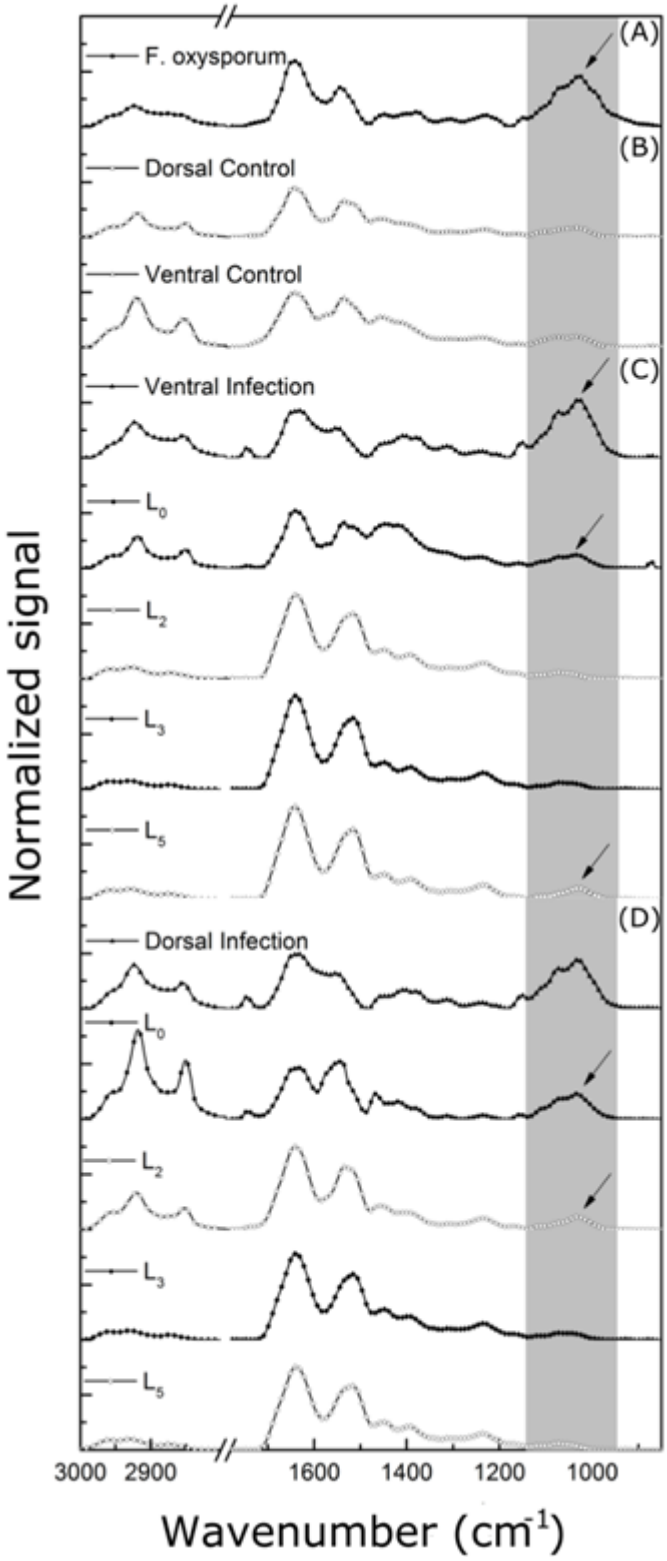

Figure 4: FTIR-ATR spectra of A) Only *F. oxysporum*; B) Only healthy human nail (dorsal and ventral controls); C) *F. oxysporum* infection in the ventral layer (ventral infection); D) *F. oxysporum* infection in the dorsal layer (dorsal infection). L_0: first measure without sanding the nail layer on the opposite side of the initial infection; L_1 to L_5: consecutives measure sanding the nail every ~50 μm.

CAPÍTULO III

CONCLUSÃO

O presente estudo sobre *Fusarium oxysporum* infectando unha humana em modelo *ex vivo* demonstrou que:

1. O fungo possui a capacidade de propagar-se em unha humana saudável;
2. Este estudo, por meio da espectroscopia FTIR-ATR em paralelo com a histopatologia, concluiu que além de infectar a unha *F. oxysporum* também é capaz de invadir a lâmina ungueal ;
3. *F. oxysporum* apresentou distintos perfis de invasão nas diferentes camadas;
4. O fungo exibiu melhor habilidade de crescimento e invasão quando colocado em contato com a unha através da camada interna;
5. A maior infecção no lado interno da unha também foi caracterizada pelo espessamento e distrofia das camadas celulares da camada interna;
6. Esse estudo colaborou para compreensão da interação que ocorre entre *F. oxysporum* e unha humana;
7. É possível concluir que *F. oxysporum* utiliza a lâmina ungueal como substrato para sobrevivência e consecutivamente ocasiona a onicomicose.

PERSPECTIVAS FUTURAS

Existe a necessidade de pesquisas adicionais sobre as interações entre *Fusarium oxysporum* e a lâmina ungueal, associando dessa etiopatogenia a técnica de FTIR-ATR. No estudo de infecções os fungos são tradicionalmente entendidos como micro-organismos planctônicos, no entanto já há indícios da formação de biofilme em unha. Dessa forma, faz-se necessário pesquisas sobre a formação biofilme em onicomicose fusarial, informações essas que seriam úteis para restabelecer o tratamento desta micose e a profilaxia da fusariose sistêmica. Visto que a infecção nas unhas é uma porta de entrada para infecções invasivas, principalmente em pacientes imunocomprometidos e apresentam altas taxas de mortalidade.