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ARTICLE

Fusarium spp. is able to grow and invade healthy human nails as a single source of nutrients

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Abstract Onychomycosis caused by *Fusarium* spp. is emerging, but some factors associated with its development remain unclear, such as whether this genus is keratinolytic. The main aim of the present study was to evaluate the ability of *Fusarium* to use the human nail as a single source of nutrients. We also performed an epidemiological study and antifungal susceptibility testing of *Fusarium* spp. that were isolated from patients with onychomycosis. The epidemiological study showed that *Fusarium* species accounted for 12.4 % of onychomycosis cases, and it was the most common among nondermatophyte molds. The most frequent species identified were *F. oxysporum* (36.5 %), *F. solani* (31.8 %), and *F. subglutinans* (8.3 %). Fluconazole was not active against *Fusarium* spp., and the response to terbinafine varied according to species. *Fusarium* was able to grow in vitro without the addition of nutrients and invade healthy nails. Thus, we found that *Fusarium* uses keratin as a single source of nutrients, and the model proposed herein may be useful for future studies on the pathogenesis of onychomycosis.

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Introduction

Onychomycosis is a common fungal infection of the nails that accounts for approximately 50 % of all onychopathies. Its prevalence in North America is 13.8 % [1], varying from 2 to 18 % in the world's population [2]. It can infect up to 50 % of people older than 70 years of age [3]. Dermatophytes have been described as the most frequent etiological agent of onychomycosis, but cases of onychomycosis that are caused by nondermatophyte molds (NDMs) are emerging, among which is *Fusarium* spp. [4]. Among NDMs, *Fusarium* spp. is the most frequently isolated fungus in nail infections in Brazil [5, 6].

Fusarium spp. infection is a serious problem because of its higher resistance compared with dermatophytes [7], and they are often considered secondary invasive species of the nail plate [8].

The pathogenesis of fusariosis in humans is still not completely clear, and few studies on onychomycosis have investigated this issue [9]. Thus, the present study utilized a novel model to investigate the pathogenesis of onychomycosis that is caused by *Fusarium* spp., demonstrating its ability to use the human nail as a single source of nutrients. We also performed an epidemiological study and antifungal susceptibility testing of *Fusarium* spp. that was isolated from patients with onychomycosis.

Materials and methods

Epidemiological study

We first performed an epidemiological study to determine the frequency of *Fusarium* and species of this genus in patients with onychomycosis. The study included data from all

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patients who attended the Teaching and Research Laboratory of Clinical Analysis (LEPAC), Division of Mycology, Universidade Estadual de Maringá (UEM), between January 2003 and December 2013 with suspected onychomycosis. This descriptive, retrospective, cross-sectional, observational study was performed in Maringá, Paraná, Brazil, and approved by the Research Ethics Committee of UEM (approval no. 615.643/2014).

Fungal strains

Fungi were isolated from nail samples of patients who attended the LEPAC in 2013. The inclusion criteria for the study were patients with paronychia and ungueal alterations that were compatible with onychomycosis. Their nails were disinfected with 70 % alcohol, and the subungueal lamina was scraped with a sterilized curette. The sample was placed in a sterilized Petri dish. Under direct microscopy, nail scrapings were clarified with 40 % potassium hydroxide (KOH) plus Evans blue. Nail scrapings were cultivated in three tubes that contained Sabouraud dextrose agar (SDA) and three tubes that contained Mycosel. The identification of fungi was performed using classic methods, including the examination of colonies and microscopic morphology, according to De Hoog and Guarro [10]. We followed the laboratory criteria that were described by Guilhermetti et al. [5]. The isolated and identified fungi were maintained in a freeze-dried state in the Mycology Collection of the Laboratory of Medical Mycology, UEM, Brazil. We selected a strain from each of the three most frequent species to perform the tests.

In vitro nail infection

This assay was performed based on the technique described by Rashid et al. [11], with some modifications. Glass slides were mounted in a sterilized Petri dish that contained a piece of filter paper soaked with distilled water to ensure a humid environment. Three clinical isolates of *Fusarium* spp. were included in this study (*F. solani*, *F. oxysporum*, and *F. subglutinans*). The isolates were subcultured on potato dextrose agar (PDA) for 7 days at 25 °C. The cellular density was adjusted to 5×10^4 conidia/mL in 0.85 % sterile saline using a Neubauer chamber. The sterilized nail fragments that were collected from healthy female volunteers were then placed on glass slides and 50 μL of conidial suspension (5×10^4 conidia/mL) were pipetted over them. They were then incubated for 30 days at 25 °C. Nail fragments without the suspension were also incubated as a negative control. The growth of the fungus in the nail was confirmed by SDA culture. Nail damage was observed through a magnifying glass, and photographs were taken. After incubation, the nail fragments were clarified with 40 % KOH, stained with Evans blue, and observed under a light microscope. The histological analysis of nails was performed according to Rashid et al., and nails were stained with Grocott [11].

In vitro susceptibility testing

In vitro antifungal susceptibility testing was performed using a microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) M38-A2 protocol [12], with some modifications. The antifungal agents included terbinafine (0.015–8 μg/mL) and fluconazole (0.125–64 μg/

Table 1 Epidemiological data from patients who attended the Teaching and Research Laboratory of Clinical Analysis at Universidade Estadual de Maringá (UEM) between January 2003 and December 2013

Year	Cases of onychomycosis	Total of cases due to *Fusarium* spp. (%)	*F. oxysporum* (%)	*F. solani* (%)	*F. subglutinans* (%)	Other *Fusarium* spp.[a]
2003	360	27 (7.5)	10	4	0	13
2004	128	15 (11.7)	6	2	0	7
2005	117	9 (7.7)	3	1	0	5
2006	124	17 (13.7)	9	1	2	5
2007	145	19 (13.1)	10	7	1	1
2008	160	24 (15)	8	10	6	0
2009	151	21 (13.9)	12	6	0	3
2010	81	11 (13.6)	3	6	1	1
2011	79	14 (17.7)	3	6	0	5
2012	71	9 (12.7)	2	6	0	1
2013	131	26 (19.8)	4	12	6	4
Total	1,547	192 (12.4)	70 (36.5)	61 (31.8)	16 (8.3)	45 (23.4)

[a] Other species: *F. incarnatum* (6.2 %), *F. sacchari* (3.1 %), *F. verticillioides* (4.7 %), *F. dimerum* (2.6 %), *F. chlamydosporum* (2.1 %), *Fusarium* spp. (1.6 %), *F. proliferatum* (1 %), *F. napiforme* (0.5 %), *F. tabacinum* (0.5 %), (45/192)

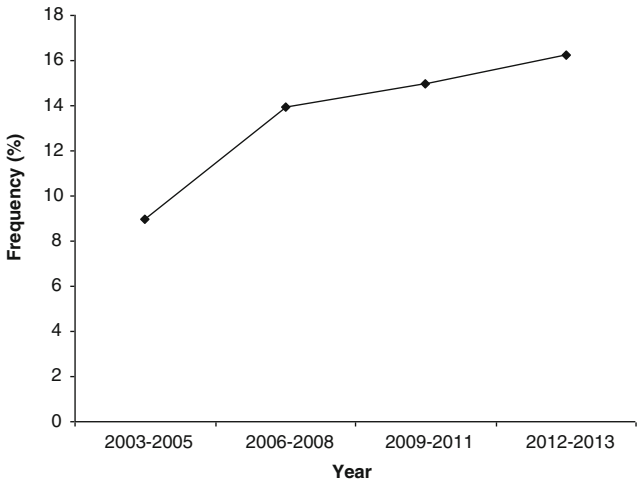

Fig. 1 Frequency of onychomycosis that was caused by *Fusarium* spp. over a period of 10 years among patients who attended the Teaching and Research Laboratory of Clinical Analysis at Universidade Estadual de Maringá (UEM)

mL). The strains of *Fusarium* spp. were grown on PDA for 7 days at 25 °C. They were then harvested with 0.85 % sterile saline. The colonies were gently scraped, and the conidia were counted in a Neubauer chamber. The inoculum was adjusted to a final concentration of 5×10^4 conidia/mL. The minimum inhibitory concentrations (MICs) were visually determined after 48 h of incubation at 35 °C. *F. solani* INCQS 40099 and *Candida albicans* ATCC 90028 were used as reference control strains in this assay. This test was performed in duplicate.

Results and discussion

There have been increasing reports of cases of nail infection caused by *Fusarium* spp. in both immunocompetent and immunosuppressed patients [5, 13]. Studies that investigate the pathogenic process of *Fusarium* species that cause onychomycosis are important. We first performed an epidemiological study to determine the frequency and identify the main species of *Fusarium* that cause onychomycosis in our region (Table 1). We then evaluated the potential for in vitro infection with *F. oxysporum*, *F. solani*, and *F. subglutinans* in sterile healthy human nails.

We found that, during a period of 10 years (2003–2013), *Fusarium* spp. was isolated in 12.4 % of the cases (Table 1), and it was the most frequently isolated NDM. Importantly, we observed an increase in the number of cases of onychomycosis caused by *Fusarium* spp., and this value increased over the years, reaching 19.8 % in 2013 (Fig. 1). The frequency of onychomycosis caused by *Fusarium* spp. in our region is higher than in other countries. Colombia, the United States, and Sri Lanka have reported rates of 6, 6.9, and 6.25 %, respectively [1, 14, 15]. Moreover, all patients who were diagnosed with onychomycosis caused by *Fusarium* spp. experienced pain, with nails that were hyperkeratotic and presented deformities and paronychia, according to observations in the literature [5].

Figure 2 shows abundant in vitro growth, within a few days, of three species of *Fusarium* on sterile healthy human nail fragments as the only organic source of nutrients. *F. solani* presented the most rapid and highest growth. Furthermore, it caused white spots that are characteristic of onychomycosis (Fig. 2b).

The microscopic analysis of growth on the nails revealed evidence of hyaline, thick, and septate hyphae in the entire nail, chlamydospores, and microconidia (Fig. 3). The presence of chlamydospores and septate hyphae with large diameters in direct examination is considered predictive of onychomycosis that is caused by *Fusarium* spp. [13]. The histological analysis showed that, beyond macroscopic and microscopic growth on

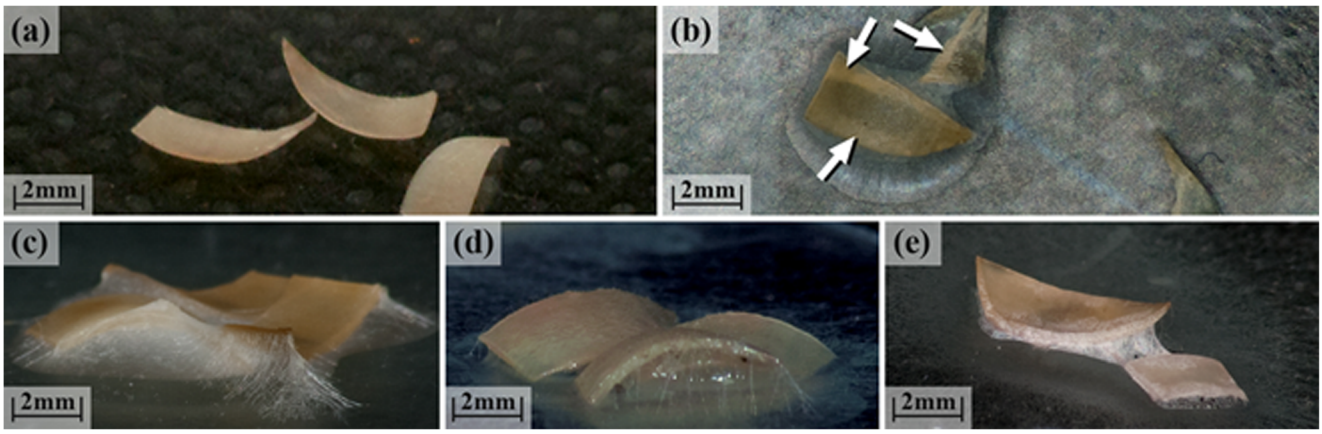

Fig. 2 Macroscopic growth of *Fusarium* spp. (initial concentration 5×10^4 conidia/mL) on nail as a single source of nutrients. **a** Sterile healthy human nail fragments as negative control. **b** Visible growth showing damage caused by *Fusarium* spp. in the nail, characteristic of onychomycosis; the *arrows* denote yellow–white discoloration that appeared after 15 days of incubation at 25 °C. Macroscopic aspects of fungal growth without nutrients added: **c** *F. solani*, **e** *F. oxysporum*, and **d** *F. subglutinans* observed after 3, 6, and 9 days, respectively

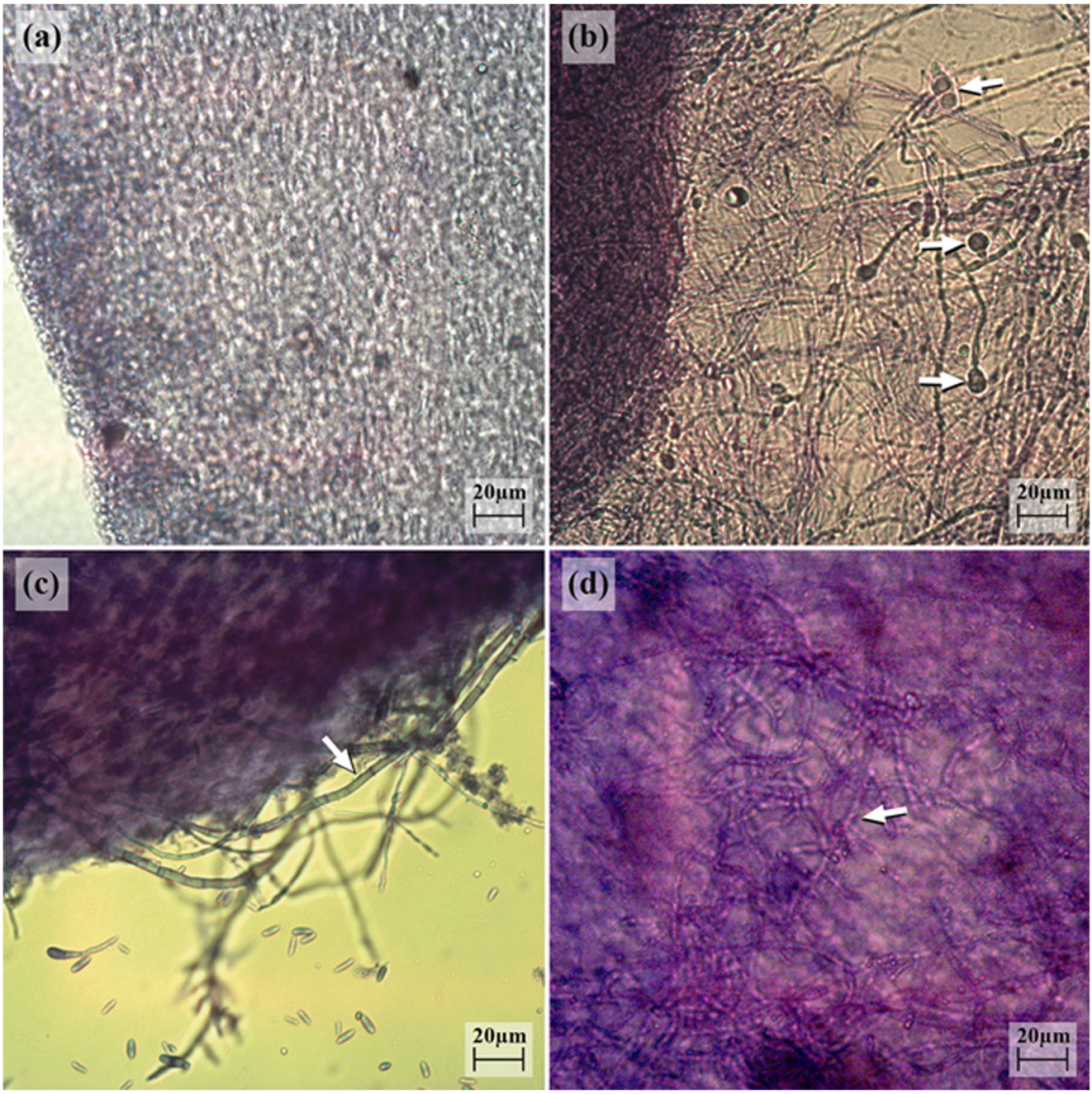

Fig. 3 Nail fragments clarified with 40 % potassium hydroxide (KOH) and stained with Evans blue, showing microscopic aspects of *Fusarium* spp. (initial concentration of 5×10^4 conidia/mL) on nails as a single source of nutrients. **a** Uninfected nail. **b** Hyphae, microconidia, and chlamydospores of *F. solani*. Thick and septate hyphae of **c** *F. subglutinans* and **d** *F. oxysporum*. The nails were observed under a light microscope (400× magnification)

the nail, *Fusarium* spp. was able to invade the nail plate. This event occurred at different times for each species. Figure 4 shows that hyphae appeared inside the nail plate at 3, 6, and 9 days for *F. solani*, *F. oxysporum*, and *F. subglutinans*, respectively. Therefore, this model of in vitro nail infection confirmed that *Fusarium* is able to use the nail as a single source of nutrients, and it can invade the nail by itself (i.e., they are not necessarily secondary invaders of the nail). These findings are consistent with those of Anbu et al. [16], who found that *F. solani* can grow in medium with chicken feathers (a source of keratin) as the sole carbon and nitrogen source.

The present results show that *Fusarium* spp. is able to destroy the stratum corneum and use it as the single source of nutrients for growth, suggesting that *Fusarium* spp. is also a keratinolytic fungus like dermatophytes. Therefore, the model proposed herein may be useful for studies on the pathogenesis of onychomycosis that is caused by *Fusarium* spp.

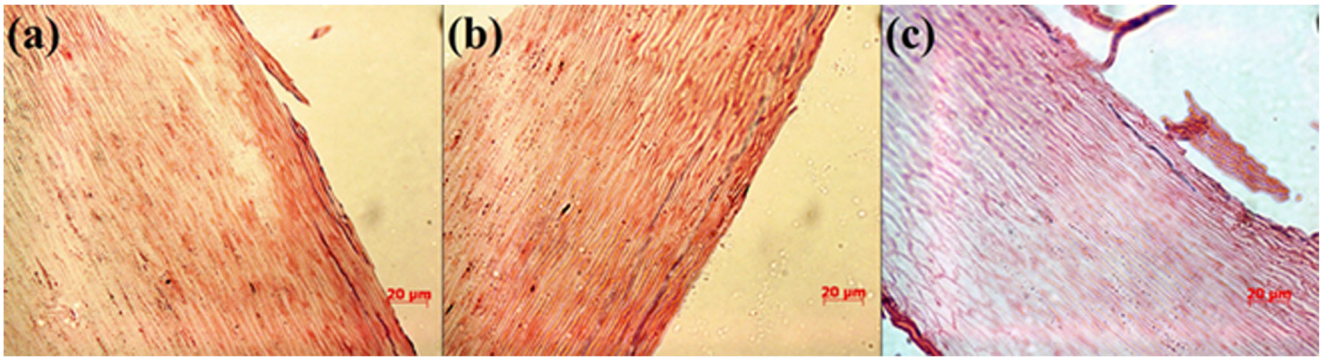

Fig. 4 Histological section of nail infected with 5×10^4 conidia/mL of: **a** *F. solani*, **b** *F. oxysporum*, and **c** *F. subglutinans*, revealing hyphae inside the nail plate. This analysis was performed 3 (**a**), 6 (**b**), and 9 (**c**) days after incubation. Light microscopy (Gomori, 400× magnification)

With regard to the susceptibility of *Fusarium* spp. to antifungal agents, all of the strains required high MICs for fluconazole (Table 2). Similar findings have been reported by Pujol et al. [17] and Bueno et al. [7]. Fluconazole is effective against some yeasts [9]. Paronychia is also observed in onychomycosis that is caused by yeasts, and the identification of etiological agents in these cases is indispensable to avoid treatment failure. Only *F. solani* had diminished susceptibility to terbinafine (Table 2). Lower MICs for terbinafine against these species suggest its possible use in vivo [7]. In vitro susceptibility testing and the identification of pathogens are important when choosing the proper treatment for onychomycosis. Attention should be paid to correct identification, including signs and symptoms (e.g., paronychia and pain) and the presence of certain structures (e.g., thick and septate hyphae and chlamydospores) in direct microscopy, which may be reliable criteria for the diagnosis of onychomycosis that is caused by *Fusarium* spp.

Conclusions

We demonstrated that the frequency of onychomycosis that is caused by *Fusarium* spp. has increased in our region of Brazil. We found that *Fusarium* is able to use the nail as a single source of nutrients, and it can invade the healthy nail by itself. This ability varies according to species, suggesting different mechanisms of invasion or at least differences in invasion kinetics. More studies are necessary to elucidate the pathogenesis of nail invasion by *Fusarium*. The model of nail infection proposed herein may be useful for future studies on the pathogenesis of onychomycosis.

Table 2 Minimum inhibitory concentration (MIC) of antifungal agents against *Fusarium* spp.

Drug	MIC (μg/mL)		
	F.oxysporum	*F.solani*	*F.subglutinans*
Terbinafine	0.5	4	0.125
Fluconazole	≥64	≥64	≥64

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Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of UEM, approved with judgment number 615.643/2014, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

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