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Received 29 November 2024; accepted 15 January 2025
Available online 24 February 2025

https://doi.org/10.1016/j.abd.2025.01.001

In vitro characterization of biofilm produced by *Fusarium oxysporum*, an onychomycosis agent☆

Dear Editor,

Onychomycosis caused by Non-Dermatophyte fungi (NDMs), such as *Fusarium* spp., is more prevalent than previously thought, especially in warmer climates.[1] Furthermore, onychomycosis has currently been attributed to fungi organizing themselves into a biofilm form.[2] Biofilm is a complex microbial community, highly adhered to the nail and surrounded by a matrix that provides protection and antifungal resistance.[2,3]

The research group has been studying the genus *Fusarium* spp. as an agent of onychomycosis in immunocompetent hosts. The authors reported its high prevalence in the studied region, established clinical and laboratory criteria for this genus as a causal agent of onychomycosis, and determined the susceptibility profile to the systemic antifungals most commonly used in Brazil.[4] Later, the authors proved that *Fusarium* spp. uses nail keratin as a single source of nutrients[5] and began studies on the etiopathogenesis of fusarial onychomycosis based on an *ex vivo* model using sterile human nail fragments.[3]

More recently the authors reported, for the first time, that *Fusarium oxysporum* is able to form biofilm on the human nail as the only nutritional source.[6,7] Also, the authors describe the volatile organic molecule 2-Ethyl-1-Hexanol (2 EH) as a quorum-sensing component capable of modulating such biofilm.[8] These findings were relevant to confirm the etiopathogenesis of fusarial onychomycosis. However, it did not reveal the proper characteristics of the biofilm formed under nutritional support. Thus, the current study aimed to characterize the *in vitro* biofilm formation of *F. oxysporum*, evaluating its natural ability, during 7-days with controlled nutrient availability.

This study was conducted with *F. oxysporum* CMRP2925 isolated from a previously described onychomycosis case.[4] The isolate was reactivated to confirm its purity and identification, before assays, and was cultured on Sabouraud Dextrose Agar (SDA; DifcoTM, MI, USA) for 7-days at 25 °C. Biofilms were prepared according to Galletti et al.,[9] with some modifications. A suspension containing 1×10^7 conidia mL^{-1} was prepared in RPMI Medium 1640 (Gibco, NY, USA), with L-glutamine, sodium bicarbonate, 0.165 M 3-(N-morpholino) propanesulfonic acid (pH 7.2), and 2% glucose. This suspension was placed into 96-well flat-bottomed microtitration plates and incubated at 35 °C in a shaker at 110 rev min^{-1}, for 7-days. Every 24 h, the culture medium was renewed by removing 100 μL of old broth and adding the same volume of fresh RPMI. During the seven days, biofilms were evaluated under different aspects, as previously described.[6,9] Briefly, cell viability was assessed by counting Colony Forming Units (CFU), quantification of total biomass by Crystal Violet, metabolic activity by the reduction assay of tetrazolium salt, 2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-([phenylamino]carbonyl)-2H Tetrazolium hydroxide (XTT), characterization of the Extracellular Matrix (ECM), and visualization of biofilm structure by Scanning Electron Microscopy (SEM).

☆ Study conducted at the Universidade Estadual de Maringá, Maringá, PR, Brazil.

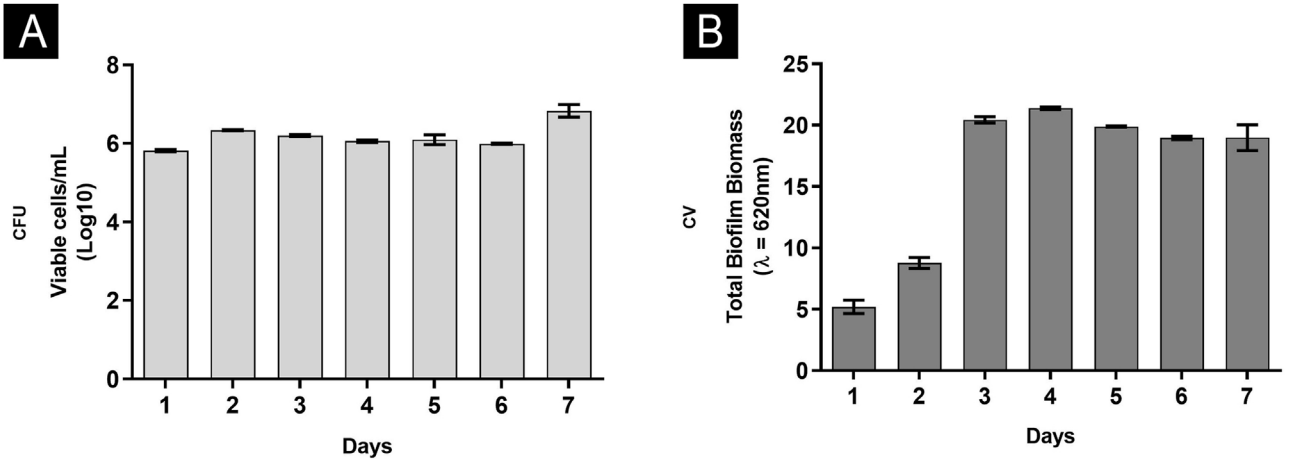

Fig. 1 Characterization of biofilm produced by *F. oxysporum* on flat-bottomed polystyrene plates from one to seven days, in RPMI medium. (A) Number of viable cells by counting Colony-Forming Units (CFU); (B) Total biomass by Crystal Violet staining (CV).

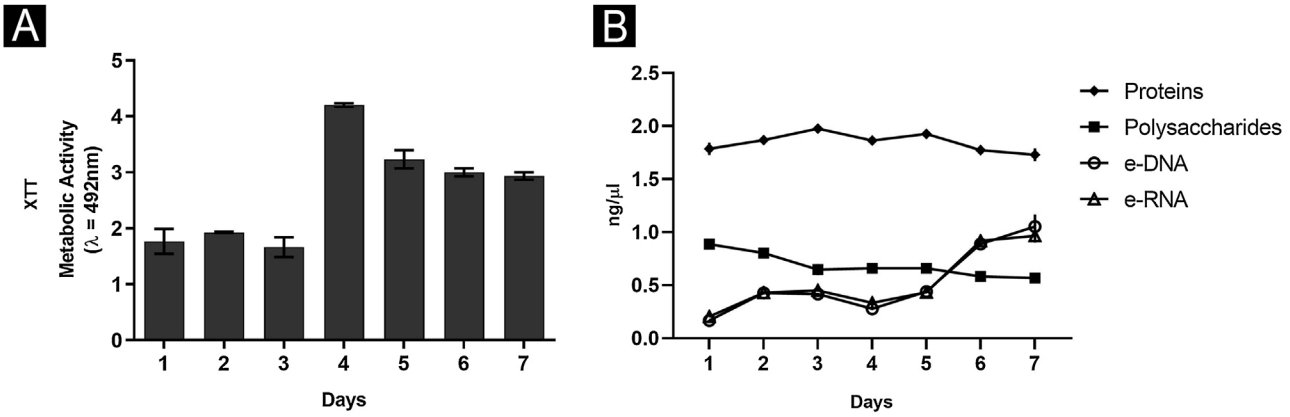

Fig. 2 Characterization of biofilm produced by *F. oxysporum* on flat-bottomed polystyrene plates from one to seven days, in RPMI medium. (A) Metabolic activity by XTT reduction; (B) Quantification of Extracellular Matrix (ECM): proteins, polysaccharides, extracellular DNA and RNA.

Overall, the biofilm's characteristics were similar, drawing a parallel between greater nutritional support with RPMI and nails only.[6] In both situations, it was possible to show that the third and fourth days were critical. In rich medium and neutral pH, the number of CFU remained constant at all times (Fig. 1A), since the first day, with an increase in metabolic activity on the third day, stabilizing from this moment. A similar profile was found for proteins and nucleic acids. Interestingly, polysaccharides were significantly consumed until the third day, continuing to decline gradually until the seventh day. On the other hand, using the nail as a nutritional source had observed an increase in CFU until the third day, a high metabolic activity only on the fourth day, while the increase in proteins and carbohydrates occurred late, corroborating the results of nucleic acids.[6] These differences can be attributed to the availability of nutrients, with daily renewal of the RPMI medium (food at will), and the fact that in the nail (restricted food), the fungus itself needed a period of adaptation.

In addition, a progressive increase in the amount of biomass was observed (Fig. 1B) between the first and third day, followed by stability, which seems to be associated with the consumption of polysaccharides (Fig. 2), a fundamental component during the development and maintenance of the biofilm. Taking into account that the CFU remained stable, this increase was attributed to the large production of ECM, in a rich environment. The increase in metabolic activity (Fig. 2) is reflected in protein and nucleic acid synthesis, mainly eRNA. Protein production appears to be crucial in the process of biofilm formation and support of the entire system.[6,10] This idea is reinforced by the high levels of XTT and eDNA, from the fourth day onwards, since this nucleic acid is needed in activities that demand cellular energy, such as the construction of a mature biofilm.[10] The biofilm structure begins by forming a cell monolayer and within seven days the highly complex three-dimensional structure was observed (Fig. 3A and B).

Thus, it is logical to assume that the ability of *F. oxysporum* to produce biofilm is intrinsic. The nutritional support seems just to facilitate the first hours of its formation, in addition to favoring the production of a greater amount of biochemical components associated with the maturation and stability of the biofilm. Therefore, the experience draws attention to the natural abilities of this fungus, pre-

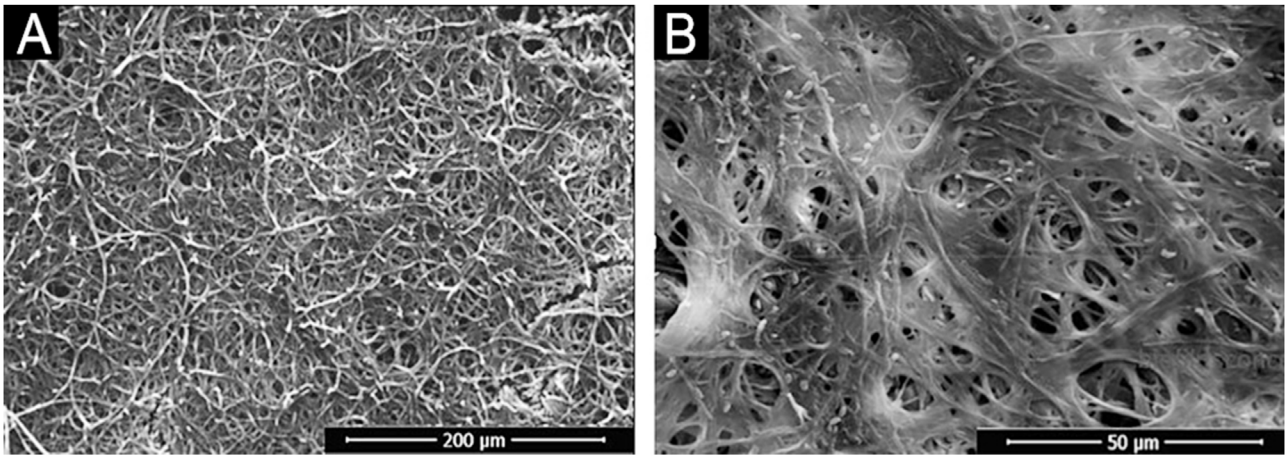

Fig. 3 SEM illustration of four-day *F. oxysporum* biofilm structure. (A) Highlighting the structural organization of the biofilm, the intertwining and compaction of the hyphae (500×); (B) Higher magnification, showing the extracellular matrix produced (2000×).

viously restricted to the environmental, agricultural, and animal interest. Probably, the natural selection induced by pesticides facilitated its adaptation to human tissues. The authors were able to show its virulence potential (here the efficiency in producing biofilm) is maintained regardless of the addition of nutritional support. This behavior therefore allows us to consider the genus *Fusarium* spp. as a primary pathogen of interest in dermatology.

Financial support

This work received partial financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) (Finance Code 001, through a scholarship awarded to ILEB); Conselho Nacional de Desenvolvimento Científico e Tecnológico – Brasil (Process number 3128262023-0 given to TIES) and INCT-CERBC, 2022, Process number 406645/2022-1.

Authors' contributions

Terezinha Inez Estivalet Svidzinski: Came up with the research idea, planned the experiments, and proofread them.

Isabella Letícia Esteves Barros: Came up with the research idea and planned the experiments; Performed the experiments and analyzed the data; Wrote the manuscript.

Flávia Franco Veiga: Performed the experiments and analyzed the data.

Conflicts of interest

None declared.

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Received 17 October 2024; accepted 6 November 2024
Available online 21 April 2025

https://doi.org/10.1016/j.abd.2024.11.005

Latent tuberculosis infection in patients with psoriasis using immunobiologicals: an observational study☆

Dear Editor,

Immunobiologicals are an increasingly important therapy in the context of inflammatory diseases, mainly in dermatology, with numerous approved medications.[1,2] However, the use of these agents is associated with immunosuppressant effects and, consequently, increases the risk of reactivation of latent infections, such as tuberculosis (TB).[2]

Recent guidelines recommend carrying out TB screening before starting treatment with any immunobiological agent, using the tuberculin skin test (TST) or interferon gama release assay (IGRA), as well as being repeated annually in high-risk patients. For patients who are not considered at high risk, this screening is particularly important for those receiving anti-tumor necrosis factor (TNF) agents.[3] Despite the recommendation, this is not done frequently in practice, so the objective of the present study was to evaluate the prevalence of TB infection (assessed via TST) in patients receiving immunobiological therapy for psoriasis.

This study was carried out at the Regional Hospital of Presidente Prudente, São Paulo, Brazil, and was started only after the project was approved by the Research Ethics Committee of the University of West Paulista (Protocol: 65581122.0.0000.5515 – December 18, 2023) and only those who signed an informed consent form were enrolled. Patients aged 18 years or older who were diagnosed with psoriasis, who were receiving immunobiological agents for at least 6 months, and who had undergone TST before the start of treatment were included. Patients with immunosuppressive diseases or a history of previous TB were excluded.

The TST result was considered negative when there was no induration formation or if it was smaller than 5 mm. The presence of an induration with a diameter greater than or equal to 5 mm when the previous TST was zero or an increase of 10 mm or more from the value of the previous TST, if it was positive, was considered to indicate a positive result. These values were used on the basis of the guidelines of the Brazilian Ministry of Health.[4,5] Although IGRA is a test with greater specificity in diagnosing latent tuberculosis infection (LTBI), it has a high cost and is currently available in Brazil through the Unified Health System (SUS) only for certain selected conditions.[6]

A total of 85 patients with psoriasis were evaluated and the immunobiologicals used were anti-Interleukin (IL) agents (77.6%), with 25 patients using ustekinumab, 30 using secukinumab and 11 using risankizumab, and anti-TNF agents (22.4%), with 18 patients using adalimumab and 1 patient using etanercept. In 46 patients (63%) there was concomitant use of methotrexate. Table 1 presents demographic and clinical characteristics of psoriasis patients. Table 2 presents the data on the characteristics of the patients with positive results for the second TST. No statistically significant associations were observed for any of the characteristics evaluated.

The second TST was positive, with a diagnosis of LTBI being made in 10 patients (11.7%), of whom 9 used an anti-IL agent (5 secukinumab, 3 ustekinumab, and 1 risankizumab) and only 1 patient used an anti-TNF agent (adalimumab). Despite the higher prevalence of LTBI in patients using anti-ILs, there was no statistical significance, probably because the vast majority of patients included in the study were using anti-ILs. This result is similar to a study also carried out in Brazil with rheumatological patients using anti-TNF, where the TST became positive in 12.3% of patients.[7]

Of the 10 patients who were positive on the second TST, 8 were negative on the first TST, suggesting that they may have acquired the infection while using immunobiological agents. This is probably due to the fact that the region where the study was carried out has a high prevalence rate of TB. Among the 9 positive patients who used anti-ILs, 4 had previously used an anti-TNF agent (1 infliximab and 3 adalimumab), which makes it impossible to infer which medication the positivity would be implicated in or whether it occurred due to the sum of the effects of the two therapies. It is important to highlight that none of the patients developed active TB.

The tuberculin skin test has certain limitations and may present false-positive results due to BCG vaccination and exposure to nontuberculous mycobacteria. The effects of BCG vaccination on TST results are smaller after 15 years. Therefore, a strong positive test, with an induration equal to or greater than 15 mm, has a greater chance of indicating TB infection than occurring as a result of the vaccine.[8] As the BCG vaccine in Brazil is administered in the first year of life and the patients included in this study were over 18 years old, TST observed positivity has to be understood as a real latent TB infection. Furthermore, most patients had results greater than 15 mm, which also strengthened that the test positivity was due to real infection. False negatives can also occur due to problems with the reagent, inaccurate administration or interpretation of the test, or tuberculin anergy in children and immunocompromised patients, including those who used methotrexate.[5] However, these possibilities were considered remote, as the PPD kits were used within their

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