

**Perfil de sensibilidade antifúngica de isolados clínicos obtidos de onicomicose aos
antifúngicos convencionais**

**Antifungal sensitivity profile of clinical isolates obtained from onychomycosis to
conventional antifungals**

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antifúngicos convencionales**

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Shellygton Lima Silva

ORCID: <https://orcid.org/0000-0001-7168-7725>

Universidade Federal da Paraíba, Brasil

E-mail: shellygton.lima@gmail.com

Maísa Evangelista de Lima

ORCID: <https://orcid.org/0000-0002-7246-4643>

Centro Universitário UNIFACISA, Brasil

E-mail: maisaevidl@gmail.com

Raissa Daniel Trajano dos Santos

ORCID: <https://orcid.org/0000-0002-2209-8710>

Centro Universitário UNIFACISA, Brasil

E-mail: raissadtrajano@gmail.com

Hermes Diniz Neto

ORCID: <https://orcid.org/0000-0003-2878-1737>

Universidade Federal da Paraíba, Brasil

E-mail: hermes.dn@hotmail.com

Daniele de Figueredo Silva

ORCID: <https://orcid.org/0000-0002-9939-0410>

Universidade Federal da Paraíba, Brasil

E-mail: danielefigueredo31@gmail.com

Edeltrudes de Oliveira Lima

ORCID: <https://orcid.org/0000-0002-9547-0886>

Universidade Federal da Paraíba, Brasil

E-mail: edelolima@yahoo.com.br

Resumo

Onicomicoses são afecções micóticas de unhas ocasionadas por fungos patogênicos, dermatófitos, não-dermatófitos e principalmente por espécies do gênero *Candida*. Fungos leveduriformes causam infecções em unhas, devido à sua capacidade de invasão ao hiponíquio, borda proximal e por disseminação vascular ou linfática, representando um importante problema de saúde pública mundial. Com o propósito de compreender a baixa eficácia de tratamento para onicomicoses e modificações fenotípicas causadas por mecanismos de resistência esse estudo teve por objetivo avaliar o perfil de susceptibilidade de isolados clínicos de onicomicoses do gênero *Candida* a antifúngicos convencionais. O ensaio de sensibilidade aos antifúngicos para diferentes cepas e isolados clínicos obtidos da micoteca do Laboratório de Atividade Antibacteriana e Antifúngica de Produtos Naturais e Sintéticos Bioativos foi realizado pelo teste de difusão em disco. Diante dos resultados observados pode-se inferir que os isolados apresentaram alto grau de resistência aos fármacos azólicos, considerados medicamentos de primeira escolha no tratamento de onicomicose. Portanto, o conhecimento prévio de análises *in vitro* pode auxiliar na farmacoterapia de onicomicose e no monitoramento de prevalência do perfil de suscetibilidade aos antifúngicos convencionais e como perspectiva deve-se utilizar outros testes de susceptibilidade aos antifúngicos e técnicas moleculares para uma melhor compreensão de fenótipos de resistência.

Palavras-chave: *Candida*; Difusão em disco; Onicomicose; Resistência a antifúngica.

Abstract

Onychomycosis are fungal infections of nails caused by pathogenic fungi, dermatophytes, non-dermatophytes and especially by species of the genus *Candida*. Yeast fungi cause infections in nails, due to their ability to invade the hyponychium, proximal border and by vascular or lymphatic dissemination, representing an important worldwide public health problem. In order to understand the low efficacy of treatment for onychomycosis and phenotypic changes caused by resistance mechanisms this study was to evaluate the susceptibility profile of onychomycosis clinical isolates of *Candida* to conventional antifungal. The antifungal sensitivity test for different strains and clinical isolates obtained from the Micoteca of the Laboratory of Antibacterial and Antifungal Activity of Natural and Synthetic Bioactive Products was carried out by the disk diffusion test. In view of the observed results, it can be inferred that the isolates had a high degree of resistance to azole drugs, considered to be drugs of first choice in the treatment of onychomycosis. Therefore, prior knowledge of *in vitro* tests can help in the pharmacotherapy of onychomycosis and

monitoring prevalence of susceptibility profile to conventional antifungal and how perspective should use other susceptibility test to antifungal drugs and molecular techniques for a better understanding of phenotypes resistance.

Keywords: *Candida*; Disk diffusion; Onychomycosis; Resistance antifungals.

Resumen

La onicomicosis son infecciones fúngicas de las uñas causadas por hongos patógenos, dermatofitos, no dermatofitos y especialmente por especies del género *Candida*. Los hongos de levadura causan infecciones en las uñas, debido a su capacidad de invadir el hiponiquio, el borde proximal y la diseminación vascular o linfática, lo que representa un importante problema de salud pública en todo el mundo. Con el fin de comprender la baja eficacia del tratamiento para la onicomicosis y los cambios fenotípicos causados por los mecanismos de resistencia, este estudio tuvo como objetivo evaluar el perfil de susceptibilidad de los aislados clínicos de onicomicosis por *Candida* a los antifúngicos convencionales. La prueba de difusión de disco llevó a cabo la prueba de sensibilidad antifúngica para diferentes cepas y aislados clínicos obtenidos de la micoteca del Laboratorio de actividad antibacteriana y antifúngica de productos bioactivos naturales y sintéticos. En vista de los resultados observados, se puede inferir que los aislamientos tenían un alto grado de resistencia a los fármacos azólicos, considerados medicamentos de primera elección en el tratamiento de la onicomicosis. Por lo tanto, el conocimiento previo de los análisis *in vitro* puede ayudar en la farmacoterapia de la onicomicosis y en el monitoreo de la prevalencia del perfil de susceptibilidad a los antifúngicos convencionales y, como perspectiva, se deben usar otras pruebas de susceptibilidad a los antifúngicos y las técnicas moleculares para una mejor comprensión de los fenotipos de resistencia.

Palabras clave: *Candida*; Difusión en disco; Onicomicosis; Resistencia antifúngica.

1. Introduction

Onychomycosis are considered mycotic infections in nails that result in impairment of the nail matrix, nail plate and adjacent tissues caused by dermatophyte fungi (*Trichophyton*, *Microsporum* e *Epidermophyton*), non-dermatophytes (*Scopulariopsis brevicaulis*, *Aspergillus* spp. e *Fusarium* spp), mainly including yeasts (*Candida albicans*, *C. tropicalis* e *C. parapsilosis*) (Lipner & Scher, 2019; Silva et al., 2020).

Yeasts fungi cause these infections, due to their ability to spread and invade keratin layers in the hyponychium region, through the surface of the nail plate through enzymatic or chemical processes by the proximal border or by systemic vascular or lymphatic dissemination (Trevisan, 2016).

Onychomycosis is a major public health problem worldwide, accounting for about 50% of all nail infections and 30% of superficial infections by skin fungi (Gregoriou et al., 2020). The worldwide prevalence of onychomycosis is estimated to be approximately 5.5% (Leung et al., 2020) and will increase as predisposing factors become more prevalent, such as persistent nail trauma, individuals suffering from psoriasis, diabetes, poor peripheral circulation, immunosuppression, obesity and smoking are also especially susceptible (Gupta et al., 2017; Gupta et al., 2020). Furthermore, the global variation in the prevalence of onychomycosis can be attributed to changes in population migration, modern patterns related to lifestyle and geographic location, such as onychomycosis caused by species of the genus *Candida* that are often reported in regions of hot and humid climates (Gupta et al., 2017).

Clinically these superficial mycoses affect healthy individuals and predominantly elderly and immunocompromised, relevant due to the high frequency and interference in quality of life, mainly due to the reduction of self-esteem and functional capacity and can be classified into four subtypes: Distal and lateral subungual onychomycosis is the most common form of this infection, characterized by hyperkeratosis of the lower surface of the plaque and distal nail bed (Freedman & Tosti, 2017; Chemello et al., 2018).

Proximal subungual onychomycosis, an unusual subtype, occurs when microorganisms invade the nails through the proximal fold, including, proximal onycholysis and leukonychia (Glinos & Tosti, 2017). White superficial onychomycosis occurs when fungi invade the superficial layers of the nails and are recognized by the presence of opaque “white islands” well-defined in the external nail plate (Chemello et al., 2018; Rigopoulos, 2018) and Total dystrophic onychomycosis represents the final stage of the disease the entire nail unit becomes thick and dystrophic (Gupta et al., 2018; Chemello et al., 2018).

The criteria for the clinical presentation of onychomycosis may favor the understanding of the degree of invasion and infecting microorganisms. However, it may not be satisfactory to treat onychomycosis based only on clinical diagnosis (Mahoney et al., 2003).

Although historical and clinical contexts are useful, mycological laboratory confirmation is considered the “gold standard” for the diagnosis of onychomycosis, composed by direct microscopic exams and cell culture, assisting in the detection of morphological

structures and confirmation of infecting microorganisms, respectively (Lipner & Scher, 2019).

In addition, more advanced laboratory techniques are used for the diagnosis of onychomycosis, such as: polymerase chain reaction technique, confocal microscopy and optical coherence tomography (Lipner & Scher, 2019). However, due to the high cost and because it requires specialized professionals are not employed in clinical practice.

Oral pharmacological treatment with imidazole class antifungal drugs, heterocyclic benzofuran triazoles and allylamines are the most common for treating onychomycoses in the last years (Aggarwal et al., 2020). However, for some patients it is medically inappropriate, what becomes necessary to implant topical therapy amorfiline 5%, cyclopyrox 8% and thioconazole solution 28% appears to be a good solution to the problem if effectiveness rates are better (Bodman & Krishnamurthy, 2019).

The implementation of methodologies to elucidate the susceptibility of microorganisms to antifungal agents has been the subject of numerous studies in recent decades. In this context, Clinical and Laboratory Standards Institute (CLSI) has developed and standardized tests to determine *in vitro* susceptibility of yeasts, such as the disk diffusion test (DD) (Demitto et al., 2012; CLSI, 2010). In addition, Agência Nacional de Vigilância Sanitária (ANVISA, 2006) recommends DD screening method for assessing antifungal susceptibility. Its practicality, low cost and easy operation facilitate the implementation of this method in clinical microbiology research, which favors a better understanding of the low efficacy of treatment available in clinical practice and genotypic modifications, caused by resistance mechanisms.

Therefore, this study aimed to evaluate the susceptibility profile of clinical isolates of species of the genus *Candida* to conventional antifungals using the Disc Diffusion methodology.

2. Methodology

2.1 Research location

Antifungal sensitivity investigation study was developed at the Laboratório de Atividade Antibacteriana e Antifúngica de Produtos Naturais e Sintéticos Bioativos, from the Departamento de Ciências Farmacêuticas of the Universidade Federal da Paraíba (UFPB).

2.2 Fungal strains

All strains and clinical isolates used in this research belong to the micoteca of the Laboratório de Atividade Antibacteriana e Antifúngica de Produtos Naturais e Sintéticos Bioativos. The microorganisms were maintained on Sabouraud dextrose agar (SDA) at 4 °C. To prepare the inoculum, the colonies obtained were suspended in sterile 0.9 % saline solution and adjusted according to the standard 0.5 on the Mc Farland scale to obtain 10⁶ CFU/mL (CLSI, 2008).

2.3 Antifungal susceptibility testing

The sensitivity test to antifungals to different strains and clinical isolates was performed using the disk diffusion test (DD), according to the experimental model described Barry & Thornsberry (1991) and document M44-A2 of Clinical Laboratory Standards Institute (CLSI, 2010). Amphotericin B discs (100 µg), ketoconazole (50 µg), clotrimazole (50 µg), fluconazole (25 µg), itraconazole (100 µg), miconazole (50 µg) and nystatin (100 IU) obtained from Centro de Controle e Produtos para Diagnóstico (CECON, São Paulo, Brazil).

Aliquots of 1mL of each fungal suspension were seeded with a disposable bacteriological loop on the solid surface of Sabouraud Dextrose Agar contained in petri dishes (90x15mm). Then, paper disks with a diameter of 6.35 mm impregnated with the antifungal patterns with their respective concentrations were applied to the seeded plates.

At the end of the whole process the plates were sealed aseptically and incubated at a temperature of 35 ± 2 °C for 48h. After that time, the growth inhibition halos of the microorganism were read against antifungal agents. The results were classified as sensitive and resistant, according to Table 1.

Table 1 - Classification of halos of inhibition formed by standard antifungals.

| Standard drugs | Classification of the zone of inhibition |
|-----------------------|--|
| Amphotericin B 100 µg | > 10 sensitive ≤ 10 resistant |
| Ketoconazole 50 µg | > 20 sensitive ≤ 20 resistente |
| Clotrimazole 50 µg | >20 sensitive ≤ 20 resistente |
| Fluconazole 25 µg | ≥ 19 sensitive < 19 resistente |
| Itraconazole 10 µg | ≥ 20 sensitive < 20 resistente |
| Miconazole 50 µg | > 20 sensitive ≤ 20 resistente |
| Nystatin 100 UI | > 10 sensitive ≤ 10resistente |

Results expressed according to the inhibition zone in mm²

Source: Authors.

3. Results and Discussion

In the present study, clinical isolates of *Candida* spp. of individuals with signs and clinical diagnoses of onychomycosis, stored in Micoteca collection, (11) 65% were *C. albicans*, (3) 17% were *C. tropicalis* and *C. parapsilosis*. While three strains standard used were American Type Culture Collection (ATCC).

In antifungal susceptibility tests, not any of the species showed resistance in the presence of amphotericin B and low degree of resistance in the presence of nystatin (4) 20% and ketoconazole (7) 35%. However, observed a high degree of resistance to azole drugs (13) 65% isolates resistant to itraconazole and (12) 60% isolates to fluconazole, the main drugs used for the treatment of onychomycosis. For other antifungal, there was variation between (7) and 35% (9) 45% resistance in disk diffusion method, as noted in Table 2.

Table 2 - Results of the susceptibility profile of strains and clinical isolates of *C. albicans*, *C.tropicalis* and *C.parapsilosis* against antifungal

| Strains and clinical isolates | Amphotericin B 100 µg | Ketoconazole 50 µg | Clotrimazole 50 µg | Fluconazole 25 µg | Itraconazole 10 µg | Miconazole 50 µg | Nystatin 100 UI | Cell viability | Sterility |
|-------------------------------------|--------------------------|-----------------------|-----------------------|----------------------|-----------------------|---------------------|--------------------|----------------|-----------|
| <i>C. albicans</i> ATCC -76645 | 15 ^(S) | 8 ^(R) | 26 ^(S) | 0 ^(R) | 0 ^(R) | 30 ^(S) | 20 ^(S) | + | - |
| <i>C. albicans</i> LM-03 | 14 ^(S) | 17 ^(R) | 20 ^(R) | 23 ^(S) | 0 ^(R) | 30 ^(S) | 20 ^(S) | + | - |
| <i>C. albicans</i> LM-26 | 16 ^(S) | 28 ^(S) | 12 ^(R) | 20 ^(S) | 0 ^(R) | 25 ^(S) | 26 ^(S) | + | - |
| <i>C. albicans</i> LM-44 | 15 ^(S) | 17 ^(R) | 22 ^(S) | 0 ^(R) | 12 ^(S) | 8 ^(R) | 18 ^(S) | + | - |
| <i>C. albicans</i> LM-74 | 13 ^(S) | 19 ^(S) | 16 ^(R) | 0 ^(R) | 0 ^(R) | 10 ^(R) | 20 ^(S) | + | - |
| <i>C. albicans</i> LM-123 | 15 ^(S) | 25 ^(S) | 25 ^(S) | 0 ^(R) | 10 ^(S) | 6 ^(R) | 23 ^(S) | + | - |
| <i>C. albicans</i> LM-157 | 14 ^(S) | 12 ^(R) | 24 ^(S) | 12 ^(S) | 11 ^(S) | 14 ^(S) | 16 ^(S) | + | - |
| <i>C. albicans</i> LM-165 | 13 ^(S) | 30 ^(S) | 11 ^(R) | 0 ^(R) | 0 ^(R) | 26 ^(S) | 20 ^(S) | + | - |
| <i>C. albicans</i> LM-175 | 20 ^(S) | 15 ^(R) | 18 ^(R) | 0 ^(R) | 10 ^(S) | 21 ^(S) | 17 ^(S) | + | - |
| <i>C. albicans</i> LM-441 | 12 ^(S) | 22 ^(R) | 13 ^(S) | 0 ^(R) | 0 ^(R) | 10 ^(R) | 19 ^(S) | + | - |
| <i>C. albicans</i> LM-600 | 13 ^(S) | 21 ^(S) | 21 ^(S) | 0 ^(R) | 0 ^(R) | 10 ^(R) | 10 ^(R) | + | - |
| <i>C. albicans</i> LM-615 | 10 ^(R) | 10 ^(R) | 21 ^(S) | 14 ^(S) | 15 ^(S) | 20 ^(S) | 10 ^(R) | + | - |
| <i>C. tropicalis</i> ATCC -13803 | 12 ^(S) | 29 ^(S) | 21 ^(S) | 15 ^(S) | 12 ^(S) | 20 ^(S) | 20 ^(S) | + | - |
| <i>C. tropicalis</i> LM-98 | 13 ^(S) | 21 ^(S) | 18 ^(R) | 15 ^(S) | 0 ^(R) | 22 ^(S) | 20 ^(S) | + | - |
| <i>C. tropicalis</i> | 15 ^(S) | 10 ^(R) | 15 ^(R) | 10 ^(R) | 0 ^(R) | 20 ^(S) | 20 ^(S) | + | - |

| | | | | | | | | | |
|--|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|---|---|
| LM-111 | | | | | | | | | |
| <i>C. tropicalis</i> LM-135 | 10 ^(R) | 28 ^(S) | 22 ^(S) | 16 ^(R) | 0 ^(R) | 08 ^(R) | 10 ^(R) | + | - |
| <i>C. parapsilosis</i> ATCC -2209 | 13 ^(S) | 30 ^(S) | 19 ^(R) | 14 ^(S) | 0 ^(R) | 20 ^(S) | 09 ^(S) | + | - |
| <i>C. parapsilosis</i> LM-78 | 17 ^(S) | 21 ^(S) | 10 ^(R) | 0 ^(R) | 0 ^(R) | 19 ^(S) | 20 ^(S) | + | - |
| <i>C. parapsilosis</i> LM-197 | 16 ^(S) | 10 ^(S) | 22 ^(S) | 16 ^(S) | 15 ^(S) | 10 ^(R) | 20 ^(S) | + | - |
| <i>C. parapsilosis</i> LM-707 | 14 ^(S) | 26 ^(S) | 23 ^(S) | 05 ^(R) | 0 ^(R) | 20 ^(R) | 10 ^(R) | + | - |

Results are expressed as inhibition halos/mm²

Our results confirm the data presented by Motta (2009) observed, clinical isolates of *C. albicans*, *C. tropicalis* and *C. parapsilosis* showed sensitivity in the presence of amphotericin B.

Similarly to the results observed in susceptibility profile standard strains and clinical isolates nail to drug classes of azole, tests conducted by Magalhães Lima et al (2009) demonstrated that a total of 23 species of the genus *Candida* isolated nail 48% were resistant to fluconazole, and itraconazole.

In a recent study conducted by Silva et al (2020) with a total of 20 species *C. albicans*, *C. tropicalis* and *C. parapsilosis*, representing the species found in most fungal infections of nails, there was a percentage of 100% of clinical isolates resistant to itraconazole, confirming a high degree of resistance shown in our study. Resistance to miconazole (7) 35% and clotrimazole (9) 45%, although poorly studied, has been demonstrated in recent years in several clinical isolates of *Candida*.

Currently, there is a reasonable number of antifungal drugs in the pharmaceutical market to control mycosis because their cellular targets are restricted and fungi can exhibit tolerance or resistance to these agents. It is noteworthy that the stress caused by antifungal and cytotoxic drugs in sub-inhibitory concentrations promotes compensatory responses to stress, with the overexpression of genes involved in cellular detoxification, drug efflux and signaling pathways between the various mechanisms that may contribute to tolerance to drugs (Martinez-Rossi et al., 2018).

Based on the results found in our study, it is possible to infer a predominance of resistance to antifungals commonly used for the treatment of onychomycosis, since a significant percentage of clinical isolates come from possible patients who were treated with medications, but relapsed to infection.

Given the above, the combination of pharmacological knowledge of each antifungal, associated with the therapeutic response in each patient, and previous knowledge of the in vitro susceptibility profile should guide the conduct of a more effective, safe and less toxic antifungal treatment. This conduct in the pharmacotherapy becomes more indispensable, mainly due to the high rate of relapse accounts and ineffective treatment.

4. Final considerations

Whereas the antifungal susceptibility testing are not required routinely in clinical practice and that our results have identified a very significant percentage of resistant yeasts,

suggest the need for a monitoring program of clinical isolates of patients with onychomycosis, in order to monitor and demarcate the prevalence of susceptibility profile to conventional antifungal. In this perspective, other methods of fungal sensitivity and molecular techniques should be used for a better understanding of resistance phenotypes.

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Percentage of contribution of each author in the manuscript

Shellygton Lima Silva - 30%

Maísa Evangelista de Lima - 10%

Raissa Daniel Trajano dos Santos - 10%

Hermes Diniz Neto - 10%

Daniele de Figueredo Silva - 10%

Edeltrudes de Oliveira Lima - 30%