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Jamile de Paiva Macedo

Unveiling antifungal resistance and biocide tolerance in clinical isolates of *Candida* spp.

Juiz de Fora, MG
2025

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Dissertation submitted to the Postgraduate Program in Biological Sciences, Federal University of Juiz de Fora, as a partial requirement for obtaining the degree of Master in Biological Sciences, with emphasis on Microbiology.

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ABSTRACT

Candidiasis is an opportunistic infection that impacts public health. In recent years, emerging *Candida* species have contributed to the increase in the disease burden exacerbated by antifungal resistance. This study aimed to evaluate the antifungal susceptibility profile of *Candida* clinical isolates, investigate resistance mechanisms, evaluate the efflux pump activity, and assess biocide tolerance. 100 *Candida* isolates from hospitalized and outpatient individuals were analyzed. Antifungal susceptibility and biocide tolerance (sodium hypochlorite, hydrogen peroxide, and benzalkonium chloride) were assessed using a disk diffusion test, and the molecular resistance mechanisms were explored through polymerase chain reaction (PCR). Efflux pump activity was performed using the Cartwheel method. A high frequency of resistance (n=87/87%) to at least one antifungal was observed, with 21% (n=21) of isolates showing simultaneous resistance to multiple antifungal agents. The fungal isolates evaluated showed the highest frequency of resistance to fluconazole (n=70), followed by voriconazole (n=68), itraconazole (n=47), and caspofungin (n=20), and the highest susceptibility to amphotericin B (n=1). The most common mutation was detected in the *ERG11* gene (n=38/43.7%), followed by *CDR2* (n=17/19.5%), *CDR1* (n=7/8%) and *MDR1* (n=6/6.9%). The *ERG2* and *FKS1* genes were not detected in any of the isolates in this study. Besides the intrinsic efflux pump activity observed in all *Candida* isolates, 14 *Candida albicans* isolates exhibited a higher MCEtBr compared to the reference strain, with 1.0 µg/l (n=10) and 1.5 µg/l (n=4). For the *non-albicans* group, 6 isolates of *Candida tropicalis,* 3 of *Candida parapsilosis,* and 1 each of *Meyerozyma guilliermondii* (syn. *Candida guilliermondii)* and *Pichia kudriavzevii* (syn. *Candida krusei*) presented an MCEtBr greater than the reference at 1.0 µg/l, those findings point towards an overexpression of the mechanism. Sodium hypochlorite was the most effective biocide tested with an inhibition zone ranging from 18.3mm (±4.4) to 34.0mm (±4.0), and the least effective was hydrogen peroxide at 4.25% and benzalkonium chloride 5% with 6.00mm (±0.0). This study highlights considerable antifungal resistance in *Candida* spp., particularly to azoles, in association with tolerance to routinely used biocides, emphasizing the need to develop new therapeutic strategies and infection control measures for healthcare-associated infections.

Keywords: *Candida*; Infections; Antifungal susceptibility; Azoles; Efflux pumps; Biocide tolerance; Sodium hypochlorite.

RESUMO

A candidíase é uma infecção oportunista que afeta a saúde pública. Nos últimos anos, espécies emergentes de *Candida* contribuíram para aumentar o impacto da doença pela resistência antifúngica. Este estudo teve como objetivo avaliar o perfil de suscetibilidade antifúngica de isolados clínicos de *Candida*, investigar mecanismos de resistência, avaliar a atividade da bomba de efluxo e avaliar a tolerância a biocidas. Foram analisados 100 isolados de indivíduos hospitalizados e ambulatoriais. A suscetibilidade antifúngica e a tolerância a biocidas (hipoclorito de sódio, peróxido de hidrogênio e cloreto de benzalcônio) foram avaliadas usando um teste de disco-difusão, e os mecanismos de resistência molecular foram explorados por meio da reação em cadeia da polimerase (PCR). A atividade da bomba de efluxo foi realizada usando o método Cartwheel. Foi observada uma alta frequência de resistência (n=87/87%) a pelo menos um antifúngico, com 21% (n=21) dos isolados mostrando simultaneamente resistência a múltiplos agentes antifúngicos. A maior frequência de resistência a agente antifúngico foi observada para fluconazol (n=70), seguido por voriconazol (n=68), itraconazol (n=47), caspofungina (n=20) e a maior suscetibilidade para anfotericina B (n=1). A mutação mais comum encontrada foi do gene *ERG11* (n=38/43,7%), seguido por *CDR2* (n=17/19,5%), *CDR1* (n=7/8%) e *MDR1* (n=6/6,9%). *ERG2* e *FSK1* não foram encontrados. Apesar da atividade intrínseca da bomba de efluxo ser encontrados em todos os isolados de *Candida*, 14 isolados de *Candida albicans* apresentaram MCEtBr maior que a linhagem de referência, com 1,0 µg/l (n=10) e 1,5 µg/l (n=4). Para o grupo *non-albicans* 6 isolados de *Candida tropicalis*, 3 de *Candida parapsilosis* e 1 de *Meyerozyma guilliermondii* (sin. *Candida guilliermondii*) e *Pichia kudriavzevii* (sin. *Candida krusei*) apresentaram MCEtBr maior que a referência em 1,0 µg/l, com índice de 1 em relação à linhagem de referência, que apontam para uma possível superexpressão do mecanismo. Biocida mais eficaz foi o hipoclorito de sódio, com uma zona de inibição variando de 18,3mm (±4,4) a 34,0mm (±4,0), e o menos eficaz peróxido de hidrogênio a 4,25% e cloreto de benzalcônio a 5% com 6,0mm (±0,0). Este estudo destaca considerável resistência aos antifúngicos em *Candida* spp., particularmente aos azóis, com associação entre a tolerância a biocidas utilizados rotineiramente.

Palavras-chave: *Candida;* Infecções; Suscetibilidade antifúngica; Azóis; Bombas de efluxo; Tolerância aos biocidas; Hipoclorito de sódio.

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LIST OF ABBREVIATIONS

NCAC- non-*Candida albicans Candida* species

CLSI- Clinical and Laboratory Standard Institute

PCR- Polymerase chain reaction

NaCl- Sodium chloride

DNA- DeoxyriboNucleic acid

dsDNA- Double string DeoxyriboNucleic acid

EtBr- Ethidium bromide

ATCC- Reference lineage

DDT- Disk diffusion technique

AFST- Antifungal susceptibility testing

ITR- Itraconazole

FLU- Fluconazole

VOR- Voriconazole

CAS- Caspofungin

ANB- Amphotericin B

SYMBOLS LIST

ml- Mililiter

ºC- Celsium

%- Porcentage

µg- Mircrogram

g/l- Gram per liter

V- Voltage

h- Hour

µL- Microliter

±- Standard Deviation

SUMMARY

1. INTRODUCTION

Candida spp. is a genus of fungi that includes several pathogenic species, with *Candida albicans, Candida tropicalis, Candida parapsilosis* and *Candida glabrata* being the most common in human infections. These infections, often healthcare-associated, are associated with high rates of morbidity and mortality, as well as significant hospitalization costs. It is estimated that invasive fungal infections caused by *Candida* spp. represent one of the main causes of hospital infections in intensive care units, with an increasing prevalence in recent decades, particularly in immuno-compromised patients, such as those with HIV, cancer patients, or those using immunosuppressive therapies. The increased prevalence of these infections has been concomitant with the emergence of antifungal-resistant strains, complicating therapeutic management and making treatment options more limited and expensive.

Antifungal resistance has become a significant challenge for treating *Candida* spp. infections, associated with different molecular mechanisms. Resistance to azole antifungal compounds, such as fluconazole and voriconazole, is particularly concerning, as these drugs are commonly used as first-line treatment. Mutations in the *ERG* genes, which are involved in the biosynthesis of ergosterol, the main target of azoles, are the most common molecular mechanisms of resistance. In addition, the over-expression of the efflux pumps, whose proteins are encoded by *CDR1, CDR2* and *MDR1,* is frequently associated with resistance to several antifungals, including azoles, echinocandins and polyenes. These efflux pumps act in the active export of drugs out of cells, decreasing the intracellular concentration of the antimicrobial agent and, consequently, its efficacy. In addition to resistance to antifungals, tolerance to biocides, such as those used for hospital disinfection and sterilization, also represents an obstacle in the control of healthcare-associated infections. Recent studies have shown that efflux pumps contribute to antifungal resistance and also play a significant role in reducing the efficacy of biocides. This phenomenon becomes an additional challenge in the control of *Candida* spp. in hospital environments, where rigorous disinfection and the use of biocides are crucial to prevent outbreaks. The ability of efflux pumps to export these substances impedes the effective action of biocides, contributing to the persistence of *Candida* spp. in hospital environments and increasing the burden of infections.

Effective management of these infections demands a clear understanding of the resistance and tolerance mechanisms in *Candida* spp., along with constant surveillance to detect multidrug-resistant strains. Molecular studies that identify specific mutations in resistance genes, conducted through polymerase chain reaction (PCR), combined with phenotypic assessments of antifungal and biocide susceptibility, are crucial for enhancing therapeutic and prevention strategies. Rapid and accurate diagnosis of resistant strains enables the selection of more effective treatments, which can reduce both mortality and the costs associated with infections. Furthermore, implementing control measures, like the rational use of antimicrobials, rigorous monitoring of resistance, and proper sanitation of hospital environments, is essential to preventing the spread of resistant strains and minimizing the clinical and economic impact of these infections.

In this context, the present study aims to analyze clinical isolates of *Candida* spp. resistance to antifungals, correlating the phenotypic findings with the genetic resistance profiles, investigating the impact of efflux pumps as a mechanism of multifactorial resistance, and accessing its tolerance to biocides.

2. LITERATURE REVIEW

2.1 *Candida* spp.

Among the most common fungal infections, candidiasis is a disease popularly caused by *Candida* spp. The genus *Candida* belongs to the *Saccharomycete*s class, phylum Ascomycota, and encompasses a group of opportunistic unicellular yeasts (Chen et al., 2020). In recent years, the genus has undergone taxonomic changes based on molecular sequences that have not yet been fully elucidated (Takashima; Sugita, 2022).

The most described etiological agent for candidiasis is *Candida albicans*. However, other *Candida non-albicans* (NCAC) species such as *Nakaseomyces glabratus (syn. Candida glabrata), Candida parapsilosis, Meyerozyma guilliermondii (syn. Candida guilliermondii), Pichia kudriavzevii (syn. Candida krusei*) and *Candida tropicalis,* have great influence as clinically emerging pathogens, accounting for more than half the cases of invasive candidiasis and candidemia worldwide since the early 2000s (Gonzalez-lara; Ostrosky-zeichner, 2020; Mccarty; White; Pappas, 2021). Additionally, *Candida auris* is an emerging multidrug-resistant species identified in the last decade, associated with a high mortality rate ranging from 33% to 73% in the United Kingdom (Rhodes; Fisher, 2019).

The first description of *Candida* spp. was made through an autopsy of the esophagus mucosa of a cadaver with typhoid fever by Professor Dr. B. Langenbeck in 1839. At the time, he described the fungus as a thick, loose, opaque, yellowish mass with a wool-like surface that strongly adhered to the mucous membrane of the esophagus. Under microscopy, it appeared as a huge number of fungi with extremely tangled and branched thread-shaped stalks. A simple cell lineage, whose cells are somewhat stretched, joins the threads (Knoke; Bernhardt, 2006).

The ability to acquire changes in the phenotype of this genus in question guarantees the ability to thrive in various host niches with different pH, metabolite and oxide profiles (Scaduto; Bennett, 2015). Such as the ability to form hyphae in *Candida albicans* as a pathogenic process (Chen et al., 2020). These microorganisms are commonly associated with healthy human intestinal, vaginal and skin microbiota (Kreulen et al., 2023). In cases of imbalance in the microorganism-host relationship, localized infection, horizontal transmission by the hands of professionals or use of invasive medical devices, they can trigger a pathological process (Lu et al., 2023). The

pathogenesis of these infections involves a series of factors, including adherence to the host surface, biofilm formation capacity, production of hydrolytic enzymes and the ability to evade the host's immune response (Lopes; Lionakis, 2022).

The main predisposing factors for infections caused by *Candida* spp. include immune suppression (e.g. neutropenia), use of immunosuppressive medications, mucositis, use of broad-spectrum antibiotics, invasive medical devices (e.g. central venous catheters), and the fungal virulence ability (Jenks et al., 2020).

In Europe, *C. albicans* is the most prevalent species, followed by *C. glabrata* and *C. parapsilosis*, with notable regional variations; for example, Austria has a high proportion of *C. albicans*, whereas *C. glabrata* is more common in the Czech Republic, France, and the UK (Arendrup et al., 2023). The Antimicrobial Surveillance Program (SENTRY) reported similar trends globally, with *C. albicans* predominating in the Asia-Pacific region, *C. glabrata* being more prevalent in North America, and *C. parapsilosis* and *C. tropicalis* more common in Latin America (Pfaller et al., 2011). Genotyping studies have shown high clonal diversity and the widespread presence of certain *Candida* genotypes, indicating that some strains are shared across distant geographical areas, potentially due to patient-to-patient transmission or limitations in genetic discrimination methods (Guinea et al., 2020).

2.2 Morphopatogenesis

Understanding the reasons and mechanisms underlying the pathogenicity of *Candida* species is of great importance, given its broad spectrum of characteristics, which include morphological variations, biofilm formation, thigmotropism, and production of virulence factors. In addition, it is essential to understand the factors that make an individual susceptible to candidiasis, as well as the elements that contribute to the severity of this condition (Farhan et al., 2024).

Yeast, hyphae, and pseudohyphae differ in their cell morphology, function, and growth condition (Chen et al., 2020). One important characteristic of *C. albicans* is that it can exist in three phases, budding yeast, pseudohyphae, and hyphae. The plasticity of the mycelial form is a determinant factor of drug resistance and is also an important form during the infection stage. In addition, the transformation of *C. albicans* from yeast to hyphae can help fungi in tissue penetration through tigmotropism leading to blood vessels and deep seated-organs colonization, and escape the phagocytosis

of macrophages, resulting in an increased likelihood of invading host tissues and causing greater damage (Thomson; Wehmeier; Byfield, 2015; Witchley et al., 2019).

Candida tropicalis can grow as pseudohyphae and *C. parapsilosis* can grow as short filaments (Silva et al., 2012). This hints toward a unique morphogenetic pathway in *C. albicans*, which is not present in more distantly related NCAC species (Bottcher et al., 2016; Gómez-gaviria; Ramírez-sotelo; Mora-montes, 2022). *In vivo* and *in vitro* studies carried out with the *C. auris* clinical isolate showed increased tissue colonization with no morphological changes documented, such as pseudohyphae or mycelium (Wang et al., 2022).

Candida albicans demonstrates remarkable morphological plasticity at both cellular and colony levels, enabling their survival in diverse host environments under varying conditions such as pH, temperature, and oxygen availability. A key feature of this adaptability is phenotypic switching, where the organism transitions between distinct white and opaque forms, a mechanism crucial for mating, survival, and pathogenicity. While significant attention has been given to the yeast-to-hyphal transition due to its role in disease, white-to-opaque switching is equally vital, influencing adaptability and infection across host niches (Takagi et al., 2019).

Since its isolation in 1839, the understanding of *C. albicans* morphology has evolved, revealing its capacity for a non-meiotic parasexual cycle involving hybridization and genetic recombination, challenging earlier beliefs of purely asexual reproduction. These morphological and reproductive traits are central to *C. albicans* pathogenicity, contributing to its genetic diversity and capacity to thrive in the human host (Mishra; Forche; Anderson, 2021).

Yeasts of the genus *Candida* are capable of synthesising various virulence factors that play a crucial role in their pathogenesis, that include: thermotolerance, adhesins, hydrolytic enzymes (e.g. proteases, lipases, hemolysins), membrane and cell wall barriers, biofilm formation, signal transduction pathway, expression of resistance genes to antifungal drugs, production of toxins and germ tube formation (Hube; Naglik, 2001; Liu, 2001; Al-dabbagh;ajah; Salman, 2023; De Souza et al., 2023; Saiprom et al.., 2023; Sundstrom, 2020; Wijaya; Halleyantoro; Kalumpiu, 2023). They also produce extracellular enzymes such as phospholipases, proteinases, and hemolysins. Besides facilitating colonization and infection of host tissues, these factors also contribute to antifungal resistance, which makes them an important target for studies about *Candida* spp. drug resistance (Staniszewska, 2020).

The virulence of a microbial species is determined by the dynamic interactions between the microorganism and the host rather than constituting an intrinsic characteristic of the microorganism. While primary pathogens can develop independently of the presence of lesions in the host, opportunistic pathogens, such as *Candida* spp., require specific conditions to cause infection. In this context, environmental factors exert a determining influence, modulating the strategies employed by microorganisms to overcome the host's innate defense mechanisms. Thus, virulence proves to be an adaptive characteristic and can be increased, reduced, or even reestablished depending on environmental conditions and interaction with the host (Méthot; Alizon, 2014; Du et al., 2020; Singh; Tóth; Gácser, 2020; Farhan et al., 2024;).

2.3 Clinical Manifestations

Candida spp. infections represent a significant challenge to global health and are favored by three main conditions: prolonged use of antimicrobials, immunosuppression, and use of invasive medical devices, such as catheters and probes (Pappas et al., 2018).

Some of the most common manifestations of candidiasis include mucocutaneous infection that affects mucosa, mainly oral or vaginal, and skin. Clinical symptoms of itching and redness, vaginal discharge or white spots on the oral mucosa or throat may be observed (Kan et al., 2020). It is estimated that millions of cases of *Candida* spp. infections occur annually worldwide, including mucocutaneous infections, candidemia and invasive candidiasis (LOCKHArt; Guarner, 2019). As part of the vaginal microbiota, it is estimated that between 5 and 10% of people may develop endogenous vulvovaginal candidiasis, with 70 to 90% of cases being caused by *C. albicans*. Other factors that influence the infection are vaginal delivery, transmission of *Candida* spp. from the rectal tract to the vulvovaginal region, or transmission through sexual intercourse (Tian et al., 2021). Cases of candiduria have been reported in 5.5% of the hospitalization in pediatric intensive care units (ICU) (Gharaghani et al., 2021). One of the main causes of onychomycosis, a fungal nail infection responsible for 50% nail disorders, is *Candida* spp., being the third most common cause of infection (Widaty et al., 2020). Despite the frequency of other clinical manifestations, candidemia and invasive candidiasis are the manifestations with the greatest clinical relevance, given the increase in the rate of candidemia cases in the

last 10 years, from 2.18 cases per 100,000 inhabitants to 3.22 cases (Oliva et al., 2023), being the second most common cause of bloodstream infection associated with healthcare services in the United States (Mccarty; White; Pappas, 2021).

Another clinical condition is invasive candidiasis or candidemia, which combines the invasive capacity and the ability to compromise organs and the bloodstream, respectively. This condition is commonly observed in hospital settings, mainly in intensive care units (ICUs), where the rate of secondary infections is high due to factors such as morbidity and immunosuppression in severely ill patients (Pappas et al., 2018; Lona-Reyes et al., 2022). Invasive candidiasis is not a single condition but rather a disorder with a myriad of clinical symptoms. It results from the colonization of internal organs by *Candida* spp., which can trigger intra-abdominal abscess, peritonitis, and osteomyelitis. This clinical condition may or may not be accompanied by candidemia (Pappas et al., 2018).

Cases of invasive candidiasis are led internationally by the species *C. albicans*, but since the early 2000s, NCAC species collectively account for more than half of cases worldwide, with those of the *C. parapsilosis* complex being the most common among them (Gonzalez-lara; Ostrosky-zeichner, 2020). Despite this shift in the scenario, *C. albicans* remain the most prevalent, having decreased from 57.4% to 46.4% over the past 20 years (Friedman; Schwartz, 2019). Nosocomial infections have the highest mortality rate due to hospital infections, with survival of up to 30 days after diagnosis in 40 to 55% of cases in intensive care units. Risk factors include the use of immunosuppressive drugs, elderly patients, severity of the condition, comorbidities, use of venous catheters, and failed antifungal treatments. With a global incidence rate of 3-5 per 100,000 people, and an incidence of 1 to 2% of all ICU admissions, an annual incidence of 75,000 cases per year worldwide (Soriano et al., 2023).

Sentry is an antifungal surveillance program that provides a database for epidemiological studies of invasive infections caused by *Candida* spp. worldwide and currently includes clinical isolates from 39 countries. They studied 1,846 clinical isolates, among them 82% were *Candida* spp. The region with the highest incidence was Europe with 41%, followed by Asia-Pacific (24.5%), North America (23.5%), and Latin America with the lowest incidence with 11% (Castanheira et al., 2016). A multicenter surveillance study in 16 hospitals distributed across five regions of Brazil found in 2,563 nosocomial bloodstream infection episodes that *Candida* spp. was the 7th most prevalent agent, with a crude mortality rate of candidemia during the hospital

admission at 72.2%. It was observed that 29 days of hospitalization in the candidemia cases (Doi et al., 2016).

One of the great challenges in controlling nosocomial infections by *Candida* spp. is in the proper management for decontamination of the hospital environment, considering that the capacity for biofilm formation allows the colonization of biotic and abiotic surfaces, such as the hands of health professionals, medical devices and surfaces in hospital environments (Dal Guedes Da Silva; Sanches; Chassot, 2022).

2.4 Laboratorial diagnosis

In addition to the patient's clinical history, an accurate laboratory diagnosis is essential for determining the treatment of the infection. This diagnosis can be made through direct examination of clinical specimens such as blood, cerebrospinal fluid, synovial fluid, peritoneal fluid, vaginal secretion, and tissue fragments, allowing the observation of fungal structures such as blastoconidia or pseudohyphae (Pfaller; Castanheira, 2015). However, the method gold standard for diagnosis in cases of suspected candidemia and invasive candidiasis is culture in specific media, capable of characterizing the fungal species involved in the infectious process (Deorukhar And Roushani, 2018; Mccarty; White; Pappas, 2021). The characterization at the species level of *Candida spp.* isolates can be performed by manual or automated methods, such as Vitek 2® (bioMérieux/France), based on physiological and biochemical tests, or through molecular identification tests such as PCR to detect the DNA of this microorganism (Patwardhan et al., 2024).

Traditional methods for identifying *Candida* species are often slow, requiring 48 to 72 hours for results, and may misidentify rare or emerging species, like *C. auris,* which can have significant clinical implications (Kathuria; Singh; Sharma, 2015). Amplification techniques, such as multiplex PCR, are increasingly used for post-culture identification of *Candida* species, offering rapid results with high sensitivity and specificity. PCR-based tests can detect *Candida* DNA in clinical samples like blood, serum, and tissues, although challenges such as nucleic acid extraction and variable analytical sensitivity persist (Pfaller; Castanheira, 2015). Moreover, others diagnostic tools, such as peptide nucleic acid (PNA)-fluorescent in situ hybridization (FISH) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), provide faster and more precise species identification within 1 to 2 hours

post-culture (Arvanitis; Anagnostou; Fuchs, 2014). However, molecular biology-based methods are not yet widely used in clinical laboratories due to their high cost, the need for specialized equipment, and the requirement for qualified analysts (Dadar et al., 2018).

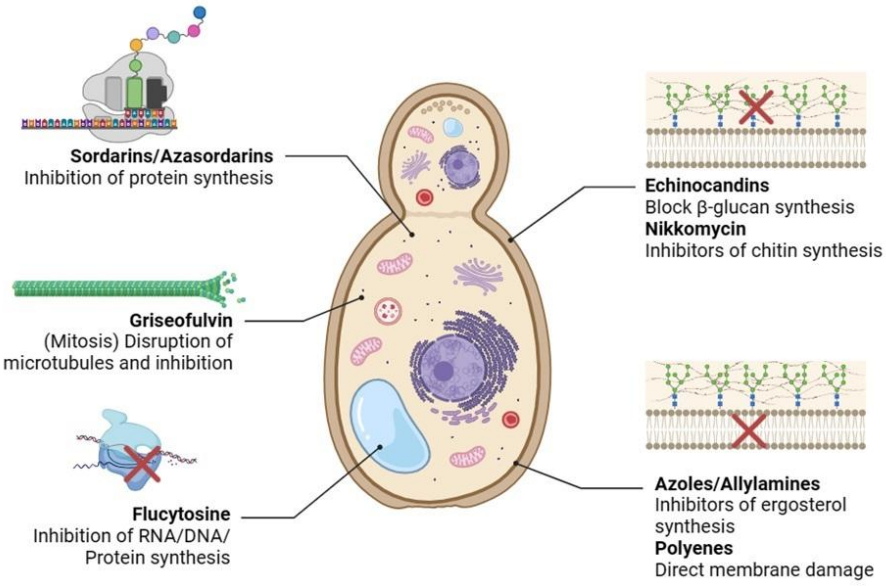
Qualitative methods are widely used in clinical laboratories due to their low cost and ease of execution, such as those based on disk diffusion, described by Kirby-Bauer (White et al., 2021). There are very important protocols used for interpreting the breakpoints of these methods: Clinical & Laboratory Standards Institute (CLSI), European Committee on Antimicrobial Susceptibility Testing *in vitro* (EUCAST), and Brazilian Committee on Antimicrobial Susceptibility Testing (BRCAST), for determining susceptibility to antifungals can be qualitative or quantitative and help determine the best therapy for *Candida* spp. infections. Among the quantitative methods, micro or macrodilution in broth are procedures considered the gold standard, in which the minimum inhibitory concentration (MIC) values are used as a parameter for implementing assertive therapy (Castanheira et al., 2016). Automated or semi-automated commercial methods are available for laboratory use: Broth microdilution (e.g., Vitek-2TM, Sensititre YeastOne®) or agar-based (e.g., MIC Test Strip® and Etest®) (Alastruey-izquierdo et al., 2015).

2.5 Antifungal agents of clinical interest

Currently, the clinical arsenal of antifungals against *Candida* spp. comprises different mechanisms of action. Azole derivatives such as itraconazole, fluconazole, voriconazole, posaconazole and isavuconazole act as inhibitors of ergosterol biosynthesis at the plasma membrane level, reducing membrane flexibility, permeability and the function of membrane enzymes, leading to inhibition of the growth of *Candida* spp. (Nivoix; Ledoux; Herbrecht, 2020). Polyene derivatives, such as amphotericin B (AmB), act by forming pores in the fungal cell membrane, altering membrane permeability. These drugs have fungistatic and fungicidal activity (Prasad; Shah; Rawal, 2016). Echinocandins, such as micafungin, anidulafungin, act as inhibitors of fungal cell wall synthesis, blocking the biosynthesis of (1,3)-β-D-glucan, leading to osmotic imbalance and cell death (Campoy; Adrio, 2017; Nivoix; Ledoux; Herbrecht, 2020).

Figure 1. Different mechanisms of action for antifungal agents in the yeast cell (Macedo; Dias,

2024).



2.5.1. Azoles

Azoles are a commonly prescribed class of antifungal drugs, with several derivatives such as itraconazole, fluconazole, voriconazole, posaconazole and isavuconazole. Azoles exert their effect by inhibiting the enzyme CYP-dependent C-14α-demethylase, involved in the conversion of lanosterol to ergosterol, which is encoded by the *ERG11* gene in yeast (Spampinato; Leonardi, 2013). This causes depletion of ergosterol, which is an essential sterol of the fungal cell membrane. It will reduce membrane flexibility, permeability, and the function of membrane enzymes, resulting in a fungistatic activity of *Candida* spp (Lewis, 2011; Perlin; Rautemaa-richardson; Alastruey-izquierdo, 2017).

2.5.2. Echinocandin

Echinocandins, like caspofungin, are a natural derivative type of medication that acts by disrupting the cell wall, blocking the biosynthesis of (1,3)-β-D-glucan. Which is not present in human cells, making it safe for patients, and leads to osmotic imbalance that ultimately kills the fungus (Lewis, 2011). These drugs are highly effective against *Candida* spp. and are recommended as a primary treatment for invasive candidiasis (Lai et al., 2008). They are administered intravenously due to poor oral absorption. Echinocandins target a heteromeric enzyme complex comprising a regulatory subunit (Rho1), that needs a chemical named guanosine triphosphate to

work, and a catalytic subunit made from the *FKS* gene. By acting on these mechanisms, echinocandins can lead to reduced yeast budding (Perlin; Rautemaa-richardson; Alastruey-izquierdo, 2017).

2.5.3. Polyenes

Polyenes are a group of antifungal drugs that act by binding to the fungal cell membrane and making it porous, which ultimately leads to the death of the cell. Amphotericin B (AmB) is a specific type of polyene that is commonly used to treat severe systemic fungal infections, such as those affecting the lungs, brain and bloodstream. This group of drugs is the oldest among antifungals, and AmB is the most popular and has excellent antifungal activity against a broad range of fungi, including several species of *Candida* spp. (Liu, 2001; Perlin; Rautemaa-richardson; Alastruey-izquierdo, 2017). Amphotericin B debuted as a therapeutic option in 1958, with a potent broad-spectrum antifungal activity and with a significant advancement for immunocompromised patients. However, its use was constrained by toxicity, paving the way for the development of alternative drugs with improved tolerability, reduced drug interactions, and complementary mechanisms of action (Quiles-melero; García-rodríguez, 2021). Lipid-based AmB formulations, introduced in the 1990s, permitted the use of a higher daily dose, provided better delivery to organs within the reticular endothelial system such as the lungs, liver, and spleen, with similar efficacy to conventional amphotericin B, and less nephrotoxic (Hamill, 2013). In contrast to azoles that target ergosterol biosynthesis through the inhibition of sterol 14α-demethylase activity (*ERG11*), Amphotericin B has both fungistatic and fungicidal activity by binding to the fungal membrane, leading to changes in membrane permeability. Vander Waals forces stabilize the interaction between AmB and ergosterol, resulting in the formation of membrane pores that leak monovalent ions (K+, Na+, H+, Cl-). Furthermore, AmB causes oxidation that results in fungal cell damage and has a stimulatory effect on phagocytic cells assisting fungal infection clearance (Prasad; Shah; Rawal, 2016; Al Balushi et al., 2018; Rybak; Fortwendel; Rogers, 2019).

Table 1. Schematic representation of different classes of antifungals and their representatives, including spectrum of action, route of administration and origin (Macedo; Dias, 2024).

Antifungal class	Spectrum of action	Administration pathway	Origin
Polyenes			
Amphotericin B	*Cryptococcus neoformans*, most *Candida spp.*, *Aspergillus spp.*	Intravenous (IV) Dose range 0.7 to 1mg/kg/day	Isolated from *Streptomyces nodosus*
Echinocandin			
Micafungin	Most *Candida spp.*	IV: 100 mg/d 150 mg/d 50mg/d	Fermentation products from *A. nidulans, A. aculeatus*, and *Zalerion arboricola*
Caspofungin	*Candida spp., Aspergillus spp.,* and some other molds and dimorphic fungi	IV: 70mg on day 1, then 50 mg/d	
Anidulafungin	Most *Candida spp.*	IV: 200mg on day 1, then 100mg/d	
Azoles			
Fluconazole	*Candida spp*. (except *C. krusei* and to a lesser extent *C. glabrata*) and other yeasts such as *Cryptococcus spp.*	Oral: 50–800mg once a day Inactive against molds	Synthetic heterocyclic compounds
Itraconazole	*Candida spp*. and *Cryptococcus spp.,* molds *Aspergillus spp*., and dimorphic fungi.	Oral solutions or capsules: 100–200mg/d	
Posaconazole	Candida spp. and Aspergillus spp.	IV and tablets: 2x300 mg on day 1, then 300mg/d Oral suspension: 100–800mg/d depending on the indication (in combination with food)	
Isavuconazole	*C. neoformans, Candida spp,,* molds *Aspergillus spp.* and Mucorales	IV: 3x200mg on days 1 and 2, then 200 mg/d	
Voriconazole	Yeasts and gold standard to *Aspergillus spp.*	IV: 2x6mg/kg on day 1 then 2x4mg/kg/d Oral: 2x400mg on day 1, then 2x200 mg/d	Natural compounds

2.6 Antifungal resistance

A repository published by the World Health Organization (WHO) discussed

a list of fungal pathogens that are a priority for health and research actions. It named 19 fungi according to their impact on public health and due to the emerging risk of antifungal resistance exhibited by these fungi. Among them, the species *C. auris* and *C. albicans* are in the critical priority group, and *C. glabrata, C. parapsilosis* and *C. tropicalis* are in the high priority group (Who, 2022).

Antifungal resistance is a growing public health concern, with a direct impact on the treatment and management of *Candida* spp. infections (Perlin; Rautemaa-richardson; Alastruey-izquierdo, 2017). Various antifungal resistance mechanisms have been described in the literature, and these may be molecular or non-molecular. For example, mutations in the *ERG* genes (involved in ergosterol biosynthesis) and overexpression genes for efflux pumps (*MDR1, CDR1* and *CDR2* genes) are the most reported molecular mechanisms of resistance in clinical isolates, mainly related to azole derivatives (Branco et al., 2017). For echinocandins, a molecular mechanism most described in the scientific literature is the mutation in the *FKS* genes that are involved in beta-glucan synthesis (Arendrup; Perlin, 2014a).

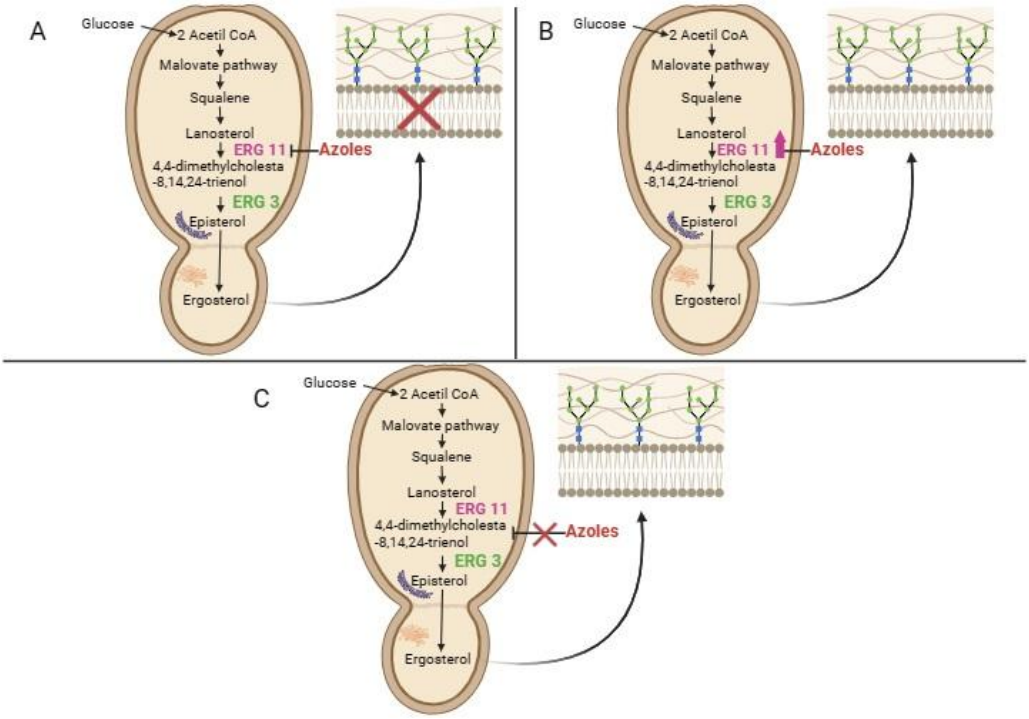
2.6.1. Ergosterol biosynthesis pathway

ERG genes are responsible for regulating the synthesis of ergosterol, which is an important constituent of the fungal cell membrane. It plays a crucial role in several cellular functions, including maintaining membrane fluidity and integrity, as well as facilitating the proper functioning of membrane-bound enzymes (Branco; Ola; Silva, 2017). Azole drugs block synthesis by inhibiting the activity of *ERG* genes, binding to and inhibiting the enzyme lanosterol demethylase, causing damage to the cell membrane. In the literature, we have many findings with overexpression and mutation of different *ERG* genes, but the most frequent is *ERG11*. Alterations in the *ERG11* gene could be due to gene overexpression or prevention of azole binding on lanosterol-4,4-dimethylcholesta-8,14,24-trienol, which could be through steric blockade of the ligand-binding pocket where azole antifungals compete with the ergosterol precursor allowing the formation of ergosterol and, consequently, losing azole susceptibility (Nishimoto; Sharma; Rogers, 2020). Fan et al., showed by Reverse Transcription Polymerase Chain Reaction (RT-PCR) that azole-resistant *C. tropicalis* isolates have higher expression of the *ERG11* gene than susceptible isolates and, in 92% of resistant isolates, amino acid substitutions in A395T and C461T that resulted in Y132C and S154C (Fan; Xiao; Zhang, 2019; Song et al., 2022). The same homozygous mutations

that result in substitutions in A395T and C461T were also found in *C. parapsilosis* (Nabili et al., 2016; Nishimoto; Sharma; Rogers, 2020).

In addition, there is evidence in the literature of the action of gene promoters of the *ERG* genes, namely, *UPC2* and ndt80. They have the role of precisely inducing the expression of the *ERG* genes, in the same way that their deletion can be a mechanism to control resistance through the overproduction of ergosterol in *Saccharomyces cerevisiae*, *C. albicans* and *C. parapsilosis* (Branco, 2017; Song et al., 2022).

Figure 2. Schematic representations of the ergosterol biosynthesis pathway, and alterations involved in azole resistance (Macedo; Dias, 2024).



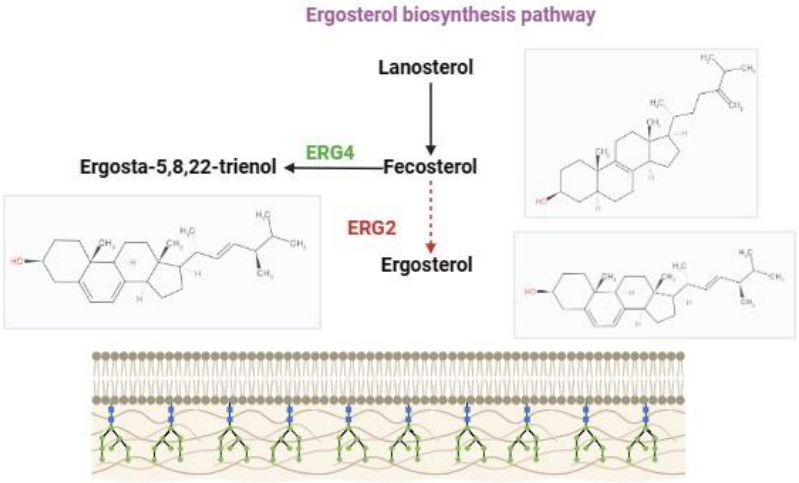
(A) A schematic of the ergosterol biosynthesis pathway, which highlights the action of Azoles in the *ERG11* gene. (B) The overexpression of *ERG11*, leads to the synthesis of ergosterol independently of the azole action. (C) A mutation in *ERG11*, which inhibits the binding of azoles in lanosterol-4,4-dimethylcholesta-8,14,24-trienol (Macedo; Dias, 2024).

2.6.2. Alternative pathway of ergosterol biosynthesis

The essential mechanism of action of AmB consists of binding to ergosterol present in the cell membrane. Non-molecular or acquired resistance is the previous use of azoles that inhibit ergosterol biosynthesis, which has been described for multiple

Candida species, including *C. albicans, C. parapsilosis, C. guilliermondii,* and *C. luisitaniae* (Vazquez et al., 1998). A molecular mechanism involved in ergosterol biosynthesis in the *ERG2* gene mutation leads to an alternative pathway that results in another formulation, Ergosta-5,8,22-trienol, which is not susceptible to AmB (Hull; Bader; Parker, 2012).

Figure 3. Schematic representations of ergosterol biosynthesis pathway and mutation in the *ERG2* gene leading to an alternative pathway as a resistance mechanism for AmB (Macedo; Dias, 2024).



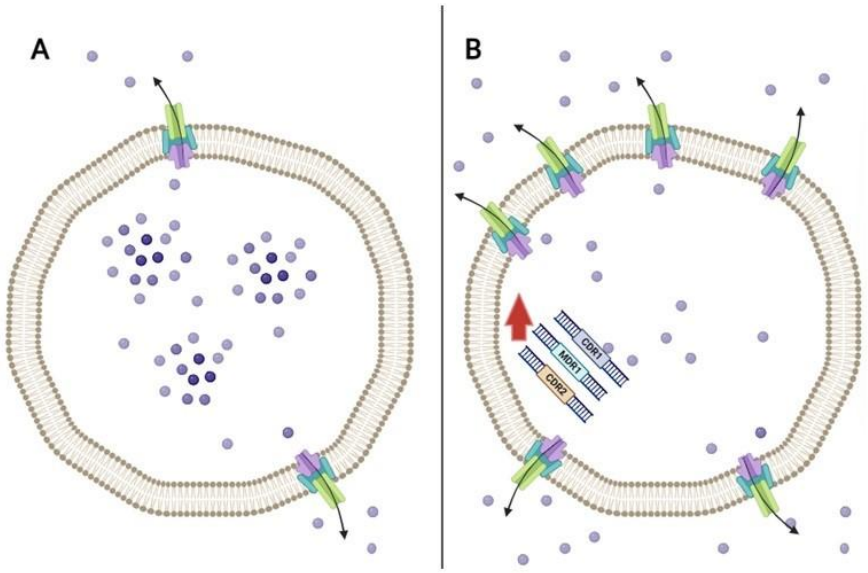
2.6.3. Overexpression of the efflux pump

Another known mechanism is the efflux pump, where pathogens can export azole drugs from inside the cell to the outside. Expression is regulated by the genes *CDR2, CDR1* and *MDR1*, which coordinate the synthesis of ABC transport proteins that act on the cell membrane by exporting azole substances. In cases of overexpression, the synthesis of ABC transporters is higher, causing greater action of the efflux pump; transcription factors, namely TAC1 and Mrr1, are also synthesized by the gain in maintenance related to the mutation (Ben-ami; Kontoyiannis, 2021).

Mrr1 and Tac1b are both transcription factors that, respectively, act on the expression of *CDR1* and positively regulate the expression of the ATP-binding cassette (ABC) transporter *CDR1*, involved in the efflux of azole resistance in *C. auris* (Fan et al., 2021). A mutation in Tac1b and increased expression in *CDR1* were found in clinical isolates of *C. auris* in synergy with fluconazole-resistant isolates (Lockhart;

Etienne; Vallabhaneni, 2017; Carolus; Pierson; Muñoz, 2021). A mutation in the *CDR4* and *MDR1* genes, involved in ABC transporters, was also described for this multidrug-resistant species (Fan et al., 2021). Homozygous alleles (A/A) in the MDR promoter confer higher levels of *CDR2* and *MDR1* expression and resistance to fluconazole in *C. albicans* (Castanheira et al., 2017). In *C. glabrata*, overexpression in *CDR1* and *CDR2*, and *PDR1* Y372C gain of function mutation, which resulted in decreased intracellular triazole accumulation (Cavalheiro; Costa; Silva-dias, 2018). As did increased expression in *MDR1* and *CDR1* in *C. parapsilosis* (Castanheira et al., 2020; Handelman; Osherov, 2022).

Figure 4. Schematic diagram illustrating the efflux pump activity and ABC transporters in the fungal membrane (Macedo; Dias, 2024).



(A) A schematic diagram illustrating the normal action of an efflux pump. (B) A schematic diagram showing the efflux pump with increased expression of *MDR1*, *CDR1* and *CDR2* genes, leading to the synthesis of more ABC transporters, and consequently, an overly active efflux pump (Macedo; Dias, 2024).

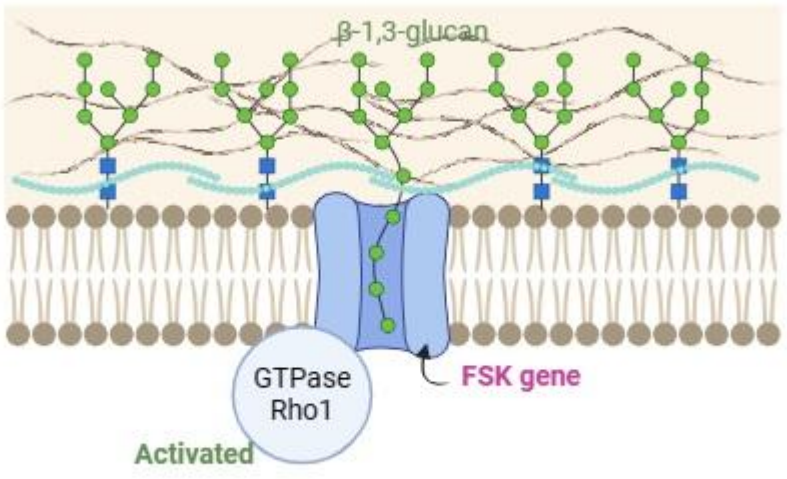
2.6.4. *FKS* genes

The fungal cell wall contains β-1,3-Glucan, which is synthesized by a membrane protein synthase encoded by *FKS* genes. This synthesis is regulated by the GTPase Rho1. Once the glucan is polymerized, the encoded protein moves to the extracellular space (Hu; Yang; Chai, 2023). The clinical advancement of echinocandin resistance is associated with an intrinsic amino acid substitution in two narrow hotspot

regions of *FKS1* for all *Candida* species and *FKS*2 in *C. glabrata* (Arendrup; Perlin, 2014b).

Alteration in *FKS* genes was described in 73.3% of resistant *C. glabrata* isolates, including *FKS2 HS1 S663P, FKS1 HS1 S629P, FKS1 HS1 F625S* and *FKS2 HS1 F659S/Y*, first in four isolates and the others in three isolates. In addition, three isolates carried mudouble mutations, they were *FKS1 HS1 S629P/FKS2 HS1 S663P* or *FKS1 HS1 F625S/FKS2 HS1 F659Y* (Fan et al., 2021; FRaser; Thorn; Lawrance, 2020; Alexander; Johnson; Pfeiffer, 2013). Diekema et al. also pointed out mutations in *HS* for 51 *C. glabrata* isolates, and for other *Candida* spp. such as *C. albicans* and *C. tropicalis* with the same *HS* mutations (Diekema et al., 2019). These types of mutations have been shown to influence the sensitivity of the GS enzyme complex to inhibition by individual echinocandins (Fraser; Thorn; Lawrance, 2020; Song et al., 2022). In a multicenter study in India, the S639F mutation in *FKS1* was described in four echinocandin-resistant isolates of *C. auris* (Chowdhary; Prakash; Sharma, 2018).

Figure 5. Schematic representations of fungal cell wall beta 1,3 glucan biosynthesis (FKS gene) (Macedo; Dias, 2024).



2.6.5. Previous exposure to azoles

A study measured the susceptibility of *Candida* species isolates to AmB alone and with previous exposure to azoles. The results showed that pre-exposure to azoles confers a decreased fungicidal effect on AmB, conferring acquired resistance

to all isolates (Vazquez et al., 1998).

2.6.6. Other factors

Many variables contribute to antifungal resistance, like the host's immune system. Little is known about the role of the host immune system in antifungal resistance in clinical conditions, but it has been described as a form of inability to sustain the action of the antifungal drug by the immune response of the immunocompromised host, resulting in prolonged use of the drug (Mancuso et al., 2022). This exposure to suboptimal levels of the drug leads to the selection of resistant strains, creating new subclinical reservoirs and infections (Revie et al., 2018). As for the battle of the host immune system against fungal infections, in some cases it may face some difficulties. This may be immunologically related, such as HIV patients who are more susceptible to fungal infections, mainly by *C. albicans,* but can also be caused by *C. tropicalis, C. krusei* or *C. parapsilosis* (Singh; Fatima; Hameed, 2015). Furthermore, in immunosuppressive therapy, such as in cancer patients or patients with hematological disorders, a risk factor for contracting fungal infections (Pappas et al., 2018).

2.7 Environment Decontamination and Hospital Fungal Infections Management

As it is a concerning topic, the healthcare-associated infections caused by *Candida* species may have a certain influence through the difficult to access a quick and assertive diagnosis. The management occurs according to each hospital or region to insert a primary empirical approach to treatment due to the lack of timely access to fungal diagnosis (Santolaya et al., 2018).

A study presented findings from a nationwide survey conducted among thirteen of the largest pediatric units in the United Kingdom and discussed the predominance of empirical treatment approaches among the surveyed centers, influenced by a lack of pediatric studies supporting pre-emptive strategies. Out of the responding hospitals, the majority reported having their own local guidelines for managing invasive fungal diseases (IFD), reflecting a strong reliance on institution-specific protocols. Among the centers that utilized established international guidelines some referenced the IDSA 2016 Practice Guidelines for the Diagnosis and Management of Aspergillosis, as well as the IDSA 2016 Practice Guideline for the Diagnosis and Management of Candidiasis, others reported following the ESCMID

Guideline for the Diagnosis and Management of *Candida* Diseases Guideline for the Use of Antimicrobial Agents in Neutropenic Patients with Cancer. This diverse adoption of guidelines highlights variations in clinical practice and the integration of both local and international standards in managing IFD across different institutions (Ferreras-antolín; Sharland; Warris, 2019).

During the COVID-19 pandemic, there has been a significant rise in interest in comparing microbial contamination of surfaces, both in community settings (Caggiano et al., 2021) and healthcare environments (Zhou et al., 2023). In healthcare, rooms previously occupied by patients with multidrug-resistant organisms pose a considerable risk of transmission to subsequent patients if not properly disinfected (Boyce, 2021). Effective surface cleaning and disinfection, particularly during patient discharge, are critical components of a robust infection prevention program. It is imperative to not only use disinfectants appropriately but also ensure they are effective biocides with a broad spectrum of activity against microorganisms to minimize the risk of infections caused by healthcare-associated pathogens (Weber; Anderson; Rutala, 2013).

2.7.1 Quaternary ammonium compounds

Quaternary ammonium compounds, such as alkyl dimethyl benzyl ammonium chlorides and dialkyl dimethyl ammonium chlorides, are widely used disinfectants. They are characterized by their bactericidal, fungicidal, and virucidal properties, although their virucidal action is limited to lipophilic or enveloped viruses, and they are not effective against non-enveloped viruses or *Mycobacterium tuberculosis*. These compounds are low in corrosiveness and toxicity, making them suitable for use on fixed surfaces, including environments like nutrition and neonatology areas, provided neonates are not present. Their mechanism of action involves the inactivation of energy-producing enzymes, denaturation of proteins and acid nucleic, and disruption of the cell membrane. However, they can be inactivated in the presence of organic matter. The concentration of these compounds varies depending on the manufacturer's formulation (Brasil, 2010).

2.7.2 Inorganic chlorite active compounds

Active chlorine-releasing compounds, particularly sodium, calcium, and lithium hypochlorite's, are commonly used disinfectants. These compounds exhibit bactericidal, virucidal, fungicidal, tuberculocidal, and sporicidal properties, depending on their concentration. Available in liquid or powder form, they offer a broad spectrum of activity, rapid action, and low cost, making them effective for the disinfection of fixed surfaces. While the exact mechanism of action remains unclear, these compounds are highly effective within their use parameters. However, they have several disadvantages, including instability when exposed to sunlight, temperatures above 25ºC, or acidic pH levels. They are also inactivated by organic matter, corrosive to metals, have an unpleasant odor, and its volatility may cause irritation to the eyes and mucous membranes, and it is high reactivity can lead to corrosion of medical equipment and delicate surfaces. Recommended concentrations for disinfection range from 0.02% to 1.0% (Brasil, 2010).

2.7.3 Hydrogen peroxide

Published reports ascribe good germicidal activity to hydrogen peroxide and attest to its bactericidal, virucidal, sporicidal, and fungicidal properties (Cdc, 2024). Hydrogen peroxide works by producing destructive hydroxyl free radicals that can attack membrane lipids, DNA, and other essential cell components. Catalase, produced by aerobic organisms and facultative anaerobes that possess cytochrome systems, can protect cells from metabolically produced hydrogen peroxide by degrading hydrogen peroxide to water and oxygen, but this defense can be overwhelmed by the concentrations used for disinfection (Rutala; Gergen; Weber, 1993; Cdc, 2024).

Commercially available 3% hydrogen peroxide is a reliable and stable disinfectant for inanimate surfaces. It is commonly used at concentrations ranging from 3% to 6% for disinfecting items such as soft contact lenses (e.g., 3% for 2–3 hours), tonometer biprisms, ventilators, fabrics, and endoscopes. It has also proven effective in spot-disinfecting fabrics in patient rooms. However, improper rinsing of hydrogen peroxide-soaked medical devices, such as tonometer tips, has been associated with corneal damage. Hydrogen peroxide has also been tested as a disinfectant for urinary drainage bags to address bladder bacteriuria and environmental contamination. While

it effectively reduced microbial contamination within the bags, this procedure did not lead to a decrease in the incidence of catheter-associated bacteriuria, highlighting its limitations in such applications (Cdc, 2024).

3. OBJECTIVE

3.1 General objective

To determine the antifungal susceptibility profile through physiological and molecular tests, evaluate efflux pump activity, and assess biocide tolerance in *Candida* spp. isolated by the microbiology service of the Côrtes Villela Laboratory, located in a tertiary hospital in the city of Juiz de Fora, Minas Gerais.

3.2 Specific objectives

- To analyze the results of the antifungal susceptibility profile of *Candida* spp. isolated from community and hospital individuals during the year 2021 and clinical and microbiological data;
- To evaluate efflux pump activity in clinical isolates of *Candida* spp.;
- To evaluate the presence of antifungal resistance genes *ERG11, MDR1, ERG2, CDR1, CDR2* and *FKS1* among clinical isolates;
- To determine the biocide tolerance profile of *Candida* spp. Isolates;

4. METHODOLOGY

4.1 Study design

This is an observational and experimental study that gathers retrospective and prospective experimental data on clinical isolates of *Candida* spp. arranged in a collection, to elucidate the antifungal and biocide sensitivity profile of the strains obtained in 2021, from community individuals and individuals admitted to a tertiary hospital in Juiz de Fora, Minas Gerais.

The hospital in question has approximately 120 beds, covering units such as intensive care for adults and neonates, coronary care unit, neurological unit, wards, surgical center and outpatient care. In addition, it offers specialized clinics and diagnostic services exclusively for the private network.

The retrospective study involved the evaluation of clinical and microbiological data, such as the determination of clinical specimen, hospitalization unit or if it was of community origin, and clinical outcome of the participants, obtained directly from electronic medical records. The prospective study involves experimental data from the same collection of clinical isolates.

This study was conducted after consultation and consent of the participants, according to a project approved by the Ethics Committee for Research Involving Human Beings of the Federal University of Juiz de Fora, under number 3.783.237, CAAE 18611019.6.0000.5147.

4.2 Assessment of the integrity of the isolates

The collection samples were kept in sterile isotonic saline solution with 0.9% NaCl (sodium chloride) (SANOBIOL, BRASIL) in 15 ml glass test tubes with screw caps and a sample identification label. There was a total of 102 samples. The clinical isolates of *Candida* spp. from the collection underwent a cultivation process in Sabouraud Agar plates (NEOGEN, BRASIL), with incubation for at least 48 hours at 36ºC. Morphological aspects were observed, and the Gram staining method was performed to assess the growth, viability and integrity of the isolates.

4.3 Antifungal susceptibility testing

The isolates of the genus were evaluated for their antimicrobial susceptibility profile, according to the current CLSI (Clinical and Laboratory Standard Institute)

(2017) guidelines, using the disk diffusion method, applied through commercial disks of fluconazole 25 µg, voriconazole 1 µg, caspofungin 5 µg and amphotericin B 20 µg (Liofilchem Diagnostics, Italy) in Agar Sabouraud plates (NEOGEN, BRASIL). The interpretation of the yeast inhibition zones was performed according to the reference described in document M44 (Clsi, 2017) and the manufacturer's Liofilchem criteria.

4.4 Identification of resistance genes

The genomic DNA of the yeasts was extracted by the phenol-chloroform digestion and purification method, according to the study by Rodrigues et al. (2013) with some modifications and subsequently stored in a freezer at -80ºC. Quantification (Nanodrop) and DNA integrity gel were performed, standardizing dsDNA to 50 g/l. The genes *ERG11* (fwd5'-CCA.AGG.CTA.GTG.CTG.TTT.CT-3';rev5'-TGT.GTC.TAC.CAC.CAC.CGA.AT-3') (Fernandes; Silva; Henriques, 2016), *CDR1* (fwd 5'-CCA.GAG.GTT.TGG.ATT.CCG.CT-3'; rev5'-TGG.CTT.TGT.CTG.CTT.TCC.CA-3') (Jin et al., 2018), *CDR2* (fwd5'-GCT.TAG.ATG.CCG.CCA.CTG-3'; rev5'-AGC.CCA.TTC.TGA.TGA.AAT.ACT.C-3') (Shao et al., 2016), *MDR1* (fwd 5'-GGGTGCATCATTCCAGCCTA-3'; rev5'-GGGATGGCAATCATCACGAG-3') (Jin et al., 2018)], *ERG2* (fwd 5'-ATGAAGTTCTTTATCAA T-3'; rev5'-TTAGAACTTTTGGTTTTG-3') (Hull; Bader; Parker, 2012), and *FKS1* (fwd 5'-GAAATCGGCATATGCTGTGTC-3'; rev5'-AATGAACGACCAATGGAGAAG-3') (Baixench et al., 2007) were investigated by PCR. The reaction was performed using 1µl at 5 µM/l of each primer in addition to 12.5µl of Master mix G2 Green (PROMEGA, USA) and 9.5µl of sterile water, for 1µl of DNA. The reaction parameters were established for each gene as described by their respective authors with modifications. The generated amplicons were subjected to electrophoresis using a 1.5% agarose gel (LGCbiotecnologia, Brazil) with ethidium bromide (EtBr) (AMRESCO, USA), followed by separation at 90V for 40 minutes. The gel was visualized by UV light using a transilluminator to identify the DNA bands. Subsequently, 10% of the positive isolates for each gene were selected for Sanger sequencing to confirm the genes investigated.

4.5 Evaluation of efflux pump activity

The expression of efflux pumps was evaluated using Ethidium Bromide (EtBr) (AMRESCO, USA) according to the Cartwheel method, with modifications (Martins et

al., 2011). The method consisted of culture on Mueller-Hinton Agar (Kasvi/Brazil) at concentrations of 0.5 µg/mL, 1.0 µg/mL, 1.5 µg/mL, 2.0 µg/mL and 2.5 µg/mL of EtBr and plates without EtBr used as control for microbial growth. Solutions equivalent to a turbidity of 0.5 on the McFarland scale were prepared for each isolate and ATCC strains, which were inoculated with the aid of a Steers replicator. Duplicates were made for each concentration level and control. The plates were incubated for 24h at 35 °C, protected from light. After incubation, the plates were read under ultraviolet light, and the presence of fluorescence emitted by the samples was a negative indication of efflux pump action.

The index for the minimum concentration of EtBr (MCEtBr) that produces fluorescence of the isolates (MDR) compared to each respective reference strain species (*C. albicans* ATCC14053, *C. parapsilosis* ATCC22019, *C. tropicalis* ATCC66029, and *C. glabrata* ATCC2001) was calculated using the following formula: Index=[MCEtBr(MDR)-MCEtBr(REF)]/MCEtBr(REF) (Martins et al., 2011).

4.6 Assessment of biocide tolerance

An adaptation of the disk diffusion technique (DDT) described by CLSI (2017) was performed to assess tolerance to biocides routinely used in the hospital where the isolates originated. From suspensions of isolates on a McFarland turbidity scale of 0.5, the isolates were inoculated uniformly with the aid of a swab in Mueller-Hinton agar culture medium (Kasvi/Brazil), to which filter paper discs soaked in 5µL of 1%, 1.5%, 2% sodium hypochlorite solution (Start/Brazil), 4.25% diluted hydrogen peroxide (Rioquímica/Brazil) and 5% benzalkonium chloride (Santos/Brazil) were added. The plates were incubated at 35ºC, and readings of the diameter of the inhibition zones were taken after 24 hours and 48 hours of incubation.

The biocides used were of commercial quality, stored under regular conditions and used within validity periods. All tests were performed in duplicate for all 100 clinical isolates and reference strains of *C. albicans* ATCC 14053, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 66029 and *N. glabratus* ATCC 2001 were used as experimental controls.

4.7 Statistical Analysis

For the statistical analysis of the data obtained by the biocide test, Student's t-test (p-value 0.05) for the mean halo diameters comparing the reference strain and

isolates in separate groups of *C. albicans* and NCAC. ANOVA two-way testing (p-value 0.05) was used to associate the results of the minimum inhibitory concentration of EtBr (MICEtBr) for positivity of the efflux pump testing and resistance genes described to overexpression of efflux pump.

5. RESULTS

5.1 Clinical and microbiolobigal data

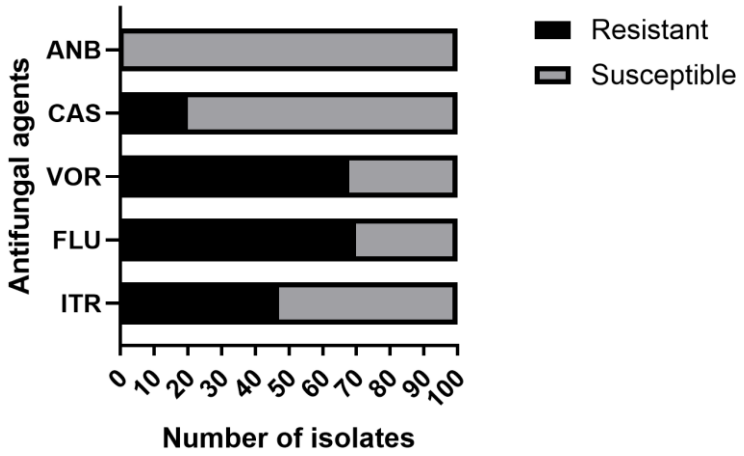
In the distribution of species, 59 (59%) *C. albicans* out of 100 isolates were found, and 41 were NCAC (41%), of which 25 were *C. tropicalis*, 12 were *C. parapsilosis*, 2 were *N. glabratus*, and one each of *M. guilliermondii* and *P. kudriavzevii*. Sixty-three individuals in this study were hospitalized. Among the clinical specimens of *C. albicans*, 39 were from hospitalized individuals and 20 from community residents, representing 66.1% of hospitalizations for the species. Within the NCAC group, 22 clinical specimens were of hospital origin and 19 from community residents, representing 53.7% of hospitalizations in the group. The median duration of hospital stay was 39.5 days (range: 6–202) for *C. albicans* infections, compared with 31.5 days (range: 7–162) for infections caused by NCAC species. Invasive medical device use was recorded in 60 patients with *C. albicans* and 29 with NCAC species. The study also reported 28 in-hospital deaths associated with *C. albicans* infections and 16 deaths related to non-*albicans*.

5.1. Antifungal susceptibility testing

Antifungal susceptibility testing was performed on a total of 100 clinical isolates of *Candida* spp. Of these, (n=87/87%) showed resistance to at least one antifungal tested, representing a high percentage of microorganisms. The highest resistance rate to antifungals exhibited by the evaluated strains was observed for Fluconazole (n=70), followed by Voriconazole (n=68), Itraconazole (n=47), Caspofungin (n=20), and the highest susceptibility was for amphotericin B (n=1).Regarding the antifungal classes tested, (n=15/15%) of the yeasts were resistant to one antifungal agent, with the vast majority being resistant to caspofungin (n=12). Resistance of the yeasts to two classes of antifungals was observed in (n=21/21%), with some isolates exhibiting combined resistance to azoles and echinocandins (Table 2). Only one yeast of *C. tropicalis* showed resistance to the azole class and amphotericin B, which was the antifungal that showed the greatest efficacy. One isolate of *C. albicans* showed resistance to four antifungal agents that combined azole and echinocandin classes.

A total of 59 *C. albicans* (n=47/79.7%) were resistant to fluconazole and voriconazole. While the non-*albicans Candida* spp. group (n=27/65.9%) was also resistant to fluconazole and voriconazole (Figure 6).

Figure 6. Antifungal susceptibility profile of *Candida* spp. isolates (n=100).



ITR- Itraconazole; FLU- Fluconazole; VOR- Voriconazole; CAS- Caspofungin; ANB- Amphotericin b.

5.2. Detection of antifungal resistance genes

The *ERG11* gene was identified in 17 isolates of *C. albicans*, 14 *C. tropicalis,* 5 *C. parapsilosis* and 2 *N. glabratus,* being the most frequent gene, representing 28.8% and 51.2% of the *C. albicans* and NCAC groups, respectively. The *CDR2* gene was the second most frequent: *C. albicans* (n=11), *C. tropicalis* (n=4), *C. parapsilosis* (n=1) and *N. glabratus* (n=1), present in 18.6% and 14.6% of the *C. albicans* and NCAC groups, respectively. The *ERG2* and *FKS1* genes were not found in any of the isolates in this study (Table 2).

Table 2. Phenotypic and genotypic antifungal resistance profile of clinical *Candida* spp. isolates (n=87).

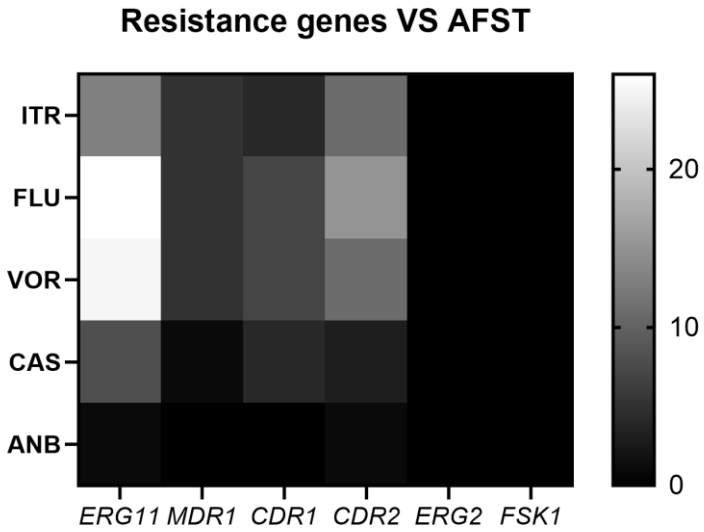
Phenotypic Antifungal resistance profile	Genotypic Antifungal Resistance Profile	Species (n)	Clinical outcome Death
ITR/FLU/VOR/CAS (n=1)	***ERG11;CDR1***	*C. albicans* (n=1)	n=1 (*100%)*
ITR/FLU/VOR (n=41)	***ERG11;CDR1***	*C. tropicalis* (n=1)	*n=18* (43.%)
	ERG11;MDR1	*C. tropicalis* (n=2)	
	ERG11	*C. albicans* (n=2); *C. tropicalis* (n=4)	
	MDR1	*C. tropicalis* (n=2)	
	CDR1;CDR2	*C. albicans* (n=1); *C. tropicalis* (n=1)	
	CDR2	*C. albicans* (n=7); *C. tropicalis* (n=1)	
	None	*C. albicans* (n=17); *C. tropicalis* (n=3)	
ITR/CAS (n=3)	***CDR2***	*C. tropicalis* (n=1)	*n=1* (33.33%)
	ERG11	*C. parapsilosis* (n=1)	
	None	*C. parapsilosis* (n=1)	
FLU/VOR/CAS (n=3)	***ERG11;CDR1;MDR1***	*C. tropicalis* (n=1)	*n=1* (33.33%)
	ERG11;CDR1	*C. tropicalis* (n=1)	
	None	*C. albicans* (n=1)	
FLU/VOR (n=22)	***ERG11***	*C. albicans* (n=10); *N. glabratus* (n=1); *C. tropicalis* (n=1)	*n=10* (45.45%)
	None	*C. albicans* (n=8); *M. guilliermondii* (n=1); *C. tropicalis* (n=1)	
FLU/VOR/ANB (n=1)	***ERG11;CDR2***	*C. tropicalis* (n=1)	*
FLU (n=1)	***ERG11;CDR2***	*N. glabratus* (n=1)	n=1 (100%)
FLU/CAS (n=1)	**None**	*P. kudriavzevii* (n=1)	n=1 (100%)
CAS (n=12)	***ERG11***	*C. parapsilosis* (n=4)	n=2 (16.67%)
	CDR1;CDR2	*C. albicans* (n=1)	
	CDR2	*C. parapsilosis* (n=1)	
	None	*C. parapsilosis* (n=4); *C. albicans* (n=1); *C. tropicalis* (n=1)	
ITR (n=2)	***ERG11;MDR1***	*C. tropicalis* (n=1)	n=2 (100%)
	ERG11	*C. tropicalis* (n=1)	

Legend: ITR- Itraconazole; VOR-Voriconazole; FLU- FLuconazole; CAS- Caspofungin; ANB- Amphotericin B. *Clinical specimen of community origin

ERG11 (n=64) was the most frequent gene found associated with phenotypic isolates resistant to azole derivatives, such as *MDR1* (n=16), *CDR1* (n=22) and *CDR2* (n=41). *FKS1* or *ERG2* were not identified, however, *ERG11* (n=8), *MDR1* (n=1), *CDR1* (n=4) and *CDR2* (n=3) were found to be associated with caspofungin-resistant isolates, and both *ERG11* and *CDR2* were associated with the amphotericin B-resistant isolate (n=1) that also presented resistance to azole derivatives in a community patient. The mortality rate was observed in 100% of cases for *C. albicans* with multiple resistance, while species such as *N. glabratus* and *P. kudriavzevii* also

presented isolated resistance with 100% mortality. Furthermore, in some resistant isolates, no mutations were detected in the genes evaluated (Figure 7).

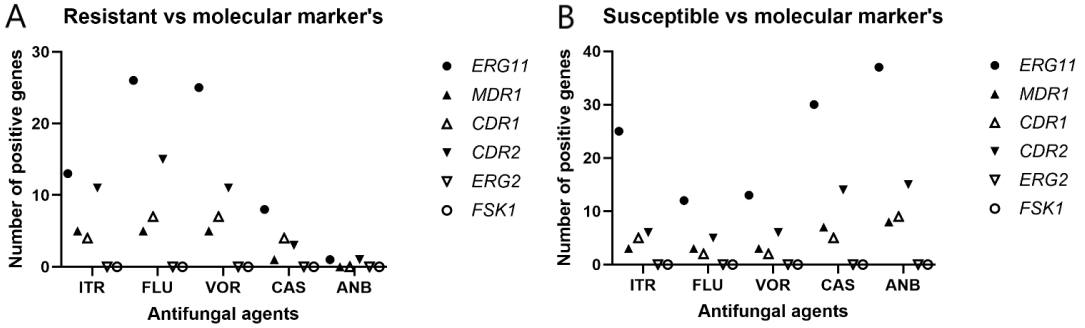
Figure 7. Heat map the pattern of distribution of resistance genes according to the antifungal susceptibility testing (AFST) results of the resistant isolates.



Legend: ITR- Itraconazole; FLU- Fluconazole; VOR- Voriconazole; CAS- Caspofungin; ANB- Amphotericin b; AFST- Antifungal susceptibility testing.

The *ERG11*, was highly identified in resistant strains, particularly with VOR and FLU, such as *CDR2* and *CDR1*, which were also more found associated with these antifungal agents (Figure 8).

Figure 8. Comparison of molecular marker findings in Resistant vs. Susceptible strains across different antifungal agents.



Legend: (A) Distribution of the number of positive genes for molecular markers (*ERG11, MDR1, CDR1, CDR2, ERG2* and *FKS1*) in resistant strains treated with various antifungal agents (ITR: Itraconazole, FLU: Fluconazole, VOR: Voriconazole, CAS: Caspofungin, ANB: Amphotericin b). (B) Distribution of the number of positive genes for molecular markers in

susceptible strains under the same conditions. Each marker is represented by its respective symbols.

5.3. Efflux pump activity assessment

The ability of clinical isolates to efflux EtBr was classified in relation to their reference strains by calculating their index for efflux activity (Table 3). The results showed that in the *C. albicans* group, the majority (n=10) followed the expected MCEtBr (0.5 µg/l) of the ATCC, however, 14 isolates presented a MCEtBr higher than the reference, such as 1.0 µg/l (n=10) and 1.5 µg/l (n=4), with an index range of 1 to 2, respectively.

Table 3. Expression of efflux pump activity among clinical isolates of *Candida* spp.

Species	Isolates	MC_{EtBr} ($\mu g/\mu l$)	**Index[a]**
Candida albicans	**ATCC 14053**	0.5	
	Clinical isolates (n=45)	0.5	**0**
	Clinical isolates (n=10)	1.0	**1**
	Clinical isolates (n=4)	1.5	**2**
non-albicans Candida spp.	***Candida tropicalis* ATCC 66029**	0.5	
	Clinical isolates *C. tropicalis* (n=19)	0.5	**0**
	Clinical isolates *C. tropicalis* (n=6)	1.0	**1**
	***Candida parapsilosis* ATCC 22019**	0.5	
	Clinical isolates *C. parapsilosis* (n=9)	0.5	**0**
	Clinical isolates *C. parapsilosis* (n=3)	1.0	**1**
	***Nakaseomyces glabratus (syn. Candida glabrata)* ATCC 2001**	0.5	
	Clinical isolates *N. glabratus (syn. C. glabrata)* (n=2)	0.5	**0**
	Clinical isolates *M. guilliermondii (syn. C. guilliermondii)* (n=1)	1.0	**1**
	Clinical isolates *P. kudriavzevii (syn. C. krusei)* (n=1)	1.0	**1**

[a]The index (defined in the Materials and Methods section) provided the range of efflux activity that can be obtained for each strain in comparison with the reference strain.

In the NCAC group, a MCEtBr of 0.5 µg/l was observed in all ATCC isolates, and most isolates also presented the same MCEtBr as their respective reference species. With a change of 6 isolates of *C. tropicalis,* 3 of *C. parapsilosis* and 1 each of

M. guilliermondii (syn. *C. guilliermondii)* and *P. kudriavzevii* (syn. *C. krusei*), as all presented a MCEtBr greater than the reference of 1.0 µg/l, with an index of 1.

An analysis between the efflux pump results and the molecular markers for efflux pump overexpression showed a higher frequence of these molecular markers at the smaller MICEtBr for efflux pump positivity. As in the isolates with MICEtBr 0.5 for positivity of efflux pump activity, n=16 presented *CDR2*, n=7 *CDR1* and n=6 for *MDR1*, with a statistical difference (0.0537) between the number of sensitive genes compared to the different concentrations of EtBr (Table 4).

Table 4. Comparison between genotypic and phenotypic profiles associated with efflux pump overexpression in *Candida* spp. strains.

Molecular marker's	MIC_{EtBr} 0.5 (n=79)	MIC_{EtBr} 1.0 (n=21)	MIC_{EtBr} 1.5 (n=4)	***p-value***
***MDR1* (n=8)**	6	2	0	Row factor 0.5533
***CDR1* (n=9)**	7	1	1	Column factor 0.0537
***CDR2* (n=17)**	16	0	1	

MIC_{EtBr} -minimum inhibitory concentration of ethidium bromide for positivity of efflux pump.

5.4. Assessment of biocides tolerance

The biocide tolerance determination test using the disk diffusion method revealed that the most effective compound was sodium hypochlorite, especially at 2% when compared to hydrogen peroxide at 4.25% (diluted 1:16) and benzalkonium chloride 5% (Table 5).

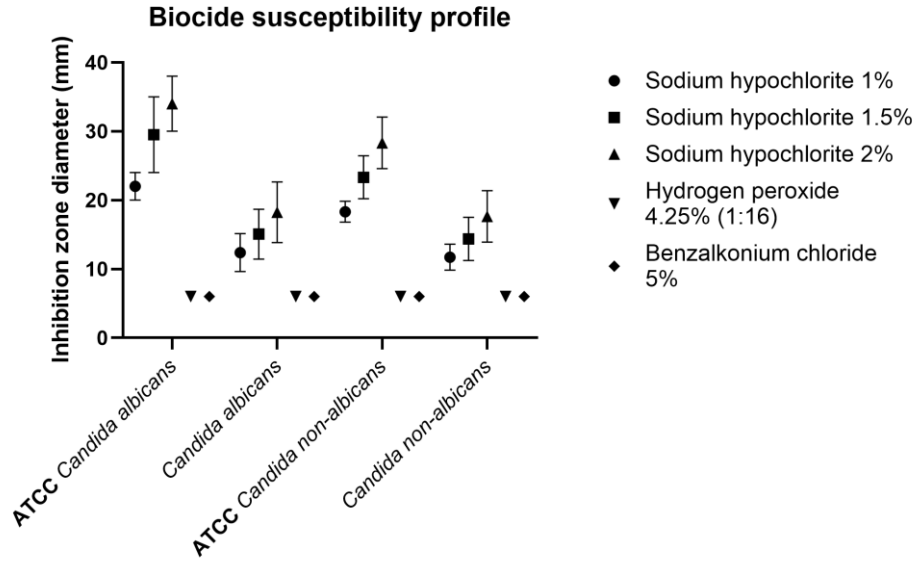
Table 5. Biocide tolerance pattern on the clinical isolates of *Candida* spp (n=100).

Isolates	Sodium hypochlorite 1%	Sodium hypochlorite 1.5%	Sodium hypochlorite 2%	Hydrogen peroxide 4.25% (1:16)	Benzalkonium chloride 5%	*p*-value
	Mean±standard deviation					
	Inhibition halo diameter (mm)					
Candida albicans ATCC 14053	22.00±2.00	29.50±5.50	34.*0* ±4.00	6.00±0.00	6.00±0.00	**0.041**
Clinical isolates *Candida albicans*	12.40±2.74	15.06±3.61	18.25±4.40	6.00±0.00	6.00±0.00	
non-albicans Candida spp. ATCC'S (reference lineages)	18.33±1.53	23.33±4.62	28.33±5.77	6.00±0.00	6.00±0.00	**0.037**
Clinical isolates *non-albicans Candida* spp.	11.73±1.88	14.38±3.12	17.66±3.73	6.00±0.00	6.00±0.00	

A significant difference was observed between the clinical isolates and the reference strain for each group. Clinical isolates had a significantly smaller meaning halo diameter than the reference strain, indicating greater tolerance to the compounds than the ATCC sample (Table 4).

C. albicans ATCC strains exhibited larger zones of inhibition compared to clinical isolates of *C. albicans* and NCAC species. Sodium hypochlorite demonstrated the greatest antifungal activity, with zones of inhibition increasing in size as concentration increased (1%, 1.5%, 2%), particularly in *C. albicans* ATCC strains. In contrast, hydrogen peroxide (4.25%; 1:16 dilution) and benzalkonium chloride (5%) showed no measurable zones of inhibition, indicating resistance in all groups tested (Figure 9).

Figure 9. Dispersion of inhibition halos (mm) exhibited by *Candida* spp isolates against the biocides sodium hypochlorite at 1%, 1.5%, and 2%, hydrogen peroxide at 4.25% (1:16), and benzalkonium chloride at 5%.



6. DISCUSSION

Some species have inherent resistance to specific genetically linked antifungals. For example, the intrinsic resistance of *P. kudriavzevii* (syn. *C. krusei*) to fluconazole, due to the low affinity of the drug to the target enzyme, lanosterol 14-α-demethylase (encoded by the *ERG11* gene), in addition to the activity of the efflux pump (Jamiu et al., 2021). Other fungi can also select resistant strains through exposure to antifungal agents, which can be genetic or epigenetic. This acquired resistance can occur through several stress factors, such as the patient's underlying condition(s), reduced drug bioavailability, increased drug metabolism, and impaired immune function, leading to alterations in the fungal genome (Jamiu et al., 2021; Macedo; Dias, 2024).

AFST plays a crucial role in assertive therapeutic decisions and in improving patient outcomes. Jeon S et al. (2021) validated the DDT method (CLSI M44-ED1 or ED2) against the BMD reference method, reporting high categorical agreement (96.3%), confirming the reliability of DDT in clinical settings. This allows for faster antifungal susceptibility profiling, cost-effective alternative when compared to the broth microdilution (BMD) allowing clinicians to choose the best antifungal treatment and improving patient prognosis (Castanheira et al., 2014; Berkow; Lockhart; Ostrosky-zeichner, 2020).

The analysis revealed a worrying level of antifungal resistance among 100 yeast isolates, with a significant resistant frequency of 87%. Among *C. albicans* isolates, 79.7% were resistant to fluconazole and voriconazole. Similarly, the *non-albicans* group also showed a high rate of resistance (65.9%) to the same antifungal agents. These findings show higher rates of phenotypic antifungal resistance compared to other studies (Castanheira et al., 2016; Bilal et al., 2022; Hernández-pabón et al., 2024).

A review by the SENTRY Antifungal Surveillance Program analyzed 1,846 fungal isolates from 31 countries and reported echinocandin resistance in *Candida* spp. ranging from 0.0% to 2.8%. Fluconazole resistance was observed in 11.9% of *C. glabrata* and 11.6% of *C. tropicalis* (Castanheira et al., 2016). Similarly, a study from mainland China (2011–2021) evaluating 44,716 *Candida* spp. isolates showed higher susceptibility to fluconazole in *C. albicans* (91.6%), compared to *C. tropicalis* (77.95%) and *C. glabrata* (79.4%). However, *C. parapsilosis* had the highest susceptibility (93.25%). Amphotericin B and anidulafungin were the most effective antifungals (Bilal et al., 2022).

In South America, a study conducted in a fourth-level hospital in Colombia highlighted 13.8% fluconazole resistance in *C. parapsilosis* and 5.9% dual resistance to fluconazole and voriconazole in *C. tropicalis*. Among 18 *C. glabrata* isolates, 5.5% were resistant to caspofungin and 11.1% were resistant to fluconazole, with another 5.5% showing dose-dependent susceptibility (DDS) to fluconazole (Hernández-pabón et al., 2024).

Although the most resistant strains in this study were *C. albicans,* the findings of antifungal resistance among NCAC species (n =37) represented 91.9% of the population. This is corroborated by findings in the literature and highlights the importance of the emergence and influence of non-*albicans* spp. in the clinical setting (Castanheira et al., 2016; Bilal et al., 2022; Hernández-pabón et al., 2024).

A synergy between this study and literature data is the predominance of resistance to azole derivatives, considered first-line drugs (Castanheira et al., 2016; Bilal et al., 2022; Hernández-pabón et al., 2024). Itraconazole is effective against several fungal pathogens, particularly those that cause systemic infections. The good bioavailability of fluconazole is often preferred for its safety profile and efficacy against *Candida* species. Voriconazole has a broader spectrum of activity among them, making it suitable for treating several fungal infections (Kriegl et al., 2024). This should

emphasize the need for continued research for associated or alternative treatments and other strategies to prevent acquired resistance to these drugs.

One strategy that can help us advance against antifungal resistance is to understand the genetic mutations involved in acquired resistance. In the literature, mutations in the ergosterol biosynthesis pathway (e.g., *ERG11*) and overexpression of efflux pumps, through transporter genes such as *CDR1, CDR2,* and *MDR1*, promoting azole resistance, are described as molecular mechanisms associated with antifungal resistance. Mutations in cell wall biosynthesis genes (*FKS1, FKS2,* and *PDR1*) also confer resistance to echinocandins (Czajka et al., 2023). Azole resistance was associated with mutations in genes such as *ERG11, PDR1*, and *MRR1*, while mutations in *FKS1* and *FKS2* contributed to echinocandin resistance in *C. auris* and *C. glabrata* (Bilal et al., 2022). Our results revealed that resistance to multiple antifungals (ITR/FLU/VOR/CAS) was associated with genetic mutations in *ERG11*, *CDR1* or *CDR2,* with *C. tropicalis* showing the highest frequency of these alterations. Furthermore, the frequency of resistance findings in *C. tropicalis* indicates that this species may be an important target for epidemiological surveillance and control strategies.

In addition, the mortality rate was high, with 100% in cases of ITR/FLU/VOR/CAS-resistant *C. albicans* and species such as *C. tropicalis*, *N. glabratus* and *P. kudriavzevii.* The high mortality associated with the resistance to multiple antifungals highlights the need for specific therapeutic approaches, especially in cases involving genetic mutations in pathways such as *ERG11* and efflux pumps (*CDR1, CDR2* and *MDR1*). One study identified non-synonymous mutations in the *ERG11* gene as a predominant mechanism of azole resistance, followed by overexpression of *CDR1* and *CDR2* to be associated with further azole resistance. It also highlighted cross-resistance with other azole derivatives, indicating that treatment options may be limited for patients infected with resistant strains (Paul et al., 2022).

In addition, our study showed 100% of *N. glabratus* and *P. kudriavzevii* phenotypically resistant to fluconazole, which has previously been described for *N. glabratus, P. kudriavzevii* and *C. auris* being resistant to fluconazole, highlighting the need for personalized treatment strategies based on species identification (Czajka et al., 2023).

Some isolates, although resistant to antifungals, did not present the genes evaluated, suggesting the existence of other resistance mechanisms and the need for

additional studies to identify new resistance mechanisms. A known phenomenon is heteroresistance, that refers to the variability in the response to a drug within a clonal cell population. Strains with heteroresistance cannot be detected with conventional susceptibility tests. Heteroresistance to fluconazole is a graded phenotype associated with upregulation of the ABC transporter and drug efflux, which may reflect cellular and/or epigenetic adaptation (Ben-ami et al., 2016). Standard antimicrobial susceptibility tests often fail to detect heteroresistance. This undetected resistance can result in treatment failures, as the susceptibility cells are killed off while the resistant ones persist and proliferate (Zhai et al., 2024). Further studies are crucial to unravel the mechanisms and clinical impact of heteroresistance, particularly in *Candida* species (Gautier et al., 2024)

Alarmingly, a *C. tropicalis* isolate showed resistance to four antifungal agents, combining azoles, echinocandins and amphotericin B in a community patient, associated with mutations in *ERG11* and *CDR2*. Although molecular markers of amphotericin B have been described for *N. glabratus* associated with mutations in *ERG2*, which leads to an alternative pathway of ergosterol biosynthesis, the presence of *ERG2* gene in this isolate wasn't found (Hull; Bader; Parker, 2012). Additionally, *C. tropicalis* has been described as presenting biofilm formation that leads to phenotypic resistance to this antifungal agent (Al-fattani; Douglas, 2006b). According to these results, phenotypic resistance to amphotericin B could be involved with other resistance mechanisms that were not explored in this study and that could be elucidated with more in-depth genomic analyses, or even the role of the efflux pump and its ability to export and neutralize the action of this antifungal agent.

Regarding efflux pumps, the calculated index provided an average classification of each isolate in relation to its reference species (Martins et al., 2011). From the calculated index, we were able to observe a total of 21 isolates that presented an index equal to 1 and 4 isolates of *C. albicans* within an index of 2. In the study by Salama et al. (2024), they investigated the efflux pump activity in *Klebsiella pneumoniae* evaluating using the Cartwheel method with variable concentrations of ethidium bromide (EtBr). An index range of 0.5 to 1.5 (n = 7) was found, with efflux pump genes overexpressed in strains classified in index 1.5 also linked to higher minimum inhibitory concentration (MIC) of antibiotics. This suggests that when isolates have higher efflux activity than the reference strain, they may be more efficient in expelling substrates, which may contribute to greater resistance to certain antifungal

agents and more tolerance to biocidal compounds. However, efflux activity can be influenced by several factors, including the presence of efflux pump inhibitors, environmental conditions, and genetic variations among strains. It is important to consider these factors when interpreting the index (Kalvass; Pollack, 2007).

A study evaluated the phenotypic expression of the efflux pump, using the method of Castelo-Branco et al. (2013), with promethazine and haloperidol, which resulted in the reversion of resistance to itraconazole and fluconazole in all *Candida* spp. isolates tested. These compounds act to inhibit efflux pump activity, resulting in the reversion of azole resistance, suggesting that azole resistance among *Candida* spp. is related to the increased activity of these pumps (Brilhante et al., 2016).

The results of the efflux pump and molecular markers for efflux pump overexpression showed a significantly higher frequency of these molecular markers at the minimum inhibitory concentration of ethidium bromide (MICEtBr) for efflux pump positivity. Studies have already described how overexpression of these genes can ensure resistance to antifungals. It was described that homozygous alleles (A/A) in the MDR promoter confer higher levels of *CDR2* and *MDR1* expression and resistance to fluconazole in *C. albicans* (Castanheira et al., 2017b). In *C. glabrata* isolates, overexpression in *CDR1* and *CDR2* gained a functional mutation, which resulted in decreased intracellular accumulation of azole derivatives (Cavalheiro et al., 2019). However, regarding the validation of the phenotypic expression of this resistance mechanism, studies are still needed to elucidate the phenomenon and its clinical implications.

Our population-based sample highlights the significant burden of *Candida* spp. infections in hospitalized patients. *C. albicans* was responsible for the majority (60%) of infections, with a median hospital stay of 39.5 days, longer than the 31.5 days observed for NCAC species. The frequent use of invasive medical devices in both groups (60 patients with *C. albicans* and 29 with NCAC) highlights the association between device use and infection risk. Additionally, the mortality rate was higher in *C. albicans* infections (28 deaths) compared to NCAC (16 deaths), reflecting the potentially more severe clinical outcomes of *C. albicans.*

Hospitalization for *Candida* spp. infections significantly burdens healthcare systems and patients, with prolonged hospital stays averaging more than 30 days and high costs associated with antifungal therapies and advanced interventions

(Armaganidis et al., 2017). The burden is further exacerbated by multidrug-resistant microorganisms (Giraldi et al., 2019).

Opportunistic fungi such as *Candida* spp. present unique challenges due to their lack of seasonality and demographic preferences, making surveillance and prediction more difficult compared to other emerging fungal pathogens (Makled, 2024). Early appropriate antifungal therapy based on local epidemiology can reduce mortality attributable to candidemia and length of hospital stay (Mencarini et al., 2018). Their ability to adapt to diverse environmental and host conditions further complicates management and emphasizes the need for robust monitoring and tailored infection control strategies in healthcare settings. On the other hand, while the focus is often on the challenges posed by opportunistic fungi, advances in genomic testing and automated surveillance methods such as Vitek 2® and MALDI-TOF can improve outbreak detection and management, potentially mitigating some of these issues (Abby, 2023). Effective biocides to prevent transmission of *Candida* spp. are crucial to mitigate some of these consequences. Investing in prevention, including research on biocide compatibility and fungal resistance mechanisms, is essential to improve these results.

An alarming finding was the absence of inhibition zones for the compound's hydrogen peroxide 4.25% and benzalkonium chloride 5%, both for reference ATCCs and clinical isolates. According to the recommendation of the National Health Surveillance Agency (Anvisa), they are officially indicated for the decontamination of fixed surfaces, including the nutrition and neonatal environments. This shows the inadequacy of the use of these compounds for surface decontamination and the influence on the 89 individuals in our population who use invasive medical devices.

The biocide tolerance test showed a statistical difference when comparing the means of the inhibition zones for sodium hypochlorite compounds between the group of clinical isolates and ATCCs, both among the *C. albicans* and NCAC species. Larger halos were observed among ATCCs, indicating greater susceptibility, while clinical isolates showed a smaller halo of inhibition compared to reference strains. Significantly, clinical isolates showed greater resistance to biocidal compounds than reference ATCCs; however, sodium hypochlorite remains the most effective among the compounds studied. The recommended disinfection dose by the Centers for Disease Control (CDC) is 5.25%–6.15% sodium hypochlorite, or 52,500–61,500 ppm available chlorine, which is almost provided by a 1:1,000 dilution, but studies have

confirmed efficacy for *Candida* in 30 seconds of exposure to 500 ppm chlorine (Silverman et al., 1999; Cdc, 2024).

Moore et al. (2017) used a European standard quantitative suspension test method (EN 13624:2013) and showed that 0.1% chlorine was effective in its fungicidal action against *C. auris* and *C. albicans*, for both clean and dirty conditions when the contact time was 5 min. The fungicidal action of chlorine-based benzalkonium chloride was also demonstrated for *C. glabrata, C. albicans* and *C. auris,* using the disk diffusion method (ASTM E-2197-02). However, quaternary ammonium-based disinfectants were significantly less effective against *Candida* species, which corroborates our findings (Moore et al., 2017; Cadnum et al., 2017). One study evaluated the susceptibility of clinical strains of *C. auris* to several biocides, including chlorine, chlorhexidine and benzalkonium chloride. Resistance has been observed with benzalkonium chloride at concentrations of 16–32 mg/L (Erganis et al., 2024).

In hospital settings, it is impractical to rely on a single biocide to effectively eliminate all microorganisms. Variability in microbial tolerance requires monitoring the efficacy of biocides used at specific concentrations. For example, in the same hospital, a study was conducted by Dias et al. (2017) on *Pseudomonas aeruginosa* and *Acinetobacter*, clinical isolates found that Carbapenem-resistant strains exhibited increased tolerance to 2% sodium hypochlorite and 5% benzalkonium chloride compared to susceptible strains. This highlights that biocides effective against one pathogen may be less effective against others that share the same hospital environment.

Investing in research to discover compounds with broad-spectrum antimicrobial activity is essential to improve infection control strategies in healthcare settings. This is particularly vital given the challenge posed by the intrinsic mechanisms of the efflux pump in the *Candida* genus, which may contribute to biocide tolerance, as has already been observed in bacterial strains (Matthew; Wand; Darby, 2022; Carmine et al., 2018). The ability of efflux pumps to actively expel biocidal compounds from fungal cells, reducing their effectiveness and complicating the management of these pathogens (Chang et al., 2018).

7. CONCLUSION

In conclusion, this study highlights the significant challenges posed by antifungal resistance among *Candida* species in clinical settings. The findings reveal high rates of resistance, particularly to azoles and echinocandins, associated mostly with *ERG11, CDR2,* and *CDR1* mutations, emphasizing the critical need for effective diagnostic tools and targeted therapeutic strategies. The study also points out the relevance of resistance mechanisms such as efflux pumps, which showed higher phenotypic activity when comparing clinical isolates with reference lineages. That can also impact biocide tolerance in those isolates that showed tolerance for quaternary ammonium and hydrogen peroxide compounds, directly impacting the hospital environment decontamination.

The results obtained reinforce the need for continuity in the surveillance of susceptibility to antifungals, highlighting the importance of monitoring changes in resistance patterns. In addition, they highlight the urgency of investments in research aimed at the development of new biocidal agents capable of offering effective alternatives in the fight against fungal infections. Finally, the findings emphasize the relevance of improving infection prevention strategies, especially in the hospital context, to minimize the spread of resistant pathogens.

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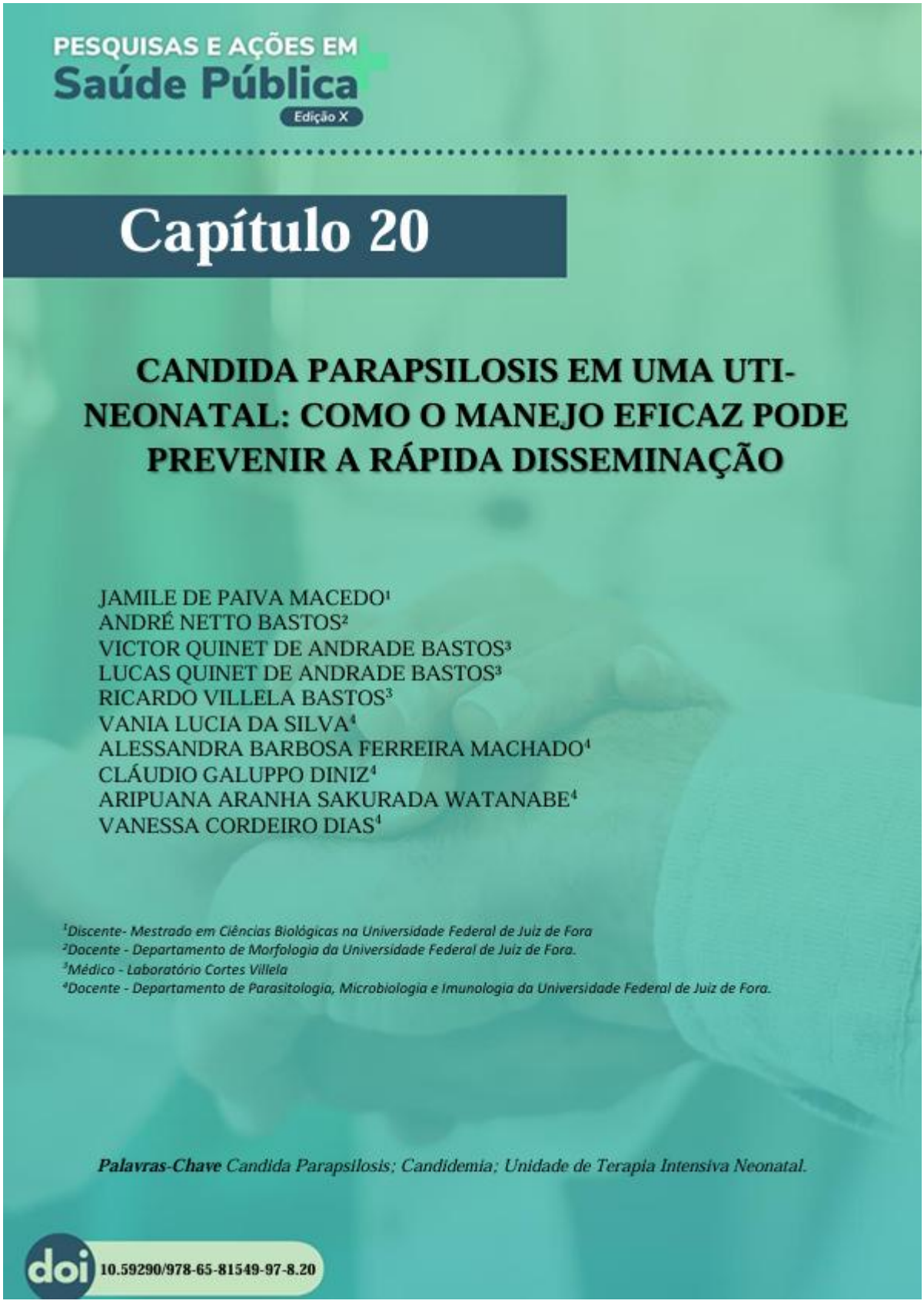
9. APPENDAGE

9.1 *Candida parapsilosis* EM UMA UTI NEONATAL: COMO O MANEJO EFICAZ PODE PREVENIR A RÁPIDA DISSEMINAÇÃO



EDITORA PASTEUR

INTRODUÇÃO

As espécies de *Candida spp.* estão frequentemente associadas a infecções fúngicas em contexto hospitalar, principalmente em unidades de terapia intensiva (UTI's) onde a taxa de infecções secundárias é alta devido a fatores como, morbilidades e imunossupressão (VINCENT *et al.*, 2009).

Esses microrganismos são comumente associados a microbiota intestinal e da pele de humanos atuando como comensais. Em casos de desequilíbrio da relação saúde e hospedeiro, infecção localizada, transmissão horizontal por mãos de profissionais ou uso de dispositivos médicos invasivos, podem promover a candidíase invasiva ou a candidemia. Essa mudança entre o comensalismo e oportunismo possui três condições principais: o uso prolongado de antimicrobianos, imunossupressão e a violação das barreiras gastrointestinais e cutâneas, como a inserção de cateteres (PAPPAS *et al.*, 2018). Essa atividade invasiva do fungo é uma característica emergente graças aos avanços tecnológicos na medicina, e indicam uma grande causa de morbilidade e mortalidade nos ambientes hospitalares (LONA-REYES *et al.*, 2022). Os casos de candidíase invasiva são liderados internacionalmente pela espécie *Candida albicans*, mas desde o início dos anos 2000 as espécies de *Candida não albicans* coletivamente representam mais da metade dos casos no mundo, sendo as do complexo *Candida parapsilosis* as mais comuns entre elas (PFALLER *et al.*, 2014; GONZALEZ-LARA & OSTROSKY-ZEICHNER, 2020).

O complexo *Candida parapsilosis* engloba as espécies *C. parapsilosis sensus stricto, C. orthopsilosis* e *C. metapsilosis*. Entretanto, clinicamente as espécies não são comumente distinguidas sendo retratadas genericamente como *C. parapsilosis* (MARTÍN-GÓMEZ & PUIG-ASENSIO, 2023). Essa é a espécie de *Candida não albicans* mais encontrada em países da América Latina, como Argentina, Peru e Brasil. No Brasil, a prevalência de *C. parapsilosis* é de 24,1%, ficando atrás somente de *C. albicans* com 34,3% dos casos de candidemia (TÓTH *et al.*, 2019).

Segundo a lista de prioridade de fungos patógenos pela Organização Mundial da Saúde (OMS), *C. parapsilosis* está na lista de alta prioridade para pesquisa, desenvolvimento e ações de saúde pública associada a uma taxa de mortalidade de 20 a 45% (WHO, 2022). Esse microrganismo é um fungo leveduriforme oportunista, capaz de proliferar em soluções de hiperalimentação, inibir a ação de fagócitos do sistema imune do hospedeiro beneficiando a dispersão de células, tem a capacidade de formar biofilme garantindo aderência a superfícies bióticas e abióticas, como dispositivos médicos, e limitando a penetração de substâncias pela matriz diminuindo o impacto de antifúngicos. Todas essas características garantem a colonização e persistência hospitalar do fungo, que também realiza a formação de biofilme especialmente em pacientes com cateter venoso central que recebem nutrição parental, sendo frequente a infecção em recém-nascidos prematuros, pacientes pediátricos e lactantes em UTI's (DAL GUEDES DA SILVA *et al.*, 2022).

Em estudos retrospectivos de distribuição de *Candida spp*, enquanto *C. albicans* se mostrou o patógeno mais frequente para populações desde neonatos a adultos, a espécie *C. parapsilosis* foi a mais predominante entre os indivíduos de UTI neonatal (UTI-N) (FALAGAS *et al.*, 2010). *C. parapsilosis* representa ⅓ (um terço) dos casos de infecção por *Candida spp.* neonatais, com aproximadamente 10% de mortalidade (BRANCO *et al.*, 2023).

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Considerando a alta capacidade de colonização e dispersão por transmissão horizontal observada em ambientes hospitalares, principalmente em UTI's, e a afinidade de ocorrência da infecção entre pacientes neonatais e pediátricos, o reconhecimento dos fatores de risco, epidemiologia e fatores de virulência do agente etiológico, são necessários para elaboração de técnicas de manejo dentro do ambiente hospitalar. Desta forma, interferir na progressão e desfecho da doença, controlar o estabelecimento de *C parapsilosis* nesse contexto e consequentemente diminuir a morbilidade e mortalidade associados a esse microrganismo no ambiente hospitalar.

O objetivo do presente capítulo é apresentar uma série de casos de sucesso na identificação e diagnóstico de infecção por *C. parapsilosis* entre pacientes de uma UTI neonatal, bem como, o manejo e tratamento, garantindo o controle do microrganismo na ala hospitalar e a recuperação dos pacientes envolvidos.

MÉTODO

Trata-se de um estudo transversal, retrospectivo, baseado em dados coletados de pacientes de um hospital terciário de Juiz de Fora/Minas Gerais, acometidos por candidemia por *C. parapsilosis*, entre março e abril de 2022.

Este estudo relata um surto envolvendo pacientes hospitalizados em uma UTI-N, com capacidade para doze leitos, independentemente do sexo, que apresentaram exame laboratorial de hemocultura (cultura de sangue) positiva para *C. parapsilosis*.

A partir de prontuários eletrônicos, foram obtidos os seguintes dados epidemiológicos e clínicos: idade, sexo, motivo e duração da hospitalização (em dia), desfecho clínico, uso de dispositivos médicos invasivos, uso prévio de antimicrobianos e tipo de parto.

Mediante análise de registros internos, foram observadas as ferramentas de gestão do surto implementadas pelo serviço de controle de infecção hospitalar.

Os ensaios laboratoriais para determinação da cultura do sangue foram realizados conforme prevê o Manual de Microbiologia Clínica para o Controle de Infecção em Serviço de Saúde (ANVISA, 2004). A identificação dos isolados de *C. parapsilosis* foi efetuada pelo sistema Vitek 2® (bioMerieux/França), de acordo com as instruções do fabricante.

Este estudo foi apreciado pelo Comitê de Ética em Pesquisa da Universidade Federal de Juiz de Fora e aprovado sob o número 3.783.237, CAAE 18611019.6.0000.5147.

RESULTADOS E DISCUSSÃO

Epidemiologia

No presente estudo, 4 indivíduos hospitalizados entre o período de março e abril de 2022 em uma UTI-N desenvolveram quadro clínico compatível com fungemia, comprovada por meio de exame laboratorial como infecção por *C. parapsilosis*. A instituição é referência em saúde no município de Juiz de Fora, Minas Gerais, e conta com estrutura para internação, centro cirúrgico, CTI, UTI, entre outros. O referido hospital possui certificado de excelência nível 3 pela Organização Nacional de Acreditação (ONA).

Os indivíduos desse estudo eram todos do sexo masculino, sendo três recém-nascidos de cesárea e um paciente pediátrico de oito anos sem identificação do parto. O período médio de hospitalização foi de 39 dias [27 a 51 dias], e todos evoluíram para alta hospitalar após tratamento adequado, conforme **Tabela 20.1**. Os resultados do diagnóstico das amostras clínicas foram positivos para *C. parapsilosis* para todos os exames de hemocultura e três dos quatro se mostraram positivos para colonização em cateter venoso central (**Tabela 20.1**). O achado laboratorial de hemocultura e cateter venoso central positivos para *C. parapsilosis* e mesmo

hemocultura positiva para *C. parapsilosis* e cateter negativo para qualquer fungo pode indicar uma infecção da corrente sangüínea relacionada ao cateter vascular.

Tabela 20.1 Aspectos clínicos e epidemiológicos dos indivíduos com candidemia por *C. parapsilosis*

Pacientes	Idade	Tempo de Hospitalização (dias)	Desfecho clínico	Tipo de Parto
01	8 anos	51	Alta hospitalar	Não aplicável
02	RN	39	Alta hospitalar	Cesárea
03	RN	27	Alta hospitalar	Cesárea
04	RN	39	Alta hospitalar	Cesárea

Legenda: RN - recém-nascido

A emergência de infecções fúngicas invasivas por patógenos nosocomiais como, *C. parapsilosis*, pode refletir a administração exacerbada de antimicrobianos, imunossupressores, quimioterápicos, aumento do uso de tecnologias de suporte à vida, nutrição parental e uso de cateter intravenoso, além do aumento na prevalência de síndrome da imunodeficiência adquirida (AIDS) (YAMIN *et al.*, 2022). Se antes *C. parapsilosis* não era considerado um possível patógeno, hoje vemos a espécie disputando o espaço de maior frequência em fungemia com *C. albicans* em algumas instituições (CHEN *et al.*, 2021). Com passar dos anos diversos estudos de casos de infecções fúngicas em neonatos e pacientes pediátricos têm apontado uma prevalência maior de espécies não *albicans* em relação a *C. albicans*, destacando entre elas a espécie *C. parapsilosis*. Um estudo de metanálise entre casos reportados de infecção por *Candida* no período de 1990 a 2012 em neonatos, constatou uma prevalência de 33,47% dos casos de infecção por *C. parapsilosis* (PAMMI *et al.*, 2013). Podemos ver essa recorrência também no estudo de Öncü e colaboradores (2019), que relatou uma prevalência de 66% dos neonatos e crianças infectados por *Candida spp* não albicans entre pacientes de um hospital terciário, representando que a maioria das infecções ocorreram por *Candida spp* não *albicans*, sendo *C. parapsilosis* a mais comum entre neonatos (45,4%) e em crianças (30,5%). Um estudo retrospectivo de 5 anos realizado com isolados clínicos de pacientes pediátricos de um hospital no Brasil, identificou uma incidência de 1,24 casos de 1000 casos anuais, 123 espécies de *Candida*, dentre elas a mais prevalente foi *C. parapsilosis*, seguida *C. albicans* e *C. tropicalis* (RODRIGUES *et al.*, 2022). Com base em evidências da literatura e os relatos do presente estudo da prevalência de infecções por *C. parapsilosis* em pacientes neonatos e pediátricos no contexto hospitalar, nos indicam que a espécie é um patógeno neonatal significativo que merece maior atenção em ações de educação em saúde, desenvolvimento e pesquisa.

Fatores de risco

Todos os pacientes observados neste estudo faziam uso dos dispositivos invasivos: cateter venoso central (CVC), sonda nasogástrica (SNG), sonda aspiração traqueal e nutrição parental.

O principal motivo de internação destes indivíduos foi sepse bacteriana. Além disso, todos estavam realizando tratamento com antibacterianos antes do diagnóstico de fungemia, sendo ampicilina, vancomicina, meropenem, gentamicina os fármacos mais utilizados, conforme demonstrado na **Tabela 20.2**.

O conhecimento a respeito dos fatores de risco para infecções por *Candida*, especialmente *C. parapsilosis*, é importante para a elaboração de estratégias preventivas e de eficiência terapêutica. Um estudo realizou uma análise univariável e encontrou que o peso de

nascimento, CVC, tempo de exposição a cateter uretral, ventilação assistida e idade gestacional foram fatores de risco para *C. parapsilosis*. E quando realizado a análise multivariada em comparação com *C. albicans* os autores relataram que paciente neonatos, transplantados e que receberam terapia antifúngica ou nutrição parenteral tiveram maior associação com infecção por *C. parapsilosis* (GARZILLO *et al.*, 2017).

Tabela 20.2 Fatores de risco associados aos indivíduos com candidemia por *C. parapsilosis*

Pacientes	Dispositivo invasivo	Antimicrobianos utilizados anteriormente ao exame microbiológico	Motivo da internação
01	CVC/SNG/sonda aspiração traqueal/ sonda uretral/ nutrição parenteral	Vancomicina/Meropenem/ Nistatina	Epilepsia
02	CVC/SNG/sonda aspiração traqueal/ nutrição parenteral	Vancomicina/Meropenem/Cefepime/ Linezolida/Ampicilina/ Gentamicina	Sepse Bacteriana
03	CVC/SNG/sonda aspiração traqueal/ nutrição parenteral	Cefotaxima/Oxacilina/Nistatina/ Ampicilina/Gentamicina	Sepse Bacteriana
04	CVC/SNG/sonda aspiração traqueal/ nutrição parenteral	Vancomicina/Meropenem/Cefepime/ Linezolida/Ampicilina/ Gentamicina	Sepse Bacteriana

Legenda: Cateter venoso central (CVC), Sonda nasogástrica (SNG).

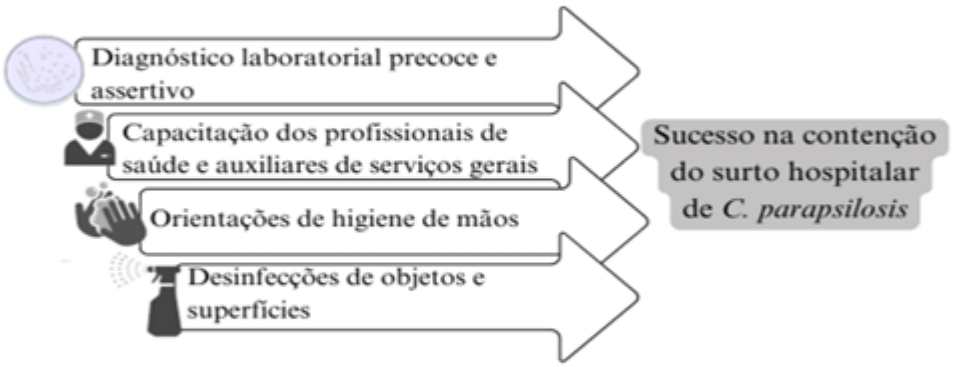
Outro estudo, em uma análise multivariada, destacou uma maior significância como fatores de risco independentes à admissão em UTI, uso da nutrição parenteral e CVC (YAMIN *et al.*, 2021). Um estudo recente apontou como fatores de risco a nutrição parenteral, exposição a antimicrobianos de amplo espectro, prematuridade e uso prévio de cateter venoso central, sendo esse último o único associado à mortalidade (DA SILVA *et al.*, 2023). Esses fatores de risco descritos coincidem aos de diversos outros estudos, que também destacam o CVC como sendo mais significativo para a infecção por *C.parapsilosis* (CONDE-ROSA *et al.*, 2010; ZAOUTIS *et al.*, 2010). A partir desses achados podemos destacar o uso de nutrição parenteral prévia, CVC, a sonda de aspiração traqueal e o uso de antimicrobianos observados no histórico de todos os pacientes diagnosticados com fungemia por *C. parapsilosis* na UTI neonatal relatados neste estudo como fatores de risco associados à infecção.

Considerando a habilidade de *C. parapsilosis* em formar biofilme em superfícies bióticas e abióticas, associado à sua capacidade de colonizar soluções de hiperalimentação, fazem do CVC e nutrição parenteral importantes fatores de risco à infecção por esta levedura (CHOW *et al.*, 2012; PRAMODHINI *et al.*, 2021). Esta observação se torna possível a partir do estabelecimento de um vínculo epidemiológico entre esses métodos invasivos e os pacientes com o surto de *C. parapsilosis* nessa ala hospitalar.

Manejo

Após diagnóstico laboratorial rápido e assertivo, capacitação da equipe multidisciplinar, incluindo auxiliares de serviços gerais, intensificando ações para correta higienização das mãos, adoção de medidas de desinfecção de objetos e superfícies, o surto foi contido como exemplificado na **Figura 20.1**.

Figura 20.1 Fluxograma de estratégias utilizadas para manejo das infecções por *C. parapsilosis* para o sucesso na contenção do surto



Um estudo realizado em uma UTI-N em Uberlândia/Minas Gerais teve como objetivo a descrição de um surto de candidemia entre neonatos, identificação da espécie e mapeamento das zonas contaminadas da ala hospitalar. Ao final do estudo os autores observaram que 46,1% dos indivíduos foram infectados por *C. parapsilosis sensu stricto*. Análises de biologia molecular permitiu elucidar a origem dos clones: mãos dos profissionais de saúde e das mães, nas pias de higienização das mãos, nos leitos da UTI, nas portas de entrada das salas de higiene e da UTI-N e um dreno (DE PAULA MENEZES *et al.*, 2020).

Outro estudo similar efetuou um levantamento das mãos dos profissionais de saúde que atuam na UTI-N e constataram uma frequência aproximada de 40% de *C. parapsilosis* (DE PAULA MENEZES *et al.*, 2019). Esses dados nos mostram um fator epidemiológico importante na transmissão horizontal de *C. parapsilosis* que são as mãos de profissionais da saúde, e trazem à tona a necessidade de realizar a orientação para a higienização correta e frequente das mãos dos profissionais de saúde envolvidos nas UTIs, especialmente neonatal.

Thomaz e colaboradores (2021) identificaram linhagens de *C. parapsilosis* nas mãos de profissionais atuantes em cuidados à saúde em UTI e superfícies nosocomiais, como leitos, carrinhos de cabeceira e monitores cardíacos.

Outro estudo que teve o objetivo de realizar sequenciamento genômico para avaliar o comportamento e evolução de *Candida* na microbiota intestinal de prematuros e no ambiente hospitalar, sugeriu que a transmissão de membros da população fúngica se deu de um reservatório do ambiente, ambiente hospitalar, especialmente a UTI-N, para o neonato (WEST *et al.*, 2021). Isso nos mostra o ambiente e as superfícies como importantes fontes de transmissão de microrganismos e a necessidade de descontaminação correta nas alas hospitalares, tendo em vista que patógenos como *C. parapsilosis* podem sobreviver dias a meses em superfícies (KRAMER *et al.*, 2006).

CONCLUSÃO

O presente estudo relatou uma série de casos de fungemia por *C. parapsilosis* em uma UTI-N de um hospital terciário, trazendo à tona a importância do diagnóstico assertivo e manejo adequado para contenção de um patógeno com inúmeros fatores de virulência, associado a elevados índices de morbilidade e mortalidade entre crianças e recém-nascidos. Considerando que o manejo adequado é essencial para o controle desse agente no ambiente hospitalar, a

capacitação dos profissionais no tocante à higienização pessoal e a desinfecção do ambiente são de grande valia.

Assim, o nosso estudo desperta a necessidade do desenvolvimento de pesquisas e ações de saúde pública para melhor compreensão a respeito da patobiologia do fungo e elaboração de futuras estratégias de prevenção e controle.

No entanto, o estudo possui algumas limitações como, a ausência de investigação da(s) fonte(s) ou origem dos casos de infecção, bem como a determinação da existência de um único clone de *C. parapsilosis* circulante na instituição.

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9.2 Antifungal resistance: why are we losing this battle?

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REVIEW

Antifungal resistance: why are we losing this battle?

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ABSTRACT

The emergence of fungal pathogens and changes in the epidemiological landscape are prevalent issues in clinical mycology. Reports of resistance to antifungals have been reported. This review aims to evaluate molecular and nonmolecular mechanisms related to antifungal resistance. Mutations in the *ERG* genes and overexpression of the efflux pump (*MDR1*, *CDR1* and *CDR2* genes) were the most reported molecular mechanisms of resistance in clinical isolates, mainly related to Azoles. For echinocandins, a molecular mechanism described was mutation in the *FSK* genes. Furthermore, nonmolecular virulence factors contributed to therapeutic failure, such as biofilm formation and selective pressure due to previous exposure to antifungals. Thus, there are many public health challenges in treating fungal infections.

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amphotericin B; antifungal agents; antifungal resistance; azole; biofilm; echinocandin

1. Introduction

The emergence of fungal pathogens and changes in scenery are a prevalent subject in clinical mycology. Species of non-albicans *Candida* spp. have been reported to cause invasive infection, surpassing the rates of *Candida albicans*, worldwide [1]. In this context, *Candida auris*, a fungus with a multiresistant profile to medical antifungals, with the potential for nosocomial infections, has been reported on all continents [2]. Another major public health challenge is invasive pulmonary aspergillosis, caused by species of the genus *Aspergillus*, mainly *Aspergillus fumigatus* [3]. This condition has been highly associated with COVID-19-positive patients after admission to the intensive care unit (ICU) [4]. Another relevant invasive fungal infection is cryptococcosis, caused mainly by *Cryptococcus neoformans* and *Cryptococcus gatti*, which has increased dramatically in the last 50 years, in association with HIV infection [5].

The fungi cell wall and membrane will play an important role in the establishment of the fungi infection, as it is vital for maintaining the structure and integrity of the cell [6]. The cell wall is a complex structure composed of an inner and outer layer. The inner layer is made of chitin and beta-glucans that are linked to chitin through beta-(1,4) linkages. The outer layer consists of glycosylated cell wall proteins decorated with mannans and phosphomannans [7].

The fungal membrane has a dual structure composed of fatty chains with various attachments like choline, serine and ethanolamine. It also contains important components like sphingolipids and ergosterol, unlike animal cells which have cholesterol as the main sterol. The plasma membrane also hosts important protein families involved in transport, energy production, signaling, cell structure and other critical functions [8].

Furthermore, there is also a constant report of resistance during the treatment of fungal infections [9,10]. Antifungal resistance can be categorized as intrinsic (primary) or acquired (secondary). Intrinsic resistance is genetically predetermined and is linked to fungal species that are inherently insensitive to specific antifungals, regardless of prior exposure. An example is the known intrinsic resistance to azole derivatives found in strains of *Candida krusei* [11]. Acquired resistance, on the other hand, results from exposure to stress factors, such as antifungals, or their analogs, and can be genetic or epigenetic. The adaptability of fungi to changing environmental conditions facilitates the emergence of resistance during exposure to sublethal concentrations of drugs [12]. An acquired resistance could be exemplified by *Candida* species that have acquired resistance through molecular or nonmolecular mechanisms against antifungal agents [13].

Presently, the clinical arsenal for systemic antifungals counts with different mechanisms of action and many classes. There are inhibitors of ergosterol biosynthe-

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sis which include: azoles, allylamines, and morpholines. Polyenes, such as amphotericin B (AmB) (Supplementary Figure S1), act by disrupting the fungal membrane. The inhibitors of the fungal cell wall synthesis can act by inhibiting the β-glucan synthesis like Echinocandins or inhibiting chitin synthesis like nikkomycins and polyoxins. Flucytosine it's a nucleic acid synthesis inhibitor is used adjunctively with AmB, primarily for treating cryptococcal meningitis. Griseofulvin it's a microtubule biosynthesis inhibitor [9]. Given the limited therapeutic targets, concerns about antifungal resistance loom large. Resistance to a single class of drugs can significantly curtail treatment options, while multidrug resistance may render fungal infections practically untreatable (Figure 1) [10].

This review aims to group the molecular and non-molecular mechanisms correlated with the resistance of nosocomial infections like *Candida* spp., *Aspergillus* spp. and *Cryptococcus* spp., to antifungals currently used in health services. To corroborate advances already made and identify gaps that still exist to direct future studies and evidence-based medicine, in combating these fungal infections that have caused major impacts on public and One health.

The articles passed through three processes: identification, screening and eligibility. The identification was carried out in the following databases: EMBASE; MEDLINE; and COCHRANE. Using advanced search tools with the following terms ('Antifungal resistance' OR 'Drug resistance' OR 'Azoles resistance' OR 'Echinocandin resistance' OR 'Amphotericin B'); ('Antifungal resistance' OR 'Drug resistance' AND 'Molecular mechanisms' OR 'ERG11' OR 'Cyp51A'); ('Antifungal resistance' OR 'Drug resistance' AND 'Biofilm' OR 'Antimicrobial drugs'). Using a date range of 5 years from 2018 to 2023 resulted in 68 articles (Figure 2).

The results were exported to the software Mendeley where the duplicates were removed, and there was a screening through the abstracts. At last, the full texts were read and for articles to meet the inclusion criteria they should, be written in English, be relevant to the topic at hand, have Open access or institutionality access, use blood or other sterile body fluid samples, and have antifungal susceptibility testing according to the CSLI or the EUCAST. The following exclusion criteria were, small sample n, not relevant to the study, single case report, articles with low impact factor and publication status. The final data consisted of 89 full-text read articles, with a few articles included in the processes as demand for the review, with a final number of 74 articles included.

2. Antifungal

2.1. Flucytosine

Flucytosine, introduced in 1973, demonstrated activity against *Candida* spp. and *Cryptococcus* spp., but faced limitations due to emerging drug resistance and toxicity (Figure 1) [11]. First-generation azole drugs like fluconazole (Supplementary Figure S2) and itraconazole emerged in the 1990s, offering oral administration and good activity against yeast pathogens, albeit with notable drug interactions. Second-generation azole drugs like voriconazole, posaconazole and isavuconazole, introduced in the 2000s, extended the spectrum of activity against filamentous fungi [13]. Echinocandins, available in the 2000s, exhibited excellent activity against *Candida* spp. with minimal drug interactions but were limited to parenteral administration (Figure 1) [14].

2.2. Polyenes

Polyenes are a group of antifungal drugs that act by binding to the fungal cell membrane and making it porous, which ultimately leads to the death of the cell. Amphotericin B (AmB) is a specific type of polyene that is commonly used to treat severe systemic fungal infections, such as those affecting the lungs, brain and bloodstream. This group of drugs is the oldest among antifungals, and AmB is the most popular and has excellent antifungal activity against a broad range of fungi, including several species of *Candida* spp. [15,16].

Amphotericin B debuted as a therapeutic option in 1958, with a potent broad-spectrum antifungal activity and with a significant advancement for immunocompromised patients. However, its use was constrained by toxicity, paving the way for the development of alternative drugs with improved tolerability, reduced drug interactions and complementary mechanisms of action [12]. Lipid-based AmB formulations, introduced in the 1990s, maintained potent broad-spectrum activity with reduced toxicity (Figure 1).

In contrast to azoles that target ergosterol biosynthesis through inhibition of sterol 14α-demethylase activity (*ERG11*), Amphotericin B works by forming extra membranes that extract ergosterol from the fungal cell membrane and act as a 'sterol sponge'. It has both fungistatic and fungicidal activity by binding to the fungal membrane, leading to changes in membrane permeability. Van der Waals forces stabilize the interaction between AmB and ergosterol, resulting in the formation of membrane pores that leak monovalent ions (K+, Na+, H+, Cl-). Furthermore, AmB causes oxidation that results in fungal cell damage [17].

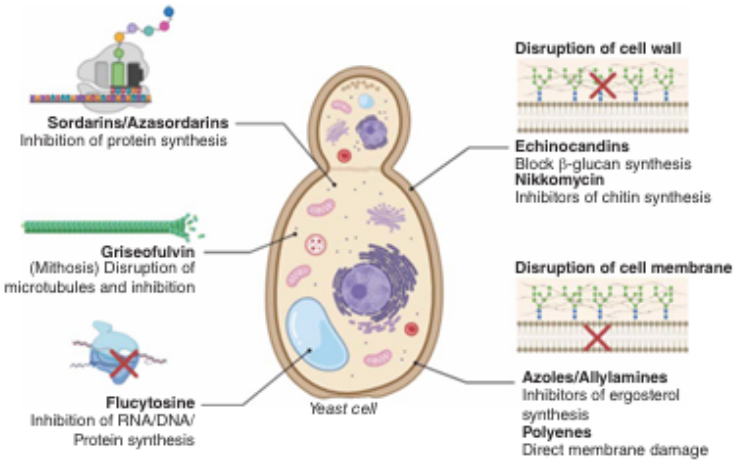

Figure 1. Different mechanisms of action for antifungal agents in the yeast cell.

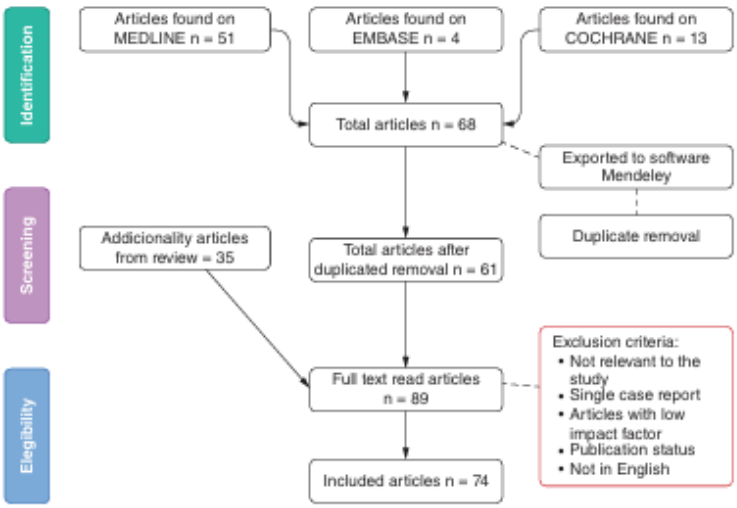

Figure 2. Search review methodology for resistance mechanisms in invasive fungal infections.

2.3. Azoles

Azoles are a commonly prescribed class of antifungal drugs, with several derivatives such as itraconazole, fluconazole, voriconazole, posaconazole and isavuconazole. They work by blocking the synthesis of ergosterol, which is a key component of the fungal cell membrane [16]. This, in turn, reduces membrane flexibility, permeability, and the function of membrane enzymes, leading to inhibition of the growth of *Candida* spp. However, it is important to note that azoles do not kill these pathogens and may promote resistance to the drug. Azoles exert their effect by inhibiting the enzyme CYP-dependent C-14 α-demethylase, which is encoded by *ERG11* in yeast and Cyp51 in molds, which is involved in the conversion of lanosterol to ergosterol [18]. This causes depletion of ergosterol, which is an essential sterol of the fungal cell membrane, ultimately compromising cell membrane integrity. As a result, azoles have a fungistatic activity in yeasts and fungicidal in molds [15].

2.4. Echinocandin

Echinocandins, like caspofungin (Supplementary Figure S3), are a natural derivate type of medication that acts by disrupting the cell wall, blocking the biosynthesis of (1,3)-β-D-glucan. This is not present in human cells, making it safe for patients, and leads to osmotic imbalance that ultimately kills the fungus [16]. These drugs are highly effective against *Candida* spp. and are recommended as a primary treatment for invasive candidiasis [19]. They are administered intravenously due to poor oral absorption. Echinocandins target a heteromeric enzyme complex comprising a regulatory subunit (Rho1), that needs a chemical named guanosine triphosphate to work, and a catalytic subunit made from the *FKS* gene. By acting on these mechanisms, echinocandins can lead to reduced yeast budding and hindered growth at the tips of hyphae in molds. It's important to note that echinocandins have poor oral bioavailability and are only administered intravenously [15].

Schematic representation of different classes of antifungals and their representatives, including spectrum of action, route of administration and origin are described in Supplementary Table S1.

3. Methods for determining susceptibility to antifungals

Once a fungus is isolated, an important step is to characterize the antifungal susceptibility profile using standardized, relevant and reproducible methods. *In vitro* susceptibility determination testing can be based on qualitative or quantitative methods that help determine the best therapy against fungal pathogens.

Quantitative methods allow the determination of the minimum inhibitory concentration (MIC), contributing to the safe administration of antifungals [20]. Broth microdilution methods are gold standard procedures, in which MIC values are used as a parameter for implementing assertive therapy [21].

Two important documents can be used for interpretation of breakpoints: *Clinical & Laboratory Standards Institute* (CLSI) and *European Committee on Antimicrobial Susceptibility Testing* (EUCAST) [21]. These two present differences that can be found in the size of the inoculum, the incubation time, and the composition of the medium, which can lead to differences in the classification and interpretation of the MIC.

Despite these differences, the breakpoints recommended by the two documents demonstrate the reality of American and European fungal isolates, respectively, including pharmacokinetic and pharmacological characteristics. Both describe standard procedures for susceptibility testing for yeasts (e.g. *Candida* spp. and *Cryptococcus* spp.) and for filamentous fungi that can also cause invasive infections (e.g. *Aspergillus* spp.) [22].

Another available method is agar-based disk diffusion used for yeast. This technique has a qualitative nature and is widely used in clinical laboratories, due to its low cost and ease of execution. It is used to determine the antifungal sensitivity profile of isolates of *Candida* spp, *Cryptococcus*, and other yeasts of clinical interest [23].

Regardless of the gold standard for broth microdilution methods, they are time consuming and sometimes inconvenient for clinical laboratory use. In those cases, there are plenty of automated or semi-automated commercial methods in use that also need to attend to the values established by CLSI or EUCAST. They can be broth microdilution (e.g., Vitek-2®, Sensititre YeastOne®) or agar-based (e.g. MIC Test Strip® and Etest®) [24].

4. Resistance to antifungals

The evolution of drug resistance in pathogenic fungi mirrors the selective pressures exerted by clinical and agricultural antifungal agent use [15]. The widespread use of certain antifungals, like fluconazole, led to an alarming rise in drug-resistant *Candida* spp. isolates. Similarly, since the mid-1990s, the global dissemination of azole-resistant *Aspergillus fumigatus* has posed a threat to the effectiveness of these drugs in preventing and treating invasive fungal diseases [25]. The advent of echinocandins, though a significant development, also saw the emergence of multidrug-resistant *Candida auris* as a serious global health concern, causing outbreaks in hospitals with high mortality rates. Its global spread, starting in 2009, has led to warnings from public healthcare institutions due to nosocomial transmissions, making it a pressing public health issue [26].

In multicenter surveillance studies, the global prevalence of *C. albicans* resistance to echinocandins is less than 1% and *C. parapsilosis* 1.2% in isolates from North America, Europe and Latin America [27]. Other epidemiological prevalence studies reported antifungal resistance of 2–4% to *C. glabrata*. Rates of non-susceptible isolates *Candida* spp. increased from 4.2% in 2008 to 7.8% in 2014 [28].

For triazole resistance in Latin America, the review showed a rate that posaconazole was most prominent among *C. albicans* isolates (6.5%), *C. glabrata* isolates (5.3%) [29]. The ARTEMIS Antifungal Surveillance Program reported an increase in *C. glabrata* as the cause of invasive candidiasis from 18 to 25% between 1992–2001 and 2001–2007, respectively. Fluconazole resistance increased from 9% to 14% during the same period [30]. A surveillance study in the Netherlands reveals that 3.2% of

Aspergillus fumigatus isolates are resistant to one or more azoles [31].

A study from the SENTRY Antifungal Surveillance Program with global fungal collections. After reviewing 1846 fungal clinical isolates from 31 countries, they found that echinocandin resistance among *Candida* spp. varied from 0.0 to 2.8%, with the highest resistance seen against anidulafungin in *C. glabrata*. Furthermore, resistance to fluconazole was detected in approximately 11% of *C. glabrata* and *C. tropicalis* isolates [21].

The application of fungicides has become an essential practice to safeguard and guarantee agricultural production. Not only crops but also farm animals such as cows, pigs and chickens are vulnerable to fungal infections due to exposure to soil containing fungal pathogens. The One Health perspective emphasizes the correlation between the use of fungicides in the field and the emergence of antifungal-resistant strains that can infect animals and humans, leading to a reduction in the therapeutic arsenal [22]. A relevant example is the finding of *A. fumigatus*, which is at the forefront of the cross-resistance profile to antifungals. A study showed a relationship of resistance to clinical azoles by this fungal strain, after exposure to agricultural azoles [32].

Therefore, it is important to disseminate information about the strategies used by emerging fungi with a resistance profile to the main antifungals of clinical interest, as they have a significant impact on public health.

4.1. Ergosterol biosynthesis pathway

ERG's genes are responsible for regulating the synthesis of ergosterol, which is an important constituent of the fungal cell membrane. It plays a crucial role in several cellular functions, including maintaining membrane fluidity and integrity, as well as facilitating the proper functioning of membrane-bound enzymes [33]. The azole drugs block the synthesis by inhibiting the activity of the genes Erg, binding and inhibiting the lanosterol demethylase enzyme, causing damage to the cell membrane (Figure 3A).

In literature, we have a lot of findings with overexpression and mutation of different ERG genes, but the most frequent is *ERG11*. Changes in the *ERG11* gene may be due to overexpression of the gene (Figure 3B) or preventing the binding of azoles in lanosterol-4,4-dimethylcholesta-8,14,24-trienol (Figure 3C), which could be through a steric blocking of the ligand-binding pocket where the azole antifungals compete with the ergosterol precursor. Another potential way is by modifying or decreasing crucial interactions, such as the hydrogen bond [27]. Allowing the formation of ergosterol and consequently losing susceptibility to the azole.

Fan et al., showed through Reverse Transcription Polymerase Chain Reaction (RT-PCR), that azole-resistant *C. tropicalis* isolates have higher expression of the *ERG11* gene than susceptible isolates, and in 92% of resistant isolates, amino acid substitutions in A395T and C461T that resulted in Y132C and S154C [34,35]. The same homozygous mutations that result in substitutions in A395T and C461T were also found in *C. parapsilosis* [27,36].

Furthermore, there is evidence in the literature of the action of gene promoters of the *ERG* genes, namely UPC2 and ndt80. They have the role of precisely inducing the expression of *ERG* genes, in the same way, that their deletion may be a mechanism to control resistance through the overproduction of ergosterol in *Saccharomyces cerevisiae*, *C. albicans* and *C. parapsilosis* [33,35].

The same mechanism is observed in *Aspergillus fumigatus* through the expression of Cyp51 genes (*ERG11* in *Candida* spp.) that synthesize CYP enzymes that catalyze lanosterol, the precursor of ergosterol. The overexpression has been noticed to be a sixfold increase in azole-resistant isolates than susceptible [37]. Also, nonsynonymous mutations in the gene have been reported in cyp51A and confer azole resistance by altering the ligand entry channel structure [38].

The main triazole resistance mechanisms found in *Cryptococcus* spp. were described in a study with 24 clinical isolates. They found aneuploidy of Chr1, which is the primary chromosome that encodes *ERG11* involved in ergosterol biosynthesize and AFR1 involved in efflux transporter, which can cause elevated expression for each gene [39].

4.2. Alternative ergosterol biosynthesis pathway

The essential mechanism of action of AmB consists of binding to ergosterol present in the cell membrane. A nonmolecular resistance or acquired it's the previous use of azoles that have action in inhibiting the ergosterol biosynthesis that was already described for multiple *Candida* spp. including, *C. albicans*, *C. parapsilosis*, *C. guilliermondii* and *C. luisitaniae* [40]. A molecular mechanism involved in ergosterol biosynthesis in the mutation of the *ERG2* gene, leads to an alternative pathway which results in another formulation the Ergosta-5,8,22-trienol (Figure 4). That isn't bound susceptible to AmB [41].

4.3. Overexpression of efflux pump

Another mechanism that is known is the efflux pump, where pathogens can export azole drugs from the inside of the cell to the outside (Figure 5A). The expression is regulated by the genes *CDR2*, *CDR1* and *MDR1*, which coordinate the synthesis of ABC transporter proteins that act on the cell membrane exporting the azole substances.

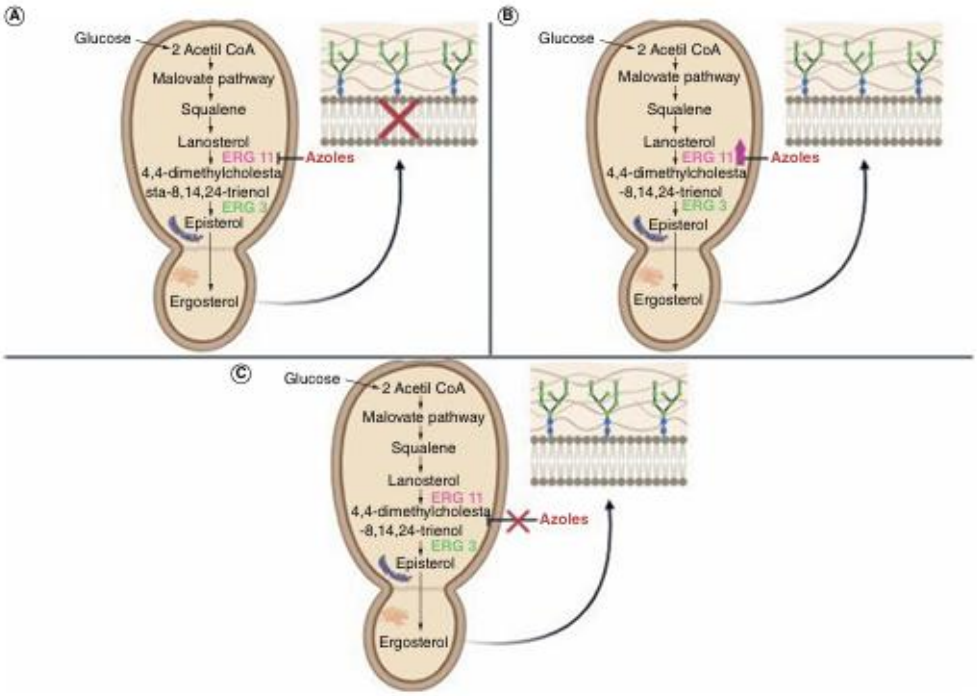

Figure 3. Schematic displays of the ergosterol biosynthesis pathway, and alterations involved in Azole resistance. **(A)** A schematic of the ergosterol biosynthesis pathway, which highlights the action of Azoles in the *ERG11* gene. **(B)** The overexpression of *ERG11*, leads to the synthesis of ergosterol independently of the azole action. **(C)** A mutation in *ERG11*, which inhibits the binding of azoles in lanosterol-4,4-dimethylcholesta-8,14,24-trienol.

In cases of overexpression the synthesis of ABC transporters is superior causing bigger action of efflux pump, also the transcription factors namely TAC1 and Mrr1 are synthesized by gain of maintenance related to mutation (Figure 5B) [42]. Mrr1 and Tac1b are both transcription factors that, respectively, work on the expression of *CDR1* and positively regulates the expression of the ATP binding cassette (ABC) transporter CDR1, involved in azole efflux and resistance in *C. auris* [43]. A mutation in Tac1b and increased expression in *CDR1* were found in clinical isolates from *C. auris* in synergy with fluconazole-resistant isolates [44,45]. It was also described a mutation in *CDR4* and *MDR1* genes, involved in ABC transporters, for this multidrug-resistant species [43].

Homozygous alleles (A/A) in the MDR promoter confer higher levels of *CDR2* and *MDR1* expression and resistance to fluconazole in *C. albicans* [46].

In *C. glabrata*, overexpression in *CDR1* and *CDR2*, and *PDR1* Y372C gained function mutation, which resulted in decreased intracellular triazole accumulation [47]. Like increased expression in *MDR1* and *CDR1* on *C. parapsilosis* [48,49].

In molds, there are only a few described mechanisms for resistance associated with increased expression of the efflux pump. A study on *A. fumigatus* isolates with a wild-type *cyp51A* gene led to the finding that mRNA encoding a particular ABC transporter was raised in 8 of 11 azole-resistant strains. It was also described that along with clinical isolates loss of *abcG1* (ABC transporter), it shown to cause acute azole hypersensitivity [50].

4.4. *FSK genes*

The fungal cell wall contains β-1,3-Glucan, which is synthesized by a membrane-integrated synthase encoded by *FKS* genes. This synthesis is regulated by the GTPase Rho1 (Figure 6). Once the glucan is polymerized, the encoded protein moves into the extracellular space [51]. The clinical breakthrough of echinocandin resistance is associated with an intrinsic amino acid substitution in two narrow

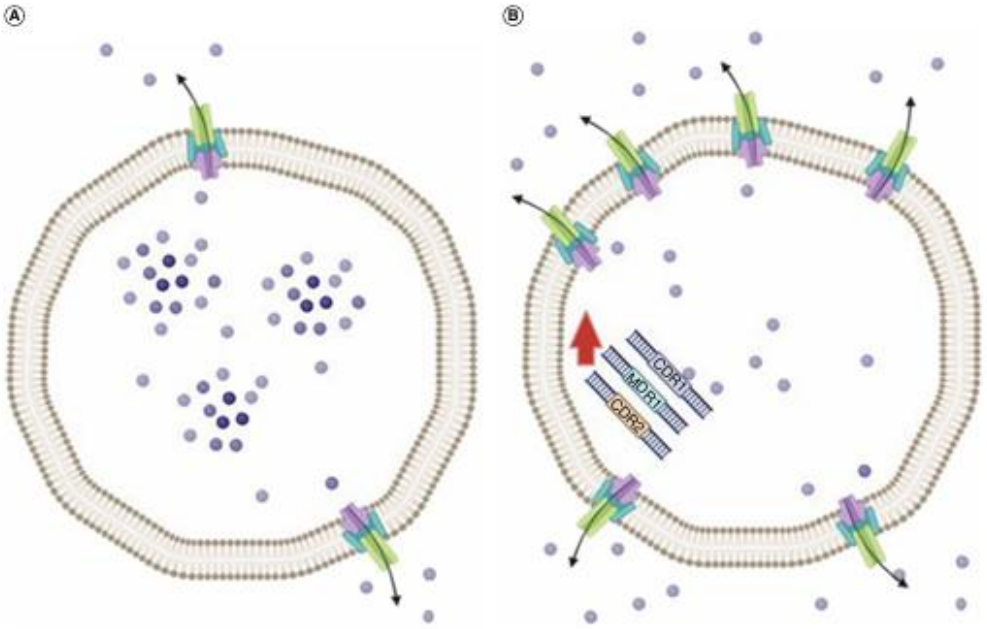

Figure 4. Schematic display of ergosterol biosynthesis pathway and mutation in the *ERG2* gene leading to an alternative pathway as a resistance mechanism for Am8.

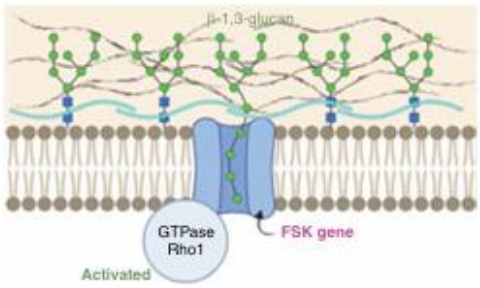

Figure 5. Schematic display of fungal cell wall beta 1,3 glucan biosynthesis (FSK gene). **(A)** A schematic diagram illustrating the normal action of an efflux pump. **(B)** A schematic diagram showing the efflux pump with increased expression of *MDR1*, *CDR1* and *CDR2* genes, leading to the synthesis of more ABC transporters, and consequently, an overly active efflux pump.

hot spot regions of *FSK1* for all *Candida* species and *FSK2* in *C. glabrata* [52].

The alteration in *FSK* genes was described in 73.3% resistant isolates of *C. glabrata*, including *FKS2 HS1 S663P, FKS1 HS1 S629P, FKS1 HS1 F625S* and *FKS2 HS1 F659S/Y*, first in four isolates and the others in three isolates. Additionally, three isolates were carrying double mutations, they were *FKS1 HS1 S629P/FKS2 HS1 S663P* or *FKS1 HS1 F625S/FKS2 HS1 F659Y* [45,53,54]. Diekema et al. also pointed out mutations in *HS* for 51 isolates of *C. glabrata*, and for another *Candida* spp. like *C. albicans* and *C. tropicalis* with the same *HS* mutations [55]. These kinds of mutations have been shown to influence the sensibility of the GS enzyme complex to inhibition by the individual echinocandins [50,53,35]. In a multicenter study in India was described S639F mutation in *FKS1* in four echinocandin-resistant isolates of *C. auris* [56].

The molecular findings of our review are organized and concatenated in Table 1.

4.5. Biofilm formation

Biofilms show complex architecture and heterogeneous in which cells form a dense network that is covered by a matrix of polysaccharides, carbohydrates, proteins and signaling molecules extracellular matrix [57]. It effectively reduces the concentration of systemic antifungal drug classes, especially azole groups, by trapping it in a glucan-rich matrix polymer synthesized by the fungal cell. It can easily colonize medical devices like catheters and prosthetic heart valves, increasing the risk of candidemia and

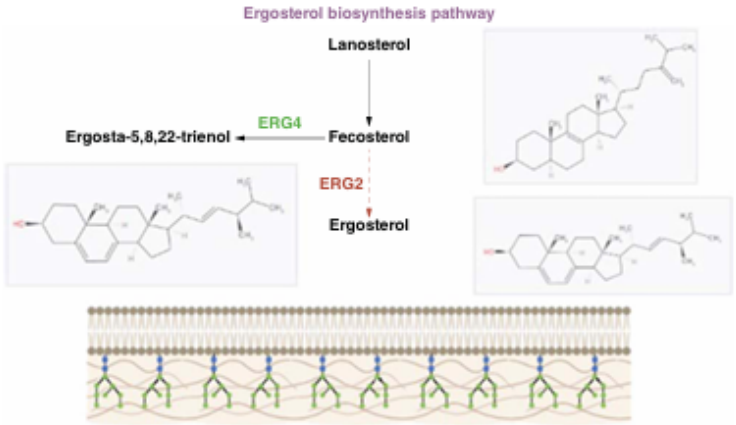

Figure 6. Schematic display of biosynthesis of beta-glucan components of the cell wall in fungi. The membrane-integrated synthase is encoded by the *FSK* gene.

Table 1. Results in literature for molecular mechanisms involved in azoles, echinocandins and amphotericin B (AmB) resistance.

Species	Molecular mechanisms	Ref.
Echinocandins		
C. albicans	Mutation *FSK1*	[46,55]
	Mutation *FSK2*	[55]
C. glabrata	Mutation *FSK1*	[46,53–55]
	Mutation *FSK2*	[55]
C. tropicalis	Mutation FSK1	[48,55]
C. auris	Mutation FSK1	[56]
	Mutations *CDR4*	[43]
	Mutations *MDR1*	[43]
	Mutation *TAC1* and *MDR1*	[43]
C. parapsilosis	Mutation *FSK1*	[49]
Azoles		
C. tropicalis	Mutation *ERG11*	[28,35]
	Mutation *ERG3*	[28]
C. albicans	Mutation *ERG11*	[35,46]
	Mutation *ERG3*	[46]
	Increase expression *MDR1*	[46]
	Increase expression *CDR2*	[46]
C. auris	Mutation *ERG11*	[45,56]
	Mutation *TAC1b*	[44]
	Increase expression *CDR1*	[44]
C. parapsilosis	Mutation *ERG11*	[48,52]
	Increase expression *MDR1*	[48,52]
	Increase expression *CDR1*	[48]
C. glabrata	Mutation *ERG11*	[36]
	Upregulation *CDR1* and *CDR2*	[47]
A. fumigatus	Overexpression cyp51A	[28]
	Mutation cyp51A	[38]
Cryptococcus spp.	Aneuploidy Ch1	[39]
Amphotericin B		
C. glabrata	Mutation *ERG2*	[41]

sepsis in critically ill patients. However, through changes in chemical modulation of the β-1,3-glucan synthase, or genetic mutation, can reduce the drug entrapment in the matrix, leading the biofilm to be susceptible to antifungal agents [58].

Biofilm formation was found in *C. glabrata*, with increased adhesin-encoding genes (especially CgEpa3), directly or indirectly leading to decreased accumulation of azole drugs. These results were unexpected, but important since they indicate the possible role player of CgEpa3 in azole resistance, might be to protect the cells from the

extracellular concentration of the drug by promoting cell aggregation [47]. *C. tropicalis* biofilms, with an extensive hexosamine-rich matrix, were poorly penetrated by AmB and fluconazole when exposed to high concentrations of both drugs [59]. Drug resistance to AmB and fluconazole of *C. albicans* biofilms may be significantly enhanced by increased production of matrix material [59,60]. The biofilms show the lowest levels of drug penetration from fluconazole by *C. parapsilosis* [60].

Biofilm formation is also a resistance factor for *Aspergillus* spp, which forms extensive exopolysaccharide matrices [15]. A study showed that amphotericin B exhibited the highest speed and effectiveness against cell populations of *A. fumigatus*. That voriconazole, while highly efficient against germinating conidia, demonstrated decreased efficacy as filamentation progressed. Contrarily, caspofungin showed consistently ineffectiveness against all populations investigated [61].

Our understanding of *C. auris* biofilm formation are still behind the knowledge of this mechanism on other *Candida* species. However, it's known that *C. auris* biofilms play a significant role in enhancing the virulence, antifungal resistance, and survival capabilities of *C. auris* both in environmental settings and presumably within host organisms [62].

4.6. Fungal melanins

It was suggested as a mechanism of resistance to the melanization of *C. neoformans* cells for echinocandins and Amb. The mechanisms could be explained with the binding of the drugs by melanin significantly decreasing the effect of pore formation in the cell wall and inhibition of synthesis of 1,3-β-d-glucan, in AmB and echinocandins, respectively [63].

4.7. Previous exposure to azoles

One study measured the susceptibility of isolates from *Candida* species to AmB alone and with previous exposure to azole. The results showed that preexposure to azoles confers a decreased AmB fungicidal effect, conferring an acquired resistance for all isolates [40].

The nonmolecular findings of our review are organized and concatenated in Table 2.

4.8. Other factors

Many variables contribute to antifungal resistance, one of them being the host immune system. Not much is known about the role of the host immune system in antifungal resistance in clinical conditions, but it was described as a way the inability to sustain the action of the antifungal drug by the immune response of the immunocompromised host, resulting in prolonged use of the drug [64]. That exposure to suboptimal drug levels leads to the selection of resistant strains, creating new subclinical reservoirs and infections [65].

As for the battle of the immune system host against fungal infections in some cases can face a few difficulties. That could be immunologically related, like HIV patients that are more susceptible to fungal infections, mostly by *C. albicans*, but can also be caused by *C. tropicalis*, *C. krusei* or *C. parapsilosis* [66]. Also, in immunosuppressive therapy like in cancer patients or patients with hematological disorders a risk factor for contracting fungal infections [67].

Another mechanism that favors the establishment of fungal infections by *Candida albicans* and non-*albicans* species, is the capability to evade the host complement proteins masking their cell membrane's pathogen-associated molecular patterns (PAMP) secreting specific proteases aimed at the opsonization of the complement, which are part of the innate immune system, protecting the host by lysing pathogens or by increasing their phagocytosis by phagocytes through opsonization [68].

One of the most studied and pathogenic species is *C. albicans*, and it has several virulence factors that contribute to the establishment of infection and an imbalance in the battle between the host and pathogen. In addition to biofilm formation, another important compound for the success of infection is adhesins [69]. These are proteins secreted by ALS genes in the cell wall of *C. albicans*, which play a crucial role in the adhesion of epithelial tissues and colonization in the host [70]. They have also been described for *C. tropicalis* [71]. Adhesins are also involved in the production of pseudo hyphae in *C. albicans*, which are prolongations of germinative tubes that penetrate through the tissue, contributing to the colonization of the host bloodstream or deep-seated organs in invasive candidiasis [72]. Another predicament for the host side is the synthesis of extracellular hydrolytic enzymes by *C. albicans*, which act in the invasion and tissue damages, help evade the host immune response, and work in the nutrient acquisition for the microorganism [73]. For other fungi species, such as *A. fumigatus* and *C. neoformans*, these proteases are mostly involved in nutrient acquisition and have not concretely described a direct pathogenic role in infections [74].

The interrelationship between resistance and other virulence factors involved in the establishment and perpetuation of fungal infections despite the treatment with antifungals, brings us to mind that standard treat-

Table 2. Results in literature for nonmolecular mechanisms involved in azoles, echinocandins and amphotericin B (AmB) resistance.

Species	Nonmolecular mechanisms	Ref.
Echinocandins		
Cryptococcus neoformans	Fungal melanins	[63]
A. fumigatus	Biofilm formation	[61]
Azoles		
C. tropicalis	Biofilm formation	[59,60]
C. albicans	Biofilm formation	[59,60]
C. parapsilosis	Biofilm formation	[60]
C. glabrata	Biofilm formation	[47]
A. fumigatus	Biofilm formation	[61]
Amphotericin b		
Candida spp.	Previous use of azoles	[40]
C. tropicalis	Biofilm formation	[59]
C. albicans	Biofilm formation	[59]
C. neoformans	Fungal melanins	[63]

ments are no longer effective for those scenerios. It is responsible for the fungus to win the battle against the host.

5. Conclusion

The ability to exhibit resistance to antifungal agents is an alarming condition in clinical practice today. Multiple molecular and nonmolecular mechanisms of resistance to these drugs have been observed in fungal pathogens, most of them of nosocomial importance, contributing to high mortality rates. In this context, the development of new antifungal compounds and new treatment strategies to win the battle against fungal resistance is of fundamental importance.

It is important to highlight the need to use tools for clinical and laboratory diagnosis of fungal infections. Antifungal therapy should be supported by laboratory evidence or validated clinical protocols. Epidemiological surveillance of multiple resistance to antifungals is relevant for the implementation of public health measures.

6. Future perspective

The post-COVID-19 pandemic period has shown little knowledge about the pathogenesis of fungal infections, difficulty in clinical and laboratory diagnosis of these diseases, as well as the existence of a reduced therapeutic arsenal.

As the irrational use of antifungals continues to be a practice and the development of survival strategies on biotic and abiotic surfaces has been observed in fungi, what we see is the loss of the battle for these microorganisms.

Therefore, robust research is needed on new antifungals, whether natural or synthetic, as well as continuing education focused on the appropriate use of these medications, associated with the use of artificial intelligence tools for diagnosis.

Article highlights

- The emergence of fungal pathogens and changes in scenery are a prevalent subject in clinical mycology.
- Species of non-*albicans Candida* spp. have been reported to cause invasive infection, surpassing the rates of *Candida albicans*, worldwide.
- Furthermore, there is also a constant report of resistance during the treatment of fungal infections.
- Presently, the clinical arsenal for systemic antifungals counts with different mechanisms of action and many classes.
- This review aims to group the molecular and nonmolecular mechanisms correlated with the resistance of nosocomial infections like *Candida* spp., *Aspergillus* spp. and *Cryptococcus* spp., to antifungals currently used in health services.
- The articles passed through three processes: identification, screening and eligibility. The identification was carried out in the following databases: EMBASE; MEDLINE; and COCHRANE. It included 74 articles.

Ergosterol biosynthesis pathway

- *ERG's* genes are responsible for regulating the synthesis of ergosterol, which is the major component of the fungal cell membrane and contributing with the fluidity and integrity of the membrane and the proper function of membrane-bound enzymes.
- The azole drugs block the synthesis by inhibiting the activity of the genes *ERG*, binding and inhibiting the lanosterol demethylase enzyme, causing damage to the cell membrane.
- In literature, we have a lot of findings with overexpression and mutation of different *ERG* genes, but the most frequent is *ERG11*.
- Mutations and super expression in the *ERG* genes, that are responsible for the ergosterol biosynthesis pathway, promote a resistance mechanism against azoles or Amphotericin b.

Overexpression of efflux pump

- The efflux pump is a mechanism where pathogens can export azole drugs from the inside of the cell.
- The expression is regulated by the genes *CDR2*, *CDR1* and *MDR1*, which coordinate the synthesis of ABC transporter proteins that act on the cell membrane exporting the azole substances.
- In cases of overexpression the synthesis of ABC transporters is superior causing bigger action of efflux pump, it contributes to a bigger exporting activite of azoles substances outside the cell conferring resistance.

FSK genes

- The fungal cell wall contains β-1,3-Glucan, which is synthesized by a membrane-integrated synthase encoded by *FKS* genes.
- For echinocandins, a molecular mechanism described was mutation in the *FSK* genes, these kinds of mutations have been shown to influence the sensibility of the GS enzyme complex to inhibition by the individual echinocandins.
- Furthermore, nonmolecular virulence factors contributed to therapeutic failure, such as biofilm formation, fungal melanins and

selective pressure due to previous exposure to antifungals, and other factors that involved virulence factors and the host immunity system. Thus, there are many public health challenges in treating fungal infections.

Conclusion

- This study warns of the emergence of fungi resistant to the main antifungals of medical interest.
- Multiple molecular and nonmolecular mechanisms of antifungal resistance have been identified in several fungal pathogens, emphasizing the need for new strategies to contain fungal resistance.
- The use of tools for accurate laboratory diagnosis of fungal infections is necessary.

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Author contributions

All authors contributed to the conception and writing of this review and approved the final version of the manuscript.

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9.3 EPIDEMIOLOGIA DE INDIVÍDUOS COM PNEUMONIA ASSOCIADA À VENTILAÇÃO MECÂNICA COM E SEM COVID-19

INTRODUÇÃO

Desde o surto inicial em 2019 até hoje, a pandemia SARS-CoV-2 acumulou mais de 770 milhões de casos notificados, incluindo 6,9 milhões de mortes, segundo a Organização Mundial da Saúde (OMS) (WHO, 2023). O sistema de saúde brasileiro ficou sobrecarregado durante a "primeira onda" (25 de fevereiro de 2020 a 5 de novembro de 2020) e, durante a segunda, apresentou recursos insuficientes, num contexto de baixa adesão a práticas não medicamentosas e dominância convergente de variantes virais de interesse médico. Estas conclusões, em retrospectiva, mostram-nos quão necessário era, naquela altura, tomar medidas urgentes para conter a transmissão viral, bem como expandir a cobertura vacinal e otimizar os cuidados intensivos para a Covid-19, uma vez que a incapacidade de realizar adequadamente essas medidas justificam o colapso observado no Sistema Único de Saúde, diante dos momentos mais graves da pandemia (BASTOS *et al.*, 2021).

Pneumonia associada à ventilação mecânica (PAV) é a designação dada à infecção pulmonar associada à assistência à saúde, que pode se manifestar após, pelo menos, 48 horas de intubação endotraqueal em pacientes internados em unidades de terapia intensiva (UTI) submetidos à ventilação mecânica invasiva. Nestes pacientes, o funcionamento das vias aéreas fica prejudicado e a produção de secreção é aumentada devido ao tubo, impedindo, assim, um reflexo de tosse eficiente. Essa condição pode criar um ambiente conveniente para a colonização de patógenos (WATSON *et al.*, 2022).

A ecologia microbiana na PAV é frequentemente caracterizada pela ocorrência de multiplicação e penetração progressiva de agentes infecciosos, principalmente bactérias e fungos multirresistentes. Os microrganismos podem induzir, como consequência, uma resposta inflamatória no parênquima pulmonar do hospedeiro com sinais e sintomas respiratórios como dispneia e produção de secreção, e sinais de infecção sistêmica como febre e leucopenia. Outros sintomas seguintes são comprometimento da imunidade e alterações na deglutição. De acordo com a literatura internacional, a incidência de PAV é de aproximadamente 15 a 35% em pacientes internados em UTI, sendo que pacientes obesos, com índice de massa corporal (IMC) ≥ 40, possuem mais chances de adquirir PAV se comparados aos não obesos (IMC < 30). As taxas de mortalidade como desfecho extremo variam entre 15 a 70% para pacientes sem Covid-19 e impactam como um problema de saúde pública os sistemas de saúde em todo o mundo, resultando em maiores custos com hospitalização e internação mais longa (WATSON *et al.*, 2022).

Pacientes gravemente enfermos com Covid-19 apresentam maior risco de desenvolver infecções nosocomiais, como PAV. A taxa de mortalidade estimada de PAV em pacientes com Covid-19 é de 42,7% (IPPOLITO *et al.*, 2021). Alguns motivos podem ser associados, considerados como fatores extrínsecos, como escassez de profissionais e falta de equipamentos de proteção individual (EPI) para a assistência aos pacientes, tratamento com agentes imunomoduladores e oxigenação por membrana extracorpórea (ECMO). Acrescenta-se a isso os fatores intrínsecos, como danos ao parênquima pulmonar, baixa adesão, desregulação imunológica, alterações no microbioma pulmonar e aumento do risco de trombose (BOYD *et al.*, 2022). Embora poucos dados da literatura considerando PAV e Covid-19 como comorbidade estejam disponíveis, foi relatada recentemente uma alta taxa de PAV em pacientes infectados por SARS-CoV-2 (50%),

com duração média de ventilação mecânica entre 12-30 dias (FUMAGALLI *et al.*, 2022). No entanto, a taxa de mortalidade descrita para pacientes com Covid-19 com PAV foi semelhante à de pacientes sem Covid-19 (NSEIR *et al.*, 2021). Em outro estudo, desta vez realizado durante a "segunda onda" de infecções por Covid-19 (6 de novembro de 2020 a 30 de abril de 2021), no Egito, todas as amostras coletadas de pacientes sob ventilação mecânica foram positivas para bactérias e/ou infecção fúngica (MEAWED *et al.*, 2021).

De modo geral, no que diz respeito à etiologia, os dados sugerem que os microrganismos mais prevalentes na PAV são bactérias gram-negativas, principalmente *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp. e Escherichia coli*, juntamente com bactérias gram-positivas, como *Staphylococcus aureus e Enterococcus faecium* (BOYD *et al.*, 2022). Por outro lado, o aumento de organismos multirresistentes devido ao grande uso de antimicrobianos empíricos, especialmente durante a pandemia de Covid-19, é uma preocupação amplamente discutida atualmente (CANTÓN *et al.*, 2020).

O aumento das internações em UTI devido à pandemia de Covid-19 contribuiu para o aumento da PAV e as consequências dessa coinfecção deveriam ser melhor abordadas. Logo, este estudo teve como objetivo avaliar e comparar as características epidemiológicas, clínicas e etiologia da PAV em pacientes com e sem coinfecção por Covid-19 entre 2021 e 2022 em um hospital terciário brasileiro.

MÉTODO

Protocolo de estudo

Trata-se de um estudo transversal retrospectivo baseado na análise de dados de pacientes de UTI com diagnóstico exclusivo de PAV, de ambos os sexos, maiores de 18 anos, com resultado positivo ou negativo para infecção por SARS-CoV-2. Informações epidemiológicas, clínicas e laboratoriais de pacientes atendidos em um hospital terciário localizado em Juiz de Fora-MG foram coletadas de prontuários eletrônicos no período de setembro de 2021 a maio de 2022. Este hospital é do tipo geral e possui aproximadamente 200 leitos, incluindo unidade de terapia intensiva (adulto e neonatologia), unidade coronariana, enfermarias, centro cirúrgico e atendimento ambulatorial. Oferece clínicas especializadas e serviços de diagnóstico, com atendimento restrito à rede privada.

O Comitê de Ética em Pesquisa da UFJF aprovou este estudo sob CAAE 50838421.1.0000.5147 Nº 4.943.773.

Coleta de dados e variáveis

Os dados epidemiológicos avaliados incluíram idade e sexo. As informações clínicas coletadas foram tempo de internação hospitalar, tempo de internação na UTI, tempo de internação na UTI antes da PAV, tempo de internação em ventilação mecânica (VM), tempo de internação em VM antes da PAV, patógeno causador da PAV e desfechos clínicos (alta hospitalar ou óbito).

Critérios/definição de VAP e Covid-19

De acordo com os prontuários, foi utilizado o exame laboratorial RT-PCR para o diagnóstico de Covid-19, a partir do achado de RNA do SARS-CoV-2 em *swabs* de nasofaringe e/ou orofaringe, após 72 horas de sintomas clínicos.

A pneumonia foi considerada PAV quando os sintomas clínicos ocorreram após 48 horas de ventilação mecânica e microrganismos estavam presentes em culturas de aspirados endotraqueais. Amostras biológicas para diag-

nóstico laboratorial de Covid-19 e PAV foram coletadas conforme suspeita médica, com base em anamnese, achados laboratoriais/radiológicos, sintomas clínicos e sinais vitais.

Análise estatística

Frequências relativas e absolutas foram utilizadas para descrever as características da amostra. Diferenças estatisticamente significativas foram avaliadas para determinar se a presença de Covid-19 influenciou o desfecho clínico, bem como a distribuição do agente microbiano causador de pneumonia entre os grupos por meio do teste qui-quadrado. Tempo de internação e tempo de ventilação mecânica, entre os grupos com e sem Covid-19, foram avaliados mediante o conceito de normalidade, por meio do emprego do teste de Shapiro-Wilk, onde valores de $p > 0,05$ indicam distribuição normal e $p < 0,05$ distribuição não normal. Como todas as variáveis apresentaram distribuição não normal, foi utilizado o teste U de Mann-Whitney para comparar as médias entre os dois grupos.

RESULTADOS E DISCUSSÃO

Neste estudo transversal retrospectivo baseado na análise de dados de pacientes de UTI com diagnóstico exclusivo de PAV, foram avaliadas características epidemiológicas, clínicas e microbiológicas considerando pacientes positivos ou negativos para SARS-CoV-2. Os participantes (n = 146) foram divididos em dois grupos: admissão em UTI com (n = 95) ou sem (n = 51) infecção por SARS-CoV-2. Durante o período avaliado (setembro de 2021 a maio de 2022), o quadro clínico da PAV foi mais comum entre indivíduos afetados pela Covid-19.

Nossos resultados indicam predomínio de indivíduos com idade avançada (idade média de 68 anos), sem diferença entre os sexos, conforme descrito na **Tabela 30.1**. Quanto à idade média avançada dos pacientes com PAV, a idade e o sexo são descritos como fatores altamente relacionados à ocorrência de PAV (IPPOLITO *et al.*, 2021). Além disso, a idade avançada e o sexo masculino são considerados um dos principais fatores de risco para o desenvolvimento desta doença (MEAWED *et al.*, 2021; WATSON *et al.*, 2022).

No que diz respeito à pandemia de Covid-19 como fator de risco de PAV, um estudo de coorte retrospectivo que analisou 230 casos de pacientes demonstrou que entre os 42 pacientes que também tiveram Covid-19, a idade média foi de 60 anos, e 67% dos os indivíduos eram do sexo masculino. No mesmo estudo, entre aqueles que tiveram PAV, mas sem Covid-19, a idade média foi de 63,4 anos e 73% eram do sexo masculino (MAES *et al.*, 2021). Em outro estudo, no qual foi realizada análise retrospectiva multicêntrica de 774 casos de pacientes internados em unidade de terapia intensiva por Covid-19, foi demonstrado que a idade mediana dos indivíduos era de 62 anos, e 77% eram homens (GARNIER *et al.*, 2023). Além disso, num estudo observacional multicêntrico em 149 hospitais localizados na Europa, foi demonstrado que em 3.338 pacientes internados na UTI por Covid-19, a idade média entre aqueles que desenvolveram PAV foi de 63 anos, sendo 79% desses indivíduos do sexo masculino (LOYOLA-CRUZ *et al.*, 2023).

Nosso estudo mostra que há diferença entre os valores médios para diagnóstico de PAV em pacientes com e sem Covid-19. Pacientes com infecção por SARS-CoV-2 desenvolveram PAV em menor média de dias de internação e VM. Por outro lado, os indivíduos sem Covid-19 permaneceram mais tempo na UTI, em VM e internados, indicando maior sobrevida (**Tabela 30.1**). A presença de PAV independente da

coinfecção com SARS-CoV-2 é caracterizada pelo tempo prolongado de permanência na UTI e também pelo aumento do tempo de internação. Neste contexto, estudos apontam para aumento dos custos de hospitalização, maior vulnerabilidade dos pacientes e maior exposição a infecções nosocomiais (WATSON *et al.*, 2022).

Em muitos casos, os pacientes com Covid-19 que evoluem para PAV apresentam intensa resposta inflamatória, além do uso de medicamentos imunossupressores e diversos dispositivos médicos invasivos, podendo necessitar de hemodiálise. Essa condição clínica está relacionada ao maior tempo de internação hospitalar e pode contribuir para o agravamento do quadro do paciente, tornando-o mais suscetível a infecções nosocomiais (MEAWED *et al.*, 2021). Nesse contexto, nosso estudo demonstrou que o grupo de indivíduos positivos para Covid-19 com PAV apresentou maior taxa de mortalidade, em consonância com os achados de Maes *et al.* (2021). Fatores como ativação hiper-inflamatória e funções antimicrobianas prejudicadas, falta de tratamento específico e baixa cobertura vacinal podem ser os contribuintes.

Neste estudo, a principal etiologia relacionada aos pacientes com PAV compreende o grupo de bactérias gram-negativas não fermentadoras de glicose, como *Acinetobacter baumannii* (n = 56; 35,22%) e *Pseudomonas aeruginosa* (n = 40; 25,15%). O desfecho clínico mais frequente entre os indivíduos avaliados foi o óbito (n = 117/81,3%) (**Tabela 30.1**). A distribuição de isolados microbianos provenientes de cultura em meios específicos de aspirado endotraqueal entre os grupos Covid-19 positivo e negativo aponta predominância de bactérias gram-negativas fermentadoras de glicose, como *P. aeruginosa* e *A. baumannii* (n = 90/61,6%), seguidas por bactérias gram-negativas fermentadoras de glicose, como *Klebsiella pneumoniae*, *Enterobacter cloacae* e *Serratia spp* (n = 37/25,3%). Os relatos de bactérias gram-positivas e fungos foram menos frequentes (n=19/13,1%) (**Tabela 30.1**).

O fato de *Acinetobacter baumanii* e *Pseudomonas aeruginosa* terem sido os microrganismos mais comuns encontrados nos aspirados endotraqueais dos pacientes do nosso estudo é consistente com estudos anteriores de PAV em pacientes com Covid-19 e sem Covid-19, que também observaram uma maior prevalência de infecções gram-negativas não fermentativas (LOYOLA-CRUZ *et al.*, 2023; ROUYER *et al.*, 2021). Essas bactérias são comumente identificadas em superfícies inanimadas, pele e mucosas da equipe médica, além de dispositivos utilizados em fisioterapia respiratória, como ventilação mecânica (GRASSELLI *et al.*, 2021). Sabe-se que as espécies de *Pseudomonas* e *Acinetobacter* apresentam inúmeros fatores de virulência, como capacidade de formar biofilme, tolerância a sanitizantes e resistência aos principais antimicrobianos utilizados na rotina médica (DIAS *et al.*, 2017). O achado dessas bactérias, em maior incidência, neste estudo está relacionado a outros fatores além da Covid-19, como estado do sistema imunológico do hospedeiro, fatores de virulência expressos por essas bactérias, além das condições de controle de infecção hospitalar (GRASSELLI *et al.*, 2021).

Embora a frequência de achados de espécies fúngicas em amostras do trato respiratório inferior tenha sido baixa em nosso estudo (n = 5/3,4%), houve predominância de isolados do gênero *Candida*, sendo relacionados apenas a pacientes com Covid-19. Outros estudos, entretanto, mostraram um padrão diversificado (MAES *et al.*, 2021; SILVA *et al.*, 2021; TROVATO *et al.*, 2021; SINGH *et al.*, 2021).

Foi relatada aspergilose pulmonar associada à Covid-19, com uma prevalência aproximada de 13%, sendo as espécies *Aspergillus niger* (TROVATO *et al.*, 2021) e *Aspergillus fumigatus* (MAES *et al.*, 2021) as mais comuns. Espécies de *Mucor, Rhizopus, Rhizomucor* e *Syncephalastrum* também foram identificadas em indivíduos hospitalizados, gravemente enfermos, afetados pela Covid-19 (SINGH *et al.*, 2021).

Tabela 30.1 Aspectos clínicos e epidemiológicos de indivíduos com pneumonia associada à ventilação mecânica com e sem infecção por SARS-CoV-2

Variáveis	Valores (todos os indivíduos)	Grupo Covid + n = 95 (61,5%)	Grupo Covid - n = 51 (34,9%)	Valor *p*
Sexo (n/%) Feminino Masculino	74 (50.7%) 72 (49.3%)	45 (47.4%) 50 (52.6%)	29 (56.9%) 22 (43.1%)	0.347
Idade (média ± desvio padrão) [Mínimo; Máx.]	67.97 ±16.05 [18; 97]	63.78± 13.68 [32; 89]	75.78 ± 17.33 [18; 97]	< 0.001
Tempo de permanência hospitalar antes da PAV (dias: média ± desvio padrão)	21.78 ± 25.68	16.12 ± 15.17	31.74 ± 36.25	< 0.001
Tempo de ventilação mecânica antes da PAV (dias: média ± desvio padrão)	18.02±23.91 [2;215]	13.12± 13.57 [2;74]	27.12±34.38 [3;215]	0.000
Grupo microbiano n (%) Gram-positivo Gram-negativo não fermentador Fermentador Gram-negativo Fungos Outros	4 (2.9%) 90 (61.6%) 37 (25.3%) 5 (3.4%) 10 (6.8%)	3 (3.1%) 59 (62.1%) 22 (23.2%) 5(5.3%) 6(6.3%)	1 (2.0%) 31 (60.8%) 15 (29.4%) 0 (0,0%) 4 (7.8%)	0.569
Tempo total em UTI (dias: média ± desvio padrão) [Mínimo; Máx.]	47.29 ± 43.19 [8;221]	33.81 ± 26.61 [8; 131]	74.49 ± 55.62 [8; 221]	< 0.001
Tempo total em VM (dias: média ± desvio padrão) [Mínimo; Máx.]	38.88 ± 38.60 [5; 221]	28.58 ± 22.65 [5; 99]	58.05 ± 52.72 [5; 221]	< 0.001
Tempo total de internação (dias: média ± desvio padrão) [Mínimo; Máx.]	54.16 ± 50.36 [8; 263]	40.42 ±.36.86 [8; 202]	79.49±.61.39 [9; 263]	< 0.001
Resultado clínico (%) Alta Óbito	27 (18.7%) 117 (81.3%)	19 (19.2%) 76 (80.8%)	9 (18.0%) 42 (82.0%)	0.986

Legenda: PAV: pneumonia associada à ventilação mecânica; UTI: Unidades de terapia intensiva; VM: Ventilação mecânica.

A análise estatística que correlaciona ausência do grupo microbiano e presença de Covid-19 não revelou associação, considerando o teste qui-quadrado (**Tabela 30.2**).

Tabela 30.2 Aspecto microbiológico de indivíduos PAV com Covid-19 positivo ou negativo

Covid-19	Grupo microbiano			Teste qui-quadrado (2x2)	
	Gram-negativa NF	Gram-negativa F	Outros	X^2	*p*
Negativo (n = 51)	31 (21.2%)	15 (10.3%)	5 (3.4%)	1.14	0.56
Positivo (n = 95)	59 (40.4%)	22 (15.1%)	14 (9.6%)		

Legenda: NF - fermentador não glicídico; F - fermentador de glicose. Outros: fungos e associações entre diferentes microrganismos relatados com menos frequência.

Embora a análise estatística aponte falta de associação entre grupos microbianos isolados de cultura e evolução clínica na população estudada, foi possível observar que um maior número de indivíduos que evoluíram a óbito apresentou PAV por bactérias gram-negativas não fermentadoras de glicose (n = 72/49,3%). Outro fato relevante apontado em nosso estudo é que, independentemente da infecção pelo SARS-CoV-2, os indivíduos avaliados apresentaram PAV por esse grupo microbiano em maior frequência (**Tabela 30.3**).

Tabela 30.3 Aspectos clínicos e microbiológicos de indivíduos com PAV com Covid-19 positivo ou negativo

Resultado clínico	Grupo microbiano		Teste qui-quadrado (2x2)	
	Gram – NF	Gram - F	X^2	*p*
Alta	18 (12.3%)	7 (4.8%)	0.33	0.846
Óbito	72 (49.3%)	31 (21.2%)		
Covid-19	**Resultado clínico**		**Teste qui-quadrado (2x2)**	
	Alta	**Óbito**	X^2	*p*
Negativo (n = 51)	9 (6.16%)	42 (28.8%)	0.43	0.986
Positivo (n = 95)	19 (13.0%)	76 (52.04%)		

Legenda: NF - não fermentador de glicose; F - fermentador de glicose.

De modo geral, é possível que o perfil etiológico da PAV esteja intimamente associado ao perfil microbiológico da instituição hospitalar, existência de comorbidades e outras patologias associadas, estado do sistema imunológico e composição da microbiota, além da presença de Covid-19, o que se mostrou fator predisponente ao aparecimento de infecção secundária. Nesse contexto, é importante destacar que a coinfecção tem potencial para agravar o quadro clínico, aumentar a mortalidade e prolongar o tempo de internação (PÓVOA *et al.*, 2020; SINGH *et al.*, 2021).

Considerando o período de pandemia em que os dados foram coletados, observou-se a falta de informações importantes nos prontuários dos pacientes, provavelmente devido ao manejo emergencial dos pacientes. Assim, como uma limitação destas observações que contribuiriam para uma discussão epidemiológica mais aprofundada, os padrões de susceptibilidade antimicrobiana das amostras de bactérias isoladas não estavam disponíveis, nem informações que permitissem à epidemiologia molecular discutir a relação clonal em linhagens de bactérias. Além disso, a situação

vacinal dos pacientes internados na UTI não estava disponível e a relação entre vacinas (tipo e doses administradas) e evolução clínica não foi determinada.

CONCLUSÃO

Apesar das limitações apontadas, os dados aqui apresentados constituem informações consistentes que permitem discussões sobre o manejo empírico da PAV, em especial a gestão de recursos humanos (profissionais de saúde), e a implementação de medidas de controle de infecção.

Nossos resultados sugerem que a infecção por Covid-19 pode estar associada a um maior risco de desenvolvimento precoce de PAV em pacientes de UTI, e também a uma maior taxa de mortalidade em comparação com pacientes sem Covid-19. Em ambos os grupos, os principais agentes da PAV foram bactérias gram-negativas não fermentadoras de glicose, como *A. baumannii e P. aeruginosa*, enquanto bactérias gram-positivas e fungos foram menos frequentes.

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9.4 Submitted article - Unveiling antifungal resistance and biocide tolerance in clinical isolates of *Candida* spp.

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Unveiling antifungal resistance and biocide tolerance in clinical isolates of *Candida* spp.

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Abstract

Background: Candidiasis, caused by *Candida* spp., is a significant opportunistic infection, particularly in immunocompromised patients. Emerging non-*albicans* species and antifungal resistance have increased invasive infections.

Objectives: This study evaluated the antifungal susceptibility of *Candida* spp., investigated resistance mechanisms, assessed efflux pump activity, and analyzed biocide tolerance.

Methods: A total of 100 *Candida* isolates from hospitalized and outpatient individuals were analyzed. Antifungal susceptibility was tested via disk diffusion, resistance mechanisms were identified using PCR, efflux pump activity was evaluated with the Cartwheel method, and biocide tolerance (sodium hypochlorite, hydrogen peroxide, benzalkonium chloride) was assessed.

Results: High resistance to at least one antifungal (87%) was observed, with 47.1% showing multidrug resistance to azoles. Fluconazole resistance was most common (70%), while amphotericin B had the highest susceptibility (99%). The *ERG11* gene mutation was detected in 43.7% of isolates. Efflux pump activity was present in both *C. albicans* and non- *albicans* species. Sodium hypochlorite showed the highest tolerance, with inhibition zones ranging from 18.3 ± 4.4 to 34.0 ± 4.0 mm.

Conclusions: *Candida* spp. exhibits substantial resistance, particularly to azoles, driven by genetic mutations. These findings underscore the need for enhanced infection control and innovative therapies to combat resistant *Candida* infections.

Keywords: *Candida*; Antifungal susceptibility; Efflux pumps; Biocide tolerance; Opportunistic infections; Azoles; Resistance.

1. Introduction

Among the most common fungal infections, candidiasis is a popularly known disease caused by yeasts *Candida* spp. The *Candida* genus belongs to the *Saccharomycetes* class and encompasses a group of opportunistic unicellular yeasts [1]. Over recent years, the genus has undergone taxonomic changes based on molecular sequences that have not yet been fully elucidated [2].

The most commonly described etiological agent for candidiasis is *C. albicans*. However, other *Candida non-albicans* species, such as *Nakaseomyces glabratus* (syn. *Candida glabrata*), *Candida parapsilosis, Meyerozyma guilliermondii* (syn. *Candida guilliermondii*), *Pichia kudriavzevii* (syn. *Candida krusei*) and *Candida tropicalis*, are also recognized as clinically emerging pathogens, accounting for more than half of the cases of invasive candidiasis and candidemia worldwide since the early 2000s [3,4]. Another emerging species in the last decade is *Candida auris*, presenting a profile of multiple resistance to antifungals and a high mortality rate (33 to 73%) [5].

These microorganisms are commonly associated with the intestinal, vaginal, and skin microbiota of humans, among other anatomical sites [6]. Pathological processes can be triggered through an imbalance in the microorganism-host relationship, localized infection, horizontal transmission by professionals' hands, or invasive medical device use [7]. It can trigger more severe clinical manifestations such as invasive candidiasis (IC), which is comprised of candidemia (bloodstream infection), and deep-seated candidiasis in organs, which may occur concurrently or independently. These conditions are commonly observed in hospital settings, especially in intensive care units (ICUs), in severely ill patients with comorbidities and immunosuppression [4]. Pathogenesis involves a series of factors, including adherence to the host surface, the ability to form biofilms, the production of hydrolytic enzymes, and the evasion of the host's immune response [8].

One of the main challenges in controlling nosocomial infections caused by *Candida* spp. is the appropriate management of hand hygiene among healthcare professionals and the decontamination of the hospital environment, which must be based on scientific evidence when choosing broad-spectrum biocides with fungicidal capacity against these microorganisms [9]. This proper management is essential due to the biofilm formation capability that allows for the colonization of biotic and abiotic surfaces, such as medical devices and instruments [10].

Antifungal resistance among human pathogens is a rising topic and a significant challenge in medical mycology, an important matter for both superficial and invasive fungal infections [11]. This rise may be due to increased antifungal use, the vulnerability of patient populations, limited treatment options, and the inherent ability of the fungi to develop resistance mechanisms [12].

Understanding antifungal susceptibility patterns can significantly improve patient outcomes and guide treatment decisions. A repository released by the World Health Organization (WHO) listed the 19 fungal pathogens, according to their impact on public health and the emerging risk of antifungal resistance exhibited by these fungi. Among them, *C. auris* and *C. albicans* are in the critical priority group, and *N. glabratus* (syn. *C. glabrata*), *C. parapsilosis,* and *C. tropicalis* are in the high priority group [13].

Mutations in the *ERG* genes (involved in ergosterol biosynthesis), FSK genes (involved in beta-glucan synthesis), and overexpression genes for efflux pumps (*MDR 1*, *CDR 1*, and *CDR 2*) are the most reported molecular mechanisms of resistance in clinical isolates of *Candida* spp., mainly related to azole derivatives [14,15]

This study aimed to explore the antifungal susceptibility profile of clinical isolates of *Candida* spp. correlating the phenotypic findings with resistome mapping, and biocide tolerance, in addition to investigating efflux pumps' impacts as a multifactorial resistance mechanism. The research seeks to contribute to understanding fungal resistance and tolerance mechanisms, providing insights to enhance diagnostic, therapeutic, and control strategies in clinical settings.

2. Material and methods

This observational and experimental study collected retrospective and prospective data, from clinical isolates of *Candida* spp. stored in a collection and patients' electronic medical records. The collection counted 100 clinical isolates from community individuals and individuals admitted to a tertiary hospital in Juiz de Fora, Minas Gerais, Brazil, in 2021. The medical records were analyzed for the patient's clinical outcomes, hospitalization and length of stay, and use of invasive medical devices.

The identification of isolates at the species level was performed previously through PCR (Polymerase Chain Reaction) in multiplex reactions, using specific oligo primers for the genus *Candida* according to Carvalho A. (2007). The species findings were *C. albicans* (n=59), *C. tropicalis* (n=25), *C. parapsilosis* (n=12), *N. glabratus* (*syn C. glabrata*) (n=2), *P. kudriavzevi* (*syn C. kruzei*) (n=1) and *M. guilliermondii* (*syn C. guilhermondii*) (n=1).

This study was carried out after consultation and consent of the participants, according to a project approved by the Ethics Committee for Research Involving Human Beings of the Federal University of Juiz de Fora, under number 3,783,237, CAAE 18611019.6.0000.5147.

2.1. Antifungal susceptibility testing

The isolates of the genus were evaluated for their susceptibility profile to antimicrobials, according to the current guidelines of the CLSI (Clinical and Laboratory Standard Institute), using the disk diffusion method, applied through commercial discs of fluconazole 25 µg, voriconazole 1 µg, caspofungin 5 µg and amphotericin B 20 µg (Liofilchem Diagnostics, Italy). The interpretation of the yeast inhibition zones was carried out according to the reference described in document M44 [17] and the manufacturer's Liofilchem criteria.

2.2. Identification of resistance genes

The genomic DNA of the yeasts was extracted using the digestion and purification method with phenol-chloroform, according to the study by Rodrigues et al. (2013) with some modifications, and subsequently stored in a freezer at -80ºC. The quantification (Nanodrop) and the integrity gel of the DNA were performed, patronizing the dsDNA to 50 $\mu g/\mu l$.

The genes *ERG11* (fwd5'-CCA.AGG.CTA.GTG.CTG.TTT.CT-3';rev5' -TGT.GTC.TAC.CAC.CAC.CGA.AT-3') [19], *CDR1* (fwd 5' -CCA.GAG.GTT.TGG.ATT.CCG.CT-3'; rev5'-TGG.CTT.TGT.CTG.CTT.TCC.CA-3') [20], *CDR2* (fwd5' -GCT.TAG.ATG.CCG.CCA.CTG-3'; rev5'-AGC.CCA.TTC.TGA.TGA.AAT.ACT.C-3') [21], *MDR1* (fwd 5'-GGGTGCATCATTCCAGCCTA-3'; rev5'-GGGATGGCAATCATCACGAG-3') [20], *ERG 2* (fwd 5'-ATGAAGTTCTTTATCAA T-3'; rev5'-TTAGAACTTTTGGTTTTG-3') [22], and *FSK1* (fwd 5'-GAAATCGGCATATGCTGTGTC-3'; rev5'-AATGAACGACCAATGGAGAAG-3') [23] were investigated by PCR. The reaction was performed using 1μl at 5μM/μl of each primer in addiction with 12.5μl of Master mix G2 Green (PROMEGA, USA) and 9.5μl of sterile water, for 1μl of DNA. The reaction parameters were established for each gene as described by their respective authors with modifications.

The generated amplicons were submitted to electrophoresis using a 1.5% agarose (LGCbiotecnologia, Brazil) gel with ethidium bromide (EtBr) (AMRESCO, USA), followed by separation at 90V for 40 minutes. The gel was visualized through UV light using a transilluminator, to identify the DNA bands. Subsequently, 10% of the positive isolates for each gene were selected for Sanger sequencing to confirm the investigated genes.

2.3. Assessment of the efflux pump activity

The expression of efflux pumps was evaluated using Ethidium Bromide (EtBr) (AMRESCO, USA) according to the Cartwheel method, with modifications [23>24]. The method consisted of the culture in Mueller-Hinton Agar (Kasvi/Brazil) at concentrations of 0.5 µg/mL, 1.0 µg/mL, 1.5 µg/mL, 2.0 µg/mL, and 2.5 µg/mL of EtBr and plates without EtBR used as a control for microbial growth. Solutions equivalent to a turbidity of 0.5 on the McFarland scale were prepared for each isolate and ATCC's strains, which were inoculated with the aid of a Steers replicator. Duplicates were made for each concentration level and control. The plates were incubated for 24 hours at 35 °C, protected from light. After incubation, the plates were read under ultraviolet light, and the presence of fluorescence emitted by the samples was a negative indication of the action of the efflux pump.

The index for the minimum concentration of EtBr (MC_{EtBr}) that produces fluorescence of the isolates (MDR) compared to the ATCC (REF) was calculated using the following formula: $Index = [MC.(MDR) - MCEtBr(REF)] \div MCEtBr(REF)$.

2.4. Assessment of biocide tolerance

An adaptation of the disk diffusion technique (DDT) described by CLSI (2021) was performed to assess tolerance to the biocides routinely used in the hospital of origin of the isolates. From suspensions of isolates on a McFarland turbidity scale of 0.5, the isolates were uniformly inoculated with the aid of a swab in Mueller-Hinton agar culture medium (Kasvi/Brazil), to which filter paper discs soaked in 5µL of 1%, 1.5%, 2% sodium hypochlorite solution (Start/Brazil), 4.25% hydrogen peroxide diluted (Rioquímica/Brazil) and 5% benzalkonium chloride (Santos/Brazil) were added. The plates were incubated at 35ºC, and readings of the diameter of the inhibition zones were performed after 24 hours and 48 hours of incubation.

The biocides used were of commercial grade, stored in regular conditions, and used within the validity periods. All the tests were performed in duplicate for all 100 clinical isolates and reference strains of *C. albicans* ATCC14053, *C. parapsilosis* ATCC22019, *C. tropicalis* ATCC66029, and *N. glabratus* ATCC2001 were used as the experimental control.

2.5. Statistical Analysis

For the statistical analysis of the data obtained by the biocide test, the Student's T-test (*p-value* 0.05) to the mean diameters of the halos comparing the reference linage and isolates in separate groups of C. *albicans* and *non-albicans*. ANOVA two-way testing (*p-value* 0.05) was used to associate the results of efflux pump testing and resistance genes described to overexpression of efflux pump.

3. Results

Sixty-three individuals in this study were hospitalized. Of these, 60% presented with infections caused by *Candida albicans*. The average length of hospital stay was 39.5 days (range: 6-202) for *C. albicans* infections, compared to 31.5 days (range: 7-162) for infections caused by *non-albicans* species. Invasive medical devices were recorded in 60 patients with *C. albicans* and 29 with *non-albicans* species. The study also reported 28 in-hospital deaths associated with *C. albicans* infections and 16 deaths related to *non-albicans* species.

3.1. Antifungal susceptibility testing

Antifungal susceptibility tests were performed on a total of 100 yeasts. Of these, (n=87/87%) showed resistance to at least one antifungal tested, representing a high percentage of microorganisms. Regarding the classes of antifungals tested, (n=13/13%) of the yeasts were resistant to one class of antifungals, with the vast majority being resistant to caspofungin. Resistance of yeasts to two classes of antifungals was observed in (n=26/26%), with some isolates exhibiting combined resistance to both azoles and echinocandins (Table 2). The resistance for three classes was observed in (n=45/45%). The majority from all azoles class, but also combined azole resistance with echinocandins, and one yeast

from the *non-albicans Candida* spp. group showed resistance to azoles class and amphotericin B, which was the antifungal that showed the greatest efficacy. One isolate presented resistance to four antifungal agents that combined azoles and echinocandins classes.

A total of 59 *C. albicans* (n=47/79.7%) were resistant to fluconazole and voriconazole. While the *non-albicans Candida* spp. group (n=27/65.9%) were also resistant to fluconazole and voriconazole (Figure 1).

3.2. Detection of antifungal resistance genes

The *ERG11* gene was identified in 17 *C. albicans* isolates, 14 *C. tropicalis* , 5 *C. parapsilosis* and 2 *N. glabratus*, being the most frequent gene, representing 28.8% and 51.2% of the *C. albicans* and *non-albicans* groups, respectively. The *CDR2* gene was the second most prevalent: *C. albicans* (n=11), *C. tropicalis*(n=4), *C. parapsilosis* (n=1) and *N. glabratus* (n=1), present in 18.6% and 14.6% of the *C. albicans* and non-albicans groups, respectively. *ERG2* and *FSK1* genes were not found in any of the isolates in this study (Table 1).

The *ERG11* (n=64) was the most prevalent gene found associated with phenotypic resistant isolates to azole derivatives, such as *MDR1* (n=16), *CDR1* (n=22), and *CDR2* (n=41). It wasn't identified *FSK1* or *ERG2,* however, it was found that *ERG11* (n=8), *MDR1* (n=1), *CDR1* (n=4), and *CDR2* (n=3) were associated with caspofungin resistant isolates, and both *ERG11* and *CDR2* associated with the amphotericin B resistant isolate (n=1) that also presented resistance to azole derivatives in a community patient. The mortality rate was observed in 100% of cases for *C. albicans* with multiple resistance, while species such as *N. glabrata* and *P. kudriavzevii* also showed isolated resistance with 100% mortality. Furthermore, in some resistant isolates, no mutations were detected in the genes evaluated (Figure 2).

Regarding the resistant strains, the detection of *ERG11, MDR1, CDR1, CDR2, ERG2*, and *FSK1* was generally low for most antifungals tested (ITR, FLU, VOR, CAS, ANB). Except for *ERG11*, which was highly identified in resistant strains, particularly with VOR and CAS, such as *MDR1* and *CDR1* which also was most found in those antifungals. For susceptible strains, *ERG11* was consistently found, while *MDR1, CDR1, CDR2, ERG2*, and *FSK1* were low or absent across all antifungal treatments (Figure 3).

3.3. Assessment of the efflux pump activity

The capacity of the clinical isolates to efflux EtBr was ranked relative to their reference strains by calculating their index for efflux activity (Table 2). The results showed in the *C. albicans* group that the majority (n=10) followed the expected MC_{EtBr} (0.5 µg/µl) from the ATCC, however, 14 isolates had presented a bigger MC_{EtBr} than the reference, such as 1.0µg/µl (n=10) and 1.5µg/µl (n=4), with an index range from 1 to 2, respectively.

In the *C. non-albicans* group, it was observed in all ATCC isolates an MC_{EtBr} of 0.5 µg/µl, and most isolates also presented the same MC_{EtBr} as their respective reference species. With alteration from 6 isolates of *C. tropicalis*, 3 of *C. parapsilosis*, and one each of *M. guilliermondii* (syn. *C. guilliermondii*) and *P. kudriavzevii* (syn. *C. krusei*), as they all showed a bigger MC_{EtBr} than the reference of 1.0µg/µl, with an index of 1.

An analysis between the efflux pump results and the molecular markers for overexpression of the efflux pump showed more prevalence of those molecular markers in the minimum inhibitory concentration of ethidium bromide (MIC_{EtBr}) for positivity of the efflux pump. As in isolates with MIC_{EtBr} 0.5 for positivity of efflux pump activity n=16 presented *CDR2*, n=7 *CDR1,* and n=6 for *MDR1, with* a statistical difference (0.0537) between the number of positive genes compared to the different concentrations of EtBr (Table 3).

3.4. Assessment of biocide tolerance

The biocide tolerance determination test using the disk diffusion method revealed that the most effective compound was sodium hypochlorite, especially at 2% when compared to hydrogen peroxide at 4.25% (diluted 1:16) and benzalkonium chloride (Table 4).

A significant difference between the clinical isolates and the reference strain for each group was observed. The clinical isolates had a significantly smaller mean halo diameter than the reference strain, indicating greater resistance to the compounds than the ATCC sample (Table 4).

Candida albicans ATCC strains exhibited larger inhibition zones compared to clinical isolates of *C. albicans* and non-*Candida albicans* species. Sodium hypochlorite demonstrated the highest antifungal activity, with inhibition zones increasing in size as the concentration increased (1%, 1.5%, 2%), particularly in *C. albicans* ATCC strains. In contrast, hydrogen peroxide (4.25%; 1:16 dilution) and benzalkonium chloride (5%) showed no measurable inhibition zones, indicating resistance across all tested groups (Figure 4).

4. Discussion

Antifungal resistance in *Candida* species represents a significant challenge in clinical settings, particularly considering nosocomial infections, where ineffective treatments can lead to poor patient outcomes. This underscores the need to continuously monitor resistance patterns for guiding therapeutic decisions and improving infection management in hospital environments.

Currently, the clinical arsenal of antifungals against *Candida* spp. has different mechanisms of action. Azole derivatives such as itraconazole, fluconazole, voriconazole, posaconazole, and isavuconazole act as inhibitors of ergosterol biosynthesis at the plasma membrane level, reducing membrane flexibility, permeability, and the function of membrane enzymes, leading to inhibition of the yeast growth [25]. Polyene derivatives, such as amphotericin B (AmB), act by extracting ergosterol from the fungal cell membrane and forming pores, altering the membrane permeability. These drugs have fungistatic and fungicidal activity, depending on the antifungal concentration [26]. Echinocandins, such as caspofungin, micafungin, and anidulafungin, act as inhibitors of fungal cell wall synthesis, blocking the biosynthesis of (1,3)-β-D-glucan, leading to osmotic imbalance and, ultimately, cell death [25, 27].

However, some species have intrinsic resistance, which possesses inherent resistance to specific genetically linked antifungals. For example the intrinsic resistance of *P. kudriavzevii* (syn. *C. krusei*) to fluconazole, due to the low affinity of the drug to the target enzyme, lanosterol 14-α-demethylase (encoded by the *ERG11* gene), besides the efflux pump activity [28]

Other fungi can also select resistant strains through exposure to antifungal agents, which can be genetic or epigenetic. This acquired resistance can occur via various stress factors, such as the patient's underlying condition(s), reduced drug bioavailability, increased drug metabolism, and impaired immune function, leading to changes in the fungal genome [28, 30]. Some acquired

mechanisms described are overexpression in the *ERG11* gene or mutations in *ERG* genes involved in the ergosterol biosynthesis pathway, overexpression in ABC protein transporters coding genes (*CDR1, CDR2,* and *MDR1*), mutations in *FSK* genes involved in the biosynthesis of (1,3)-β-D-glucan, and biofilm formation [30, 31].

The AFST plays a crucial role in assertive therapeutic decisions, and for improving patient outcomes. The Clinical & Laboratory Standards Institute (CLSI) provides standardized guidelines for interpreting susceptibility via the Disk Diffusion Test (DDT), which is a cost-effective and quick alternative when compared to the microdilution broth method (BMD) for clinical use [32, 33]. Jeon S et al. (2021) validated the DDT method (CLSI M44-ED1 or ED2) against the BMD reference method, reporting a high categorical agreement (96.3%), confirming DDT's reliability in clinical settings. This allows for a quicker antifungal susceptibility profile, enabling clinicians to choose the best antifungal treatment and improving patient prognosis.

The analysis revealed a concerning level of antifungal resistance among 100 yeast isolates, with a significant 87% resistant strains. Among *C. albicans* isolates, 79.7% were resistant to fluconazole and voriconazole. Similarly, the *non-albicans Candida* species group also showed a high rate of resistance (65.9%) to the same antifungal agents. These findings show higher rates of phenotypic antifungal resistance compared to the data available [35, 36, 37].

A review from the SENTRY Antifungal Surveillance Program analyzed 1,846 fungal isolates from 31 countries and reported echinocandin resistance in *Candida* spp. ranging from 0.0% to 2.8%. Fluconazole resistance was observed in 11.9% of *C. glabrata* and 11.6% of *C. tropicalis* [35]. Similarly, a study from Mainland China (2011–2021) evaluating 44,716 *Candida* isolates showed higher fluconazole susceptibility in *C. albicans* (91.6%), compared to *C. tropicalis* (77.95%) and *C. glabrata* (79.4%). However, *C. parapsilosis* had the highest susceptibility (93.25%). Amphotericin B and anidulafungin were the most effective antifungals. Azole resistance was associated with mutations in genes like *ERG11*, *PDR1*, and *MRR1*, while mutations in *FKS1* and *FKS2* contributed to echinocandin resistance in *C. auris* and *C. glabrata* [36].

In South America, a study conducted in a fourth-level hospital in Colombia highlighted 13.8% fluconazole resistance in *C. parapsilosis* and 5.9% dual resistance to fluconazole and voriconazole in *C. tropicalis*. Among 18 *C. glabrata* isolates, 5.5% were resistant to caspofungin, and 11.1% were resistant to fluconazole, with another 5.5% showing dose-dependent susceptibility (SDD) to fluconazole [37].

Even though most of the resistant strains in this study were *C. albicans*, the findings of antifungal resistance among *non-albicans* species (n=37) represented 91.9% of the population stand out. This is corroborated by the findings in the literature and highlights the importance of the emergence and influence of the *Candida non-albicans* species in the clinical scenario [35, 36, 37].

A synergy between this study and the literature data is the predominance of resistance to azole derivatives, considered first-line drugs. Itraconazole has efficacy against various fungal pathogens, particularly those causing systemic infections. Fluconazole's good bioavailability is often preferred for its safety profile and effectiveness against *Candida* species. Voriconazole has a broader spectrum of activity between them, making it suitable for treating various fungal infections [38]. This should emphasize the need for continuous research for associated or alternative treatments, and other strategies to prevent acquired resistance to these drugs.

A strategy that may help in our advance against antifungal resistance is the understanding of the genetic mutations involved in the acquired resistance. In literature are described as molecular mechanisms associated with antifungal resistance, mutations in the ergosterol biosynthesis pathway (e.g., *ERG11*), and overexpression of efflux pumps, through transporter genes such as *CDR1, CDR2*, and *MDR1*, promoting azole resistance. And also mutations in cell wall biosynthesis genes (*FSK1, FSK2,* and *PDR1*) confer resistance to echinocandins [39]. Azole resistance was associated with

mutations in genes like *ERG11*, *PDR1*, and *MRR1*, while mutations in *FKS1* and *FKS2* contributed to echinocandin resistance in *C. auris* and *C. glabrata* [36].

Our results revealed that resistance to multiple antifungals (ITR/FLU/VOR/CAS) was associated with genetic mutations in *ERG11, CDR1/CDR2,* and, *C. tropicalis* showing the highest prevalence of these alterations. Furthermore, the frequency of resistance findings in *C. tropicalis* indicates that this species may be an important target for epidemiological surveillance and control strategies.

In addition, the mortality rate was high, with 100% in cases of *C. albicans* resistant to ITR/FLU/VOR/CAS and species such as *C. tropicalis*, *N. glabrata,* and *P. kudriavzevii*. The high mortality associated with multiple resistance highlights the need for specific therapeutic approaches, especially in cases involving genetic mutations in pathways such as *ERG11* and efflux pumps (*CDR1, CDR2, MDR1*).

A study identified nonsynonymous mutations in the *ERG11* gene as a predominant mechanism of azole resistance, followed by overexpression of *CDR1* and *CDR2* to be associated with more azole resistance. It also highlighted the cross-resistance with other azole-derivates indicating that treatment options may be limited for patients infected with resistant strains [40].

In addition, our study showed 100% of *N. glabratus* and *P. kudriavzevii* phenotypically resistant to fluconazole, which already was described as *N. glabratus*, *P. kudriavzevii* and *C. auris* being fluconazole-resistant [39]. It shows an important matter of how different *Candida* species exhibit unique molecular mechanisms of antifungal resistance. As *N. glabrata*, *C. krusei*, and *C. auris* are noted for their high resistance to fluconazole, highlighting the need for tailored treatment strategies based on species identification [39]. Some isolates, although resistant, did not present the genes evaluated, suggesting the existence of other resistance mechanisms. The presence of resistant isolates without known mutations suggests the need for additional studies to identify new resistance mechanisms.

Alarmingly, one *C. tropicalis* isolate showed resistance to four antifungal agents, combining azoles, echinocandins, and amphotericin B in a community patient, associated with mutations in *ERG11* and *CDR2*. Even though amphotericin b molecular markers have been described for *N. glabratus* associated with mutations in *ERG2*, that lead to an alternative ergosterol biosynthesis pathway [41], *C. tropicalis* was described as presenting biofilm formation that leads to phenotypic resistance to this antifungal agent [42]. According to these results, the phenotypic amphotericin b resistance could be involved with other resistance mechanisms that were not explored in this study that could be elucidated with further genomic analysis, or even the role of the efflux pump and its capacity to export and neutralize the action of this antifungal agent.

The efflux pump is one of the many mechanisms that the *Candida* genus presents, which guarantees the ability to export compounds, such as antifungal agents and biocidal compounds, from the inside of the cell. The expression of this mechanism is regulated by several genes such as *CDR2*, *CDR1*, and *MDR1*, which coordinate the synthesis of ABC transport proteins, acting on the cell membrane by exporting azole substances [43].

Concerning efflux pumps, the calculated index provided a mean of ranking each isolate relative to its reference species [24]. From the calculated index we were able to observe a total of 21 isolates that presented an index equal to 1, and 4 *C. albicans* isolates within an index of 2. In the Salama et al. (2024) study, they investigated the efflux pump activity in *Klebsiella pneumoniae* by assessing using the Cartwheel method with varying concentrations of ethidium bromide (EtBr). It was found an index range of 0.5 to 1.5 (n=7), with overly expressed efflux pump genes in 1.5 index-ranked strains also linked to higher minimum inhibitory concentration (MIC) of antibiotics.

This suggests that when the isolates have higher efflux activity than the reference strain, it might be more efficient at expelling substrates, which could contribute to higher resistance to certain antifungal agents and also more tolerance to biocide compounds. However, the efflux activity can be

influenced by various factors, including the presence of efflux pump inhibitors, environmental conditions, and genetic variations among strains. It's important to consider these factors when interpreting the index [45].

A study evaluated the phenotypic expression of efflux pump, using the Castelo-Branco et al. (2013) method, with promethazine and haloperidol, which resulted in the reversion of resistance to itraconazole and fluconazole in all *Candida* isolates tested. These compounds act to inhibit the activity of the efflux pump, resulting in the reversal of azole resistance, suggesting that azole resistance among *Candida* spp. is related to the enhanced activity of these pumps [47].

The efflux pump results and the molecular markers for overexpression of the efflux pump showed a significantly bigger frequency of those molecular markers in the minimum inhibitory concentration of ethidium bromide (MIC_{EtBr}) for positivity of the efflux pump. Studies have already described how overexpression of these genes can guarantee resistance to antifungals. It was described that homozygous alleles (A/A) in the *MDR* promoter confer higher levels of *CDR2* and *MDR1* expression and resistance to fluconazole in *C. albicans* [48]. In *C. glabrata* isolates, overexpression in *CDR1* and *CDR2* gained a functional mutation, which resulted in decreased intracellular accumulation of azole derivatives [49]. However, regarding the validation of the phenotypic expression of this resistance mechanism, studies are still lacking to elucidate the phenomenon and its clinical implications.

Opportunistic fungi such as *Candida* spp. present unique challenges due to their lack of seasonality and demographic preferences, making surveillance and prediction more difficult compared to other emerging fungal pathogens [50]. Their ability to adapt to diverse environmental and host conditions further complicates management and emphasizes the need for robust monitoring and tailored infection control strategies in healthcare settings. Conversely, while the focus is often on the challenges posed by opportunistic fungi, advancements in genomic testing and automated surveillance methods, such as Vitek 2 and MALDI-TOF, may enhance outbreak detection and management, potentially mitigating some of these issues [51].

Besides that, nosocomial infections, particularly those caused by opportunistic fungi, have profound health, economic, and social impacts. These infections often affect vulnerable patient populations, leading to prolonged hospital stays, increased morbidity and mortality, and higher healthcare costs [52].

Our population sample highlights the significant burden of *Candida* infections in hospitalized patients. *C. albicans* accounted for a majority (60%) of infections, with an average hospital stay of 39.5 days, longer than the 31.5 days observed for non-*C. albicans* species. The frequent use of invasive medical devices in both groups (60 patients with *C. albicans* and 29 with non-*C. albicans*) underscores the association between device use and infection risk. Additionally, the mortality rate was higher in *C. albicans* infections (28 deaths) compared to non-*C. albicans* (16 deaths), reflecting the potentially more severe clinical outcomes of *C. albicans*.

Hospitalization for *Candida* infections significantly burdens healthcare systems and patients, with prolonged stays averaging over 30 days and high costs associated with antifungal therapies and advanced interventions [53]. The burden is most exacerbated by multidrug-resistant microorganisms [54]. An early adequate antifungal therapy based on local epidemiology can reduce candidemia-attributable mortality and the length of hospitalization [55]. Effective management of invasive candidiasis, including timely initiation of antifungal therapy and appropriate risk assessment, can significantly reduce the length of hospital stays by decreasing morbidity and mortality associated with delayed treatment in critically ill patients [56]. Effective biocides to prevent *Candida* transmission are crucial to decrease some of these consequences. Investing in prevention, including research into biocide compatibility and fungal resistance mechanisms, is essential to improve those outcomes.

An alarming data was the absence of inhibition halos for the compounds hydrogen peroxide 4.25% and benzalkonium chloride 5%, both for reference ATCCs and for clinical isolates. As

recommended by the Brazilian Health Regulatory Agency (Anvisa), they are officially indicated for the decontamination of fixed surfaces, including the nutrition and neonatology environment [57]. This shows the inadequacy of the use of these compounds for surface decontamination and the influence on the 89 individuals of our population with the use of invasive medical devices.

The biocide tolerance test showed a statistical difference when comparing the means of the inhibition halos for the sodium hypochlorite compounds between the group of clinical isolates and ATCCs, both between the *C. albicans* and non-albicans species. Larger halos were observed among the ATCCs, indicating greater susceptibility, while the clinical isolates showed lower inhibition halo compared to the reference strains. Significantly the clinical isolates showed a higher resistance to biocidal compounds than the reference ATCCs, however, sodium hypochlorite continues to be the most effective among the studied compounds. The recommended by center of disease and control (CDC) for disinfection in household bleach of 5.25%–6.15% sodium hypochlorite, or 52,500–61,500 ppm available chlorine, wich is nearly provided by a 1:1,000 dilution, but studies have confirmed effectiveness for *Candida* at 30 seconds exposure to 500ppm of chlorine [58, 59].

Moore et al. (2017) used a quantitative suspension test method of European standard (EN 13624:2013) and showed that 0.1% chlorine was effective in its fungicidal action against *C. auris* and *C. albicans*, for clean and dirty conditions when the contact time was 5 minutes. The fungicidal action of chlorine-based benzalkonium chloride was also demonstrated for *C. glabrata, C. albicans,* and *C. auris*, using the disk diffusion method (ASTM E-2197-02). However, quaternary ammonium-based disinfectants were significantly less effective against *Candida* spp. species, which corroborates our findings [60, 61]. A study assessed the susceptibility of clinical *C. auris* strains to various biocides, including chlorine, chlorhexidine, and benzalkonium chloride. Resistance was observed with benzalkonium chloride at concentrations of 16–32 mg/L [62]

In hospital environments, it is impractical to rely on a single biocide to eliminate all microorganisms effectively. Variability in microbial tolerance necessitates monitoring the efficacy of biocides used at specific concentrations. For instance, at the same hospital, it was conducted a study by Dias et al. (2017) on *Pseudomonas aeruginosa* and *Acinetobacter,* clinical isolates found that carbapenem-resistant strains exhibited greater tolerance to 2% sodium hypochlorite and 5% benzalkonium chloride compared to susceptible strains. This highlights that biocides effective against one pathogen may be less so against others sharing the same hospital environment. Such findings underline the importance of routine surveillance by large centers to assess biocide tolerance patterns, ensuring tailored and effective infection control strategies.

Investing in research to uncover compounds with broad-spectrum antimicrobial action is critical for enhancing infection control strategies in healthcare settings. This is particularly vital given the challenge posed by the intrinsic efflux pump mechanisms in the *Candida* genus, which can contribute to biocide tolerance, as was already observed in bacteria strains [64, 65].

The capacity of efflux pumps actively expel biocidal compounds from fungal cells, reducing their efficacy and complicating the management of these pathogens [66]. Understanding these resistance mechanisms is essential for developing biocides capable of overcoming efflux-mediated tolerance, ensuring effective decontamination, preventing the spread of resilient fungal strains within hospital environments and decreasing health, social, and economic impacts caused by prolonged hospitalization. Enhanced research efforts can lead to innovative solutions, bolstering both prevention and treatment approaches against opportunistic pathogens.

5. Conclusion

In conclusion, this study highlights the significant challenges posed by antifungal resistance among *Candida* species in clinical settings. The findings reveal high rates of resistance, particularly to azoles and echinocandins, emphasizing the critical need for effective diagnostic tools and targeted

therapeutic strategies. The study also underscores the role of other resistance mechanisms, such as efflux pumps, in complicating the management of infections and biocide efficacy. These results advocate for ongoing surveillance of antifungal susceptibility, investments in research to develop broad-spectrum and effective biocidal agents, and enhanced infection prevention measures.

6. Article highlights

- The study reveals a high prevalence (87%) of antifungal resistance among clinical isolates of Candida to at least one antifungal agent. Notably, 47.12% exhibited multiple resistance to three azole derivatives, indicating a serious challenge in treatment options;
- The study highlights high mortality rates associated with resistant strains, particularly in cases of C. albicans resistant to multiple antifungals. The mortality rate reached 100% for certain resistant strains, underscoring the urgency for targeted therapeutic approaches;
- Assessment of Biocide Tolerance using disk diffusion technique into those clinical isolates, evaluated that sodium hypochlorite was the most effective biocide, particularly at a concentration of 2%, while hydrogen peroxide and benzalkonium chloride showed no measurable inhibition zones, indicating resistance across all tested groups;
- The clinical isolates exhibited significantly smaller mean inhibition zone diameters compared to reference strains, suggesting that the clinical isolates had greater tolerance to the biocides tested;
- The research identifies overexpression of efflux pump genes (MDR1, CDR1, and CDR2) as significant contributors to resistance in clinical isolates of Candida spp. This multifactorial resistance mechanism complicates treatment and highlights the importance of targeting these pathways in future therapeutic strategies;
- The intrinsic efflux pump mechanisms in Candida species, can also contribute to biocide tolerance. These pumps actively expel biocidal compounds from fungal cells, reducing their efficacy and complicating the management of these pathogens;

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The authors have no competing interests or relevant affiliations with any organization or entity related to the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Author´s contribuitions

Study design: all authors

Data collection: all authors

Data analysis: all authors

Manuscript writing: all authors

Manuscript revision: all authors

Study supervision: all authors

Declaration: Declare that "the database that originated the article is available in an open repository (Tabela Resultados Mestrado .xlsx) or upon request, with a corresponding author".

Figure 1. Antifungal Susceptibility Profile of *Candida* spp. Isolates (n=100)

ITR- Itraconazole; FLU- Fluconazole; VOR- Voriconazole; CAS- Caspofungin; ANB-Amphotericin b.

Figure 2. Heat map the pattern of distribution of resistance genes according to the antifungal susceptibility testing (AFST) results of the resistant isolates.

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Legend: ITR- Itraconazole; FLU- Fluconazole; VOR- Voriconazole; CAS- Caspofungin; ANB-Amphotericin b; AFST- Antifungal susceptibility testing.

Figure 3. Comparison of molecular marker findings in Resistant vs. Susceptible strains across different antifungal agents.

Legend: (A) Distribution of the number of positive genes for molecular markers (*ERG11, MDR1, CDR1, CDR2, ERG2* and *FSK1*) in resistant strains treated with various antifungal agents (ITR: Itraconazole, FLU: Fluconazole, VOR: Voriconazole, CAS: Caspofungin, ANB: Amphotericin b).
(B) Distribution of the number of positive genes for molecular markers in susceptible strains under the same conditions. Each marker is represented by its respective symbols.

Figure 4. Dispersion of inhibition halos (mm) exhibited by *Candida* spp isolates against the biocides sodium hypochlorite at 1%, 1.5%, and 2%, hydrogen peroxide at 4.25% (1:16), and benzalkonium chloride at 5%.

Table 1. Phenotypic and genotypic antifungal resistance profile of clinical *Candida* spp. isolates (n=87)

Phenotypic Antifungal resistance profile	Genotypic Antifungal Resistance Profile	Species (n)	Clinical outcome Death
ITR/FLU/VOR/CAS (n=1)	*ERG11;CDR1*	*C. albicans* (n=1)	n=1 (*100%*)
ITR/FLU/VOR (n=41)	*ERG11;CDR1*	*C. tropicalis* (n=1)	*n=18* (43.%)
	ERG11;MDR1	*C. tropicalis* (n=2)	
	ERG11	*C. albicans* (n=2); *C. tropicalis* (n=4)	
	MDR1	*C. tropicalis* (n=2)	
	CDR1;CDR2	*C. albicans* (n=1); *C. tropicalis* (n=1)	
	CDR2	*C. albicans* (n=7); *C. tropicalis* (n=1)	
	None	*C. albicans* (n=17); *C. tropicalis* (n=3)	
ITR/CAS (n=3)	*CDR2*	*C. tropicalis* (n=1)	*n=1* (33.33%)
	ERG11	*C. parapsilosis* (n=1)	
	None	*C. parapsilosis* (n=1)	
FLU/VOR/CAS (n=3)	*ERG11;CDR1;MDR1*	*C. tropicalis* (n=1)	*n=1* (33.33%)
	ERG11;CDR1	*C. tropicalis* (n=1)	
	None	*C. albicans* (n=1)	
FLU/VOR (n=22)	*ERG11*	*C. albicans* (n=10); *N. glabratus* (n=1); *C. tropicalis* (n=1)	*n=10* (45.45%)
	None	*C. albicans* (n=8); *M. guilliermondii* (n=1); *C. tropicalis* (n=1)	
FLU/VOR/ANB (n=1)	*ERG11;CDR2*	*C. tropicalis* (n=1)	*Na*
FLU (n=1)	*ERG11;CDR2*	*N. glabratus* (n=1)	n=1 (100%)
FLU/CAS (n=1)	None	*P. kudriavzevii* (n=1)	n=1 (100%)
CAS (n=12)	*ERG11*	*C. parapsilosis* (n=4)	n=2 (16.67%)
	CDR1;CDR2	*C. albicans* (n=1)	
	CDR2	*C. parapsilosis* (n=1)	
	None	*C. parapsilosis* (n=4); *C. albicans* (n=1); *C. tropicalis* (n=1)	
ITR (n=2)	*ERG11;MDR1*	*C. tropicalis* (n=1)	n=2 (100%)
	ERG11	*C. tropicalis* (n=1)	

Legend: ITR- Itraconazole; VOR-Voriconazole; FLU- FLuconazole; CAS- Caspofungin; ANB-Amphotericin B.

Species	Isolates	MC_{EtBr} ($\mu g/\mu l$)	Index*
Candida albicans			

Table 2. Expression of efflux pump activity among clinical isolates of *Candida* spp.

	ATCC14053	0.5	
	Clinical isolates (n=45)	0.5	0
	Clinical isolates (n=10)	1.0	1
	Clinical isolates (n=4)	1.5	2
Candida non-albicans			
	ATCC66029 *Candida tropicalis*	0.5	
	Clinical isolates *C. tropicalis* (n=19)	0.5	0
	Clinical isolates *C. tropicalis* (n=6)	1.0	1
	ATCC22019 *Candida parapsilosis*	0.5	
	Clinical isolates *C. parapsilosis* (n=9)	0.5	0
	Clinical isolates *C. parapsilosis* (n=3)	1.0	1
	ATCC2001 *Nakaseomyces glabratus (syn. Candida glabrata)*	0.5	
	Clinical isolates *N. glabratus (syn. C. glabrata)* (n=2)	0.5	0
	Clinical isolates *M. guilliermondii (syn. C. guilliermondii)* (n=1)	1.0	1
	Clinical isolates *P. kudriavzevii (syn. C. krusei)* (n=1)	1.0	1

[a]The index (defined in the Materials and Methods section) provided the range of efflux activity that can be obtained for each strain in comparison with the reference strain.

Table 3. Correlation between genotypic and phenotypic profiles associated with efflux pump overexpression in *Candida* spp. strains.

Molecular marker's	MC_{EtBr} 0.5 (n=79)	MC_{EtBr} 1.0 (n=21)	MC_{EtBr} 1.5 (n=4)	***p-value***
***MDR1* (n=8)**	6	2	0	Row factor 0.5533
***CDR1* (n=9)**	7	1	1	Column factor 0.0537
***CDR2* (n=17)**	16	0	1	

MIC_{EtBr} -minimum inhibitory concentration of ethidium bromide for positivity of efflux pump.

Table 4. Biocide tolerance pattern of *Candida* spp (n=100)

Isolates	Sodium hypochlorite 1%	Sodium hypochlorite 1.5%	Sodium hypochlorite 2%	Hydrogen peroxide 4.25% (1:16)	Benzalkonium chloride 5%	*p*-value
	Mean ± standard deviation					
	Inhibition halo diameter (mm)					
ATCC *Candida albicans*	22.00 ± 2.00	29.50 ± 5.50	34.0 ± 4.00	6.00 ± 0.00	6.00 ± 0.00	**0.041**
Clinical isolates *Candida albicans*	12.40 ± 2.74	15.06 ± 3.61	18.25 ± 4.40	6.00 ± 0.00	6.00 ± 0.00	
ATCC'S *Candida non-albicans*	18.33 ± 1.53	23.33 ± 4.62	28.33 ± 5.77	6.00 ± 0.00	6.00 ± 0.00	**0.037**
Clinical isolates *Candida non-albicans*	11.73 ± 1.88	14.38 ± 3.12	17.66 ± 3.73	6.00 ± 0.00	6.00 ± 0.00	

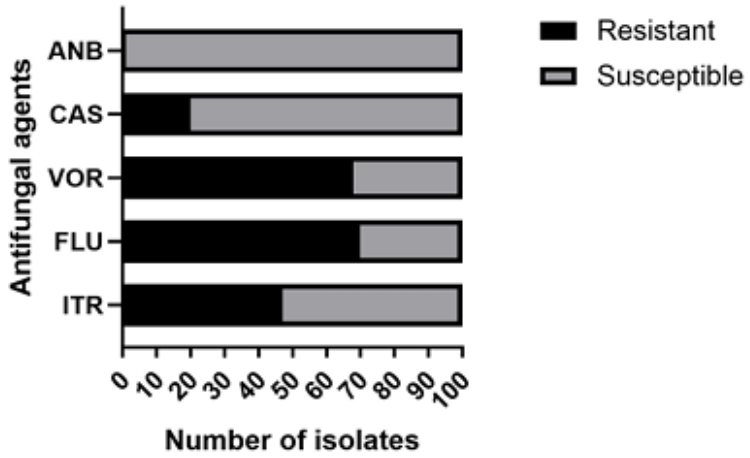

Figure 1

298x188mm (118 x 118 DPI)

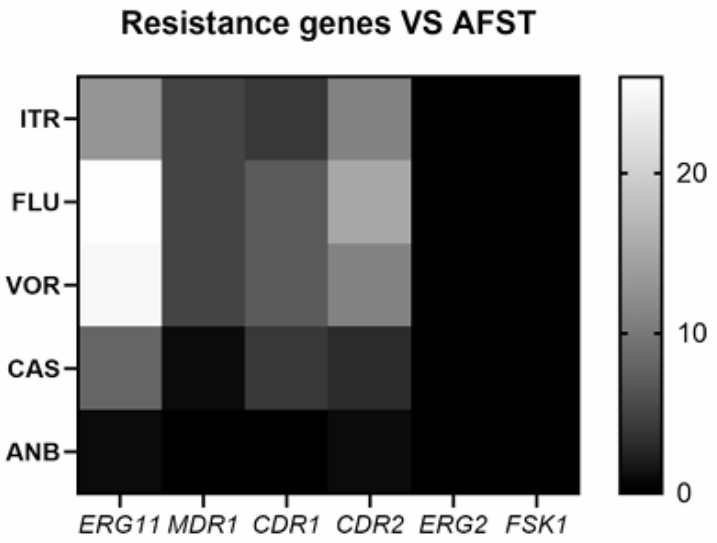

Figure 2

288x224mm (118 x 118 DPI)

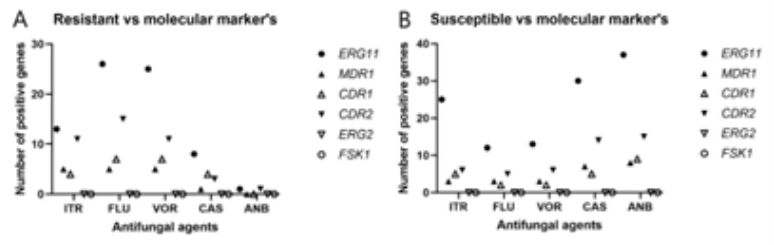

Figure 3

1034x335mm (38 x 38 DPI)

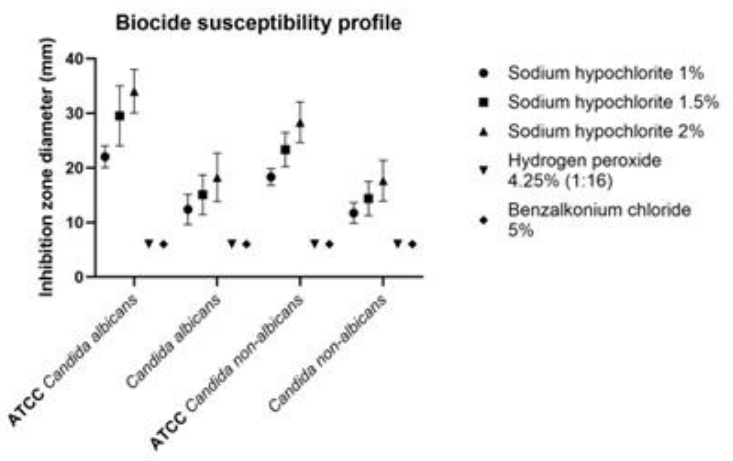

Figure 4

440x278mm (118 x 118 DPI)

9.5 Submitted article- Respiratory Syncytial Virus in Early Childhood: Post COVID-19 Case Re-emergence

Jornal de Pediatria

Respiratory Syncytial Virus in Early Childhood: Post-COVID-19 Case Re-emergence

--Manuscript Draft--

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Article Type:	Original article
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Abstract:	Objective This study investigates the frequency and clinical impact of Respiratory Syncytial Virus (RSV) among pediatric patients in Brazil, focusing on the post-pandemic context and its implications for epidemiological surveillance. Methods We conducted a descriptive, cross-sectional analysis of electronic medical records from March to July 2024, involving 36 children under 8 years of age examined for RSV at a public health service in Brazil. RSV, SARS-CoV-2, and Influenza A/B were tested using RT-qPCR in respiratory samples. Epidemiological aspects of the patients, clinical symptoms, hospitalization, and viral load, correlating these data with clinical outcomes. Results Of the 104 examined children, 36 (34.6%) tested positive for RSV. The majority (77.8%) were infants under 1 year old. Common symptoms included cough, fever, and dyspnea, with 72.2% requiring hospitalization. Three patients had comorbidities, and 14 were admitted to the ICU. Hospitalized patients had a lower mean Cycle Threshold (CT) value (26.6) compared to non-hospitalized individuals (28.1), indicating higher viral load. Conclusions RSV continues to be a significant respiratory pathogen, particularly affecting infants. Our findings highlight hospitalizations and severe cases related to RSV in the post-pandemic period, with co-circulation of SARS-CoV-2, its variants, and subvariants. Enhanced epidemiological surveillance, early diagnosis, and preventive measures are essential for effectively managing RSV and reducing its impact on vulnerable populations. Support for scientific research, alongside public health initiatives, is crucial for improving outcomes and addressing the challenges posed by RSV.
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Respiratory Syncytial Virus in Early Childhood: Post-COVID-19 Case Re-emergence

Abstract

Objective: This study investigates the frequency and clinical impact of Respiratory Syncytial Virus (RSV) among pediatric patients in Brazil, focusing on the post-pandemic context and its implications for epidemiological surveillance.

Methods: We conducted a descriptive, cross-sectional analysis of electronic medical records from March to July 2024, involving 36 children under 8 years of age examined for RSV at a public health service in Brazil. RSV, SARS-CoV-2, and Influenza A/B were tested using RT-qPCR in respiratory samples. Epidemiological aspects of the patients, clinical symptoms, hospitalization, and viral load, correlating these data with clinical outcomes.

Results: Of the 104 examined children, 36 (34.6%) tested positive for RSV. The majority (77.8%) were infants under 1 year old. Common symptoms included cough, fever, and dyspnea, with 72.2% requiring hospitalization. Three patients had comorbidities, and 14 were admitted to the ICU. Hospitalized patients had a lower mean Cycle Threshold (CT) value (26.6) compared to non-hospitalized individuals (28.1), indicating higher viral load.

Conclusions: RSV continues to be a significant respiratory pathogen, particularly affecting infants. Our findings highlight hospitalizations and severe cases related to RSV in the post-pandemic period, with co-circulation of SARS-CoV-2, its variants, and subvariants. Enhanced epidemiological surveillance, early diagnosis, and preventive measures are essential for effectively managing RSV and reducing its impact on vulnerable populations. Support for scientific research, alongside public health initiatives, is crucial for improving outcomes and addressing the challenges posed by RSV.

Keywords: Respiratory Syncytial Virus (RSV); Pediatric Infections; Viral Load; Hospitalization; RT-qPCR

INTRODUCTION

In 1956, it was identified a highly contagious microorganism from a chimpanzee's nasal secretion [1]. Later, it was found to cause infections in humans, primarily in children, and was named respiratory syncytial virus (RSV) [2]. RSV belongs to the genus Orthopneumovirus within the family Pneumoviridae [3]. The virus exhibits pleomorphic morphology, existing in the forms filamentous (50 nm in diameter) and spherical (150-250 nm in diameter). It's a single-stranded negative-sense RNA, enveloped, that replicates intracellularly and spreads by apical budding [4].

RSV transmission occurs through direct contact with respiratory droplets expelled by an infected person when coughing, sneezing, or talking, or indirect transmission may occur through contact with contaminated surfaces and objects, on which the virus can survive for several hours [5].

The interaction between the host and the pathogen elicits a robust immune response, which can often result in collateral damage that exceeds the harm caused by the virus itself. This immune response can lead to excessive mucus production and subsequent airway obstruction, exacerbating the clinical manifestations of the infection [6]. Symptoms usually appear within 4 to 6 days of transmission and include runny nose, nasal congestion, cough, fever, sneezing, sore throat, headache, and decreased appetite [5].

RSV is considered the major etiologic agent of viral infections of the lower respiratory tract such as bronchiolitis and pneumonia in children under two years of age; however, mild and asymptomatic infections in infants are uncommon [3]. Besides that, the damage inflicted by RSV can render the airways more vulnerable to secondary bacterial infections.

Globally, RSV tends to cause seasonal outbreaks each year. In the Southern Hemisphere, infections are most frequent between March and June, while in the Northern Hemisphere, outbreaks typically occur between September and December, largely influenced by climatic conditions [7]. In northern regions, such as the United States and China, RSV generally starts circulating in the fall, peaks in December or January and continues through the spring. In southern regions like Brazil, RSV circulation varies by area, with peaks occurring between April and September, during the fall and winter months [8].

In children and infants showing consistent clinical symptoms, RSV infection should be suspected. In older individuals, suspicion arises particularly in cases of immunocompromise accompanied by acute lower respiratory tract illness [9]. For otherwise healthy newborns and young children with bronchiolitis, clinical suspicion is often sufficient, and virologic confirmation is not always necessary [10]. However, laboratory confirmation of RSV is important, as it influences clinical management decisions, such as the use of antiviral therapy, continuation of palivizumab prophylaxis, or infection control measures [11].

Therefore, in light of the aforementioned considerations, this study aims to describe re-emergence cases of respiratory syncytial virus (RSV) by correlating clinical and epidemiological parameters observed during

the fall/winter period. These data serve as a critical alert regarding the circulation of RSV in the post-COVID-19 pandemic context, thereby contributing to the epidemiological surveillance of this microorganism with an emphasis on human health.

MATERIALS AND METHODS

This descriptive, observational, cross-sectional study involved a retrospective analysis of electronic medical records from pediatric patients with acute respiratory syndrome (ARS), hospitalized and non-hospitalized, who were treated at a public health service. The study was conducted in Brazil, focusing on samples processed by a public laboratory specializing in molecular diagnosis of viral infections, during the period from March to July 2024. This laboratory attends around twenty-six cities, covering a population of 740,617 people.

Laboratory diagnostics were performed on clinical specimens, including nasopharyngeal swabs and tracheal aspirates. Reverse transcriptase-polymerase chain reaction (RT-qPCR) was used to detect SARS-CoV-2, Respiratory Syncytial Virus (RSV), and Influenza A/B. The testing was carried out using Thermo Fisher Scientific kits (Sweden), and analysis was performed on the Quantstudio 5 system (Thermo Fisher Scientific, Sweden) according to the manufacturer's instructions.

Characteristics, including gender, age, symptoms, hospitalization status, vaccination history, and comorbidities, were extracted from electronic medical records. The primary inclusion criteria for participant selection were age, specifically children in early childhood, ranging from 0 months to 8 years old, who tested positive for Respiratory Syncytial Virus (RSV). The cycle threshold (CT) values obtained from RT-qPCR were analyzed for mean, maximum, and minimum values, stratified by hospitalization status (hospitalized vs. non-hospitalized).

Figure 1. Experimental design of the study and inclusion criteria

The research was conducted in compliance with the approved protocol by the Research Ethics Committee of UFJF (CAAE 70133723.3.0000.5147).

RESULTS

Among 226 ARS-related examinations, 104 were conducted on early childhood individuals, of which 36 tested positives for RSV, yielding a positivity rate of 34.6%. Of the 36 RSV-positive cases, the majority were infants under 1-year-old to 0-6 months (55.5%) and 7-24 months (30.5%), followed by children aged

2 to 6 years (14.0%). Twenty (55.6%) of the affected individuals were male. The clinical specimens, include thirty-one nasopharyngeal swabs and five tracheal aspirates. The most common symptoms included cough, low oxygen saturation, fever, and respiratory discomfort. Notably, 72.2% (n=26) of these cases resulted in hospitalization, among them n=20 (76.9%) used ventilatory support (Figure 2).

Figure 2. Distribution of clinical symptoms among RSV-positive cases (n=36).

Three had underlying comorbidities: one with chronic lung disease and two with cardiovascular disease (Table 1). The study also identified 14 cases requiring intensive care unit (ICU) admission, including one 1-year-old with chronic lung disease and one 11-month-old with chronic cardiovascular disease.

Table 1. Clinical-epidemiological parameters from the positive cases of Respiratory Syncytial Virus.

Clinical and epidemiological parameters	Respiratory Syncytial Virus (n=36)
Hospitalization (n=36): n (%)	
Yes	26 (72.2)
No	10 (27.8)
Type of hospitalization (n=26): n (%)	
Yes- Intensive Care Unit	14 (53.8)
Yes- Infirmary	12 (46.2)
Use of ventilatory support (n=36): n (%)	
Yes	20 (55.6)
No	16 (44.4)
Type of ventilatory support (n=20): n (%)	
Non-invasive	16 (80.0)
Invasive	4 (20.0)

Days of Symptoms: n (%)		
	<5 days	5 (13.9)
	5-9 days	25 (69.4)
	10-20 days	5 (13.9)
	>30 days	1 (2.8)
Vaccine: n (%)		
	No	28 (77.8)
	Yes (Influenza and/or COVID-19)	8 (22.2)
Comorbidities: n (%)		
	No	33 (91.7)
	Chronic lung disease	1 (2.8)
	Chronic cardiovascular disease	2 (5.5)
Clinical specimen: n (%)		
	Nasopharyngeal swab	31 (86.1)
	Tracheal aspirate	5 (13.9)

The mean Cyclic Threshold (CT) value for hospitalized individuals was 26.6; with a standard deviation of 5.82, with a minimum of 15.7 and a maximum of 37.5, significantly lower when compared to non-hospitalized individuals, with a mean of 28.1; a standard deviation of 8.35, minimum of 18.2 and maximum of 43.1 (Table 2).

Table 2. The Cycle Threshold (CT) mean, minimum, and maximum according to the hospitalization data of individuals positive for Respiratory Syncytial Virus.

Hospitalization	CT means	Minimum	Maximum
Yes (n=26)	26.6 ± 5.82	15.7	37.5
No (n=10)	28.1 ± 8.35	18.2	43.1

DISCUSSION

As we discuss the epidemiological scenery of RSV globally, we could highlight an exchange in incidence reports and surveillance as we compare before and after the COVID-19 pandemic. That could also be extended to other ARS-correlated viral infections.

A study evaluated worldwide age-specific surveillance data for global epidemiology of RSV in hospitalized and community care cases from 15 countries, in which, in two decades from 2000-2020, only 1641 cases were reported. In it, they found 55% of the cases from children under 1 year of age [12]. In an official epidemiological report from the Secretary of Health and Environment Surveillance in Brazil, until August of 2024, they reported 1182 cases of ARS associated with diverse viral pathogens, of which 12% had been confirmed for RSV infections [13]. In our study, we assessed 226 SARS-related examinations, as 104 were of children in early childhood, of which 36 were positive for RSV (a positivity rate of 34.6%). This incidence is of high value for RSV epidemiology surveillance. This shows the underreporting of RSV infections before the pandemic and the impact of the COVID-19 pandemic on RSV surveillance.

Most of the clinical symptoms reported by RSV-infected individuals in this study were nonspecific, including cough, low oxygen saturation, fever, and respiratory discomfort. This finding underscores the importance of laboratory diagnosis, allowing the initiation of appropriate therapy and preventing the severity of the cases, decreasing the direct impact on hospital bed management. Laboratory diagnosis of respiratory viruses is of great importance for epidemiological databases and implementing public health policies. Furthermore, knowledge of the viral load allows for monitoring and predicting the prognosis of the infectious process caused by RSV [14].

In the present study, the test was performed using real-time RT-qPCR, a highly sensitive technique and accurate in diagnosing RSV infection. It assists in the detection of RSV RNA in various specimen types, whose sensitivity exceeds that of other methods such as rapid antigen detection tests and viral culture, making it the preferred diagnostic tool for adult RSV infection. Challenges with RT-qPCR, include selection of housekeeping genes, but these can be mitigated through standardization of protocols and gene research [15].

Fourteen cases (53.8%) of infants hospitalized in the ICU were identified in this research. Studies have shown that patients in the Neonatal Intensive Care Unit (NICU) with RSV infections may experience complications such as acute respiratory distress syndrome (ARDS) and septic shock, leading to increased mortality rates, emphasizing the importance of infection control measures [16].

We observed a frequency of 76.9% of the hospitalized patients who used ventilatory support. A comparison of hospital length of stay (LOS) and disease severity for RSV cases shows that younger children, especially those under six months, have longer hospital stays and higher severe outcome rates, including ICU admissions and ventilation needs [17, 18] Additionally, children with comorbidities experience significantly longer LOS than those without [19]. The length of hospital stay (LOS) directly

impacts hospitalization costs and the risk of acquiring healthcare-associated infections (HAIs), particularly those caused by multidrug-resistant organisms (MDROs). Extended LOS increases expenses due to the need for intensive care and costly treatments, with antimicrobial-resistant infections prolonging stays by an average of 18.8 days and adding approximately €11,549 per patient [20].

In this study, the cycle threshold (CT) was lower in children who had been hospitalized, with a mean of 26.6 ± 5.82, against a mean CT of 28.1 ± 8.35 for non-hospitalized cases (Table 2). The CT value indicates the number of amplification cycles required to detect viral RNA. Therefore, the CT value indicates that the viral load of hospitalized individuals was higher than that of non-hospitalized individuals. Data on RSV viral load in children are limited, and the findings of this study highlight the importance of laboratory tests to determine viral load for establishing clinical prognosis.

Higher viral loads in children with RSV infections have been associated with increased severity and need for intensive care unit (ICU) admission. A recent study showed that children with higher RSV viral loads were more likely to be hospitalized, require ICU admission, and receive oxygen compared to those with lower viral loads [21]. In addition, a high viral load (RT-PCR RSV CT <25) was associated with a greater need for supplemental oxygen, suggesting an association between viral load and disease severity in RSV-infected children [22]. These findings underscore the importance of monitoring viral load in RSV infection to identify children at higher risk of severe disease and the potential need for intensive care interventions (Tables 2 and 3).

The RSV has a spread that is independent of gender, with a higher incidence among individuals at both ends of the age spectrum (children and the elderly), due to the fragility of the immune system. In 2019, approximately 33 million cases of acute lower respiratory tract infections and 101,400 deaths in young children associated with RSV infections were reported [23]. Globally, an average of 1,641 RSV cases per season was estimated from 2000 to 2020 across 15 countries, with the majority occurring in children under 1 year of age [12].

In the European Union, there are approximately 245,244 yearly hospital admissions associated with RSV in children under 5 years, with a significant portion occurring in infants under 1 year [24]. In the United States, RSV causes approximately 58,000-80,000 hospitalizations in children <5 years and 60,000-160,000 in adults ≥65 years annually, following seasonal patterns [25].

The findings of this study showed that RSV infections are more frequent in infants less than one year old (75.7%). According to literature data, almost 90% of children are infected by the age of 2 [21]. This corroborates with more findings, the RSV-attributable respiratory hospitalization incidence is found to be the highest among infants aged 0-5 months, representing 72% of all respiratory hospitalizations. This study also observed mortality rates ranging between 0 and 0.6 deaths per 100,000 person-years in children aged 1 year [26].

Regarding comorbidities, this study presented three cases of children with comorbidities, one with chronic lung disease and two with cardiovascular disease, at 1 year and two at 11 months, respectively. There is a consensus on the definition of high-risk groups that are more likely to experience severe clinical outcomes in the presence of infections caused by respiratory syncytial virus (RSV). These high-risk groups include preterm infants, particularly those with a gestational age of less than 35 weeks and 6 days, as well as individuals with chronic lung disease and congenital heart disease [27].

A recent study highlighted the increased severity and risk of morbidity and mortality associated with viral respiratory infections in immunocompromised children compared to immunocompetent children and underscored the clinical impact of these infections, with hospitalization rates ranging from 28% to 58% in affected cases [28].

The implications of these discoveries could greatly influence the strategic planning for RSV passive immunization, through pregnant mothers to babies. This underscores the importance of disseminating and reinforcing preventive measures against this viral infection. The presented case reports in this study provide essential information on the epidemiological and clinical parameters of the RSV, that can be used for future planning of the disease control.

COVID-19, caused by SARS-CoV-2, presents a wide range of symptoms, from mild flu-like signs to severe complications, often resembling RSV. These non-specific symptoms can complicate the diagnosis and monitoring of other viral infections like RSV. A recent study analyzed RSV seasonal patterns in the U.S. from 2017 to 2023, showing how these patterns were impacted by the COVID-19 pandemic as the RSV epidemics before the pandemic typically followed a winter pattern, but the 2020-21 season saw no epidemic, and subsequent seasons shifted earlier with longer durations, particularly in 2021-22 (33 weeks). The 2022-23 season showed intense RSV circulation, peaking at 19% positivity, with consistent regional variations. It emphasizes the need for ongoing monitoring of RSV circulation to guide the timing of immunoprophylaxis and clinical trials for new preventive products. [25].

RSV remains one of the leading causes of hospitalization, morbidity, and mortality in young children. Therefore, prevention is the most effective strategy to combat the effects of this virus. Among the preventive measures currently available are proper hygiene such as proper hand hygiene, breastfeeding, and passive immunization using palivizumab, which together constitute the front line of protection against RSV.

Palivizumab, a monoclonal antibody immunoprophylaxis against RSV, has neutralizing and fusion-inhibitory activities against RSV and demonstrated efficacy in preventing the disease and is the only one approved for use in certain high-risk pediatric populations. These include infants born at or below 35 weeks gestational age (GA), infants with hemodynamically significant congenital heart disease (HCHD), and infants with chronic lung disease of prematurity (CLD) [29].

The RSV vaccine landscape is rapidly evolving. Recent developments include the approval by the U.S. Food and Drug Administration (FDA) and the European Union of the world's first RSV vaccines for individuals aged 60 years and older, such as Arexvy and Abrysvo. These vaccines target the pre-fusion form of the viral F protein and have shown significant efficacy in preventing RSV-associated lower respiratory tract disease in older adults [30, 31]. In addition, a monoclonal antibody named nirsevimab (Beyfortus®), has demonstrated efficacy in preventing RSV disease in infants and is already licensed in Brazil [32].

The advances in immunization represent a critical step in combating RSV infection and reducing associated morbidity and mortality

CONCLUSION

The resurgence of respiratory syncytial virus (RSV) as a respiratory pathogen underscores the critical need for ongoing research, surveillance, and preventive measures. The findings presented in this study emphasize the necessity for heightened awareness among healthcare providers regarding the clinical manifestations of RSV and the importance of early diagnosis and appropriate management strategies.

Estimating the impact of RSV remains a challenge. However, vaccination against this virus has the potential to prevent adverse clinical outcomes in adults at the extremes of age. The similarities in clinical symptoms between RSV and other respiratory viral infections, combined with a lack of awareness about the need for assertive diagnosis and limited access to laboratory testing, contribute to the underestimation of RSV-related morbidity and mortality.

Continued collaboration among researchers, clinicians, and public health authorities is essential to address the challenges posed by RSV.

Author contributions

All the authors contributed to the study conception, and writing and approved the final version to be published.

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Competing interests' disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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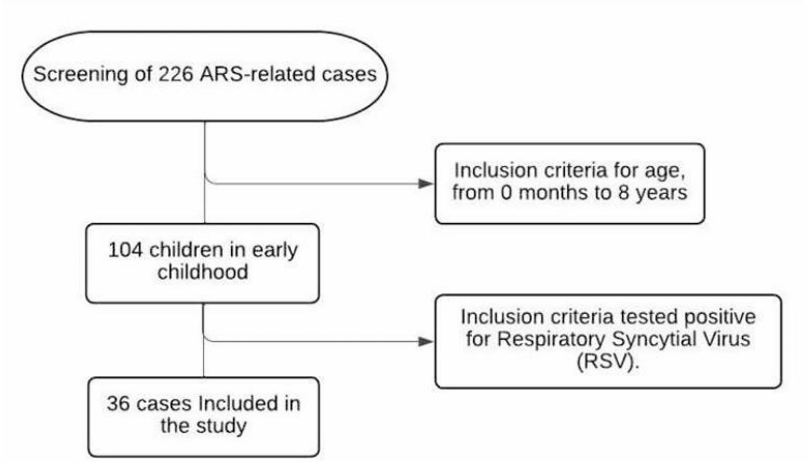

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