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# *Saprochaete clavata* invasive infection: characterization, antifungal susceptibility, and biofilm evaluation of a rare yeast isolated in Brazil

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## ABSTRACT

Rare emerging pathogens such as *Saprochaete clavata* are associated with invasive fungal diseases, high morbidity, mortality, rapidly fatal infections, and outbreaks. However, little is known about *S. clavata* infections, epidemiology, risk factors, treatment, biofilms, and disease outcomes. The objective of this study was to describe a new case of severe *S. clavata* infection in a patient diagnosed at a referral children's hospital in Brazil, including antifungal minimal inhibitory concentration, *S. clavata* biofilm characterization, and molecular characterization. The *S. clavata* isolated from an immunocompromised 11-year-old male patient was characterized using MALDI-TOF, Gram staining, scanning electron microscopy (SEM), and next generation sequencing (NGS) of genomic DNA. Biofilm production was also evaluated in parallel with determining minimal inhibitory concentration (MIC) and biofilm sensitivity to antifungal treatment. We observed small to medium, whitish, farinose, dry, filamentous margin colonies, yeast-like cells with bacillary features, and biofilm formation. The MALDI-TOF system yielded a score of ≥ 2,000, while NGS confirmed *S. clavata* presence at the nucleotide level. The MIC values (in mg $L^{-1}$) for tested drugs were as follows: fluconazole = 2, voriconazole ≤ 2, caspofungin ≥ 8, micafungin = 2, amphotericin B = 4, flucytosine ≤ 1, and anidulafungin = 1. Amphotericin B can be active against *S. clavata* biofilm and the fungus can be susceptible to new azoles. These findings were helpful for understanding the development of novel treatments for *S. clavata*-induced disease, including combined therapy for biofilm-associated infections.

**KEYWORDS:** Biofilm. Antifungal. Fungal morphology. Host susceptibility. Treatment. Infection.

## INTRODUCTION

Rare emerging pathogens such as *Saprochaete clavata* (formerly *Geotrichum clavatum)* are associated with invasive diseases and outbreaks[1,2]. *S. clavata* is an ascomycetous arthroconidia yeast-like filamentous fungus, and recent reports have identified it as the etiological agent in several clinical presentations, including post-organ transplantation infection[3], fungemia[4], central line infection[2], and disseminated infection with nodular lesions[5].

However, it is still poorly understood several *S. clavata* characteristics, including its habits, profile, origins, epidemiology, risk factors, sources, treatment, and disease outcomes. Although *S. clavata* has been found in dishwashers, a potential biofilm source[6,7], few studies have examined its biofilm formation[8].

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Additionally, the early stages of *S. clavata*-induced infections remain unclear. Some studies have suggested that the main risk factors are hematological malignancies and immunosuppressive treatments[8]. There may also be a role for subsequent gut translocation in patients with bloodstream infections who had diarrhea before or were associated with fungemia[4].

Invasive fungal infections represent an increasing threat to immunosuppressed individuals. Limited studies have suggested that *S. clavata* infection is associated with high morbidity, mortality, and rapidly fatal infections, but no data are available on its epidemiological, diagnostic, or therapeutic characteristics[4]. Specific diagnostic methods are scarce, and there are only studies on specific breakpoints for antifungal susceptibility test values and optimal therapeutic protocols[9]. Furthermore, *S. clavata* is sometimes misidentified as *Magnusiomyces capitatus* and *Trichosporon asahii*, given the similarities in their features, epidemiology, diagnosis, phylogeny, and phenotype[10].

The aim of this study is to report a case of severe infection caused by *S. clavata* with a complete description of the yeast, including antifungal minimal inhibitory concentration, biofilm characterization, antifungal activity in biofilm, morphological characterization, and genomic DNA sequencing.

## MATERIALS AND METHODS

### Microbiological characterization using MALDI-TOF, scanning electron microscopy, and DNA sequencing

Using the automated system BACTEC FX 40 (Becton Dickinson, Franklin Lakes, NJ, USA), the microorganism was first isolated from a positive blood culture incubated for 33 h. An aliquot of the blood culture was then subjected to Gram staining. Another aliquot was seeded onto Sabouraud dextrose agar (Laborclin, PR, Brazil) and incubated for 48 h at 36 °C for identification. The isolated was recovered in two separate blood cultures and urine. However, only the isolated from the first blood culture was used for *in vitro* analysis. The clinical case report may be found in Supplementary Case Report S1.

The resultant colony was subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) analysis. First, an isolated colony was placed on an agar plate along with 1 µL of 70% formic acid. The plate was dried at room temperature before adding 1 µL of alpha-Cyano-4-hydroxycinnamic acid matrix solution. After drying, the plate was placed in an equipment ionization chamber. Identification criteria followed the manufacturer's recommendations, where a score of ≥ 2,000 indicates highly reliable identification.

For biofilm production, we used a previously described method[6]. An isolated colony was dissolved in Sabouraud dextrose broth (Laborclin) and incubated for 48 h at 36 °C. The broth was then centrifuged for 5 min at 207 ×g. Upon discarding the supernatant, the pellet was washed three times with sterile 0.9% NaCl. In the last wash step, the pellet was dissolved in new Sabouraud dextrose broth. A nephelometer was used to obtain a suspension that matched the 0.5 McFarland turbidity standard. More Sabouraud dextrose broth was added to achieve a 1:10 dilution of the suspension and a concentration of $10^5$ colony forming units (CFU)/mL. Ten milliliters of the diluted suspension were poured into sterile six-well plates until they covered the titanium-alloy device, then left for 2 h to allow adequate cell adhesion. To remove planktonic cells, the device was transferred to a new sterile six-well plate containing 0.9% NaCl. Subsequently, the specimens were transferred to another sterile six-well plate and submerged in 10 mL of Sabouraud dextrose broth at 36 °C for 24 h, allowing the adherent cells to form a biofilm. The device was then submerged in 0.9% NaCl to remove the residue and unadhered (planktonic) cells. Finally, the device was placed under a scanning electron microscope (SEM).

Qualitative SEM sample preparation followed previously described protocols[11]. Titanium-alloy devices with growing biofilm were allocated into sterile glass Petri dishes filled with primary fixative agent (0.68 $g^{-1}$ sucrose, 0.42 $g^{-1}$ sodium cacodylate, 0.6 $mL^{-1}$ 30% glutaraldehyde) (Merck, Darmstadt, Germany) and 19.4 $mL^{-1}$ of deionized water, fully covering specimens for 45 min. The biofilm-covered devices were then transferred to a buffer (containing sucrose and sodium cacodylate in the same concentrations as the fixative agent) for 10 min. Afterwards, they were dehydrated in an increasing ethanol series (35%, 50%, 70%, and 100%), followed by 100% hexamethyldisilane (HMDS) (Merck, Darmstadt, Germany) for fixation; each step lasted for 10 min. Devices with biofilm were then coated with gold particles in a metalizing instrument with a Q150R ES rotary pump (Quorum Technologies, Lewes, UK), and fixed on a metal base for SEM observation under a PentaFET Precision (Oxford Instruments, Abingdon, UK) at 5.0 kV. Observations were made at magnifications between 2,000× and 6,000×.

Genomic DNA (gDNA) was obtained from a 48h culture on Sabouraud dextrose agar (Laborclin, PR, Brazil) at 36 °C. Pure and isolated colonies were selected for gDNA extraction using the Wizard® Genomic DNA Purification Kit (Promega, WI, USA). Genomic DNA libraries were prepared using the Illumina DNA Prep kit (Illumina, CA,

USA) and the NovaSeq SP kit (300 cycles) (Illumina, San Diego, CA, USA), then sequenced using Illumina NovaSeq 6000 (llumina, San Diego, CA, USA).

### Bioinformatics analysis of gDNA sequences

We performed bioinformatics analysis on raw NGS files of *S. clavata* gDNA to confirm the identification of non-redundant fungi at nucleotide level. We developed and applied an *in silico* computational pipeline using publicly available software and a reference database of the *S. clavata* genome (Supplementary Figure S1). In the first step, raw sequenced reads were subjected to quality control (QC) analysis in FastQC v0.11.5, using default parameters to generate a QC report (per base and average read QC, sequencing duplicate level, average read length). Subsequently, based on QC reports, reads were trimmed in Trimmomatic v0.39 to remove borders or entire reads that did not pass the quality threshold (parameters: CROP:100 HEADCROP:20 SLIDINGWINDOW:5:20 MINLEN:70 LEADING:3 TRAILING:3)[12]. High-quality trimmed reads were then mapped to the *S. clavata* reference genome (NCBI accession code: CBXB000000000) using Burrows-Wheeler Aligner (BWA) version 0.7.17-r1188[4]. BWA was configured to use the algorithm "mem" with the following parameters: "-t 40" (number of threads) and "-M" to mark shorter split hits as secondary (keeping the analysis output compatible with the tool Picard MarkDuplicates). Generated output SAM files from the mapping step were sorted in Picard v2.26.2 using the following parameters: "SORT_ORDER = coordinate" and "VALIDATION_STRINGENCY = SILENT." Picard was also used to locate and tag duplicate reads within the SAM file using the following parameters: "METRICS_FILE = metrics.txt" (output duplication metrics) "ASSUME_SORTED = true" and "VALIDATION_STRINGENCY = SILENT." To gather mapping statistics of NGS data, an index file for fast random data access and flagstat configuration was created in Samtools v1.11[13].

### Antifungal sensitivity test in planktonic cells and *S. clavata* biofilm

To determine the minimal inhibitory concentration (MIC) of planktonic cells, we used an automated VITEK®2 system (bioMérieux, Marcy-l'Etoile, France). The following drugs were tested: fluconazole, voriconazole, caspofungin, micafungin, amphotericin B, flucytosine, and anidulafungin. Isolated colonies were diluted in a sterile saline solution, and a suspension was prepared to match the 0.5 McFarland turbidity standard. The inoculum was evaluated using the fungal susceptibility card AST-YS08 (bioMérieux). Additionally, the anidulafungin was manually tested using the broth microdilution method, following protocol M27-A2 and in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines[14]. *Candida albicans* ATCC® 24433 was used for internal control (previously evaluated MIC was 0.5mg/L for amphotericin B, 0.5mg/L for fluconazole, and 1mg/L for flucytosine). Positive control (PC) was considered as the yeast without antifungal.

To analyze the biofilm susceptibility to antifungals, isolated colonies were cultivated in Sabouraud dextrose broth (Laborclin) and shaken (120 rpm) for 24 h at 36 °C as previously described[15]. The broth was then centrifuged (1,500 rpm for 5 min), and after discarding the supernatant, the pellet was washed with phosphate-buffered saline (PBS) at pH 7.5, centrifuged again to discard the supernatant, washed, and centrifuged for a final time. The resulting pellet was suspended in RPMI 1640 broth to match the turbidity of the 0.5 McFarland standard.

After that, 200 $\mu L^{-1}$ of the suspension was discharged into each well of a 96-well flat-bottom microplate and incubated for 24 h at 36 °C under continuous agitation (120 rpm). The growth control was prepared with 200 $\mu L^{-1}$ of each yeast-like suspension, while the sterility control was prepared with 200 $\mu L^{-1}$ of RPMI-1640 broth. After biofilm development, the medium was aspirated, and 200 $\mu L^{-1}$ of RPMI-1640 with 0.5–16 mg/L amphotericin B (Sigma-Aldrich, St. Louis, Missouri, USA), 0.5–32 mg $L^{-1}$ anidulafungin (Wyeth-Pfizer, Kalamazoo, Michigan, USA), or 2–64 mg $L^{-1}$ fluconazole (Sigma-Aldrich) were added. Microplates were shaken (120 rpm) for 24 h at 36 °C. The medium was aspirated again and the biofilm was washed with 200 $\mu L^{-1}$ of PBS.

### Determination of minimum biofilm eradication concentration (MBEC)

To determine the minimal antifungal concentration that decreases at least 2 log of biofilm, 200 $\mu L^{-1}$ of PBS (pH 7.5) was first added to each well of the 96-well microplate containing biofilm specimens[16]. The plate was then subjected to sonication using an ultrasonic bath for 5 min at 36 ºC and 40 kHz (Sanders Medical, MG, Brazil), disrupting the biofilm matrix cells through cavitation. Samples were then serially diluted with sterile saline and plated on Sabouraud dextrose agar. After incubation (24 h at 36 °C), the CFU number was determined, and CFU per area unit ($Log_{10}$ CFU/$cm^2$) was calculated. A strong reduction in biofilm was considered when > 2log CFU/$cm^2$, as previously described[16].

### Quantification of viable cells in biofilm – staining with MTT

This analysis quantifies cellular metabolism by converting MTT into formazan (a bluish-purple compound) using mitochondrial dehydrogenases from living cells. Firstly, 200 µL of 1 mg $mL^{-1}$ MTT [3-(4.5-dimethylthiazol-2yl)-2.5-diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO) was added to each well before incubating the microplate at 36 °C for 2 h. The medium was aspirated and the wells were washed three times with PBS. Formazan adhering to the biofilm was dissolved with 200 $\mu L^{-1}$ of isopropanol and homogenized. The absorbance assay was performed in Versa-Max (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm[17]. Interpretation of MTT tests was previously described[18].

### Quantification of total biomass in biofilm – violet crystal staining

To quantify total biomass, biofilm was fixed to a microplate using 200 $\mu L^{-1}$ of sterile 100% methanol for 15 min. Subsequently, the methanol was aspirated and discarded. The wells were allowed to dry at room temperature before 1% crystal violet was added to each, and the microplate was incubated at 36 ºC for 5 min. The wells were then washed three times with ultrapure water and homogenized with 200 $\mu L^{-1}$ of 33% acetic acid using a pipette. Total biomass was analyzed in Versa-Max at 570 nm. The percentage of biomass reduction was calculated.

### Statistical analysis

Yeast-like characterization (colony features, Gram staining, sequencing, and SEM) used both qualitative and descriptive data. Quantitative data were expressed as medians with 10% and 90% percentiles. The Mann–Whitney U test was used to compare medians. Significance was set at $p < 0.05$.

## RESULTS

### Microbiological characterization using MALDI-TOF, SEM, and DNA sequencing

Colonies on Sabouraud dextrose agar were small to medium in size, whitish, had a farinose surface, and exhibited a dry and filamentous margin (Figures 1A and 1B). The MALDI-TOF system identified them as *S. clavata* with a score of ≥ 2,000. Microscopic morphology after Gram-staining of positive blood cultures showed yeast-like cells with bacillary and varied size features (Figure 1C). Using SEM, we visualized biofilm formation on the titanium-alloy device, successfully identifying the biofilm (porous structures), yeast-like cells, and arthroconidia-like hyphal fragments (Figure 2).

Afterwards, we performed gDNA sequencing on *S. clavata* isolates to confirm the species at nucleotide resolution. Using NGS, we generated four digital files (two pairs of paired ends) from gDNA samples that were subjected to an *in silico* bioinformatics pipeline. In total, 461.8 million high-quality reads were sequenced (Supplementary Table S1).

The QC analysis yielded a per base sequence quality and per sequence quality scores of above Q30 (high quality) for 99% of sequences (Supplementary Figure S2A and S2B). All samples had <1% of reads comprising overrepresented sequences and no samples were found with any adapter contamination (>0.1%). Additionally, the read duplication was high (average of 82%), possibly due to the number of sequenced reads (Supplementary Figure S2C). Based on QC results, we trimmed the sequenced digital libraries (last 10 base pairs and first 20 base pairs) to


**Figure 1 -** Culture of *S. clavata* on Sabouraud dextrose agar: A) Colony streaks; B) Colony details; C) Yeast-like cells of *S. clavata* morphology observed via microscopy (Gram-staining, magnification: 100x).

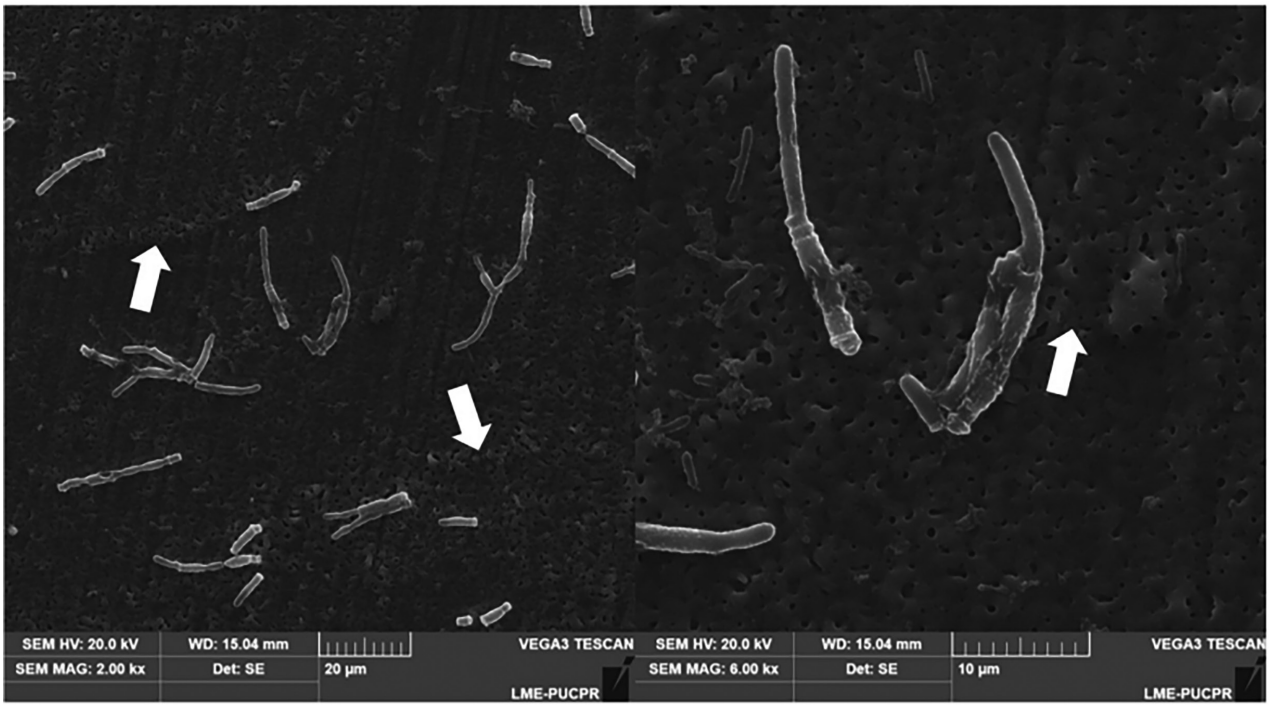


**Figure 2 -** Scanning electron microscopy images of *S. clavata* (magnification: 2,000x; 6,000x). The white arrows indicate the matrix of biofilm structure.

generate an average read length of 80 bp (Supplementary Figure S2D).

After mapping high-quality reads to the *S. clavata* reference genome (accession number CBXB01), over 98% were aligned to all reference sequences (Figure 3). The read depth/coverage was high for all genomic sequences.

## Minimal inhibitory concentration of planktonic cells

We tested *S. clavata* antimicrobial susceptibility using the automated VITEK®2 system and broth microdilution tested with CLSI guidelines. The results are shown in Table 1.

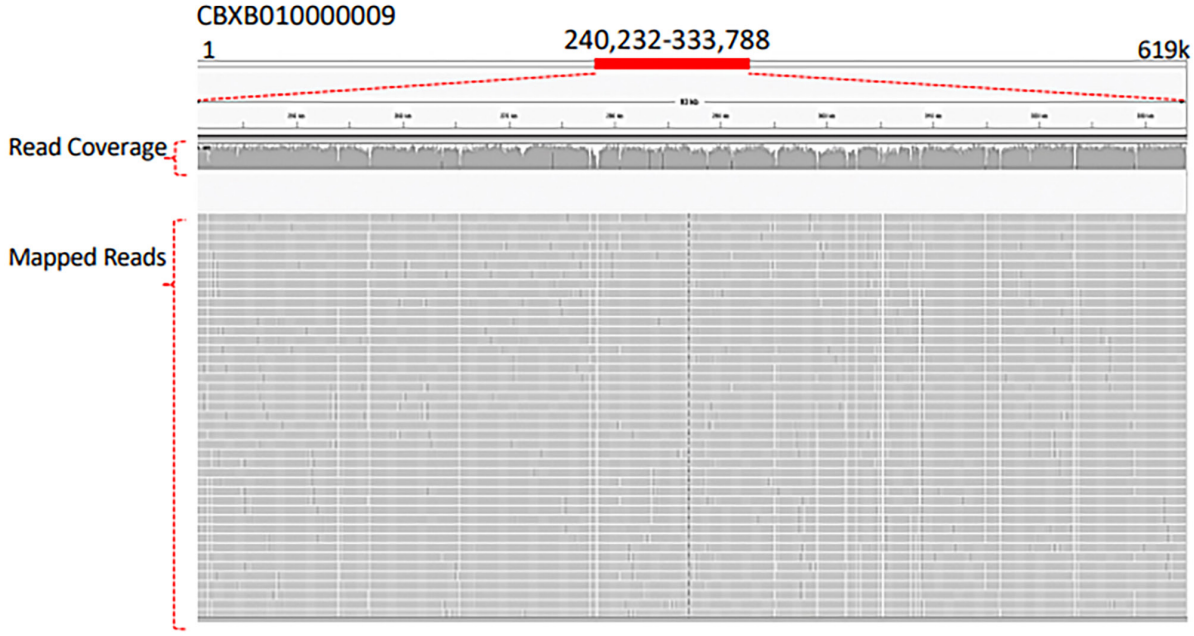


**Figure 3 -** Sequenced gDNA mapped to *S. clavata* sequence of the reference genome. An illustrative region of 93k (240,232–333,788) (red box) of the reference sequence CBXB010000009 from *S. clavata* genome is shown, with read coverage and mapped reads (gray boxes represent sequenced reads).

**Table 1 -** MIC results performed by VITEK®2 system and broth microdilution (CLSI).

| Antifungal | MIC mg $L^{-1}$ |
|---|---|
| Fluconazole | 2 |
| Voriconazole | ≤ 2 |
| Caspofungin | ≥ 8 |
| Micafungin | 2 |
| Amphotericin B | 4 |
| Flucytosine | ≤ 1 |
| Anidulafungin* | 1 |

*Anidulafungin was performed by broth microdilution.

## Antifungal sensitivity test in *S. clavata* biofilm Determination of MBEC

We defined MBEC as the minimal concentration (mg $L^{-1}$) of antifungal drugs that can reduce the biofilm cell count by 2 $\log_{10}$ per $cm^2$. Among the tested drugs, fluconazole failed to reduce the biofilm cell quantity, leading to a constant CFU $mL^{-1}$ $\log_{10}$ per $cm^2$ regardless of drug concentration (Figure 4A). Anidulafungin caused a slight reduction of CFU/mL $\log_{10}$ per $cm^2$ (Figure 4B), but not enough to be considered as eradication activity. In contrast, amphotericin B led to a strong reduction (more than 2 $\log_{10}$ per $cm^2$) of biofilm cell count, with an MBEC concentration of 8–16 mg $L^{-1}$ (Figure 4C).

## Quantification of viable cells in biofilm – staining with MTT

The MTT assays only quantified live biofilm cells and metabolic activity was measured colorimetrically. The MTT assay of fluconazole revealed a slight variation in live biofilm cell percentage depending on concentration (Figure 4D). Anidulafungin caused live biofilm cell percentage to increase in a concentration-dependent

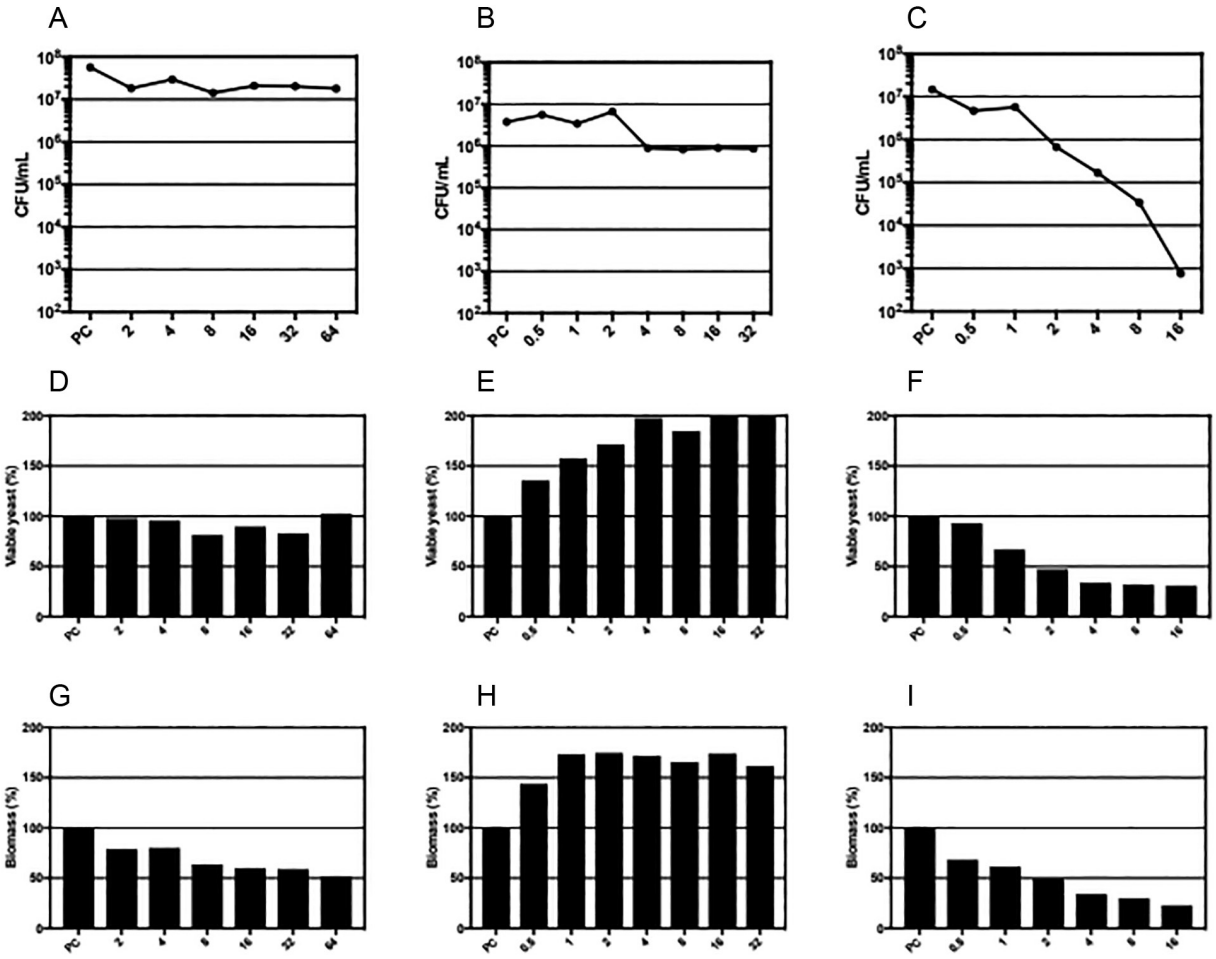


**Figure 4 -** Evaluation of minimum biofilm eradication concentration (MBEC) of *S. **clavata***. A: fluconazole. B: anidulafungin. C: amphotericin B. PC: positive growth control without antifungal. Quantification of viable cells in the biofilm via MTT staining. D: fluconazole. E: anidulafungin. F: amphotericin B. PC: positive growth control without antifungal. Quantification of total biomass in biofilm via crystal violet staining. G: fluconazole. H: anidulafungin. I: amphotericin B. PC: positive growth control without antifungal.

manner (Figure 4E). Finally, the assay results showed that amphotericin B exhibited an antifungal activity against the biofilm, progressively reducing the cell count with increasing antifungal concentration (Figure 4F).

### Quantification of total biomass in biofilm – violet crystal staining

Violet crystal staining showed that fluconazole slightly reduced total biomass in a concentration-dependent manner (Figure 4G), whereas anidulafungin did not. However, total biomass was higher when antifungal concentrations were higher than those of the positive control (Figure 4H). Amphotericin B was the most effective against biofilm formation, reducing total biomass by 77% at a concentration of 16 μg $mL^{-1}$ (Figure 4I).

## DISCUSSION

Data regarding the virulence mechanisms and biofilm production of *S. clavata* are extremely scarce. One study indicated a relationship between the central venous catheter and a positive blood culture, while another identified microbial biofilm communities on reverse osmosis membranes in whey water[6,8].

In our study, we induced biofilm formation *in vitro* using *S. clavata* isolated from a patient and verified its presence using SEM. However, we could not correlate the biofilm formation with the patient's infection. We also observed that fluconazole and anidulafungin did not have strong effects against *S. clavata* biofilm, whereas amphotericin B showed a significant effect. Overall, increasing amphotericin B concentration caused a significant reduction in biofilm cell count, biofilm live cells, and biomass reduction, with a MIC of 4 mg $L^{-1}$. The MIC value of fluconazole was 2 mg $L^{-1}$, but the antifungal sensitivity test showed that *S. clavata* biofilm did not differ significantly across different fluconazole concentrations. Notably, our *S. clavata* strain yielded relatively low MIC values against voriconazole, caspofungin, flucytosine, and anidulafungin when compared to other studies (Table 1). In contrast, micafungin and amphotericin B led to higher MIC values than those reported in other studies (Supplementary Table S2)[2,4,8,10,19-25].

*S. clavata* is resistant to echinocandins and fluconazole (high minimal inhibitory concentration not achieved by drug levels), but can be susceptible to amphotericin and new azoles, considering the minimal inhibitory concentration and pharmacokinetics parameters[26,27]. Although known to have low pathogenicity, rare yeasts still cause 1.1–5.1% of fungemia cases[28]. We highlight the fact that invasive infections caused by arthroconidia fungi, such as *S. clavata*, are more likely to occur in immunocompromised patients[8], particularly those with immunosuppression and neutropenia. Furthermore, breakpoints and epidemiological cut-offs have not been defined for *S. clavata,* therefore, the susceptibility test results must be carefully interpreted. Due to difficulties in identification and diagnosis, along with its rarity, the optimal therapy for fungi such as *S. clavata* remains a challenge[29]. The correct identification of atypical fungi is important, as assertive, and agile treatment leads to positive outcomes. Rapid molecular tools, such as nucleotide sequencing, whole-genome sequencing, and mass spectroscopy (MALDI-TOF system) are fast, reliable, and sensitive methods for identifying unusual yeasts[16].

Discrepancies in terms of *S. clavata* antifungal susceptibility are highlighted; however, voriconazole showed optimal *in vitro* results when susceptibility tests were performed[8]. Liposomal amphotericin B has also shown positive outcomes[2]. Our study only tested three drugs and we did not test for synergistic or antagonistic effects with other antifungals. Despite this observation in planktonic cells, we observed that amphotericin B demonstrated the best performance against *S. clavata* biofilm, whereas neither fluconazole nor anidulafungin showed efficient biofilm eradication. We also highlight that anidulafungin not only increased the number of viable cells in the biofilm but also increased total biofilm biomass.

In this study, the *S. clavata* strain was isolated from the blood culture and urine (>100.00 CFU $mL^{-1}$) of an immunosuppressed patient with B-cell acute lymphoblastic leukemia. In general, such cases are the usual profile and exhibit the risk factors of those who develop *S. clavata*-related infections. In Brazil, *S. clavata* had only been previously reported in 2016, isolated from a 6-year-old female patient with hemophagocytic lymphohistiocytosis. This case exhibited neutropenia and was treated with amphotericin B deoxycholate and voriconazole, but the outcome was death[30]. In our study, *S. clavata* was identified using MALDI-TOF and SARAMIS™ from blood cultures and isolated colonies, and then confirmed with NGS of gDNA using bioinformatics tools. Our case was restricted to only one patient and any other cases or outbreaks were reported after his/her death.

## CONCLUSION

In conclusion, we developed an *in vitro* biofilm of *S. clavata*. Moreover, we observed that amphotericin B was efficient against *S. clavata* biofilm, with a MIC value of 4 mg $L^{-1}$, whereas fluconazole and anidulafungin, despite both having a MIC value of 2 mg $L^{-1}$, were ineffective against *S. clavata* biofilm. These findings are extremely

helpful in elucidating the efficacy of these three drugs against *S. clavata* biofilm, given that an optimal therapy has yet to be established. We note that our study was an *in vitro* study with results from only one isolated strain in Brazil.

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## AUTHORS' CONTRIBUTIONS

Conceptualization: FAM, LK and VSTR; Writing (original draft preparation): FFT, LK, VSTR, ALMR, RHH and LPP; Writing (review and editing): FFT and VSTR; analysis: LK, VSTR, RHH, LPP, KCP, and DLAF.

## CONFLICT OF INTERESTS

FFT is a CNPq researcher. The other authors declared no conflict of interests.

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