Emerging Microbes & Infections

Candida blankii: an emergent opportunistic yeast with reduced susceptibility to antifungals

João Nobrega de Almeida, Jr1,2, Silvia V. Campos3, Danilo Y. Thomaz1, Luciana Thomaz4, Renato K. G. de Almeida1, Gilda M. B. Del Negro1, Viviane F. Gimenes2, Rafaela C. Grenfell5, Adriana L. Motta1, Flávia Rossi1 and Gil Benard2

In 1968, Buckley and van Uden described the non-fermenting yeast Candida blankii (C. blankii) found in the organs of a mink1. Until recently, this microorganism had only been the subject of biotechnological research2,3. However, in 2015, Zaragoza et al. reported a 14-year-old male patient with cystic fibrosis (CF), who had pulmonary exacerbations with repeated isolation of C. blankii from respiratory samples4. This finding raised the hypothesis that this yeast could be a relevant pathogen for CF patients5. This paper corroborates this initial observation by describing a bloodstream infection by C. blankii in a CF patient who underwent lung transplantation.

A 16-year-old female with CF was referred to our lung transplant center in March 2016. Her recent medical history was notable for severe pulmonary exacerbations, which required prolonged hospitalization and mechanical ventilation. Her most recent preadmission sputum cultures collected by the referring hospital showed positive for Staphylococcus aureus, Pseudomonas aeruginosa, Burkholderia cepacia, Aspergillus sp., including positive samples for Candida sp. (negative germ tube, isolates not available) collected after itraconazole therapy (200 mg/day) used to treat the pulmonary exacerbations. In June 2016, she underwent bilateral lung transplantation at the Hospital das Clínicas, University of São Paulo, Brazil, under antimicrobial prophylaxis consisting of teicoplanin, meropenem, cotrimoxazole and liposomal amphotericin B (L-AMB, 200 mg/day). However, during the infusion of L-AMB, the patient presented hypotension, leading to the discontinuation of the antifungal treatment. On the first postoperative day (POD), the patient developed sepsis, and so blood cultures were collected (Bactec aerobic and anaerobic/Plus, BD). Peripheral and central venous catheter blood cultures became positive for yeasts after 41 (anaerobic bottle) and 72 (aerobic bottles) hours of incubation, respectively. On the third POD, due to the provisional report of yeasts on blood cultures, micafungin became negative; transthoracic echocardiography and fundoscopic eye exam did not show any significant results. The yeast isolate showing pale pink colonies on chromogenic medium (BBL CHROMagar, BD, Sparks, USA) were not identified by MALDI-TOF mass spectrometry (Vitek MS™, IVD library, bioMérieux, Marcy-L’Etoile, France). Because clinical improvement was observed, micafungin was maintained for 14 days. The patient was discharged on the 39th POD. The clinical isolate (HCFMUSP01) was later identified as C. blankii after sequence analysis of the internal transcribed spacer 1 (ITS1, GenBank accession no. MF573785) and D1D2 region from the 26S subunit (D1D2, GenBank accession no. MF940140) of the rRNA5,6. Since little is known about this microorganism as an opportunistic pathogen, we further characterized this species in terms of genetic and proteomic diversity, antifungal susceptibility, biofilm production, and in vivo virulence by analyzing the clinical isolate and the strains (IIC1M.1, BX90C, BX81A) from the yeast collection at the Federal University of Minas Gerais, Brazil.

DOI 10.1038/s41426-017-0015-8

© The Author(s) 2018

Correspondence: João N. de Almeida (jnaj99@gmail.com)

1Central Laboratory Division- LIM-03, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo 05403-010, Brazil
2Laboratory of Medical Mycology - LIM-53, Hospital das Clínicas FMUSP and Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, São Paulo 05403-000, Brazil

Full list of author information is available at the end of the article.
The organisms had identical D1D2 rRNA sequences, but the clinical isolate showed a different ITS allele (single nucleotide deletion) when compared to the other strains (Table 1 and supplementary Table S1). Proteomic analysis by MALDI-TOF mass spectrometry (MS), this time using both Bruker’s (Microflex™, Bruker Daltonics, Bremen, Germany) and bioMérieux instruments (Vitek MS™, bioMérieux, Marcy L’Etoile, France), revealed distinct spectral profiles between clinical and environmental organisms (Supplementary Figure S1). As an observation, the Bruker’s IVD and RUO libraries lacked this species and were unable to identify the organisms, whilst the Vitek MS™ RUO library correctly identified all of them (Table 1).

Antifungal susceptibility testing was carried out using the EUCAST reference broth microdilution method. All organisms showed high minimal inhibitory concentrations (MICs) of fluconazole (Sigma-Aldrich, St. Louis, MO, USA) (16 mg/L) and voriconazole (Sigma-Aldrich) (0.5 mg/L), whereas amphotericin B (Sigma) exhibited potent activity (0.25–0.5 mg/L). Both anidulafungin (Pfizer, Groton, CT, USA) and micafungin (Astellas Pharma US, Northbrook, IL, USA) showed limited activity against C. blankii organisms, with MICs ranging from 0.25–1 mg/L and 0.25–0.5 mg/L, respectively (Table 1).

Biofilm formation and metabolic activity were measured by using crystal violet staining and XTT reduction assay, respectively. Based on previously reported cutoff values, all organisms were classified as low biofilm producers, showing low metabolic activity (Supplementary Figure S2).

Killing assays in Galleria mellonella model were performed to compare the pathogenicity of C. blankii organisms with Candida albicans (strain SC5314). Sixteen G. mellonella larvae (LFD/F1CB/USP) were used for each test group. Larvae were inoculated (1 × 10^5 and 1 × 10^6 cells/larva) and incubated at 37°C. Pathogenicity was assessed, monitoring the percent survival over 7 days and plotted using Kaplan–Meier and the Log-rank test (GraphPadPrism 4, GraphPad Software, Inc., La Jolla, USA). The C. blankii organisms HCFMUSP01 and IIC1M.1 were significantly less virulent than the C. albicans strain (P < 0.0001) in the G. mellonella model (Supplementary Figure S3). However, the strains BX 90C and BX 81 A showed similar pathogenicity when compared to C. albicans (P = 0.13). Moreover, G. mellonella killing by different C. blankii organisms were accentuated when larvae were inoculated with 1 × 10^6 organisms (Supplementary Figure S3).

Evidence is provided here that C. blankii is an opportunist pathogen for lung transplant and/or CF patients. Clinical laboratories from referral centers of CF and lung transplant should be aware of this Candida species, and the fact that not all MALDI-TOF MS libraries are able to identify it. Based on our case report and the in vitro data presented here, we suggest that CVC was not the primary source of the infection and that the patient’s respiratory tract was

| Strain name | GenBank accession no. (ITS; D1/D2) | ITS allele | Correct species identification by MALDI-TOF mass spectrometry | Antifungal susceptibility testing (minimal inhibitory concentration) |
|-------------|-----------------------------------|------------|---------------------------------------------------------------|---------------------------------------------------------------|
| HCFMUSP01   | MF573773.1; MF940140              | A          | No                                                            | Braket RUO^a Library Vitek MS RUO Library                      |
| IC1M.1      | MF590148; MF590137                | B          | No                                                            | Yes                                                          |
| BX 90C      | MF590141; MF590139                | B          | No                                                            | Yes                                                          |
| BX 81A      | MF590142; MF590138                | B          | No                                                            | Yes                                                          |

* In vitro diagnostics. **Research use only. † Fluconazole. ‡ Voriconazole. § Anidulafungin. ¶ Micafungin. " Sand of Copacabana’s beach, Rio de Janeiro, Brazil. S ugar cane bagasse, Paraíba, Brazil.

The organisms had identical D1D2 rRNA sequences, but the clinical isolate showed a different ITS allele (single nucleotide deletion) when compared to the other strains (Table 1 and supplementary Table S1). Proteomic analysis by MALDI-TOF mass spectrometry (MS), this time using both Bruker’s (Microflex™, Bruker Daltonics, Bremen, Germany) and bioMérieux instruments (Vitek MS™, bioMérieux, Marcy L’Etoile, France), revealed distinct spectral profiles between clinical and environmental organisms (Supplementary Figure S1). As an observation, the Bruker’s IVD and RUO libraries lacked this species and were unable to identify the organisms, whilst the Vitek MS™ RUO library correctly identified all of them (Table 1).

Antifungal susceptibility testing was carried out using the EUCAST reference broth microdilution method. All organisms showed high minimal inhibitory concentrations (MICs) of fluconazole (Sigma-Aldrich, St. Louis, MO, USA) (16 mg/L) and voriconazole (Sigma-Aldrich) (0.5 mg/L), whereas amphotericin B (Sigma) exhibited potent activity (0.25–0.5 mg/L). Both anidulafungin (Pfizer, Groton, CT, USA) and micafungin (Astellas Pharma US, Northbrook, IL, USA) showed limited activity against C. blankii organisms, with MICs ranging from 0.25–1 mg/L and 0.25–0.5 mg/L, respectively (Table 1).

Biofilm formation and metabolic activity were measured by using crystal violet staining and XTT reduction assay, respectively. Based on previously reported cutoff values, all organisms were classified as low biofilm producers, showing low metabolic activity (Supplementary Figure S2).

Killing assays in Galleria mellonella model were performed to compare the pathogenicity of C. blankii organisms with Candida albicans (strain SC5314). Sixteen G. mellonella larvae (LFD/F1CB/USP) were used for each test group. Larvae were inoculated (1 × 10^5 and 1 × 10^6 cells/larva) and incubated at 37°C. Pathogenicity was assessed, monitoring the percent survival over 7 days and plotted using Kaplan–Meier and the Log-rank test (GraphPadPrism 4, GraphPad Software, Inc., La Jolla, USA). The C. blankii organisms HCFMUSP01 and IIC1M.1 were significantly less virulent than the C. albicans strain (P < 0.0001) in the G. mellonella model (Supplementary Figure S3). However, the strains BX 90C and BX 81 A showed similar pathogenicity when compared to C. albicans (P = 0.13). Moreover, G. mellonella killing by different C. blankii organisms were accentuated when larvae were inoculated with 1 × 10^6 organisms (Supplementary Figure S3).

Evidence is provided here that C. blankii is an opportunist pathogen for lung transplant and/or CF patients. Clinical laboratories from referral centers of CF and lung transplant should be aware of this Candida species, and the fact that not all MALDI-TOF MS libraries are able to identify it. Based on our case report and the in vitro data presented here, we suggest that CVC was not the primary source of the infection and that the patient’s respiratory tract was...
heavily colonized by *C. blankii* (assuming that the *Candida* sp. from the referring hospital was *C. blankii* and based on *G. mellonella* killing assays). This probably led the patient to develop fungemia due to the lack of antifungal prophylaxis.

Further studies of different strains are warranted to increase knowledge of genetic diversity and antifungal susceptibility profile of *C. blankii* organisms. However, while more microbiological data are pending, it is prudent to avoid azoles for the treatment or prophylaxis of *C. blankii* infections. Like *Candida parapsilosis*, despite the reduced antifungal action of echinocandins against *C. blankii*, successful treatment may be achieved with these compounds, as illustrated in this report. However, amphotericin B showed strong in vitro activity against *C. blankii*, and its formulations should be the first line therapy for deep-seated infections by this emergent species while antifungal susceptibility testing is ongoing.

Acknowledgements
We would like to thank Ana Raquel O. Santos and Carlos A. Rosa from Federal University of Minas Gerais who kindly provided us the environmental strains.

Author details
1Central Laboratory Division– LIM-03, Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo 05403-010, Brazil. 2Laboratory of Medical Mycology - LIM-53, Hospital das Clinicas FMUSP and Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, São Paulo 05403-000, Brazil. 3Heart Institute, Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo 05403-000, Brazil. 4Institute of Biomedical Science, Universidade de São Paulo, São Paulo 05508-900, Brazil. 5Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo 04039-000, Brazil

Conflict of interest
The authors declare no conflict of interest.

Supplementary Information
accompanyes this paper at (https://doi.org/10.1038/s41426-017-0015-8).

References
1. van Uden, N. & Buckley, H. R. Five new Candida species. Mycopathol. Mycol. Appl. 36, 257–266 (1968).
2. Meyer, P. S., Du Preez, J. C. & Kilian, S. G. Chemostat cultivation of Candida blankii on sugar cane bagasse hemicellulose hydrolysate. Biotechnol. Bioeng. 40, 353–358 (1992).
3. Arlyapov, V., Kamanin, S., Ponamoreva, O. & Reshetilov, A. Biosensor analyzer for BOD index express control on the basis of the yeast microorganisms Candida maltosa, Candida blankii, and Debaryomyces Hansenii. Enzym. Microb. Technol. 50, 215–220 (2012).
4. Zaragoza, S. et al. 318 Candida blankii: New agent in cystic fibrosis airways? J. Cyst. Fibros. 14, 5140 (2015). Supplement 1.
5. Fujita, S. I., Sendai, Y., Nakaguchi, S. & Hashimoto, T. Multiplex PCR using internal transcribed spacer 1 and 2 regions for rapid detection and identification of yeast strains. J. Clin. Microbiol. 39, 3617–3622 (2001).
6. Schuch, C. L. et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc. Natl Acad. Sci. USA 109, 6241–6246 (2012).
7. Arendrup M. C. et al. For the S on AST (AFST) of the EEC, (EUCAST)* AST. 2015. EUCAST DEFINITIVE DOCUMENT E.DEF 7.3 Method for the determination of broth dilution minimum Inhibitory concentrations of antifungal agents for yeasts. http://www.eucast.org.
8. Melo, A. S., Bizerra, F. C., Freymüller, E., Arthington-Skaggs, R. A. & Colombo, A. L. Biofilm production and evaluation of antifungal susceptibility amongst clinical Candida spp. isolates, including strains of the Candida parapsilosis complex. Med. Mycol. 49, 253–262 (2011).
9. Pierce, C. G. et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat. Protoc. 3, 1494–1500 (2008).
10. Marcos-Zambrano, L. J., Escrivanho, P., Bouza, E. & Guineu, J. Production of biofilm by Candida and non-Candida spp. isolates causing fungemia: comparison of biomass production and metabolic activity and development of cut-off points. Int. J. Med Microbiol 304, 1192–1198 (2014).
11. Cotter, G., Doyle, S. & Kavanaugh, K. Development of an insect model for the in vivo pathogenicity testing of yeasts. Fems. Immunol. Med. Microbiol. 27, 163–169 (2000).