Brazilian Journal of Microbiology (2020) 51:851–860
https://doi.org/10.1007/s42770-020-00232-1

BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

# Candidemia by *Candida parapsilosis* in a neonatal intensive care unit: human and environmental reservoirs, virulence factors, and antifungal susceptibility

Ralciane de Paula Menezes[1] · Sávia Gonçalves de Oliveira Melo[2] · Meliza Arantes Souza Bessa[3] · Felipe Flávio Silva[4] · Priscila Guerino Vilela Alves[4] · Lúcio Borges Araújo[5] · Mário Paulo Amante Penatti[1] · Vânia Olivetti Steffen Abdallah[4] · Denise von Dollinger de Brito Röder[4,6] · Reginaldo dos Santos Pedroso[1,4]

Received: 18 September 2019 / Accepted: 18 January 2020 / Published online: 15 February 2020
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## Abstract

The *Candida parapsilosis* complex has emerged as one of the main causes of candidemia worldwide. This study aims to evaluate possible *C. parapsilosis* sensu stricto reservoirs in a NICU, the expression of virulence factors, and antifungal susceptibility, and to analyze their genetic and phenotypic similarity. The study included 17 isolates of *C. parapsilosis*: seven environmental, one from a newborn's mother, and nine samples from six newborns. We used molecular and phenotypic tests to characterize the isolates and to trace possible routes of infection. The genetic similarity was determined by random amplified polymorphic DNA. The hemolytic and DNAse activity was determined using sheep's blood and DNAse agar, biofilm production by XTT method, and the susceptibility to antifungals through microdilution methodology. Two environmental strains isolated in the same month had high similarity. The 17 isolates expressed at least one of the three virulence factors studied, and one environmental isolate was resistant to fluconazole. This study shows that environmental contamination can be an important reservoir of potentially pathogenic microorganisms, since isolates of *C. parapsilosis* sensu stricto collected from the hospital environment were able to express virulence factors. Therefore, we emphasized the importance of determining the transmission routes in NICU in order to detect pathogen sources and reservoirs, as well as to establish prevention measures, such as adequate disinfection of the environment.

**Keywords** Antifungals · *Candida parapsilosis* · Neonates · NICU · Virulence · RAPD-PCR

Responsible Editor: Rosana Puccia.

✉ Ralciane de Paula Menezes
ralciane@ufu.br

[1] HealthTechnical School, Federal University of Uberlandia, Av. Amazonas s/no - Block 4K – 111–Campus Umuarama, Umuarama, Uberlândia, MG ZIP 38400-902, Brazil

[2] Undergraduation in Nursing, Faculty of Medicine, Federal University of Uberlandia, Uberlandia, Minas Gerais, Brazil

[3] Undergraduation in Biology - Institute of Biology, Federal University of Uberlandia, Uberlandia, Minas Gerais, Brazil

[4] Health Sciences Postgraduate Program, Faculty of Medicine, Federal University of Uberlandia, Uberlandia, Minas Gerais, Brazil

[5] Mathematics College, Federal University of Uberlandia, Uberlandia, Minas Gerais, Brazil

[6] Institute of Biomedical Sciences, Federal University of Uberlandia, Uberlandia, Minas Gerais, Brazil

## Introduction

Newborns (NB) with severe health conditions are susceptible to the development of invasive fungal infections due to prematurity, extremely low weight, surgical pathologies (such as gastroschisis and malformations), and an immature immune system. Prolonged hospitalization, the use of invasive devices, parenteral nutrition, and broad-spectrum antimicrobial therapy are risk factors for the development of nosocomial infections [1, 2].

Bloodstream infections (BSI) caused by *Candida* spp. are common causes of late sepsis in neonatal intensive care units (NICUs) [3]. The incidence rate varies from one to three cases per 1000 hospitalizations, while the mortality rate is higher than 40% in Brazilian public tertiary care hospitals and may reach 30% in other countries [4, 5].

The colonization of NB with severe health condition occurs in the first days after birth: 10% are colonized in the first week of hospitalization, and this level can reach 64% in the fourth

week [6]. Up to 60% of neonates with an extremely low birth weight can be colonized by *Candida* in their first month of life and, generally, 7–20% of colonized NB can develop invasive candidiasis [2].

The *C. parapsilosis* complex has emerged as a major cause of candidemia worldwide among non-*albicans* species [7, 8]. In addition, it is associated with several outbreaks of NICU and high morbidity and mortality rates [6, 9, 10]. This situation becomes more relevant considering that *C. parapsilosis* is able to produce biofilm and proliferate in solutions that are rich in nutrients, including total parenteral nutrition [3, 8]. Invasive candidiasis caused by *C. parapsilosis* can occur without previous colonization and is transmitted horizontally through contaminated external sources such as hospital environment, medical devices, and health professionals' hands [3, 11].

The molecular typing of isolates involved in nosocomial infections has contributed to understanding the epidemiology of infections and the cross-transmission of pathogenic microorganisms [11, 12]. The presence of identical genotypes infecting different patients suggests a horizontal transmission of the microorganism from patient to patient or from a common source [11].

This study aims to evaluate possible *C. parapsilosis* reservoirs in a NICU, the expression of virulence factors, and antifungal susceptibility, and to analyze genetic and phenotypic similarity.

## Patients and methods

### Place and period of study

The study was carried out at the highly complex hospital, located at the Triângulo Mineiro region, Minas Gerais state, southeastern Brazil. The hospital has 520 beds, with 20 belonging to the NICU. The studied samples were collected between March 2016 and May 2017.

### Sample selection and collection

Environmental and biological samples were collected from the NICU environment (inanimate surfaces), perianal and oral mucosa, hands, and bloodstream. The collection of samples from inanimate surfaces, mucosa, and hands was performed when there was some case of candidemia in a hospitalized neonate positive to *Candida* spp. in blood culture. Blood cultures were collected based on clinical criteria established by the medical team.

Figure 1 shows the sites, the number of samples collected, and the isolation spot of *C. parapsilosis* species.

### Microorganism isolation and identification

Blood culture isolation of microorganisms was performed by the BACT/Alert® and identification by Vitek® systems (both bioMérieux–Durham, USA).

The samples from mucosal and invasive devices of patients with blood cultures positive for *Candida* spp., as well as oral mucosa samples from the mothers of each of the six neonates, were collected with a sterile swab and soaked in a physiological solution (NaCl 0.9%) that was also sterilized, after consent was taken by those responsible for participating in the research.

Environmental samples were also collected using a sterile swab, soaked in sterile physiological solution, 30 min before the unit's hygiene procedure in the morning, afternoon, and evening. After collection, the swab was placed in a tube containing 1 mL of sterile 0.9% saline solution. Then, the material contained in the tubes was homogenized in a vortex-type stirrer and 0.1 mL was inoculated in plates of Sabouraud Dextrose Agar (SDA) (Isofar, Duque de Caxias, RJ, Brazil) plus chloramphenicol and chromogenic agar plates (Himedia, Mumbai, India) and incubated at 30 °C for 72 h.

Hand washing was simulated in a sterilized polypropylene bag containing 30 mL of physiological solution to the samples collected from the hands of the mothers of the six neonates who had candidemia [13]. In the laboratory, this material was transferred to sterile Falcon-type tubes and centrifuged at 4000*g* for 10 minutes. Subsequently, the supernatant was discarded, the sediment resuspended in 0.9% physiological solution, and 0.1 mL was plated on chloramphenicol SDA plates and on plates with chromogenic agar which were then incubated at 30 °C for up to 72 h [14].

The identification of the yeast species was performed by the classical established methodology [15]. The differentiation between *C. parapsilosis* sensu stricto, *C. metapsilosis*, and *C. orthopsilosis* was performed by restriction fragment length polymorphism analysis (RFLP-PCR), according to Tavanti et al. [16].

### Virulence factors

For research into hemolytic and DNAse actives, each isolate in SDA culture (24–48 h) was suspended in physiologic saline solution (NaCl 0.9%) with turbidity equivalent to tube two of the McFarland scale ($1 \times 10^8$ to $5 \times 10^8$ CFU/mL). Subsequently, 5 μL of each suspension was deposited at equidistant points in Petri dishes (90 × 15 mm) containing sheep's blood agar 7% and DNAse agar, respectively (Hexis, São Paulo, Brazil). Incubation was performed at 30 °C for 7 days for DNAse research and for 48 h for the analysis of hemolytic activity [17, 18]. All tests were performed in duplicate.

Hemolytic activity was analyzed by hemolysis around the colony. The hemolytic activity was measured by the ratio of

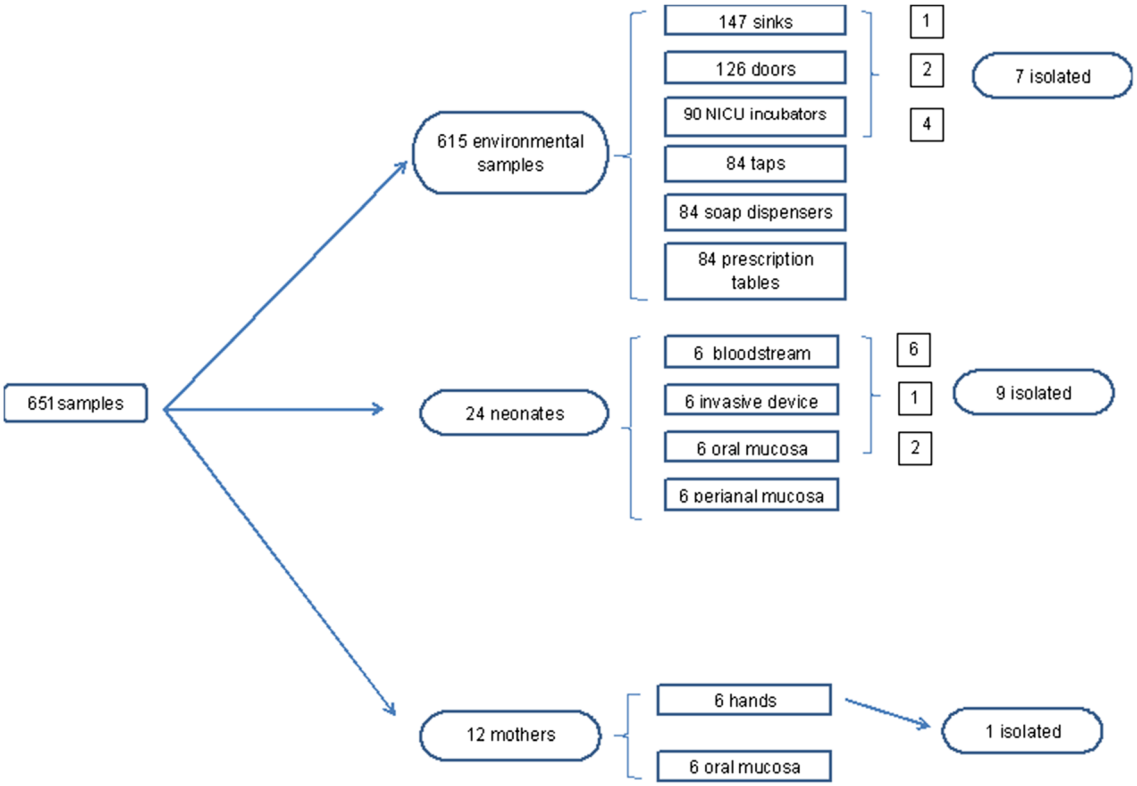


**Fig. 1** Flowchart of study NICU sample collection sites and *C. parapsilosis* sensu stricto isolation sites

colony diameter (dc) per hemolysis zone plus the colony diameter (dcp). The results were expressed as dc/dcp: Hi (hemolytic index) and classified as negative (Hi = 1), moderate (0.63 < Hi < 1), and marked (Hi ≤ 0.63) [19].

DNAse activity was expressed as positive or negative according to the presence or absence of a whitening halo around the colony [20].

Evaluation of biofilm formation capacity was done according to the methodology proposed by Pierce et al. [21], with some modifications. Briefly, 10 μL of a 24-h culture in SDA from each isolate was inoculated in 15 mL of yeast, peptone, and dextrose broth (YPD) and incubated for 12 to 18 h at 35 °C. Subsequently, the material was centrifuged at 4000 rpm for 5 minutes, the supernatant discarded, and the pellet washed three times with phosphate-buffered saline (PBS) (1:10), centrifuged again and resuspended in PBS. Then, the suspension was diluted in RPMI 1640 (Himedia, Mumbai, India) supplemented with glucose and buffered with MOPS ([N-morpholino] propane sulfonic acid) (Hexis, São Paulo, Brazil) with turbidity equivalent to 0.5 of the McFarland scale. One hundred microliters was dispensed into each well of 96-well plates. After incubation at 35 °C for 24 h, the samples were washed three times with 1× PBS to remove planktonic cells and added 100 μL of 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (Sigma-Aldrich, Darmstadt, Germany) and 0.5 mg/mL (0.1 mM solution) of menadione (Sigma-Aldrich, Darmstadt, Germany). The plates were wrapped with aluminum paper and incubated again at 35 °C for 2 h. After this time, 80 μL of each sample was transferred to another plate and read in a spectrophotometer with wave compliance of 490 nm. The test was done four times, and results were expressed according to the mean of the absorbance observed in each test. The results of the metabolic activities of biofilms were classified according to the absorbance: (1) low metabolic activity (OD < 0.097), (2) moderate metabolic activity (0.097 < OD < 0.2), and (3) high metabolic activity (OD > 0.2) [22].

## Antimicrobial susceptibility testing

The evaluation of susceptibility to antifungals fluconazole (Fluoxol, La Paz, Bolivia), amphotericin B (Cristalia, São Paulo, Brazil), and micafungin (Raffo, Buenos Aires, Argentina) was done by the broth microdilution method, as described in documents M27-A3 and M27-S4 [23, 24]. From a recent culture on SDA, a suspension was made in RPMI 1640 supplemented with glucose (2%) and buffered with MOPS containing $1 \times 10^6$ cells/mL. Then, 100 μL was pipetted in 96-well plates containing different concentrations of each antifungal, resulting to a final concentration in each well, ranging from 0.03 μg/mL to 16 μg/mL for amphotericin B and 0.015 to 8 μg/mL for micafungin, and ranging from 0.125 to 64 μg/mL for fluconazole. After 18–24 h of incubation at 35 °C, the plates

were read in a spectrophotometer with a wavelength of 490 nm. Tests were done in duplicate and results expressed by the mean absorbance observed for each antifungal concentration. Tests were performed with azole and micafungin, and the lowest concentration capable of inhibiting around 50% of the microorganism growth evaluated in relation to the control well was classified as the minimal inhibitory concentration (MIC) of the drug. However, the MIC for amphotericin B and micafungin was the lowest concentration capable of inhibiting around 80% of the microorganism growth. The MIC interpretation and cut-off points for each antifungal were considered according to CLSI documents M27-A3 and M27-S4: for fluconazole, isolates with MIC ≤ 8 μg/mL were considered sensitive; MIC between 16 and 32 μg/mL, sensitive dose dependent; and MIC ≥ 64 μg/mL, resistant. For amphotericin B, isolates were sensitive with MIC ≤ 1 μg/mL and resistant with MIC > 1 μg/mL. For micafungin, MIC ≤ 2 μg/mL was considered sensitive and MIC ≥ 4 μg/mL resistant. [23, 24].

For the quality control of the susceptibility tests, we used the strains *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019.

### RAPD-PCR analysis of isolates

The extraction of genomic DNA from each isolate was made from SDA culture (24h) according to Bolano et al. [25].

For the RAPD-PCR analysis, the oligonucleotide primers OPA09 and OPB11 (Operon Technologies Inc.) were used, with the following sequences: OPA09: 5′-GGGTAACGCC-3′, OPB: 5′-GTAGACCCGT-3′. The reactions and amplification products were performed according to Riceto et al. [26].

The profile of each sample was analyzed visually. Bands were represented as present (1) or absent (0). Genetic relationships (coefficients of similarity) were calculated using the Jaccard coefficient (Sj), which is based on the position of the fragments [26]. A value of Sj of 1.00 and 0.99 represents the same genotype, values between 0.80 and 0.99 represent clonally related samples (highly similar but not identical), and values less than 0.80 represent distinct samples. The dendrogram based on the Sj values was generated by the UPGMA (unweighted pair group method with arithmetical averages) method using the MultiVariate Statistical Package (MVSP) version 3.22 (Kovach Computing Services, UK). For a more precise determination of the similarity and discrimination of the studied isolates, a combined analysis of the results obtained with the two primers was done.

### Statistical analysis

In order to evaluate the similarity of samples included in the study, a group analysis technique (cluster analysis) was applied considering the numerical variables (susceptibility to antifungal factors and expression of virulence) and genetic variables (genotype test). Since this was a data set with numerical and categorical variables, the Gower coefficient [27] was used to determine the dissimilarity between the elements through the distances between them. This coefficient varies from 0 to 1, so the closer to zero, the smaller the distance between them and, therefore, the greater the similarity. The isolates grouped to the left of the dashed line are considered highly similar, due to cluster formation near zero. Cluster names were A, B, C, D, E, and F. Furthermore, Ward's hierarchical method was used for grouping samples. The dendrogram was constructed using the R program, version 3.5.1.

### Ethics committee

The Committee of Ethics in Research approved the research with human participants at the Federal University of Uberlandia (Approval number 989.139/2015).

## Results

Seventeen isolates of *C. parapsilosis* sensu stricto were identified, of which seven (41.1%) were from the NICU environment, one (5.9%) from a newborn's mother, and nine (53%) samples from six newborns.

Of the six newborns that had positive blood cultures (35.3% of the isolates) for *C. parapsilosis* sensu stricto, two also were positive for an oral mucosa sample (11.7% of the isolates) for the same microorganism. This specie was also isolated from the hands of the mother of a newborn who had *C. parapsilosis* candidemia.

During the study period, 194 newborns were admitted to the NICU, with 13 (6.7%) having BSI caused by *Candida* species, and *C. parapsilosis* sensu stricto being responsible for six (46.1%) of these cases. The six newborns included in the study were preterm (gestational age < 37 weeks) and had very low birth weight (< 1500 g), with four (66.6%) weighing less than 1000 g. Regarding the use of invasive devices, all had a central peripheral insertion catheter (PICC) and parenteral nutrition, four (66.6%) used an umbilical central venous catheter (CVC), four (66.6%) required mechanical ventilation, and a drain was inserted in three (50%).

Four (66.6%) neonates used three or more classes of antimicrobial agents, and two (33.3%) had previous bacterial infection. The mean length hospitalization of neonates who had *C. parapsilosis* sensu stricto was 42 days. Two newborns (33.3%) died (NB2 and NB3). NB2 presented an unstable clinical condition and spontaneous intestinal perforation, requiring drainage in the abdominal cavity and drain insertion. At the time of surgery, NB2 was using fluconazole, piperacillin/tazobactam, and teicoplanin. Isolates of *C. parapsilosis* sensu stricto were grown from the cultures collected from

**Table 1** Clinical characteristics of the six newborns that had candidemia by *C. parapsilosis* sensu stricto included in the study

| Newborn | Place | Site/ isolation place | Isolation date | PICC (days) | CVC umbilical (days) | NP (days) | Drain (days) | ATM use (days) | Length of hospital stay (days) | Evolution |
|---|---|---|---|---|---|---|---|---|---|---|
| NB1 | Bed 34 | Oral mucosa<br>Blood | March 2016<br>March 2016 | 53 | 0 | 13 | 9 | 27 | 62 | Discharge |
| NB2 | Bed 39 | Blood<br>Drain | July 2016<br>July 2016 | 27 | 12 | 34 | 6 | 33 | 39 | Death |
| NB3 | Bed 32 | Blood | Sep 2016 | 14 | 8 | 21 | 8 | 19 | 22 | Death |
| NB4 | Bed 35 | Blood<br>Bed | Jan 2017<br>Jan 2017 | 32 | 8 | 18 | 0 | 18 | 36 | Discharge |
| NB5 | Bed 32 | Blood<br>Oral mucosa | March 2017<br>March 2017 | 30 | 2 | 13 | 0 | 5 | 33 | Discharge |
| NB6 | Bed 41 | Blood | March 2017 | 9 | 0 | 8 | 0 | 17 | 46 | Discharge |

*PICC*, peripheral insertion central catheter; *CVC*, central venous catheter; *NP*, parenteral nutrition; *ATM*, antimicrobial; *Jan*, January; *Sep*, September

the bloodstream and abdominal drain samples of this newborn.

Table 1 shows the clinical characteristics of the six neonates included in the study who had candidemia by *C. parapsilosis* sensu stricto.

## Expression of virulence factors

All *C. parapsilosis* sensu stricto expressed at least one of the three virulence factors investigated. Hemolytic activity was observed in 100% of the isolates. Six (54.5%) of the 11 isolates with moderate activity were from the environment, two from BSI, and the remaining three from oral mucosa, abdominal drain, and from their mother's hands. Six isolates presented high activity, of which four (66.6%) were from the bloodstream, one from the environment (16.7%), and the last from the oral mucosa (16.7%).

Regarding the enzyme DNAse, 14 (82.3%) isolates were positive, five from BSI, five from the environment of the NICU (three from bed and two from the doors), two from the oral mucosa of the newborns who had candidemia, one from the hands of the mother of the NB that had BSI, and the last one from an invasive device (abdominal drain). The three isolates that did not present DNAse enzyme activity were from blood cultures and the NICU environment.

Concerning the biofilm formation, only one isolate of the bloodstream (5.9%) did not form a biofilm. The other isolates were able to express biofilm, with the majority being classified as high metabolic activity (76.5%). Table 2 shows the expression of hemolysin, DNAse, and biofilm production.

## Minimal inhibitory concentration

All isolates were susceptible to amphotericin B and micafungin. One environmental isolate (CP14) had MIC above 64 μg/mL for fluconazole. The MIC values of each sample are presented in Table 3.

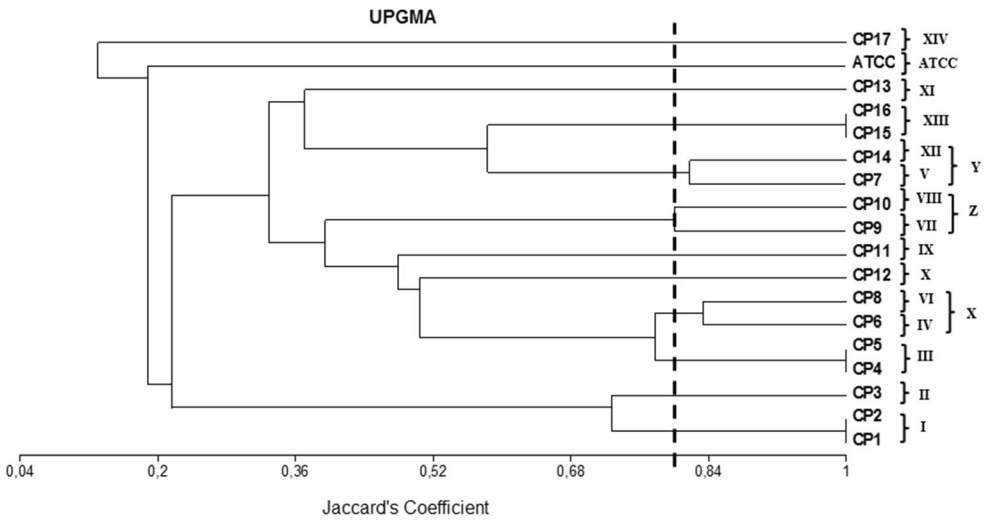


**Fig. 2** RAPD-PCR dendrogram of *C. parapsilosis* sensu stricto with combined analysis of primers OPA09 and OPB11. Note: Numbers I–XIV: Genotypes observed after combined analysis of RAPD-PCR

**Table 2** Hemolytic activity, DNAse, and biofilm production of the isolates of *C. parapsilosis* sensu stricto

| Isolation | Isolates, *N* (%) | Hemolytic activity score[a], *n* (%) | | | DNAse expression, *n* (%) | | Biofilm score[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | Negative | Positive | 1 | 2 | 3 |
| Bloodstream | 6 (100%) | 0 | 2 (33.4%) | 4 (66.6%) | 1 (16.7%) | 5 (83.3%) | 1 (16.7%) | 1 (16.7%) | 4 (66.6%) |
| Colonization | 4 (100%) | 0 | 3 (75%) | 1 (25%) | 0 | 4 (100%) | 0 | 1 (25%) | 3 (75%) |
| Environment | 7 (100%) | 0 | 6 (85.7%) | 1 (14.3%) | 2 (28.6%) | 5 (71.4%) | 0 | 1 (14.3%) | 6 (85.7%) |
| Total | 17 (100%) | 0 | 11 (64.7%) | 6 (35.3%) | 3 (17.7%) | 14 (82.3%) | 1 (5.9%) | 3 (17.6%) | 13 (76.5%) |

[a] Hemolytic activity scores: 1, did not show enzymatic activity (Hi = 1); 2, moderate enzymatic activity (0.63 < Hi < 0.99); 3, high enzymatic activity (Hi ≤ 0.63)

[b] Biofilm score: 1, low metabolic activity; 2, moderate metabolic activity; 3, high metabolic activity

## Molecular profile: RAPD-PCR

RAPD-PCR of *C. parapsilosis* sensu stricto with the combined analysis of primers OPA09 and OPB11, together with the analysis of microbiological identification and isolation sites, revealed the existence of fourteen different genetic profiles for *C. parapsilosis* sensu stricto (Fig. 2 and Table 3). The two isolates from NB1 (CP1 and CP2) showed the same

**Table 3** Phenotypic and genotypic characterization of the isolates of *C. parapsilosis* sensu stricto

| *C. parapsilosis* strains | Site/isolation | Molecular profile OPA09/OPB11 | Phenotypic profile | | | | | | Phenotype/ genotype |
|---|---|---|---|---|---|---|---|---|---|
| | | | Hemolytic activity | DNAse | Biofilm | MIC | | | |
| | | | | | | Fluco (μg/mL) *S* ≤ 8 SDD: 16–32 *R* ≥ 64 | AmB (μg/mL) *S* ≤ 1 *R* ≥ 1 | Mica (μg/mL) *S* ≤ 2 *R* ≥ 2 | |
| CP1 | Oral mucosa | I | Moderate | + | HMA | 0.5 | 0.06 | 0.5 | A |
| CP2 | BS | I | Moderate | + | HMA | 0.5 | 0.06 | 0.015 | A |
| CP3 | Mother's hands | II | Moderate | + | HMA | 1.0 | 0.06 | 0.5 | G |
| CP4 | Bed 33 | III | Moderate | + | MMA | 0.5 | 0.125 | 1.0 | E |
| CP5 | Bed 35 | III | Moderate | Negative | HMA | 1.0 | 0.125 | 1.0 | E |
| CP6 | Entrance door of NICU workers | IV | Moderate | + | HMA | 0.5 | 0.06 | 0.5 | H |
| CP7 | Bed 35 | V | Moderate | + | HMA | 1.0 | 0.06 | 1.0 | F |
| CP8 | Entrance door to the hand hygiene room | VI | Moderate | + | HMA | 0.5 | 0.125 | 0.5 | F |
| CP9 | BS | VII | High | + | HMA | 0.5 | 0.125 | 0.5 | B |
| CP10 | Invasive device: drain | VIII | Moderate | + | HMA | 1.0 | 0.06 | 0.5 | B |
| CP11 | BSI | IX | Moderate | + | HMA | 0.25 | 0.06 | 0.25 | C |
| CP12 | BSI | X | High | + | LMA | 0.5 | 0.06 | 0.5 | C |
| CP13 | Bed 35 | XI | | + | HMA | 0.5 | 0.25 | 0.5 | I |
| CP14 | Sinks used for hand hygiene | XII | High | Negative | HMA | >64 | 0.125 | 0.015 | J |
| CP15 | BS | XIII | High | + | HMA | 2.0 | 0.06 | 2.0 | D |
| CP16 | Oral mucosa | XIII | High | + | HMA | 2.0 | 0.06 | 1.0 | D |
| CP17 | BS | XIV | High | Negative | HMA | 0.5 | 0.06 | 0.5 | K |

*BS*, bloodstream; +, positive; *LMA*, low metabolic activity; *MMA*, moderate metabolic activity; *HMA*, high metabolic activity; *Fluco*, fluconazole; *AmB*, amphotericin B; *Mica*, micafungin. *S*: sensible; *SDD*, sensible dose dependent; *R*: resistant. MIC values of control strains: *C albicans* ATCC90028 and *C. parapsilosis* ATCC22019: Fluco, 2 μg/mL; AMB, 1 μg/mL; MICA, 0.03 μg/mL. Numbers I–XIV: genotypes observed after combined analysis of RAPD-PCR results with OPA09 and OPB11 primers. Letters A–K: denomination of clusters observed after combined analysis of phenotypic and genotypic tests

genetic profile represented by genotype I. Similarly, two environmental isolates, CP4 and CP5, both obtained from the same collection but in different beds, were considered identical since they presented the same genotype III. Finally, molecular analysis revealed that CP15 and CP16 isolates from the oral mucosa and bloodstream of NB5, respectively, are identical—genotype XIII.

The environmental isolates (CP6, CP7, CP8, and CP14) show high similarity and formed clusters X and Y, respectively. The isolates of NB2 (CP9 and CP10) form cluster Z (Fig. 2).

Table 3 shows the phenotypic and genotypic characterization of the 17 isolates of *C. parapsilosis* sensu stricto. When the associated analyses of phenotypic and genotypic data were performed to determine the similarity between the 17 isolates (Fig. 3), the isolates obtained from the same newborn, such as CP1 and CP2, CP9 and CP10, and CP15 and CP16, showed high similarity: clusters A, B, and D, respectively. The bloodstream and abdominal drain tip isolates of NB2 are represented by the abbreviation CP9 and CP10; the clinical features are summarized in Table 1, suggesting intestinal translocation. In addition, environmental isolates obtained from nearby collections, such as CP4 and CP5, and CP7 and CP8, were also considered highly similar (clusters E and F, respectively).

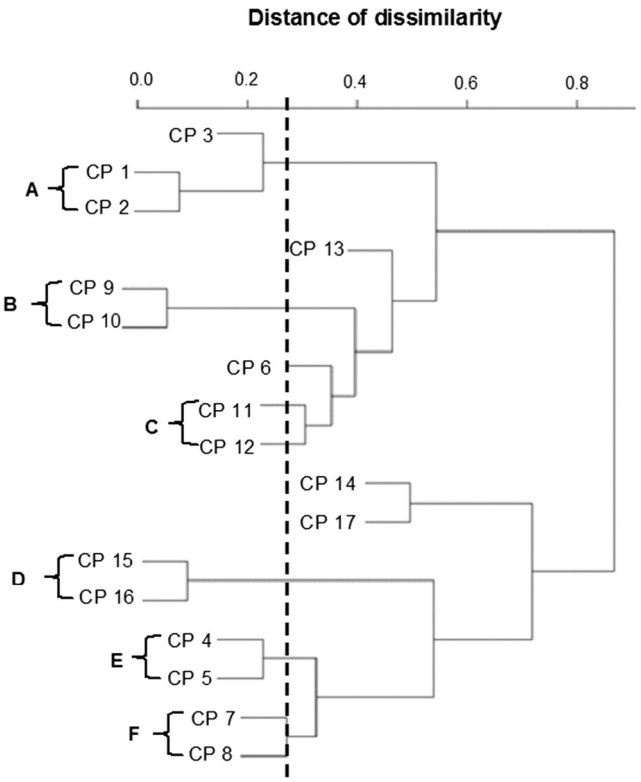


**Fig. 3** Dendrogram of the combined analysis of the phenotypic tests (DNAse and hemolysin expression, biofilm production, and antifungal susceptibility profile) and genotypic tests (RAPD-PCR) performed with the 17 isolates of *C. parapsilosis* sensu stricto, showing the similarity between them**.** Note: The isolates grouped to the left of the dashed line are considered highly similar, due to cluster formation near zero. A, B, C, D, E, and F: name of the clusters

The isolate from the mother's hands of NB1 was considered similar to cluster A, formed from the isolates collected from this newborn. Figure 3 shows the similarity relationship presented among the isolates after the combined analysis of all tests performed.

Figure 4 shows a layout of the NICU. It also shows the location of the bed in which each newborn was hospitalized during the period when they developed candidemia. It also indicates the isolation positions of the environmental samples of *C. parapsilosis* sensu stricto. The location at which strains with similar phenotypes and genotypes have been isolated is indicated.

## Discussion

Nosocomial infections by *Candida* species are a public health problem due to their severity and high morbidity and mortality rates [5, 28]. It is important to evaluate the presence of *C. parapsilosis* in the hospital environment, especially in sectors where severe patients are admitted, since many invasive infections are exogenous. Therefore, in this study, we emphasized the importance of determining the transmission routes in NICU in order to detect pathogen sources and reservoirs, as well as to establish prevention measures, such as adequate disinfection of the environment.

During the study period, *C. parapsilosis* sensu stricto was responsible for 46.1% of the candidemia cases, which corresponds to 12.2% of the total bloodstream infections. These results are higher than those observed in previous studies conducted in the same NICU, where the frequency of *Candida* spp. was below 10% [7, 29]. This can be related to the admission of newborns with severe health condition, especially because most newborns of the study were extremely low weight. *C. parapsilosis* complex has been responsible for more than one-third of invasive fungal infections in extremely low birth weight newborns in Brazil and other countries [30–32].

Invasive infections are related to both the virulence of microorganisms and the host immune system. The virulence profile of environmental isolates is important to estimate the pathogenicity of microorganisms. In this study, all environmental samples expressed at least one of the three virulence factors investigated. Similar observations were reported by Menezes et al. [33], in isolates of *Candida* spp. from health professionals' hands. These results show the need for studies aimed at evaluating the virulence of microorganisms in the hospital environment, especially those receiving severe health condition patients, such as neonates, as well as emphasizing the importance of correct and constant hygiene both in the hospital environment and in the professionals' hands as a way of minimizing the circulation, maintenance, and horizontal transmission of these microorganisms.

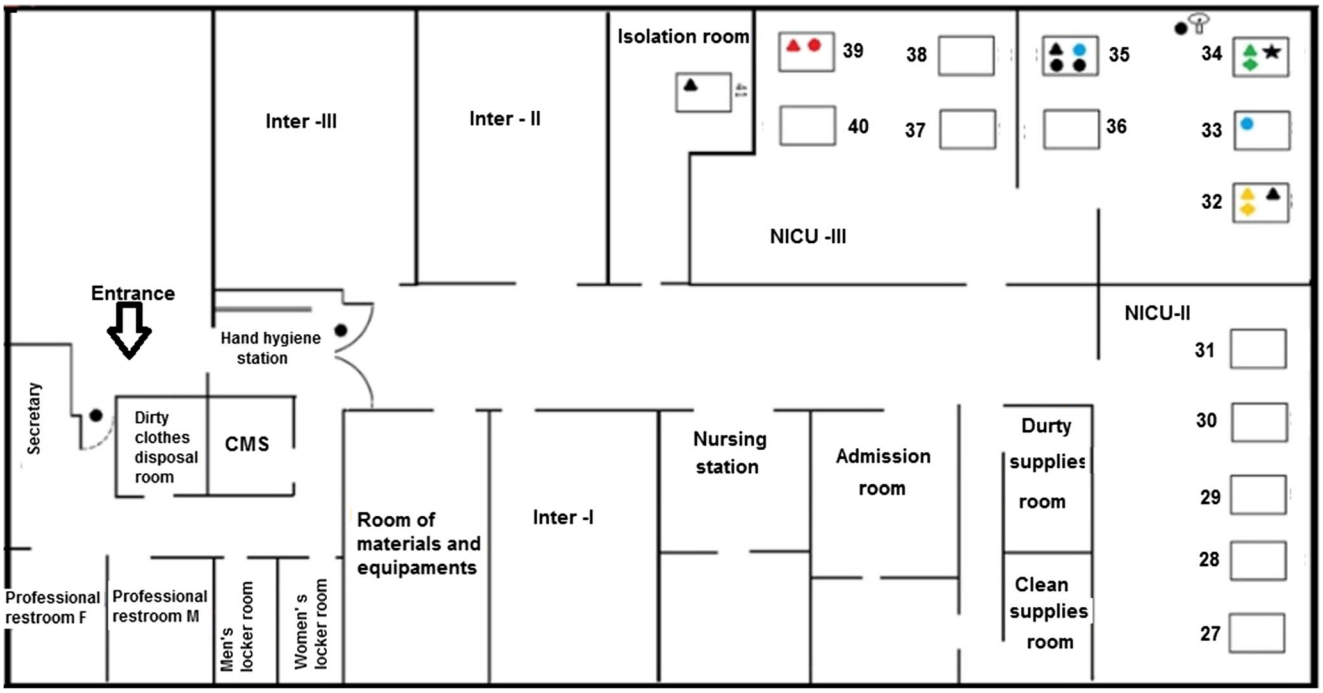


**Fig. 4** Schematic representation of the NICU and location of the isolates of *Candida parapsilosis* sensu stricto from the bloodstream, oral mucosa of neonates and mothers, and also from the environment. ▭ : incubators. ▲ : samples from the neonates' bloodstream. ◆ : isolated samples from the neonates' oral mucosa. ★ : isolated from the mother of a neonate who has BSI. ● : isolated from the environment, including the NICU's entrance door, entrance door to the hand hygiene room, two incubators, a hand wash sink located on unit III and a drain. Green: cluster A. Red: cluster B. Gold: cluster D. Blue: cluster E, according to similarity dendrogram

Azole-resistant *C. parapsilosis* sensu stricto is emerging in Brazilian hospitals, causing outbreaks in adult ICUs; there are reports of the permanence of the isolate in these units for up to 2 years [34, 35]. Furthermore, *C. parapsilosis* is reported to be able to persist on plastic healthcare surfaces for up to 28 days, presenting greater viability for culture than *C. auris* and sufficient time for transmission in critical units [36]. Thus, the isolation of *C. parapsilosis* sensu stricto from the NICU environment under study, which produces biofilm and is resistant to fluconazole, draws attention because fluconazole is the best choice for the treatment of invasive candidiasis [37] and because this microorganism can remain in the NICU for a long period or be carried to both neonates and other hospitals, causing serious infections. In addition, the presence of this resistant microorganism in the hygiene sink suggests that it came from a professional's hands who worked there.

Molecular analysis revealed a high similarity between the isolates of *C. parapsilosis* sensu stricto from oral colonization and candidemia of two newborns, suggesting infections of endogenous origin. Sabino et al. [11] also demonstrated that most candidemia originate endogenously and are preceded by colonization of the gastrointestinal tract, mucous, or skin. The genetic similarity by molecular analysis of the isolate collected from the bloodstream and abdominal drain tip suggests a possible intestinal translocation. This was seen in a newborn with spontaneous intestinal perforation, with posterior abdominal drain insertion, and following the use of parenteral nutrition and fluconazole.

The joint analysis of phenotypic and molecular data showed a similarity between the two isolates coming from NB1 and that of the mother's hands. Despite the fact that contact between the mother and newborn should be stimulated [38], this finding reveals the need for constant campaigns to parents of neonates with severe health condition about the importance of hand hygiene before and after contact with their neonate in order to decrease the chance of the transmission of pathogenic microorganisms.

High similarity was found between isolates from incubators, the NICU access door, the door of the hand hygiene room, and especially between CP4 and CP5 isolates, obtained in the same collection period in nearby beds. This similarity suggests that they were taken from one bed to another through the hands, drawing attention to the importance of awareness of those professionals who are in direct contact with the patient and the need for correct hand hygiene before and after contact with patients. These campaigns should also focus on the correct cleaning of the hospital environment in order to prevent such microorganisms from being carried from one hospital unit to another, or even brought from outside to inside the hospital, leading to contact with already critically ill neonates.

This study shows that environmental contamination can be an important reservoir of potentially pathogenic

microorganisms, since isolates of *C. parapsilosis* sensu stricto collected from the hospital environment were able to express virulence factors, such as hemolysins and DNAse and biofilm production, and one showed fluconazole resistance. In addition, the phenotypic and genetic similarity observed between some isolates revealed that microorganisms have the capacity to remain in the hospital environment for months and to disseminate within it, suggesting cross-transmission or even intestinal translocation. We also highlight the importance of care with those preterm newborns who have high chances of intestinal translocation when colonized previously, which was confirmed in this study by genetic and phenotypic similarity between isolates of blood cultures and abdominal drains.

**Acknowledgments** We would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the Scientific Scholarship Initiation, Curso Técnico em Análises Clínicas (ESTES-UFU) for providing the necessary equipment to carry out all the tests, Professor Regina Helena Pires of the Franca University for assigning the ATCC strains of the *C. parapsilosis* complex required to carry out the molecular differentiation of these isolates through the RFLP, and finally, Rodrigo Augusto Machado de Moraes for help in translating and correcting the article into English.

**Funding information** This study received financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Financing Code 001.

## Compliance with ethical standards

The Committee of Ethics in Research approved the research with human participants at the Federal University of Uberlandia (Approval number 989.139/2015).

**Conflict of interest** The authors declare that they have no conflict of interest.

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