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Candida species isolated from pigeon (*Columbia livia)* droppings may express virulence factors and resistance to azoles

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ABSTRACT

Even though it is widely known that *Cryptococcus* spp. may transmit cryptococcosis trough aerosol formed when dried birds (mainly pigeons) droppings are dispersed and become airborne, little is known about the role of these birds in harboring other pathogenic yeasts in their gastrointestinal tract, feathers and beaks, specifically because these animals often stay and reproduce close or even above air conditioner units. Here we evaluated the prevalence of pathogenic yeasts isolated from pigeon droppings collected in the outside area of a University Hospital in Brazil. We also aimed to investigate the pathogenic potential and antifungal susceptibility of *Candida* species of medical interest isolated from these samples. Therefore, we performed the evaluation of virulence factors attributes expression *in vitro*, including the ability to adhere to human buccal epithelial cells and biofilm formation and to produce lytic enzymes, such as phospholipases, proteinases and hemolysins. Antifungal susceptibility testing against fluconazole, itraconazole, amphotericin and micafungin was also performed. The *Candida* genus was the most prevalent in our study, with several medically important species being isolated. Of note, these strains were able to express several virulence factors *in vitro*, clearly showing their pathogenic potential. Our study was able to demonstrate that *Candida* spp. isolated from pigeon droppings may express virulence factors in the same manner of clinical isolates, suggesting a pathogenic potential for these yeasts. The fact these strains were collected from the outside area of a tertiary hospital may be of interest, because they may be a source of infection, specifically to immunocompromised hosts.

1. Introduction

Wild birds, including pigeons (*Columbia livia*), have long been considered a major source of pathogenic yeasts (Rosario et al., 2017). In Brazil, the domestic pigeon population has increased significantly, becoming an environmental problem and influencing public health (Costa et al., 2010). In addition to microbial colonization in their beaks, feet and feathers, pigeons can spread pathogenic yeasts to the environment and humans mainly from aerosol present in dried droppings (Rosario et al., 2017).

Several epidemiological data have been published regarding the isolation of yeasts from pigeon droppings around the world (Rosario et al., 2017; Wu et al., 2012). Although most studies in this area focus on the evaluation of the prevalence of yeasts belonging to the genus *Cryptococcus* (Teodoro et al., 2013), yeasts of other genera, such as *Candida*, *Rhodotorula* and *Trichosporon* may be isolated from pigeon droppings and could have the potential to cause fungal infections including invasive life-threatening diseases (Cafarchia et al., 2006a; Costa et al., 2010).

In a study performed by Jang et al. (2011) in Seoul, Korea, 306 feces samples were collected from public squares and courts. One hundred and twenty six samples leaded to yeasts isolation. *Candida glabrata* was the most prevalent species (34.1%), followed by *C. famata* (12.7%). *C. krusei* and *Trichosporon asahii* (1.16%) were isolated in lower numbers. More recently, another study performed in Gran Canaria Island (Spain) focused on the isolation of non-*Cryptococcus* yeasts from 83 pigeon droppings samples and found *C. guilliermondii* and *C. albicans* as the most frequently isolated species (49.4 and 15.7% of the samples, respectively). Other important yeasts of medical interest, such as *Trichosporon* spp. and *Rhodotorula mucilaginosa* were also recovered

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(Rosario et al., 2017).

Because *Candida* spp. may be part of the normal human microbiota, the development of invasive candidiasis is mostly of endogenous origin. However, exogenous infections are gaining notoriety (Nucci et al., 2010). In this context, domestic and wild birds may serve as reservoirs for yeasts with high pathogenic potential, dispersed through the air and may further colonize and cause severe infections with high mortality rates (Rosario et al., 2017).

The main *Candida* spp. virulence factors include the ability to adhere to human buccal epithelial cells (CEBH) and endothelial cells, the yeast-to-hyphae transition (morphogenesis, a typical phenotype of *C. albicans*), the production of extracellular hydrolytic enzymes (proteinases, hemolysins and phospholipases), biofilm formation, and the ability to evade the attack of immune system cells (Zuza-Alves et al., 2016).

Studies concerning the investigation of the *in vitro* expression of virulence factors by *Candida* spp. isolated from pigeon droppings are practically nonexistent. However, Jang et al. (2011) performing a similar investigation found that 6 isolates of *C. glabrata* and a single isolate of *C. krusei*, isolated from pigeon's fecal samples in Seoul, Korea, showed proteinase activity.

Because of the previously mentioned factors, it is important to know the pathogenic potential of yeasts that may be a source of colonization and further cause invasive candidiasis, specifically evaluating the expression of virulence factors *in vitro* and its possible relationship with the pathogenesis process of infections caused by *Candida* spp. Besides, the determination of drugs susceptibility profiling of these isolates obtained from the environment nearby a tertiary University hospital are of fundamental importance in order to implement more effective therapeutics and to establish nosocomial infection control approaches. Therefore, the main objectives of this study were to evaluate the pathogenicity and antifungal susceptibility of *Candida* species isolated from pigeon droppings from an external area of a tertiary hospital in the city of Natal, Rio Grande do Norte State, Brazil. To the best of our knowledge, this is the first study in the world to demonstrate that non-*Cryptococcus* yeasts isolated from pigeon droppings may express virulence factors *in vitro* and exhibit resistance to azole antifungal drugs.

2. Materials and methods

2.1. Collection and processing of fecal samples from pigeons

The study included 19 samples of pigeon droppings collected outside the building of a tertiary university hospital, including the windows of the wards, located in Natal City, Rio Grande do Norte State, Brazil, from April 2012 to March 2014.

Approximately 1 g of dried and/or fresh pigeon stools were collected and diluted in saline solution (NaCl 0.9%) in a ratio of 1: 9, shaken on a mechanical stirrer for 10 min and allowed to stand for 1 h. Subsequently, 100 µL of the supernatant was removed and inoculated into two CHROMagarCandida® plates (CHROMagar ™ *Candida*, Difco, USA) with the aid of a Drigalski loop. Each plate was incubated at 30 °C and 37 °C for 96 h, respectively. Colonies with distinct phenotypic characteristics (appearance, color, size) were selected for subsequent identification, proportionally to the total amount presented in each sample.

2.2. Identification of isolated yeasts

After cultures growth, yeasts were again plated on CHROMagar *Candida* to check for purity and screening for different color colonies (Baumgartner et al., 1996). Species identification was based on the characteristics of the cells observed microscopically after cultivation on corn meal agar added Tween 80, as well as by classical methodology (Yarrow, 1998). The most representative pathogenic *Candida* species used for the virulence tests studies were also identified by PCR using species-specific primers (Arastehfar et al., 2019; Ge et al., 2012; Li et al., 2014). After identification, all yeast strains were cultured in YPD broth (Yeast Peptone Dextrose, yeast extract 10 g / L, dextrose 20 g / L, peptone 20 g / L) overnight at 30 °C, and then were transferred to cryotubes containing 20% glycerol and stored at -80 °C. The isolates belong to the fungi culture collection of the Medical and Molecular Mycology Laboratory, Department of Clinical and Toxicological Analyses, Federal University of Rio Grande do Norte.

2.3. Strains used in the present study for the determination of virulence factors expression and antifungal susceptibility testing

We chose 59 isolates representing the most important pathogenic *Candida* species to perform the evaluation of the expression of virulence factors in *vitro* as follows: *C. parapsilosis* complex (n = 23), *C. tropicalis* (n = 18), *C. krusei* (n = 15), *C. glabrata* (n = 2) and *C. rugosa* (n = 1). Of note, *C. parapsilosis* ATCC22019, *C. tropicalis* ATCC13803, *C. krusei* ATCC6258, *C. glabrata* ATCC 2001 and *C. rugosa* ATCC10571 and were used as controls for all the identification tests and the determination of virulence attributes *in vitro* and antifungal susceptibility testing (totalizing 64 isolates with the controls included). Of note, control values were not used to calculate averages for each species, but represented a reference to compare the results.

2.4. Inoculum standardization for Candida spp. virulence factors evaluated in vitro

For the phenotypic characterization of the different isolates, the strains were initially grown in NGY broth medium (Difco Neopeptone 1 g/L, Dextrose 4 g/L; Difco yeast extract 1 g/L). When cells are inoculated by "wet looping" in this medium (with a ring loop loaded with a yeast suspension film rapidly immersed in the medium and removed) and incubated for 18–24 h in shaker at 30 °C, 200 rpm, an inoculum of approximately 2×10^8 cells/mL is produced (Chaves et al., 2013). Cultures were spectrophotometrically measured at a wavelength of 600 nm ranging from 0.8 and 1.2 (Biochrom Libra S32). Subsequently, *Candida* spp. cells were diluted to obtain the specific inoculum needed for each attribute of virulence evaluated *in vitro*.

2.5. Candida spp. adherence to human buccal epithelial cells (HBEC)

Candida spp. cells were grown overnight to stationary phase in NGY (0.1% Neopeptone [Difco], 0.4% glucose and 0.1% Yeast Extract [Difco]) at 30 °C and were mixed with human buccal epithelial cells (HBEC) from healthy volunteers at a ratio of 10 yeast cells per HBEC. The mixtures were incubated at 37 °C for 1 h with shaking; then cells were vortexed, formalin-fixed and transferred to a microscope slide. The number of *Candida* spp. cells adhered to 150 HBEC was determined with the operator blinded to the nature of the material on the slide. Tests were done in triplicate (Zuza-Alves et al., 2016).

2.6. Candida spp. biofilm formation

Biofilm formation assays were performed according by Melo et al. (2011). At first, 100 µL aliquots of a standardized cell suspension (10^7 cells/mL) were transferred to flat bottom 96 well microtiter plates and incubated for 1.5 h at 37 °C in a shaker at 75 rpm. As controls, eight wells of each microtiter plate were handled in an identical fashion, except that no *Candida* cell suspensions were added. Following the adhesion phase, cell suspensions were aspirated and each well was washed twice with 150 µL of PBS to remove loosely adherent cells. A total of 100 µL of YNB medium ("Yeast Nitrogen Base", DifcoTM) with 50 mM of glucose (D-glucose monohidratada P.A., Cinética) was added to each of the washed wells and incubated at 37 °C in a shaker at 75 rpm. Biofilms were allowed to develop for 66 h and quantified by the crystal violet assay. Briefly,

the biofilm-coated wells of microtiter plates were washed twice with 150 µL of PBS and then air dried for 45 min. Subsequently, each of the washed wells was stained with 110 µL of 0.4% aqueous crystal violet solution for 45 min. Afterward, each well was washed four times with 350 µL of sterile distilled water and immediately distained with 200 µL of 95% ethanol. After 45 min, 100 µL of destaining solution was transferred to a new well and the amount of the crystal violet stain in the referred solution was measured with a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltda.) at 570 nm. The absorbance values for the controls were subtracted from the values for the test wells to minimize background interference. Interpretation of biofilm production was according to the criteria described by Stepanovic et al. (2007).

2.7. Candida spp. proteinase production

Proteinase activity was determined by a method Zuza-Alves et al., 2016. Fifty-microliter samples from NGY cultures were grown in 5 mL YCB + BSA medium (11.7 g/L Yeast Carbon Base [Difco]; 10 g/L glucose; 5 g/L bovine serum albumin, fraction V [Sigma–Aldrich]) rotated in a rotator shaker at 30 °C for 72 h, 200 rpm. Proteolytic activity was determined by measuring the increase in trichloroacetic acid soluble products absorbing at 280 nm in triplicate after 1 h incubation of the culture supernatant with BSA substrate at 37 °C. Specific activity was expressed as OD_{280} nm/OD_{600} nm of the culture. OD readings equal to or below 0.02 were considered below the limit of detection of the technique and were represented as negative.

2.8. Candida spp. hemolysin production

In order to evaluate hemolysin production, we followed the methodology proposed by Luo et al. (2001) with some adaptations. *Candida* spp. cells were initially cultured on SDA at 35 °C for 18 h. Strains were next grown overnight in NGY broth. Ten microliters of cell culture were seeded in triplicate on the surface of SDA containing 7% fresh sheep blood (Ebe-Farma) and 3% glucose, contained in Petri dishes of 155 mm of diameter. The plates were incubated for 48 h at 37 °C in an atmosphere with 5% CO_2. After the incubation period, the presence of a clear halo around the inoculum indicated positive hemolysis. The diameter of colonies and zones of hemolysis were measured in order to obtain the hemolysis index (HI) for each strain. HI was determined by dividing the colony diameter by the precipitation zone plus colony diameter, which allowed classification of isolates in strong, moderate and weak producers, according to Linares et al. (2007). As a positive control we used a beta hemolytic strain of *Streptococcus pyogenes* (Group A). The reference strain of *Candida parapsilosis* ATCC22019 was used as a negative control (Luo et al., 2001).

2.9. Candida spp. phospholipase production

For detection of the phospholipase activity, the method of Price et al. (1982) was used. Overnight NGY cultures were diluted and standardized to a concentration of 2×10^5 cells/mL; and the suspension of cells was inoculated in triplicate on the surface of Phospholipase agar (10 g peptone, 40 g dextrose, 16 g agar, 80 mL Egg Yolk Emulsion [Fluka] was added to 1000 mL of distilled water 1000 mL). The plates were incubated at 30 °C for 72 h. After the incubation period, the diameters of the colonies and the halo formed around them were measured. The Pz (phospholipase zone) was determined by dividing the colony diameter by the precipitation zone plus colony diameter. The isolates were classified as follows, according to tertiles distribution: $Pz = 1$ as negative phospholipase activity; $0.82 \leq Pz \leq 0.88$ as weak; $0.75 \leq Pz \leq 0.81$ as moderate; $0.67 \leq Pz \leq 0.74$ as strong phospholipase producers.

2.10. Antifungal susceptibility profile of Candida spp

Solutions of fluconazole (FLU), itraconazole (ITC), micafungin (MCF), and amphotericin B (AMB) were prepared in accordance with the guidelines of M27-A3 CLSI document, being diluted in RPMI 1640 (Roswell Park Memorial Institute) (Angus buffers and Biochemical, Niagara Falls, NY, USA) buffered 3-(N-morpholino) propanesulfonic acid (MOPS) to pH 7.0. Antifungal drugs tested were diluted serially in 10 different concentrations, namely: FLU (Pfizer Incorporated, New York, NY, USA) to 0.125–64 µg/mL; ITC (Pfizer Incorporated, New York, NY, USA), MCF (Merck, Rahway, NJ, USA); and AMB (Sigma Chemical Corporation, St. Louis, MO, USA) to 0.015–8 µg/mL. The inoculum of all strains tested were obtained from 2 h cultivation in SDA at 35 °C and an initial suspension prepared with 90% transmittance determined spectrophotometrically at 530 nm. Then, two serial dilutions were made, the first in saline solution (1:100) and the second in RPMI (1:20), in order to obtain final concentration of 10^3 cells/mL. Susceptibility to antifungal agents was evaluated by broth microdilution, as recommended within document CLSI M27-A3. Aliquots of 100 µL of the final inoculum solution were dispensed in microtiter plates of 96 wells containing 100 µL of various concentrations of the tested drugs. Finally, the plates were incubated at 37 °C and test reading taken after 24 h incubation for echinocandins and fluconazole, and after 48 h for the other azoles and AMB. All strains were tested in duplicate. MIC was defined for azoles and echinocandins to the lowest drug concentration which showed about 50% reduction in turbidity as compared to the positive control well. For AMB, the MIC was defined as the lowest concentration able to inhibit any growth visually perceptible (Zuza-Alves et al., 2016).

2.11. Statistical analysis

Data were analyzed using the statistical software “Graph Pad, Prism” version 6.0 and “Stata” version 11.0. Results were presented as mean ± standard deviation, and differences were analyzed by the One-sample t-test, while the Spearman coefficient was used to assess the correlation between virulence factors. For all the analyses, P-values less than 0.05 were considered significant and the confidence interval of 95% was selected. In addition all the values obtained for some of virulence attributes tests in *vitro* were divided onto tertile categories as weak, moderate or strong producers.

3. Results

3.1. Microbiological profiling of Candida spp

Of the 19 samples collected, 184 yeast environmental isolates were obtained from pigeon dropping collected around the University Hospital. *Candida* was the most prevalent genus (77/184, 41.8%), followed by *Trichosporon* spp. (44/184, 23.9%), *Rhodotorula* spp. (43/184, 23.4%), *Cryptococcus* spp. (13/184, 7.1%) and other genera (7/184, 3.8%). Therefore, we decided to follow up this investigation including the characterization of the attributes of virulence *in vitro* with 59 *Candida* spp. strains of medical interest as follows: *C. parapsilosis* complex, *C. tropicalis*, *C. krusei*, *C. glabrata* and *C. rugosa* (Tables 2–4).

3.2. Adherence of Candida spp. to human buccal epithelial cells (HBEC)

All the strains were able to adhere to HBEC. However, the isolates showed variable expression of this specific virulence factor *in vitro* (Table 2). *C. tropicalis* showed a remarkable ability to adhere to the buccal epithelia. These data can be observed by both comparing the strains of this species with reference strain ATCC13803 and when the average values of adhesion for all the strains of this species were compared to the strains belonging to all the other *Candida* spp. evaluated ($P < 0.05$; Tables 2 and 3). Tertile analysis revealed that most of

the isolates of the other *Candida* spp. showed low ability to adhere to HBEC. In addition, most of them were also statistically significant more adherent than the reference strains of each species (Table 2). Strains belonging to *C. parapsilosis* species complex were generally statistically more adherent than those belonging to *C. krusei* (Table 3). When we evaluated each species separately, for the *C. parapsilosis* species complex, LMMM525 was the lowest adherent strain (19 ± 2 *Candida* cells/ 150 HBEC), while LMMM348 was the most adherent strain (274 ± 2 *Candida* cells/ 150 HBEC; Table 2). As previously mentioned, *C. tropicalis* was represented by highly adherent strains, including the most adherent isolate of the study (LMMM496; 487 ± 2 *Candida* cells/ 150 HBEC). The lowest value for adhesion of this species was found to the strain LMMM312 (75 ± 1 *Candida* cells/ 150 HBEC). Tertile analysis of strains belonging to *C. krusei* revealed low adhesion to HBEC, with number ranging from 27 ± 1 *Candida* cells/ 150 HBEC (LMMM503) to 91 ± 2 *Candida* cells/ 150 HBEC (LMMM493). *C. glabrata* also showed limited adhesion capacity, (53 ± 2 *Candida* cells/ 150 HBEC (LMMM180) to 58 ± 4 *Candida* cells/ 150 HBEC (LMMM327). The only isolate of *C. rugosa* was considered a weak adherent strain.

3.3. Evaluation of biofilm formation in Candida spp

Approximately half of the isolates of the present study was able to form biofilm on polystyrene microtiter plates, while 25 isolates were considered negative. In addition, several strains belonging to the *C. parapsilosis* species complex and *C. tropicalis* were not able to produce biofilm. Even so, most of the biofilm forming isolates of *C. tropicalis* together with *C. krusei* were considered either moderate or strong biofilm producers, while *C. glabrata* and *C. parapsilosis* species complex strains showed weak biofilm formation (Table 2). This data was reinforced with average values comparisons, which showed statistically significant different results between *C. tropicalis* and *C. krusei* strains with the other *Candida* spp. (Table 3). For C. *parapsilosis* species complex strains, the OD readings ranged from OD_{570nm} of 0.07 ± 0.00 (LMMM346, LMMM513, LMMM530) to OD_{570nm} of 0.3 ± 0.00 (LMMM500). *C. tropicalis* reading ranges were as follows: OD_{570nm} of 0.09 ± 0.01 (LMMM332) to OD_{570nm} of 0.59 ± 0.03 (LMMM331). Tertile analysis showed that *C. krusei* showed very variable levels of biofilm formation, with the lower biofilm producing strain showing an OD_{570nm} of 0.1 ± 0.01 (LMMM503), whereas the higher biofilm producer strain, showed a remarkable optical density of OD_{570nm} of 0.8 ± 0.01(LMMM212). The isolate LMMM180 of *C. glabrata* was the only biofilm producer of this species (OD 570 nm 0.06 ± 0.00). The only strain represented by *C. rugosa* was considered a strong biofilm producer, according to tertile analysis (Table 2).

3.4. Determination of production of proteinase in Candida spp

From the 64 (59 strains plus 5 controls) isolates included in the present study, only 18 isolates did not produce the enzyme. Of note, OD readings lower than 0.02 were considered negative, because they represent an amount below the limit of detection of this technique. The lower amount of proteinase activity was OD_{280nm}/ OD_{260nm} of 0.03 ± 0.00 (LMMM314), while the higher level was OD_{280nm}/ OD_{260nm} of 0.38 ± 0.00 (LMMM212), respectively, both from *C. krusei* (Table 2). This species, together with *C. parapsilosis* species complex had the strongest proteinase producer strains (Table 2). This result was also confirmed with the average values obtained for all the strains of each species, except when this comparison was performed between the two previously mentioned strains and *C. glabrata* (Table 3), Fifty six percent of *C. parapsilosis* species complex isolates produced more proteinase than the reference strain ATCC22019 (OD_{280nm}/ OD_{260nm} of 0.03 ± 0.00 (Table 2). Most *C. tropicalis* isolates had lower proteinase activity than the reference strain *C. tropicalis* ATCC13803, while the opposite happened for the *C. rugosa* control strain (Table 1). Tertile analysis classified most of the isolates of *C. parapsilosis* complex as moderate and strong producers, while isolates of *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. rugosa* were classified as moderate and weak producers of the enzyme. The unique *C. rugosa* strain produced the following amount of proteinase: OD_{280nm}/OD_{600nm} of 0.05 ± 0.00 (Table 2).

Table 1
Prevalence of yeasts obtained from pigeon droppings samples collected at the outside area of a University Hospital in the city of Natal, Rio Grande do Norte, Brazil.

Genus or species of yeast	Number of environmental strains (n)
C. parapsilosis species complex	23
C. tropicalis	18
C. krusei	15
C. glabrata	2
C. rugosa	1
Candida spp.	18
Cryptococcus spp.	13
Exophiala spp.	3
Geotrichum sp.	1
Rhodotorula spp.	43
Sporobolomyces spp.	3
Trichosporon spp.	44
Total	184

3.5. Determination of production of phospholipase in Candida spp

Phospholipase production was detected in 96.6% of the isolates when all the *Candida* spp. were evaluated. Besides, the levels of enzyme production varied among isolates of each species. Most *C. parapsilosis* species complex strains were considered weak to moderate phospholipase producers (Table 1), while *C. krusei* and *C. glabrata* were mostly classified as strong producers (Table 2). Once again, the amount of secretion of this enzyme was generally lower for the reference strains. For the *Candida parapsilosis* species complex, 65.2% of the strains produced low levels of phospholipase (15 out of 23 isolates), but the isolate LMMM348 stands out for showing a high production of the enzyme (0.49 ± 0.03, Table 2).

3.6. Determination of production of hemolysins by Candida spp

Most of the strains in the present study were able to produce moderate to high beta hemolysis on sheep blood agar According to Linares et al. (2007) classification, 30 isolates (50.8%) presented strong hemolytic activity (HI ≤ 0.43) which is inversely proportional to the HI, while only seven of them (11.7%) showed low production (HI ≥ 0.56; Table 2). The environmental isolates also produced equal or above levels of hemolysins than the reference strains of each species. We could detect only two isolates that did not produce the referred enzyme (LMMM346 and LMMM348, both belonging to the *C. parapsilosis* species complex). Of note, when all the isolates were analyzed together, *C. glabrata* strains average values for the HI was statistically significant lower than the HI found for the others species (meaning high hemolytic activity) and a statistically significant difference was also observed between strains belonging to the *C. parapsilosis* species complex and *C. tropicalis* (Table 3). In C. *parapsilosis* species complex strains the hemolytic activity ranged from 0.34 ± 0.01 (LMMM524) to 0.60 ± 0.04 (LMMM522) and 10 isolates of *C. parapsilosis* were strong hemolysins producers (Table 2). *C. tropicalis* hemolytic activity ranged from 0.23 ± 0.00 (LMMM496) to 0.65 ± 0.04 (LMMM518) and all *C. glabrata* isolates showed lower HI than the reference strain ATCC2001. Interestingly, the single isolate of *C. rugosa* (LMMM 216) had a moderate hemolytic enzyme production, while its reference strain *C. rugosa* ATCC10571 did not produce the enzyme.

Table 2
Virulence factors of *Candida* spp. isolates obtained from pigeon droppings collected at the outside area of a University Hospital in the city of Natal, Rio Grande do Norte State, Northeast Brazil.

	Nº of *Candida* cells adhered to 150 HBEC	Biofilm Formation ($O.D._{570nm}$)	Proteinase Activity ($O.D._{280nm}/O.D._{600}$ nm)	Phospholipase zone (Pz)	Hemolysis Index (HI)
C.parapsilosis ATCC22019	65 ± 3	0.04 ± 0.00	0.03 ± 0.00	0.83 ± 0.00	Negative
LMMM208	94 ± 4*	Negative	0.06 ± 0.01*	0.62 ± 0.03	0.47 ± 0.01*
LMMM339	105 ± 4*	Negative	Negative	0.73 ± 0.06	0.52 ± 0.02*
LMMM346	131 ± 10*	0.07 ± 0.00	0.03 ± 0.01	0.81 ± 0.03	Negative
LMMM347	34 ± 17*	Negative	0.09 ± 0.03*	0.69 ± 0.04*	0.39 ± 0.01*
LMMM348	274 ± 2*	0.16 ± 0.00*	0.05 ± 0.01*	0.49 ± 0.03*	Negative
LMMM349	143 ± 3*	Negative	0.12 ± 0.01*	0.71 ± 0.04*	0.48 ± 0.02*
LMMM494	52 ± 3*	Negative	0.13 ± 0.01*	0.77 ± 0.04	0.39 ± 0.03*
LMMM500	59 ± 7	0.3 ± 0	Negative	0.78 ± 0.03	0.53 ± 0.04*
LMMM502	49 ± 15	0.06 ± 0	0.08 ± 0.00*	0.71 ± 0.04*	0.43 ± 0.06*
LMMM506	32 ± 3*	Negative	0.06 ± 0.01*	0.73 ± 0.04	0.40 ± 0.03*
LMMM507	45 ± 3*	Negative	0.11 ± 0.01*	0.73 ± 0.04	0.39 ± 0.02*
LMMM508	104 ± 3*	Negative	0.08 ± 0.01*	0.73 ± 0.03	0.39 ± 0.02*
LMMM510	50 ± 2	Negative	0.07 ± 0.01*	0.78 ± 0.04	0.38 ± 0.02*
LMMM512	26 ± 4*	Negative	0.07 ± 0.02*	0.59 ± 0.03*	0.50 ± 0.00*
LMMM513	33 ± 3*	0.07 ± 0.01	0.13 ± 0.00*	0.69 ± 0.03*	0.46 ± 0.01*
LMMM521	68 ± 2*	Negative	0.04 ± 0.01	0.83 ± 0.00	0.46 ± 0.01*
LMMM522	82 ± 3*	Negative	0.04 ± 0.01	0.68 ± 0.04*	0.60 ± 0.04*
LMMM523	25 ± 2*	0.09 ± 0.01	Negative	0.70 ± 0.00*	0.44 ± 0.01*

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Table 2 (*continued*)

LMMM524	208 ± 7*	0.18 ± 0.02*	Negative	0.69 ± 0.03*	0.34 ± 0.01*
LMMM525	19 ± 2*	Negative	0.03 ± 0.00	0.78±0.00	0.44 ± 0.01*
LMMM527	23 ± 3*	0.19 ± 0.03*	Negative	0.78 ± 0.00	0.43 ± 0.03*
LMMM528	30 ± 3*	Negative	0.10 ± 0.00*	0.53 ± 0.00*	0.56 ± 0.01*
LMMM530	93 ± 2*	0.07 ± 0.00	0.07 ± 0.00*	0.71 ± 0.068	0.36 ± 0.01*
C.tropicalis ATCC13803	32 ± 2	0.07 ± 0.00	0.04 ± 0.00	0.73 ± 0.05	0.44 ± 0.01
LMMM179	262 ± 2*	0.16 ± 0.04*	0.04 ± 0.00	0.70 ± 0.05	0.39 ± 0.01*
LMMM182	193 ± 2*	0.21± 0.04*	0.03 ± 0.00	0.75 ± 0.04	0.43 ± 0.01
LMMM310	103 ± 2*	Negative	0.05 ± 0.00	0.73 ± 0.01	0.39 ± 0.02*
LMMM312	75 ± 1*	0.59 ± 0.03*	0.03 ± 0.00	0.68 ± 0.04	0.38 ± 0.01*
LMMM325	95 ± 2*	Negative	0.03 ± 0.00	Negative	0.37 ± 0.01*
LMMM331	420 ± 1*	Negative	0.03 ± 0.00	0.73 ± 0.03	0.38 ± 0.04
LMMM332	120 ± 9*	0.09 ± 0.01	Negative	0.67 ± 0.05	0.37 ± 0.04
LMMM333	123 ± 5*	Negative	Negative	0.73 ± 0.03	0.48 ± 0.01*
LMMM335	128 ± 7*	Negative	Negative	0.71 ± 0.03	0.39 ± 0.04
LMMM337	132 ± 6*	Negative	0.04 ± 0.00	0.62 ± 0.00*	0.39 ± 0.02*
LMMM468	127 ± 6*	Negative	0.03 ± 0.00	0.66 ± 0.04	0.38 ± 0.00*
LMMM476	151 ± 11*	Negative	0.04 ± 0.00	0.55 ± 0.02*	0.35 ± 0.02*
LMMM483	115 ± 8*	0.28 ± 0.01*	Negative	0.60 ± 0.03*	0.38 ± 0.00*
LMMM489	168 ± 2*	0.26± 0.02*	Negative	0.61 ± 0.02*	0.36 ± 0.00*
LMMM495	154 ± 8*	0.2 ± 0.02*	0.05 ± 0.01*	0.57 ± 0.04*	0.36 ± 0.00*
LMMM496	487 ± 2*	0.5 ± 0.01*	Negative	0.57 ± 0.04*	0.33 ± 0.00*
LMMM509	165 ± 7*	Negative	Negative	0.68 ± 0.05	0.43 ± 0.04
LMMM518	121 ± 4*	Negative	Negative	0.68 ± 0.05	0.65 ± 0.04*
C. krusei ATCC6258	35 ± 1	0.11 ± 0.00	0.19 ± 0.03	0.49 ± 0.04	0.48 ± 0.02

(*continued on next page*)

Table 2 (*continued*)

LMMM166	32 ± 3*	0.15 ± 0.00	Negative	0.41 ± 0.06	0.48 ± 0.06
LMMM174	52 ± 4	0.14 ± 0.00*	0.04 ± 0.01*	0.33 ± 0.02*	0.33 ± 0.03*
LMMM212	27 ± 2	0.8 ± 0.01*	0.38 ± 0.00*	0.44 ± 0.02	0.55 ± 0.02*
LMMM311	30 ± 2	0.29 ± 0.03*	0.04 ± 0.00*	0.38 ± 0.04*	0.47 ± 0.02
LMMM313	64 ± 1*	0.41 ± 0.01*	0.05 ± 0.00*	0.45 ± 0.01	0.46 ± 0.01
LMMM314	46 ± 2*	0.35 ± 0.03*	0.03 ± 0.00*	0.44 ± 0.04	0.52 ± 0.02
LMMM315	50 ± 2*	0.21 ± 0.03*	0.06 ± 0.00*	0.51 ± 0.03	0.61 ± 0.01*
LMMM334	47 ± 3*	0.34 ± 0.04*	Negative	0.38 ± 0.04*	0.45 ± 0.04
LMMM336	49 ± 5*	0.2 ± 0.03*	Negative	0.78 ± 0.04*	0.38 ± 0.02*
LMMM338	38 ± 3	0.18 ± 0.00*	0.04 ± 0.01*	0.39 ± 0.02*	0.44 ± 0.01*
LMMM490	35 ± 4	0.14 ± 0.03*	0.04 ± 0.01*	0.48 ± 0.02	0.55 ± 0.01*
LMMM493	91 ± 2*	0.21 ± 0.00*	0.03 ± 0.00*	0.51 ± 0.02	0.57 ± 0.01*
LMMM497	35 ± 1	0.36 ± 0.01*	0.03 ± 0.00*	0.37 ± 0.03*	0.53 ± 0.00*
LMMM503	27 ± 1	0.1 ± 0.01	Negative	0.45 ± 0.03	0.55 ± 0.02*
LMMM505	32 ± 3	0.14 ± 0.01	Negative	0.40 ± 0.03*	0.49 ± 0.03
C. glabrata ATCC2001	54 ± 6	0.07 ± 0.00	0.05 ± 0.02*	0.52 ± 0.05	0.41 ± 0.01
LMMM180	53 ± 2	0.06 ± 0.00	0.05 ± 0.00*	0.36 ± 0.04*	0.39 ± 0.02
LMMM327	58 ± 4	Negative	0.10 ± 0.01*	0.46 ± 0.00	0.38 ± 0.01*
C. rugosa ATCC10571	78 ± 5	0.34 ± 0.00	0.02 ± 0.00*	Negative	Negative
LMMM216	30 ± 2*	0.61 ± 0.00*	0.05 ± 0.00*	Negative	0.46 ± 0.02*

*P < 0.05 through Student *t*-test
**Not Tested.
Dark grey dashed borders stand for strong production, whereas light grey mean moderate production.
Non-colored numbers mean weak or negative expression of virulence factors.

3.7. Evaluation of antifungal susceptibility profiling of Candida spp. by the broth microdilution method

All MIC values obtained by the control microorganisms were compatible with the values expected by the CLSI methodology (2012), ensuring the reliability of the results obtained for the isolates tested. We observed that all *Candida* spp. isolates were susceptible to amphotericin B and Micafungin (Table 4).

Regarding the susceptibility profiling to fluconazole, all the environmental isolates of the *C. parapsilosis* complex were susceptible to this drug. However, only 1 out 18 isolates of *C. tropicalis*, (LMMM 325, 5.6%) was considered susceptible to this antifungal drug, while 17 of them (94.4%) were considered resistant with MICs higher than 64 μg/mL. In addition, all isolates of *C. krusei* were considered resistant (as expected), where 13 strains had MIC ≥ 64 μg/mL, while for two of them the MIC ≥ 8 μg/mL. For *C. glabrata,* the isolate LMMM180 was resistant to this drug, whereas the isolate LMMM327 was considered susceptible. The single isolate of *C. rugosa* was susceptible to fluconazole (Table 4).

Regarding the susceptibility profiling to itraconazole, all the isolates belonging to the *C. parapsilosis* species complex, and *C. rugosa* were considered susceptible to this antifungal drug. However, only a single isolate of *C. tropicalis* was susceptible to this drug (5.6%; LMMM325), while 94.4% of isolates were resistant (MIC = ≥64 μg / ml). Regarding the isolates of *C. krusei*, the isolate LMMM314 showed resistance against itraconazole (MIC = ≥1 μg/mL) and the isolate LMMM338 was susceptible dose-dependent (SDD; MIC = 0.25 μg/mL), while all the others were susceptible. One isolate of *C. glabrata* was susceptible (LMMM180; MIC ≤ 0.125), while the isolate LMMM327 was considered SDD (MIC = 0.25 μg /mL).

4. Discussion

Most of the studies regarding yeasts isolation from pigeon excreta are focused on *Cryptococcus*. In our study, *Candida* spp. together with *Trichosporon* spp. and *Rhodotorula* spp. were more frequently isolated than *Cryptococcus* spp. from pigeon droppings. This fact reinforces the potential role of pigeons as a reservoir of other opportunistic yeasts previously isolated from human infections. Actually, a few other studies have obtained similar results. For instance, Jang et al. (2011), found *C. glabrata* and other *Candida* spp. (including C. *albicans*) more frequently than *Cryptococcus* spp. in a study performed in Seoul, Korea. In the study of Costa et al., 2010 performed in Northeast Brazil (the same region of the present study), *Candida* spp. were also the more frequently

Table 3
Average virulence factors comparisons of *Candida* spp. strains isolated from pigeon droppings collected at the outside area of a University Hospital in the city of Natal, Rio Grande do Norte State, Northeast Brazil.

	Nº of *Candida* cells adhered to 150 HBEC	Biofilm Formation ($O.D._{570nm}$)	Proteinase Activity ($O.D._{280nm}/O.D._{600nm}$)	Phospholipase zone (Pz)	Hemolysis Index (HI)
C. parapsilosis x *C. tropicalis*	77 ± 4 versus 174 ± 4*	0.13 ± 0.00 versus 0.26 ± 0.02*	0.07 ± 0.01 versus 0.03 ± 0.00*	0.71 ± 0.03 versus 0.68 ± 0.03*	0.49 ± 0.03 versus 0.4 ± 0.02*
C. parapsilosis x *C. krusei*	77 ± 4 versus 44 ± 2*	0.13 ± 0.00 versus 0.27 ± 0.02*	0.07 ± 0.01 versus 0.06 ± 0.00	0.71 ± 0.03 versus 0.44 ± 0.03*	0.49 ± 0.03 versus 0.49 ± 0.02
C. parapsilosis x *C. glabrata*	77 ± 4 versus 55 ± 3	0.13 ± 0.00 versus 0.06 ± 0.0	0.07 ± 0.01 versus 0.08 ± 0.01	0.71 ± 0.03 versus 0.41 ± 0.02*	0.49 ± 0.03 versus 0.39 ± 0.02*
C. tropicalis x *C. krusei*	174 ± 4 versus 44 ± 2*	0.26 ± 0.02 versus 0.27 ± 0.01	0.03 ± 0.00 versus 0.06 ± 0.00*	0.68 ± 0.03 versus0.44 ± 0.03*	0.4 ± 0.02 versus 0.49 ± 0.02*
C. tropicalis x *C. glabrata*	174 ± 4 versus 55 ± 3*	0.26 ± 0.02 versus 0.06 ± 0.00*	0.03 ± 0.00 versus 0.08 ± 0.01*	0.68 ± 0.03 versus0.41 ± 0.02*	0.4 ± 0.02 versus 0.39 ± 0.02*
C. krusei x *C. glabrata*	44 ± 2 versus 55 ± 3	0.27 ± 0.01 versus 0.06 ± 0.00*	0.06 ± 0.00 versus 0.08 ± 0.01	0.44 ± 0.03 versus0.41 ± 0.02	0.49 ± 0.02 versus 0.39 ± 0.02*

Table 4
Results of antifungal susceptibility testing of *Candida* spp. strains isolated from pigeon droppings collected at the outside area of a University Hospital in the city of Natal, Rio Grande do Norte State, Northeast Brazil.

	Minimal Inhibitory Concentrations (μg / mL) for the antifungal drugs tested			
Strains	Fluconazole	Itraconazole	Micafungin	Amphotericin B
C. parapsilosis ATCC22019	1 (S)	≤0.125 (S)	0.125 (S)	0125 (S)
LMMM208	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.031 (S)
LMMM339	0.25(S)	≤0.125 (S)	0.125 (S)	0.031 (S)
LMMM346	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.031 (S)
LMMM347	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.031 (S)
LMMM348	0.25(S)	≤0.125 (S)	0.06 (S)	0.062 (S)
LMMM349	0.5(S)	≤0.125 (S)	0.06 (S)	0.031 (S)
LMMM494	0.5(S)	≤0.125 (S)	0.06 (S)	0.125 (S)
LMMM500	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.062 (S)
LMMM502	0.5(S)	≤0.125 (S)	≤0.03 (S)	0125 (S)
LMMM506	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.25 (S)
LMMM507	0.5(S)	≤0.125 (S)	0.06 (S)	0.25 (S)
LMMM508	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.25 (S)
LMMM510	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.5 (S)
LMMM512	0.5(S)	≤0.125 (S)	≤0.03 (S)	0.5 (S)
LMMM513	0.25(S)	≤0.125 (S)	≤0.03 (S)	0.125 (S)
LMMM521	1(S)	≤0.125 (S)	≤0.03 (S)	0.25 (S)
LMMM522	1(S)	≤0.125 (S)	≤0.03 (S)	0.5 (S)
LMMM523	0.5(S)	≤0.125 (S)	≤0.03 (S)	0.125 (S)
LMMM524	1(S)	≤0.125 (S)	≤0.03 (S)	0.5 (S)
LMMM525	0.5(S)	≤0.125 (S)	≤0.03 (S)	0.25 (S)
LMMM527	0.5(S)	≤0.125 (S)	≤0.03 (S)	0.25 (S)
LMMM528	0.5(S)	≤0.125 (S)	≤0.03 (S)	0.25 (S)
LMMM530	0.5(S)	≤0.125 (S)	≤0.03 (S)	0.25 (S)
C. tropicalis ATCC13803	0.25 (S)	0.5 (SDD)	≤ 0.03 (S)	0.1 (S)
LMMM179	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM182	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM310	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM312	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM325	0.125 (S)	≤0.125 (S)	≤ 0.03 (S)	0.062 (S)
LMMM331	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM332	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.125 (S)
LMMM333	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.125 (S)
LMMM335	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.125 (S)
LMMM337	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM468	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.125 (S)
LMMM476	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM483	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM489	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM495	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM496	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM509	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
LMMM518	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.25 (S)
C. krusei ATCC6258	≥ 64 (R)	≥ 64 (R)	≤ 0.03 (S)	0.062 (S)
LMMM166	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.25 (S)
LMMM174	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)
LMMM212	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)
LMMM311	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)
LMMM313	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)
LMMM314	≥ 64 (R)	≥1 R	≤ 0.03 (S)	0.5 (S)
LMMM315	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)
LMMM334	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)
LMMM336	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	1 (S)
LMMM338	≥ 64 (R)	0.25 (SDD)	≤ 0.03 (S)	1 (S)
LMMM490	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	1 (S)
LMMM493	8 (R)	≤0.125 (S)	≤ 0.03 (S)	0.25 (S)
LMMM497	8 (R)	≤0.125 (S)	≤ 0.03 (S)	≤0.031(S)
LMMM503	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.062 (S)
LMMM505	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.25 (S)
C. glabrata ATCC2001	0125 (S)	≤0.125 (S)	≤ 0.03 (S)	0.062 (S)
LMMM180	≥ 64 (R)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)
LMMM327	2 (S)	0.25 (SDD)	≤ 0.03 (S)	0.5 (S)
C. rugosa ATCC10571	≤0.031 (S)	≤0.125 (S)	≤ 0.03 (S)	≤0.031(S)
LMMM216	≤0.031 (S)	≤0.125 (S)	≤ 0.03 (S)	0.5 (S)

isolated species, mainly *C. albicans*. *Rhodotorula* spp. and *Trichosporon* spp. were also recovered from the samples in a lower proportion.

In this study, it is possible to observe that *Candida* spp. strains were able to express the following virulence factors (*in vitro*): adhesion to HBEC, biofilm formation (most, but not all the strains) and the secretion of lytic enzymes, such as hemolysins, proteinases and phospholipases (Table 2).

In general, the isolates evaluated in this study had a variable capacity of adhesion to HBECs. A study performed by Modrzwska & Kurn, emphasizes that adhesion to the surface of host cells is variable according to the different *Candida* species. The strains identified as *C. tropicalis* were characterized by high adhesion capacity. This species is recognized as highly adherent to mammalian epithelial and endothelial cells, only preceded by *C. albicans*. This phenomenon has been widely described with *C. tropicalis* clinical isolates studies from different anatomic sources, including the ones performed in our group (Chaves et al., 2013). In fact, *C. tropicalis* shares adhesins also present in *C. albicans*, including Alsp ('Agglutinin-like sequence; (Punithavathy and Menon, 2012 and Hwp1p ("Hyphal wall protein"; Wan Harun et al., 2013) which may justify similar high adherence for both species.

Likewise, we found in our study that *C. parapsilosis* clinical isolates show moderate adhesion to epithelial cells, while *C. glabrata* and *C. krusei* are weakly adherent. Regarding *C. glabrata*, it is speculated that the reduced adhesion capacity of this species results from an absent adhesin that occurs in *C. albicans* (Silva et al., 2012; Wan Harun et al., 2013). Our findings suggest that the strains obtained from fungal droppings behave similarly to clinical isolates, when adhesion to epithelial cells is investigated.

We also found a remarkable variability to form biofilms in polystyrene microplates for the strains of all species evaluated. *C. krusei* was the species with the highest biofilm formation followed by *C. tropicalis*. Several studies have demonstrated that *C. tropicalis* strains obtained from different anatomical sites and clinical conditions are strongly biofilm producers (Chin et al., 2013).

Data from the literature on biofilm formation of other *Candida* species, such as *C. parapsilosis*, *C. krusei* and *C. glabrata*, are very variable, since some authors report these species as highly biofilm producing, others describe them as low producers of biofilms. It is noteworthy that the great variability on biofilm formation is probably related the methodology employed, including the culture medium, incubation time, plastic material and staining. However, our *C. krusei* strains were markedly strong biofilm producers, which is relatively uncommon among clinical isolates (Treviño-Rangel et al., 2018). Nevertheless, a recent study proposed that this species may form a well-defined cellular community biofilm on a stainless steel, simulating the hydrodynamic strength that they are exposed during food processing in industry, proving that environmental strains of this species may form a strong biofilm (Brugnonia et al., 2012).

The only isolate of *C. rugosa* showed a strong biofilm formation. Interestingly a study with six isolates of *C. rugosa* obtained from blood, urine, bronchoalveolar lavage and vaginal secretion has shown that the isolates are strong biofilm producers (Biasoli et al., 2010) corroborating our results.

Contrary to all expectations, *C. krusei* showed proteinase production, despite this species does not have in its genome the *SAP* gene family (Parra-Ortega et al., 2009). However, recently a study of strains from vulvovaginal candidiasis in Iran reported the production of proteinase by isolates of this species (Shirkhani et al., 2016). Therefore, other proteinases (rather than Saps) mat be present in this species.

Comparing the proteinase activity among the species, *C. krusei, C. glabrata* and *C. parapsilosis* showed no significant difference between them. The isolates of the *C. parapsilosis* complex showed a moderate to strong proteolytic activity, whereas *C. tropicalis* isolates were classified as weak producers. The data obtained in the present study agree with those found by Chaves et al (2013) in a study carried out with clinical strains of kidney transplant recipients with oral candidiasis, from the same area of study.

Several isolates of the present study were able to produce phospholipase, being mostly classified as moderate to strong producers. This is contradictory to several studies that only report the production of this enzyme by *C. albicans* clinical isolates (Tellapagrada et al (2014). Actually, Chin et al. (2013) reported that *C. parapsilosis* and C. *glabrata* strains isolated from blood cultures did not produce the enzyme. Interestingly, our *C. glabrata* strains showed strong phospholipase production. In fact, Ghannoum (2000) reported a positive correlation between *C. glabrata* isolates that showed phospholipase activity and remained in the blood despite therapy with antifungal agents and removal of all intravenous catheters and persistent candidemia. Therefore, such ability to secrete this enzyme may be an important attribute of virulence produced by our pigeon dropping strains.

Although some studies claim that the production of hemolysins is only observed in *C. albicans* (Negri et al., 2010b). Luo et al. (2001) reported that NCAC species may produce this enzyme. These data confirm our findings, since most isolates were able to produce hemolysins.

The highest hemolytic activity occurred in *C. glabrata* isolates, followed by *C. tropicalis*. Our results corroborate with the findings of Riceto et al. (2015) in a study that evaluated hemolytic activity of different *Candida* species, which described a remarkable hemolytic activity for *C. glabrata* and *C. tropicalis* species. In a recent study performed by our group, we were able to demonstrate high hemolysin activity in *C. tropicalis* isolated from environmental sources (Zuza-Alves et al., 2016) corroborating with our findings.

The isolates of *C. krusei* and *C. parapsilosis* complex showed moderate hemolytic activity being contradictory to the findings of Pakshir et al. (2013) with *Candida* species isolated from onychomycosis and buccal mucosa, where most of the isolates of *C. parapsilosis* did not produce the enzyme. The only isolate of *C. rugosa* showed high hemolytic activity, however there are no studies in the literature to compare this result, reinforcing the novelty of our findings.

We find a considerable number of strains resistant to fluconazole and itraconazole, specifically in *C. tropicalis*. Of note, *C. krusei* is intrinsically resistant to fluconazole (Silva et al., 2012). Therefore, our results were expected. Although most isolates of *C. tropicalis* obtained from human patients are susceptible to azoles, some studies have described the occurrence of resistance in some clinical isolates of this species to fluconazole (Guinea, 2014), corroborating with the present study. High Fluconazole resistance was observed among *C. tropicalis* strains isolated from beach sand in Brazil (Zuza-Alves et al., 2016). Fluconazole and Itraconazole cross-resistance have also been previously reported in clinical (Jiang et al., 2013) and environmental investigations (Zuza-Alves et al., 2016).

Of note, *C. glabrata* may frequently develop acquired resistance to fluconazole (Silva et al., 2012) and cross-resistance between this drug and itraconazole have also been previously reported (Denardi et al., 2015).

5. Conclusion

In conclusion we observed the importance of non-*Cryptococcus* yeasts isolated from pigeon droppings. Of note, *Candida* spp. strains were able to express several virulence factors *in vitro*, clearly showing their pathogenic potential. Most importantly, quite a few numbers of strains were resistant to the azole antifungal drugs, specifically *C. tropicalis*, without any proved previous exposition to these antifungal compounds. However, a limitation of our study is that we did not include an outgroup of droppings collected from pigeons living distant of the hospital, to verify if recovered yeasts could also express virulence factors. In addition, the fact that the pigeons may have been in contact with hospital waste containing either antifungal drugs or patients' samples may not be ruled out. These findings, together with the fact that they were collected from the outside of the most important

University Hospital in our city, where air conditioners were present and may throw contaminated pigeon droppings aerosols into the wards, could be important for environment and patients colonization and further infection.

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Competing interests

The authors declare no competing interests.

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References

Arastehfar, A., Fang, W., Pan, W., Lackner, M., Liao, W., Badiee, P., Zomorodian, K., Badali, H., Hagen, F., Lass-Flörl, C., Boekhout, T., 2019. YEAST PANEL multiplex PCR for identification of clinically important yeast species: stepwise diagnostic strategy, useful for developing countries. Diagn. Microbiol. Infect. Dis. 93 (2), 112–119.

Baumgartner, C., Freydiere, A.M., Gille, Y., 1996. Direct identification and recognition of yeast species from clinical material by using albicans ID and CHROMagar *Candida* plates. J. Clin. Microbiol. 34, 454–456.

Biasoli, M.S., Tosello, M.E., Luque, A.G., Magaró, H.M., 2010. Adherence, colonization and dissemination of *Candida dubliniensis* and other *Candida* species. Med. Mycol. 48, 291–297.

Brugnonia, L.I., Cubittoa, M.A., Lozano, J.E., 2012. *Candida krusei* development on turbulent flow regimes: biofilm formation and efficiency of cleaning and disinfection program. J. of Food Engineering. 111, 546–552.

Cafarchia, C., Camarda, A., Romito, D., Campolo, M., Quaglia, N.C., Tullio, D., Otranto, D., 2006a. Occurrence of yeasts in cloacae of migratory birds. Mycopathologia 161, 229–234.

Chaves, G.M., Diniz, M.G., da Silva-Rocha, W.P., de Souza, L.B., Gondim, L.A., Ferreira, M.A., Svidzinski, T.I., Milan, E.P., 2013. Species distribution and virulence factors of *Candida* spp. Isolated from the oral cavity of kidney transplant recipients in Brazil. Mycopathologia 175, 255–263.

Chin, V.K., Foong, K.J., Maha, A., Rusliza, B., Norhafizah, M., Ng, K.P., Chong, P.P., 2013. *Candida albicans* isolates from a Malaysian hospital exhibit more potent phospholipase and haemolysin activities than non-albicans *Candida* isolates. Trop. Biomed. 30, 654–662.

Costa, A.K., Sidrim, J.J., Cordeiro, R.A., Brilhante, R.S., Monteiro, A.J., Rocha, M.F., 2010. Urban pigeons (Columba livia) as a potential source of pathogenic yeasts: a focus on antifungal susceptibility of *Cryptococcus* strains in Northeast Brazil. Mycopathologia 169, 207–213.

Denardi, L.B., Mario, D.A., Loreto, É.S., Santurio, J.M., Alves, S.H., 2015. Synergistic effects of tacrolimus and azole antifungal compounds in fluconazole-susceptible and fluconazole-resistant *Candida glabrata* isolates. Braz. J. Microbiol. 46, 125–129.

Ge, Y.P., Boekhout, T., Zhan, P., Lu, G.X., Shen, Y.N., Li, M., Shao, H.F., Liu, W.D., 2012. Characterization of the *Candida parapsilosis* complex in East China: species distribution differs among cities. Med. Mycol. 50 (1), 56–66.

Ghannoum, M.A., 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13, 122–143.

Guinea, J., 2014. Global trends in *Candida* species distribution. Clin. Microbiol. Infect. 20, 5–10.

Jang, Y.H., Lee, S.J., Lee, J.H., Chae, H.S., Kim, S.H., Choe, N.H., 2011. Prevalence of yeast-like fungi and evaluation of several virulence factors from feral pigeons in Seoul. Korea. Lett. Appl. Microbiol.

Jiang, C., Dong, D., Yu, B., Cai, G., Wang, X., Ji, Y., Peng, Y., 2013. Mechanisms of azole resistance in 52 clinical isolates of *Candida tropicalis* in China. J. Antimicrob. Chemother. 68, 778–785.

Li, J., Shan, Y., Fan, S., Liu, X., 2014. Prevalence of *Candida nivariensis* and *Candida bracarensis* in vulvovaginal Candidiasis. Mycopathologia 178 (3–4), 279–283.

Linares, C.E.B., Loreto, E.S., Silveira, C.P., Pozzatti, P., Scheid, L.A., Santurio, J.M., Alves, S.H., 2007. Enzimatic and hemolytic activities of *Candida dubliniensis* strains. Rev. Inst. Med. Trop. S. Paulo. 49, 2003–2006.

Luo, G., Samaranayake, L.P., Yau, J.Y.Y., 2001. *Candida* species exhibit differential in vitro hemolytic activities. J. Clin. Microbiol. 39, 2971–2974.

Melo, A.S., Bizerra, F.C., Freymüller, E., Arthington-Skaggs, B.A., Colombo, A.L., 2011. Biofilm production and evaluation of antifungal susceptibility amongst clinical Candida spp. isolates, including strains of the *Candida parapsilosis* complex. Med. Mycol. 49, 253–262.

Negri, M., Martins, M., Henriques, M., Svidzinski, T., Azeredo, J., Oliveira, R., 2010b. Examination of potential virulence factors of *Candida tropicalis* clinical isolates from hospitalized patients. Mycopathologia. 169, 175–182.

Nucci, M., Queiroz-Telles, F., Tobon, A.M., Restrepo, A., Colombo, A.L., 2010. Epidemiology of opportunistic fungal infections in Latin America. Clin. Infect. Dis. 51, 561–570.

Pakshir, K., Zomorodian, K., Karamitalab, M., Jafari, M., Taraz, H., Ebrahimi, H., 2013. Phospholipase, esterase and hemolytic activities of Candida spp. Isolated from onychomycosis and oral lichen planus lesions. J. Mycol. Med. 23, 113–118.

Parra-Ortega, B., Cruz-Torres, H., Villa-Tanaca, L., Hernández-Rodríguez, C., 2009. Phylogeny and evolution of the aspartyl protease family from clinically relevant *Candida* species. Mem. Inst. Oswaldo Cruz 104, 505–512.

Price, M.F., Wilkinson, I.D., Gentry, L.O., 1982. Plate method for detection of phospholipase activity in Candida albicans. Sabouraudia 20, 7–14.

Punithavathy, P.M., Menon, T., 2012. Characterization of gene family that mediates the adhesion of biofilms formed by *Candida tropicalis* isolated from HIV and non-HIV patients. BMC Infect. Dis. 12, O8.

Riceto, É.B., Menezes, R.P., Penatti, M.P., Pedroso, R.S., 2015. Enzymatic and hemolytic activity in different *Candida* species. Rev. Iberoam. Micol. 32, 79–82.

Rosario, M.I., Román, F.L., Batista, A.M., Real, V.F., Acosta, A.F., Padilla Del Castillo, D., Déniz, S.S., Ferrer, Q.O., Veja, G.B., Silva, S.F., Acosta-Hernández, B., 2017. Pigeons and their droppings as reservoirs of *Candida* and other zoonotic yeasts. Rev. Iberoam. Micol. 34 (4), 211–214.

Shirkhani, S., Sepahvand, A., Mirzaee, M., Anbari, K., 2016. Phospholipase and proteinase activities of *Candida* spp. Isolates from vulvovaginitis in Iran. J. Mycol. Med. 26, 255–260.

Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 115, 891–899 Iberoam. Micol. 34, 211–214.

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W., Azeredo, J., 2012. *Candida glabrata, Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol. Rev. 36, 288–305.

Teodoro, V.L., Gullo, F.P., SardiJde, C., Torres, E.M., Fusco-Almeida, A.M., Mendes-Giannini, M.J., 2013. Environmental isolation, biochemical identification, and antifungal drug susceptibility of Cryptococcus species. Rev. Soc. Bras. Med. Trop. 46, 759–764.

Treviño-Rangel, R.J., Peña-López, C.D., Hernández-Rodríguez, P.A., Beltrán-Santiago, D., González, G.M., 2018. Association between *Candida* biofilm-forming bloodstream isolates and the clinical evolution in patients with candidemia: an observational nine-year single center study in Mexico. Rev Iberoam. 35, 11–16.

Yarrow, D., 1998. In: Kurtzman, C.P., Fell, J.W. (Eds.), Methods for the Isolation, Maintenance and Identification of Yeasts, 4th ed. Elsevier Science B.V, Amsterdam.

Wan Harun, W.H.A., Jamil, N.A., Jamaludin, N.H., Nordin, M., 2013. Effect of Piper betle and *Brucea javanica* on the differential expression of hyphal wall protein (HWP1) in Non-*Candida albicans Candida* (NCAC) species. Evid. Based Complement. Altern. Med., 397268.

Wu, Y., Du, P.C., Li, W.G., Lu, J.X., 2012. Identification and molecular analysis of pathogenic yeasts in droppings of domestic pigeons in Beijing. China. Mycopathologia. 174, 203–214.

Zuza-Alves, D.L., de Medeiros, S.S., de Souza, L.B., Silva-Rocha, W.P., Francisco, E.C., de Araújo, M.C., Lima-Neto, R.G., Neves, R.P., Melo, A.S., Chaves, G.M., 2016. Evaluation of virulence factors *in vitro*, resistance to osmotic stress and antifungal susceptibility of *Candida tropicalis* isolated from the coastal environment of Northeast Brazil. Front Microbiol. Nov 15 (7), 1783.