Antifungal susceptibility and virulence attributes of animal-derived isolates of *Candida parapsilosis* complex

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This study aimed to identify strains of the *Candida parapsilosis* complex isolated from animals, as well as to assess their *in vitro* antifungal susceptibility profile and *in vitro* production of virulence attributes. We used 28 isolates of *C. parapsilosis sensu lato* recovered from clinically healthy animals. The strains were characterized phenotypically, followed by molecular identification of the species through PCR-restriction enzyme analysis. The susceptibility of the strains to amphotericin B, itraconazole, voriconazole, fluconazole and caspofungin was assessed through broth microdilution. Additionally, the ability of the strains to produce biofilm, phospholipases and proteases was analysed. Molecular analysis showed 13 *C. parapsilosis sensu stricto*, 10 *Candida orthopsilosis* and five *Candida metapsilosis* strains. *In vitro* resistance to fluconazole was observed in three strains of *C. parapsilosis sensu stricto* and two *C. metapsilosis*. All tested strains were able to form biofilms and 23/28 isolates presented protease production, whilst none was able to produce phospholipases. Our study showed that *C. parapsilosis sensu stricto* and *C. orthopsilosis* are the most common species of the *C. parapsilosis* species complex and that these cryptic species present no significant phenotypical differences.

INTRODUCTION

Yeasts of the genus *Candida* are considered commensal of skin and mucosal membranes of humans and other animals (Gómez et al., 2009; Sidrim et al., 2010) and they are among the main fungi involved in opportunistic mycoses in humans. Although *Candida albicans* is the main medically important species of this genus, other non-*albicans Candida* species have been gaining importance as pathogens, including *Candida parapsilosis sensu lato* (Spampinato & Leonardi, 2013).

Based on the genetic diversity among clinical isolates of the *C. parapsilosis* complex, Tavanti et al. (2005) reclassified these yeasts into three distinct species: *C. parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis*. Studies have demonstrated that these species differ in virulence and susceptibility to antifungals (Cantón et al., 2010). Although a large number of studies have been performed with *C. parapsilosis sensu stricto*, there is an increasing number of reports of *Candida orthopsilosis* and *Candida metapsilosis* in clinical isolates.
Characterization of C. parapsilosis from animals

parapsilosis sensu lato recovered from humans, there is a notable lack of data on these yeasts isolated from animals. Therefore, this study aimed at identifying strains of the C. parapsilosis complex isolated from animals, as well as to assess their in vitro antifungal susceptibility profile and in vitro production of virulence attributes.

METHODS

Origin of the studied micro-organisms. We investigated 28 strains of the C. parapsilosis complex recovered from clinically healthy animals: 15 dogs, 10 psittacines (granivorous/frugivorous birds), two raptors (carnivorous birds) and one Macrobrachium amazonicum prawn. The dogs were kept in different households and hence were raised under different dietary conditions. The psittacines were also from different facilities where they were fed with birdseeds and fruits. The raptors were from a rehabilitation centre, where they were fed with meat or mice. The M. amazonicum prawn was raised in a prawn farming laboratory and fed with commercial ration for carnivorous aquatic animals. The strains obtained from these animals were deposited in the collection of the Specialized Medical Mycology Center (CEMM) of Ceará Federal University. The cultures were maintained in saline at room temperature (18 °C) or in glycerol at −20 or −80 °C.

Phenotypical and molecular identification. The strains of the C. parapsilosis complex selected for this study were recovered by seeding on potato agar followed by incubation at 28 °C for 48 h. The strains were then analysed through morphological (growth on cornmeal agar supplemented with Tween 80) and biochemical assays (carbon and nitrogen assimilation and carbon fermentation) as described by Brilhante et al. (2010).

Molecular identification of the C. parapsilosis strains was performed according to the protocol defined by Tavanti et al. (2005), using primers S1F (5’-GTGATGTCGGTAAGATGTT-3’) and S1R (5’-CAATGCGC-AATCTTCCCCA-3’) for amplification of the partial sequence (716 nt) of the gene that encodes the secondary alcohol dehydrogenase (SADH). The amplified DNA was subsequently digested for 90 min with the Banl enzyme (New England Biolabs). The digestion products were submitted to electrophoresis on 2 % (w/v) TAE/agarose gel containing ethidium bromide (0.05 µg ml⁻¹) and were then visualized with a transilluminator. The results obtained were compared with the digestion patterns of the control strains C. parapsilosis ATCC 22019, C. orthopsilosis ATCC 96139 and C. metapsilosis ATCC 96143.

In vitro antifungal susceptibility assay. The MICs of amphotericin B (AMB; Sigma), itraconazole (ITC; Janssen Pharmaceutica), voriconazole (VRC; Pfizer), fluconazole (FLC; Pfizer) and caspofungin (CAS; Merck Sharp & Dohme) were assessed through the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI, 2008). The standard strains C. parapsilosis ATCC 22019, C. orthopsilosis ATCC 96139 and C. metapsilosis ATCC 96143 were included in the tests for quality control. Microdilution plates were incubated at 35 °C and read after 24 h for CAS or 48 h for AMB, ITC, VRCZ and FLC. The MIC for AMB was defined as the lowest concentration capable of inhibiting 100 % of the yeast growth, whilst for azole derivatives and CAS, the MICs were defined as the lowest concentration capable of inhibiting 50 % of yeast growth, when compared with the control well (CLSI, 2008). MICs of >1, ≥1, ≥8, ≥1 and ≥2 µg ml⁻¹ indicated resistance to AMB, ITC, FLC, VRCZ and CAS, respectively (CLSI, 2008; Pfaller et al., 2010).

Phospholipase and protease production assay. The production of phospholipases by the isolates was assessed on egg yolk agar, according to Sidrim et al. (2010). Phospholipase activity (Pz) was determined by calculating the ratio between the diameter of the fungal colony and the total diameter, including the colony and the precipitation zone. Thus, Pz=1 indicated that the isolate was phospholipase-negative, 1>Pz≥0.64 indicated that the isolate was positive for phospholipase activity and Pz<0.64 indicated that the isolate was strongly positive for the secretion of this enzyme (Price et al., 1982).

The same strains were also assessed for the production of proteases, after growth in yeast extract peptone glucose broth at 35 °C for 24 h. The test was performed on BSA agar as described by Vidotto et al. (2004). The protease activity (PA) was determined by calculating the ratio between the diameter of the yeast colony and the total diameter (colony + proteolysis zone around the colony). PA values of 1 indicated the absence of enzymic activity, whilst a PA value <1 indicated protease secretion.

In vitro biofilm formation assay. All the strains were evaluated for biofilm formation, according to the method developed by Peeters et al. (2008) and Ravi et al. (2009), with some modifications. The isolates were grown at 30 °C, initially on Sabouraud agar for 48 h and subsequently in Sabouraud broth for 24 h with 150 r.p.m. of rotary agitation. The cultures were then centrifuged at 6.04 g for 10 min. The pellet was washed three times with sterile PBS and resuspended in RPMI 1640 to a concentration of 0.5 on the McFarland scale. Afterwards, 100 µl aliquots of the inoculum were dispensed into the wells of a 96-well flat-bottomed polystyrene microtitre plate and maintained at 37 °C for 48 h with 150 r.p.m. of rotary agitation. Negative growth control wells were used. After incubation, the supernatant was aspirated from the wells and three washings were performed with PBS/0.05 % Tween 20. Wells were then washed with 100 µl 100 % methanol. Once dried, 100 µl 0.3 % crystal violet was added and left for 20 min. After this period, the crystal violet was removed and two washings with 200 µl sterile distilled water were performed. Finally, 150 µl 33 % acetic acid was added to the stained wells and left for 30 s. After this period, the volume was transferred to another plate, and the absorbance was read immediately using a spectrophotometer at a wavelength of 590 nm (A590). The absorbance data were corrected by subtracting the absorbance values obtained for the negative control. The cut-off value (A590) for the assay was defined as three SDs above the mean A590 of the negative control. At the end, all strains were classified, according to Stephanovic et al. (2000), as non-producers of biofilm (A590 ≤ A590c), weak producers (A590c < A590 ≤ 2 × A590c), moderate producers (2 × A590c < A590 ≤ 4 × A590c) and strong producers (A590 > 4 × A590c).

RESULTS

PCR amplification and restriction digestion analysis identified the 28 C. parapsilosis complex strains tested as the following: 13 strains of C. parapsilosis sensu stricto, 10 C. orthopsilosis and five C. metapsilosis (Fig. 1). C. parapsilosis sensu stricto was found to be more prevalent in the canine samples (12/15), whilst C. orthopsilosis was more prevalent in the avian samples (9/12) (Table 1).

Concerning the susceptibility tests, the MIC range was 0.125–1 µg ml⁻¹ for AMB, 0.03125–0.5 µg ml⁻¹ for ITC, 0.03125–0.125 µg ml⁻¹ for VRC, 0.5–16 µg ml⁻¹ for FLC and 0.0625–2 µg ml⁻¹ for CAS. Resistance to fluconazole was observed against three strains of C. parapsilosis sensu stricto and two of C. metapsilosis, whilst high MICs (2 µg ml⁻¹) were observed for caspofungin against one strain of C. parapsilosis sensu stricto and five of C. orthopsilosis (Table 1).
Regarding the virulence attributes, 23/28 strains (10 C. parapsilosis sensu stricto, 10 C. orthopsilosis and three C. metapsilosis) presented proteolytic activity, whilst none was able to produce phospholipases (Table 1). Furthermore, all the strains were able to form biofilms: one was a weak producer (C. parapsilosis sensu stricto), 20 were moderate producers (eight C. parapsilosis sensu stricto, seven C. orthopsilosis and five C. metapsilosis) and seven were strong producers (four C. parapsilosis sensu stricto and three C. orthopsilosis) (Table 1).

**DISCUSSION**

The importance of *C. parapsilosis* as a cause of candidaemia and invasive candidiasis in humans has increased in recent years. In some European and South American countries, *C. parapsilosis* is the first or second most common aetiological agent of candidaemia in humans (Gómez et al., 2009). In animals, even though *C. parapsilosis sensu lato* can be a component of the microbiota of healthy individuals (Brito et al., 2009; Brilhante et al., 2010) and can cause opportunistic infections (Brito et al., 2009), no study on identification of the cryptic species of the *C. parapsilosis* complex has been published previously.

In our study, 13 out of 28 strains originally classified as *C. parapsilosis* were classified as *C. parapsilosis sensu stricto*, 10 were *C. orthopsilosis* and five were *C. metapsilosis*. It has been reported previously that *C. parapsilosis sensu stricto* is the most prevalent *C. parapsilosis* complex found in humans (Cantón et al., 2011; Feng et al., 2012; Tosun et al., 2013). Similarly, even though the isolates we obtained were from animals raised in different households or facilities, sampled at different moments and on different diets, we found a host-specific distribution of *C. parapsilosis* strains, whereby 12/15 isolates from dogs were identified as *C. parapsilosis sensu stricto*, 9/10 from psittacines were identified as *C. orthopsilosis* and all five *C. metapsilosis* were recovered from carnivore animals (three dogs and two raptors). We believe these findings are probably associated with the feeding habits of the different animal species and their diets, but further studies concerning the ecology of these three cryptic species are necessary to better understand these associations.

Concerning the antifungal susceptibility, in this study we observed five FLC-resistant strains (three *C. parapsilosis sensu stricto* and two *C. metapsilosis*) and six strains with high CAS MIC values (one *C. parapsilosis sensu stricto* and five *C. orthopsilosis*). In contrast, Tosun et al. (2013) did not observe FLC-resistant isolates among the tested strains of the *C. parapsilosis* complex. Therefore, despite the small number of isolates, our results show a higher FLC resistance rate among strains of the *C. parapsilosis* complex recovered from animals. On the other hand, our findings corroborate those of Silva et al. (2012) who reported that the MIC values for echinocandins against human isolates of the *C. parapsilosis* complex tend to be higher than those for other common *Candida* species.

As for the production of virulence attributes, in this study we observed that 23/28 (82%) of the strains of the *C. parapsilosis* complex presented proteolytic activity, whilst none secreted phospholipases. These findings are in contrast to those of Tosun et al. (2013), who obtained a lower rate of protease-producing human strains of the *C. parapsilosis* species complex (31%). We believe that either this difference in protease secretion was observed due to different testing conditions or *C. parapsilosis sensu lato* strains from animals produce more extracellular proteases when compared with human isolates. Concerning the lack of phospholipase secretion among our isolates, it is important to note that non-*albicans* *Candida* species are
also able to produce extracellular phospholipases, but this production is low compared with that of *C. albicans* (Silva et al., 2012) and seems to be highly dependent on the strain (Sidrim et al., 2010; Brilhante et al., 2011). Moreover, all the studied isolates were able to form biofilms, which contrasts with the findings of Tosun et al. (2013), who found a low percentage (26.2%) of biofilm-producing *C. parapsilosis sensu lato* human strains. Once again, either this difference in biofilm production was observed due to different testing conditions or *C. parapsilosis sensu lato* strains from animals produce more biofilm when compared with human isolates. Finally, it is important to highlight that the capacity of *C. parapsilosis sensu lato* to form biofilms has been associated with higher morbidity and mortality in humans when compared with infections by strains that do not form biofilms (Silva et al., 2012).

## CONCLUSIONS

Our study shows that *C. parapsilosis sensu stricto* and *C. orthopsilosis* are the most common species among strains of the *C. parapsilosis* species complex recovered from different animals and that these cryptic species were not different regarding their *in vitro* antifungal susceptibility features and production of virulence attributes. To the best of our knowledge, this is the first study that approaches the specific identification of strains of the *C. parapsilosis* species complex recovered from animals.
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