International Journal of Medical Microbiology 305 (2015) 848–859

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Candida parapsilosis (*sensu lato*) isolated from hospitals located in the Southeast of Brazil: Species distribution, antifungal susceptibility and virulence attributes

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ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 28 July 2015
Accepted 10 August 2015

Keywords:
Candida parapsilosis complex
Antifungal susceptibility
Virulence factors
Pseudohyphae
Hydrolytic enzymes
Biofilm

ABSTRACT

Candida parapsilosis (*sensu lato*), which represents a fungal complex composed of three genetically related species – *Candida parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis*, has emerged as an important yeast causing fungemia worldwide. The goal of the present work was to assess the prevalence, antifungal susceptibility and production of virulence traits in 53 clinical isolates previously identified as *C. parapsilosis* (*sensu lato*) obtained from hospitals located in the Southeast of Brazil. Species forming this fungal complex are physiologically/morphologically indistinguishable; however, polymerase chain reaction followed by restriction fragment length polymorphism of *FKS1* gene has solved the identification inaccuracy, revealing that 43 (81.1%) isolates were identified as *C. parapsilosis sensu stricto* and 10 (18.9%) as *C. orthopsilosis*. No *C. metapsilosis* was found. The geographic distribution of these *Candida* species was uniform among the studied Brazilian States (São Paulo, Rio de Janeiro and Espírito Santo). All *C. orthopsilosis* and almost all *C. parapsilosis sensu stricto* (95.3%) isolates were susceptible to amphotericin B, fluconazole, itraconazole, voriconazole and caspofungin. Nevertheless, one *C. parapsilosis sensu stricto* isolate was resistant to fluconazole and another one was resistant to caspofungin. *C. parapsilosis sensu stricto* isolates exhibited higher MIC mean values to amphotericin B, fluconazole and caspofungin than those of *C. orthopsilosis*, while *C. orthopsilosis* isolates displayed higher MIC mean to itraconazole compared to *C. parapsilosis sensu stricto*. Identical MIC mean values to voriconazole were measured for these *Candida* species. All the isolates of both species were able to form biofilm on polystyrene surface. Impressively, biofilm-growing cells of *C. parapsilosis sensu stricto* and *C. orthopsilosis* exhibited a considerable resistance to all antifungal agents tested. Pseudohyphae were observed in 67.4% and 80% of *C. parapsilosis sensu stricto* and *C. orthopsilosis* isolates, respectively. The secretion of phytase (93% *versus* 100%), aspartic protease (88.4% *versus* 90%), esterase (20.9% *versus* 50%) and hemolytic factors (25.6% *versus* 40%) was detected in *C. parapsilosis sensu stricto* and *C. orthopsilosis* isolates, respectively; however, no phospholipase activity was identified. An interesting fact was observed concerning the caseinolytic activity, for which all the producers (53.5%) belonged to *C. parapsilosis sensu stricto*. Collectively, our results add new data on the epidemiology, antifungal susceptibility and production of potential virulence attributes in clinical isolates of *C. parapsilosis* complex.

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http://dx.doi.org/10.1016/j.ijmm.2015.08.003
1438-4221/

1. Introduction

Candida parapsilosis (*sensu lato*) is the second most frequently opportunistic yeast isolated from bloodstream infections in different clinical settings around the world, especially in Latin America and Asia (Pfaller et al., 2008; Diekema et al., 2009; Nucci et al., 2010; Lockhart et al., 2012). In a relevant way, *C. parapsilosis* (*sensu lato*) is responsible for 20–30% of all fungal infections, particularly those related to the usage of catheter and other medical devices. For all these reasons, *C. parapsilosis* (*sensu lato*) is considered an important public health concern (Lockhart et al., 2008; Diekema et al., 2009; Gonçalves et al., 2010; Nucci et al., 2010; Garcia-Effron et al., 2012). Taking into account the current taxonomy, *C. parapsilosis* (*sensu lato*) was reclassified as a fungal complex formed by three genotypically distinguishable species, *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis*, which reflects the great heterogeneity of isolated strains at both biochemical and genetic levels (Tavanti et al., 2005). Within the complex, *C. parapsilosis sensu stricto* represents the most predominant species, while *C. orthopsilosis* and *C. metapsilosis* are recovered at a much lower incidence (Lockhart et al., 2008; Silva et al., 2009; Bertini et al., 2013). Although the number of epidemiologic investigations is increasing, the current literature still contains little information about the distribution of these opportunistic pathogenic yeasts in clinical samples. In the same way, there is a paucity of data on the effect of different classes of antifungal agents against these three species of *Candida*.

Opportunistic fungal pathogens, such as *Candida* spp., have developed sophisticated means to establish the infectious process. In this sense, the expression of a set of virulence attributes, including surface adhesins, phenotypic switching, morphogenesis, biofilm formation and production of hydrolytic enzymes (*e.g.*, lipases, proteases and phosphatases), decisively contributes to the pathogenesis of candidiasis, allowing the fungal cells to escape and/or overcome the host defenses (Schaller et al., 2005; Cuéllar-Cruz et al., 2012; Singh and Mukhopadhyay, 2012; Deorukhkar et al., 2014; Singaravelu et al., 2014). However, these determinants of pathogenicity remain largely unexplored for the species of the *C. parapsilosis* complex. Aggravating this scenario, many contradictory results have been generated regarding the production of potential virulence traits in species belonging to the *C. parapsilosis* complex. The ability to form biofilm on polystyrene surface is a good example of this conflicting subject (Song et al., 2005; Tavanti et al., 2007; Lattif et al., 2010; Toro et al., 2011; Abi-chacra et al., 2013).

In light of the knowledge, with no doubt, there is a scarcity of information concerning the epidemiology and antifungal susceptibility of *C. parapsilosis* complex worldwide, especially in our country (Brazil), as well as the capability of the clinical isolates in producing virulence attributes associated to the infectious process. These reasons led us to perform a study focused on the most developed areas of Brazil, located in the Southeast region. In this context, 53 clinical isolates were collected from hospitals located at three Brazilian States (São Paulo, Rio de Janeiro and Espírito Santo), which were initially identified as *C. parapsilosis* (*sensu lato*), in order to evaluate the prevalence of species forming the *C. parapsilosis* complex as well as their antifungal susceptibility patterns and the production of well-known phenotypic characteristics associated with fungal pathogenesis, including filamentation capability, secretion of hydrolytic enzymes (*e.g.*, proteases, lipases and phytases) and hemolysins as well as biofilm formation capacity. In addition, the effects of antifungal drugs on biofilm-forming cells were also assessed. To finalize, a survey of the epidemiology of *C. parapsilosis* complex was summarized in this paper in order to present a global perspective of these clinically relevant *Candida* species during the last 20 years.

2. Materials and methods

2.1. Yeast strains

Fifty-three non-duplicate fungal clinical isolates were recovered between 2002 and 2012 from three hospitals located at three distinct States of Southeast Brazil (Espírito Santo, Rio de Janeiro and São Paulo) (Fig. 1A) and used in all parts of the present study. In addition, *C. parapsilosis sensu stricto* (ATCC 22019), *C. metapsilosis* (ATCC 96143) and *C. orthopsilosis* (ATCC 96141) were obtained from American Type Culture Collection (ATCC, Rockville, USA) and used as controls in both biochemical and molecular identification experiments.

2.2. Growth conditions and biochemical identification

Fungal cells were cultured into Sabouraud dextrose agar (Difco, Becton, Dickinson and Company, USA) plate preparation (glucose, 20 g; peptone, 10 g; yeast extract, 5 g; agar, 20 g) and phenotypically identified by plating onto CHROMagar Candida® medium (HiCrome Candida Differential Agar – HiMedia Laboratories, India), which was incubated for 48 h at 37 °C under aerobic conditions. Additionally, the carbohydrate assimilation and metabolic enzymatic profiles were evaluated by VITEK 2® system (bioMérieux, France) using yeast (YST) card, according to the manufacturer's guidelines.

2.3. Molecular identification of C. parapsilosis complex

Fungal cells were recovered from Sabouraud dextrose agar and used for genomic DNA extraction with the Gentra® Puregene® Yeast and G+ Bacteria Kit (Qiagen®, Maryland, USA), according to the manufacturer's protocol. DNA concentration was estimated with a spectrophotometer (Nanodrop ND-2000, Thermo Scientific) at A_{260}, and its integrity was verified under UV light through a 1% agarose gel electrophoresis stained with ethidium bromide (Sigma–Aldrich, USA) at a final concentration of 0.5 g/ml. DNA extracts were stored at −20 °C until use. We standardized 250 ng as DNA amount for our PCR experiments. Gene primers REA-F (5′-GATGACCAATTYTCAAGAGT-3′) and REA-R (5′-GTCAACATAAATGTAGCATTCTAGAAATC-3′), which amplify a fragment of β (1,3)-glucan synthase subunit 1 (*FKS1*) gene, were used for the polymerase chain reaction (PCR) (Garcia-Effron et al., 2011). The expected 1032-bp amplicon was subsequently digested with the restriction endonuclease *Eco*RI according to the manufacturer's instructions (Sigma–Aldrich, USA). The resulting DNA fragments of the enzymatic digestion were separated and visualized on a 2% agarose gel stained with ethidium bromide. *C. orthopsilosis*, *C. metapsilosis* and *C. parapsilosis sensu stricto FKS1* fragments showed three bands (474, 306, and 252 bp), two bands (474 and 558 bp) and one band (1032 bp), respectively.

2.4. Antifungal susceptibility assay for planktonic cells

Antifungal susceptibility testing was performed according to the standardized broth microdilution technique described in the M27-A3 (CLSI, 2008) and was interpreted according to the M27-S4 (CLSI, 2012) document published by the Clinical and Laboratory Standards Institute (CLSI). The antifungal drugs tested were amphotericin B, fluconazole, itraconazole, voriconazole and caspofungin (Sigma-Aldrich, USA). As recommended by CLSI, *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control isolates in each test. The clinical breakpoints defined for *Candida* spp. were used for the interpretation of minimum inhibitory concentration (MIC) data as follows: susceptible (S) ≤2 mg/l, susceptible-dose dependent (S-DD) 4 mg/l, resistant (R) ≥8 mg/l for fluconazole; S ≤0.125 mg/l, S-DD 0.25–0.5 mg/l,