Acta Scientific Microbiology (ISSN: 2581-8635)

Volume 3 Issue 9 September 2020

Research Article

Antifungal Activity of Fluconazole, Itraconazole, Voriconazole, Amphotericin B and Caspofungin against *Candida parapsilosis* Blood Isolates

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Received: June 19, 2020

Published: August 26, 2020

Abstract

Candida albicans is considered the most frequent etiological agent of candidemia worldwide. However, in the last decades there has been a rise in non-albicans *Candida* spp. causing candidemia. For example, in some Latin American countries *Candida parapsilosis* is replacing *C. albicans* as the main species isolated from blood samples. There is also an emerging concern regarding the decrease in susceptibility of non-albicans *Candida* spp. to first-line antifungals. In Costa Rica, there is limited information about this emerging problem. Thus, the objective of the present investigation was to study the susceptibility pattern of *C. parapsilosis* blood isolates to fluconazole, itraconazole, voriconazole, caspofungin and amphotericin B. Sixty-nine isolates collected from three Type A hospitals were studied. The isolates are part of the Fungal collection of the School of Microbiology, University of Costa Rica. Two reference methods were used to determine antifungal susceptibility. The EUCAST was performed for the azoles and amphotericin B. The end-points for caspofungin are not established by the EUCAST, thus the CLSI method was used instead. Results showed that 29% of the isolates were resistant to fluconazole, 25% to voriconazole, 6% to itraconazole and 3% to caspofungin. As to amphotericin no resistance was found; however, it is noteworthy that 42% of the isolates had minimal inhibitory concentrations of 1 μg ml^{-1}, the upper limit to be considered as sensible. In conclusion, these results highlight the importance of vigilance programs for susceptibility testing of *C. parapsilosis* involved in candidemia in Costa Rica.

Keywords: Amphotericin B; Azoles; *Candida parapsilosis;* Candidemia; Caspofungin; Fluconazole; Itraconazole; Susceptibility Testing; Voriconazole

Abbreviations

CLSI: Clinical Laboratory and Standards Institute; EUCAST: European Committee for Antimicrobial Susceptibility Testing; MIC: Minimal Inhibitory Concentration; MIC_{50}: Minimal Inhibitory Concentration, which inhibits 50% of the isolates; MIC_{90}: Minimal Inhibitory Concentration, which inhibits 90% of the isolates; MFC: Minimal Fungicidal Concentration.

Introduction

Candida parapsilosis is an opportunistic yeast that causes both superficial infections in fingernails and feet, skin, middle ear and systemic infections; mainly in low weight premature neonates, catheter patients or receiving parenteral hyperfeeding [1]. This yeast is associated with the hands of the health area staff, including Costa Rica [2], which contributes to its nosocomial dissemination. *Candida albicans* is reported worldwide for approximately half of the cases of candidemia, followed by *C. parapsilosis* [1]. Even, in

some Latin American countries, *C. parapsilosis* has displaced *C. albicans* as the main isolated species from blood cultures [3-5]. This is of clinical importance since species *C.* no-*albicans* such as *C. parapsilosis, C. tropicalis*, *C. glabrata* and *C. krusei*; are generally less susceptible to antifungals than *C. albicans,* implying different therapeutic management for this group of patients [1]. Clinical management of invasive candidosis with *C. parapsilosis* includes catheter removal and administration of systemic antifungals, such as amphotericin B, 5-fluorocyteine, echinocanine and some azoles such as fluconazole, itraconazole and voriconazole [6]. Amphotericin B is the most commonly used systemic antifungal; however, its use can cause nephrotoxicity, which forces to lower the doses or stop therapy [7,8]. The lipid formulation of amphotericin B is better tolerated [9-11] but for its high cost is not available in many countries such as Costa Rica. Therefore, although (2 - 3)% *in vitro* resistance of *C. parapsilosis* to this antifungal [12] has been reported the biggest problem of the use of amphotericin B therapy is mainly its toxicity [13]. Fluconazole is the most common alternative treatment for amphotericin B. *In vitro* resistance to fluconazole has been demonstrated among the species of *Candida* no-*albicans*, particularly *C. glabrata* and *C. krusei,* the latter being intrinsically resistant to this medicine [14-16]. In addition, clinical resistance has been reported in *C. parapsilosis* in the case of this antifungal [17,18]. As for itraconazole, *in vitro* resistance of 1.5% [19] to 4% [12] in isolations of *C. parapsilosis* has been observed. Voriconazole is effective for invasive disease and its clinical use has mainly been in oral therapies for patients with *C. krusei* and *C. glabrata* fluconazole-resistant and voriconazole-susceptible infection. Fluconazole and itraconazole have been shown to induce cross-resistance in *C. parapsilosis* and mutations have been identified in fluconazole and voriconazole resistant strains [20]. Due to this pattern, it is of relevance to carry out further studies on the *in vitro* sensitivity of these yeasts against the most widely used antifungals and thus to be able to guide the treating physician on what would be a better treatment scheme for patients with systemic thrush, decisive decision in the outcome of the clinical picture.

Materials and Methods

Candida parapsilosis isolations

The isolates studied are part of the collection of the Micoteca of the School of Microbiology of the University of Costa Rica. Sixty-nine blood culture isolates were collected during the period of time between 1995 and 2018. The fungi were kept in Sabouraud glucose agar tubes at room temperature ((20 - 30) C). Control strains of the American Type Culture Collection: *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as controls for the susceptibility studies.

Susceptibility tests

TThe cutting points set by this method for amphotericin B are sensitive ≤ 1μg ml^{-1} and resistant > 1μg ml^{-1}; for fluconazole, sensitive ≤ 2 μg ml^{-1} and resistant > 4 μg ml^{-1}; for itraconazole, sensitive ≤ 0,125 μg ml^{-1} and resistant > 0,125 μg ml^{-1} and for voriconazole, sensitive ≤ 0,125 μg ml^{-1} and resistant > 0,25 μg ml^{-1}. Because the cut-off points for caspofungin are not established by the EUCAST, the Clinical Laboratory and Standards Institute (CLSI) M27-A3 Broth Microdilution Method Standards (CLSI) [22] was used. The cutting points by this method are: sensitive ≤ 2 μg ml^{-1} and resistant > 2 μg ml^{-1}.

Statistical analysis

Each trial was conducted in triplicate. For statistical analysis, the SPSS program for Windows, version 20 (SPSS Inc., Chicago, Ill, USA) was used. An average of the minimal inhibitory concentratio (MIC) was obtained with its respective standard error and the MIC_{50} and MIC_{90} for each antifungal. A variance analysis (ANOVA) was then performed to determine whether there are statistically significant differences between the values of MICs between antifungals and between hospitals, along with Tukey testing.

Results and Discussion

C. parapsilosis is a major cause of fungemia worldwide ((20 - 30)%), the incidence of which has increased dramatically over the past two decades [4]. *C. parapsilosis* is found within the species of the genus *Candida* that are increasing as causal agents of candidemias in Australia, Latin America, Canada, Asia, Africa and Europe [23]. It is one of the most common species causing candidemia in hospitalized patients. In Europe; for example, it is the second or third most common agent by country, being responsible for (15 - 23)% of all candidomic episodes. On the other hand, in the United States, *C. parapsilosis* is the third most common agent in patients over 12 years (12%) and the second most common in younger patients (34%). In Brazil and other Latin American countries, *C. parapsilosis* is responsible for (20 - 30)% of candidemias, showing similar prevalence in adult and paediatric populations [24]. In some cases, *C. parapsilosis* has surpassed *C. albicans* as a causal species of candidemia, with a mortality rate ranging from 4 to 45%, with an average of 28.5% [23].

Despite the growing relevance of candidemias, to date there are only three publications in Costa Rica in this regard, two from Hospital San Juan de Dios and one from Hospital México. In the first study [25], the clinical records of 47 adult patients, treated at the Hospital San Juan de Dios and diagnosed with systemic thrush be-

tween 1996 and 1998, were analyzed. All had some risk factor for candidemia (previous use of antibiotics, central venous catheter, among others). *C. albicans* was reported to be the most common species (47%), followed by *C. tropicalis* (19%) and *C. parapsilosis* (11%). In the second study conducted, five years later, *C. parapsilosis* ranked first [26]. In the latest study, between 2007 and 2010 [5] *Candida* species other than *C. albicans* are reported to have accounted for 62% of the causes of candidemia at Hospital México, which coincides with what is reported in other centers in Latin America and the world [5]. However, although the percentage of *Candida* no *albicans* so high coincides with what is reported for the region (collectively the *non-albican species* found outweigh *C. albicans),* the difference was found that *C. parapsilosis* was the most common cause of candidemia, displacing *C. albicans* as the most common species of all and its predominance in 3 of the 4 years analyzed show it as a problem endemic in that hospital. This differs from what is described in most reports, including Latin America, where *C. albicans* is the most common species (although in total percentage of candidemias it is exceeded by the sum of the percentage of *non-albican species)* and *C. parapsilosis* occupies the second or third place, with an incidence ranging from 10% to 25% [5]. In addition, in recent decades multiple studies have reported the emergence and increase of resistance to the most widely used ampfungals, where case *C. parapsilosis* [1] is of importance.

This study analysed 69 isolations from blood cultures in three hospital centers in the metropolitan area. 65% (n = 45) of the isolates came from hospital A, 32% (n = 22) from hospital B and 3% (n = 2) from hospital C. When analysing the isolates of the three hospitals as a whole, 29% (n = 20) of resistance to fluconazole was found, 25% (n = 17) to voriconazole, 6% (n s 4) to itraconazole and 3% (n = 2) to caspofungin. It is important to note that statistically significant differences were found between the geometric means of the MICs of the antifungals ($F = 19,992$; $df = 4$; $p < 0.001$). The Tukey test distributed the treatments in two groups, in the first were itraconazole, voriconazole, caspofungin and amphotericin B, which had the lowest MICs and in the second included fluconazole, with significantly higher MICs. Table 1 presents the distribution of geometric means, the range and the Inhibitory Minimum Concentrations (MICs) of the isolates.

In hospital A two fluconazole-resistant isolates were found, two to itraconazole, two to voriconazole and two to caspofungin (4.44% resistance for each of the four antifungals) and one of the isolates was resistant to both fluconazole and voriconazole, but not

	Fluconazole ($\mu g\ mL^{-1}$)	**Itraconazole ($\mu g\ mL^{-1}$)**	**Voriconazol ($\mu g\ mL^{-1}$)**	**Amphotericin ($\mu g\ mL^{-1}$)**	**Caspofungina ($\mu g\ mL^{-1}$)**
Geometric mean	9,56 (± 16,76)	0,09 (± 0,07)	0,25 (±0,43)	0,66 (±0,31)	1,54 (±0,70)
Range	0,25-64,00	0,03-0,50	0,03-2,00	0,19-1,00	0,25-4,00
MIC_{50}	1,50	0,06	0,03	0,50	2,00
MIC_{90}	32,00	0,13	1,00	1,00	2,00

Table 1: Distribution of the minimum inhibitory concentrations (MICs) of *C. parapsilosis* isolations from three public Class A hospitals in Costa Rica, obtained from blood cultures, against fluconazole, itraconazole, voriconazole, amphotericin B and caspofungin (n = 69).

to itraconazole. In addition, itraconazole-resistant insulation was observed, but sensitive to fluconazole and voriconazole. The two caspofungin-resistant insulations were shown to be sensitive to the three azoles studied. In hospital B, 82% (n = 18) of resistance to fluconazole was observed, 68% (n = 15) to voriconazole and 9% (n = 2) to itraconazole and all were sensitive to caspofungin. The 15 voriconazole-resistant isolates were also resistant to fluconazole. The two isolates from hospital C were sensitive to the antifungals studied. It should be noted that statistically significant differences were found between fluconazole and voriconazole MICs between hospitals ($F = 15,590$; $df = 2$; $p < 0.001$) and ($F = 12,042$; $df = 2$; $p < 0.001$), respectively). In both cases, tukey-tested grouped hospitals A and C into one group and B in the other.

Se has reported Decreased sensitivity has been reported to *C. parapsilosis* to first-line antifungals, such as fluconazole [27]. Different studies have correlated the presence of resistance to fluconazole and of the pathogen isolation site, where yeasts obtained from the bloodstream are those that have such resistance. Interestingly, prolonged use of fluconazole to control *C. parapsilosis* candidemia in Finland led to the emergence of a resistant strain responsible for cross-infections for a period of 12 years [17]. However, the prophylaxis of this antifungal has not caused resistant strains in studies conducted over a period of 14 to 30 months [28,29]. As mentioned above, significant resistance to voriconazole (25%) was also observed and to a lesser extent itraconazole (6%). Although not common, resistance to itraconazole and voriconazole have also been reported [30].

In this study, 3% resistance to caspofungin was observed. Compared to other species, *C. parapsilosis* tends to have high CMIs for echinocandines, which can lead to greater use of fluconazole for treatment and thus lead to resistance to azol. Also, it raises concern resistance to equinocandins, so that none of the first-line treatments [31] can be counted. Currently, the cause of decreased sensitivity to equinocandins is under study, but it has been seen that this species has polymorphisms occurring naturally in the *FKS* gene, which leads to changes in the corresponding subunit of glucan-synthetase and, therefore, a reduced action of the antifungal. In the case of amphotericin B, both increased MICs and resistance have been reported [31].

All the isolates in the present study were sensitive to amphotericin. Amphotericin B resistance is not common, so amphotericin B MICs for *C. parapsilosis* are reported at average MIC values MIC_{50} and MIC_{90} between (0.13 - 1) µg ml^{-1} and (0.5 - 1) µg ml^{-1} respectively [16,32-34]. In our study, it was observed that 42% (n = 29) of the isolates analysed had MICs equal to 1 g ml^{-1} for amphotericin B, the upper limit to be classified as sensitive. Initially, it could be argued that strains are developing resistance mechanisms that allow them to survive in increasing concentrations of amphotericin B. However, we cannot suggest a decrease in the sensitivity of amphotericin B because tolerance to this antifungal by this species has been described. The reason for such behaviour has not been clarified; however, it was described since 1983, where a comparative study was conducted between seven different species of *Candida* with different antifungals. In this work it was observed that the minimum fungicide concentration (MFC) for C. *parapsilosis* isolations was up to 32 or more times its corresponding MICs, while the other species had MFCs less than 16 times their corresponding CMIs, so only *C. parapsilosis* met the definition of tolerance to amphotericin B (the same criterion was used as defining tolerance for bacterial agents). The author proposes that this tolerance may be due to the composition of cell membrane sterols of this species, very similar to that observed in other *Amphotericin* resistant *Candida* spp. [35]. The same characteristic was observed in 2003, except that *Candida dubliniensis* was also listed as tolerant. In another study conducted in 1998, it was observed that in cultures of density between (0.5 - 1.0) x 10^6 cells ml^{-1} supplemented with 2 µg ml^{-1} amphotericin B, *C. parapsilosis* together with *C. krusei* were the species that had the lowest susceptibility to the antifungal, with a decrease of viable cells of only 1 to 2 magnitudes. *C. parapsilosis* was the least sensitive to amphotericin B of all, with a survival rate of 1% even after 24-hour exposure [36]. Another example of this behavior was reported in 2004, in a study comparing the patterns of the rate of elimination of amphotericin B over different *Candida* species. Unlike the other species where the fungicide point was reached between 2 to 4 hours of incubation with 4 times the antifungal MIC, a 48-hour incubation was required to reach it with *C. parapsilosis* MIC [37]. One study reported that *C. parapsilosis* is the species with the highest percentage of hydrophobicity in its cell wall compared to the other species analyzed and the second with the most biofilm formation capacity (only after *C. albicans*). The authors argue that these two characteristics play an important role in resistance to antifungals, disinfectants and even factors in the host [38]. This could help to clarify the mechanisms of tolerance to amphotericin B of *C. parapsilosis;* however, more studies are needed in order to determine the reason for that characteristic.

A link has been established between proper hand washing and decreased incidence of candidemia for *C. parapsilosis.* Since this species is in the hands of humans, iti is important to diminish the amout of yeast in the hands of health personnel [5,39] . A proper decrease in the amount of yeast through this practice would lead to less exposure of this species to antifungals administered to patients, reducing the chances of resistance development and the selection of already resistant strains themselves. Another factor to consider is a good protocol and guidelines for the administration of antifungals established for the treatment of candidemias. In Costa Rica, there is no protocol given by the Ministry of Health or the Costa Rican Social Security Fund, considering the results of the present study", it is recommended to carry out tests of sensitivity to first-line antifungals in the candidemia caused by *C. parapsilosis* to provide adequate treatment and thus reduce mortality in patients, prolonged hospital stay and increase in care costs.

Conclusion

In the present study, the data obtained from a collection of 69 *C. parapsilosis* bloodstream isolates from three public hospitals in Costa Rica demonstrated that 29% were resistant to fluconazole, 25% to voriconazole, 6% to itraconazole and 3% to caspofungin. These results emphasize the need for surveillance programs to include first line antifungal profiles of *C. parapsilosis* involved in candidemia in Costa Rica.

Acknowledgements

We would like to thank the University of Costa Rica for financial support (Projects VI-803-B9-024 and VI-430-B7-732) and Alejandra Gómez Arrieta, Assistant of the Medical Mycology Laboratory, School of Microbiology, University of Costa Rica for her valuable support.

Conflict of Interest

The authors have no conflicting interests to declare.

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