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Original article

Killing kinetics of anidulafungin, caspofungin and micafungin against *Candida parapsilosis* species complex: Evaluation of the fungicidal activity

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ABSTRACT

Background: *Candida parapsilosis*, *Candida metapsilosis* and *Candida orthopsilosis* are emerging as relevant causes of candidemia. Moreover, they show differences in their antifungal susceptibility and virulence. The echinocandins are different in terms of in vitro antifungal activity against *Candida*. Time-kill (TK) curves represent an excellent approach to evaluate the fungicidal activity of antifungal drugs.
Aims: To compare the fungicidal activities of anidulafungin, caspofungin and micafungin against *C. parapsilosis* species complex by TK curves.
Methods: Antifungal activities of three echinocandins against *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* were studied by TK curves. Drug concentrations assayed were 0.25, 2 and 8 μg/ml. CFU/ml were determined at 0, 2, 4, 6, 24 and 48 h.
Results: Killing activities of echinocandins were species-, isolates- and concentration-dependent. Anidulafungin reached the fungicidad endpoint for 6 out of 7 isolates (86%); it required between 13.34 and 29.67 h to reach this endpoint for the three species studied, but more than 48 h were needed against one isolate of *C. orthopsilosis* (8 μg/ml). Caspofungin fungicidal endpoint was only achieved with 8 μg/ml against one isolate of *C. metapsilosis* after 30.12 h (1 out of 7 isolates; 14%). Micafungin fungicidal endpoint was reached in 12.74–28.38 h (8 μg/ml) against one isolate each of *C. parapsilosis* and *C. orthopsilosis*, and against both *C. metapsilosis* isolates (4 out of 7 isolates; 57%).
Conclusions: *C. metapsilosis* was the most susceptible species to echinocandins, followed by *C. orthopsilosis* and *C. parapsilosis*. Anidulafungin was the most active echinocandin against *C. parapsilosis* complex.

Cinéticas de letalidad de la anidulafungina, la caspofungina y la micafungina frente a las especies del complejo *Candida parapsilosis*: evaluación de la actividad fungicida

Palabras clave:
Candida parapsilosis
Anidulafungina
Caspofungina
Micafungina
Curvas de tiempo-letalidad

RESUMEN

Antecedentes: *Candida parapsilosis*, *Candida metapsilosis* y *Candida orthopsilosis* son causas relevantes de candidemia. Además, muestran diferencias en la sensibilidad a los fármacos antifúngicos. Las equinocandinas muestran diferente actividad antifúngica in vitro frente a *Candida*. Las curvas de tiempo-letalidad (TK) representan una excelente aproximación para evaluar la actividad fungicida de los fármacos antifúngicos.
Objetivos: Comparar la actividad fungicida de la anidulafungina, la caspofungina y la micafungina frente al complejo *C. parapsilosis* mediante las curvas de TK.

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Métodos: Se estudió la actividad de tres equinocandinas frente a *C. parapsilosis*, *C. metapsilosis* y *C. orthopsilosis* mediante las curvas de TK. Las concentraciones ensayadas fueron 0,25, 2 y 8 μg/ml. Se determinaron las UFC/ml a las 0, 2, 4, 6, 24 y 48 h.
Resultados: La actividad de las equinocandinas fue especie-, aislamiento- y concentración-dependiente. La anidulafungina alcanzó el límite fungicida frente a 6 de 7 aislamientos (86%), y necesitó 13,34-29,67 h para alcanzar este límite en las tres especies estudiadas; para un aislamiento de *C. orthopsilosis*, requirió más de 48 h (8 μg/ml). El límite fungicida de la caspofungina solo se alcanzó con 8 μg/ml frente a un aislamiento de *C. metapsilosis* después de 30,12 h (1 de 7 aislamientos; 14%). La micafungina alcanzó este límite en 12,74-28,38 h (8 μg/ml) frente a un aislamiento de *C. parapsilosis* y *C. orthopsilosis* y frente a ambos aislamientos de *C. metapsilosis* (4 de 7 aislamientos; 57%).
Conclusiones: *C. metapsilosis* fue la especie más sensible a las equinocandinas, seguida de *C. orthopsilosis* y *C. parapsilosis*. La anidulafungina fue la equinocandina más activa frente al complejo *C. parapsilosis*.

Invasive candidiasis is an important cause of morbidity and mortality worldwide. Incidence of infections due to non-*Candida albicans* species is increasing, although *C. albicans* remains the most common etiology in most countries.[37] *Candida parapsilosis* is an emerging and relevant cause of candidemia in both adults and children, being the second or third most frequent species depending on the geographical area.[21] Moreover, *Candida orthopsilosis* and *Candida metapsilosis* are newly recognized members of *C. parapsilosis* complex. These three species show substantial differences in their antifungal susceptibility and virulence, and their epidemiology is a matter of increasing interest.[26,33,44]

The echinocandin drugs, anidulafungin, caspofungin and micafungin, inhibit 1,3-β-D-glucan synthesis of fungal cell wall, causing fungistatic as well as fungicidal effects. Echinocandins are considered front-line antifungal agents for the therapy of candidemia and invasive candidiasis.[30,31] Despite the mechanism of action and chemical structure of these echinocandins are very similar, there are subtle differences among them in terms of in vitro antifungal activity against many *Candida* species.[20,29] Although traditionally dose and drug selection in antimicrobial therapy is based on a single static in vitro parameter, such as the minimum inhibitory concentration (MIC), the in vivo antimicrobial effect is the result of a dynamic exposure of the infective agent to the antifungal drug. Time-kill (TK) curves describe the drug-microorganism interactions in a more dimensional way by a dynamic integration of concentration and time, being a more rational approach than the classical MIC value.[28] The aims of our study were to compare and to widen the knowledge on the TK curves of anidulafungin, caspofungin and micafungin against *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*.

Materials and methods

Microorganisms

Seven clinical isolates and strains from the *C. parapsilosis* complex were studied, including 3 *C. parapsilosis sensu stricto* (1 blood isolate -UPV/EHU 09-378- and 2 reference strains -ATCC 22019 and ATCC 90018-); 2 *C. metapsilosis* (1 blood isolate -UPV/EHU 07-045- and ATCC 96143 strain) and 2 *C. orthopsilosis* (1 blood isolate -UPV/EHU 07-035- and ATCC 96139 strain). Blood isolates were obtained from the fungal culture collection of the Laboratorio de Micología Médica, Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Bilbao, Spain. Isolates were identified by metabolic properties (ATB ID 32C, bioMérieux, France) and by molecular methods, as previously described.[27,32]

Antifungal agents

Caspofungin (Merck Sharp & Dohme, Spain), micafungin (Astellas Pharma, Spain) and anidulafungin (Pfizer SLU, Spain) were dissolved in dimethyl sulfoxide to obtain a stock solution of 5120 μg/ml. Further dilutions were prepared in RPMI 1640 medium with L-glutamine and without $NaHCO_2$ buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, Spain). Stock solutions were stored at −80 °C until use.

In vitro susceptibility testing

MICs, defined as minimum concentrations that produce ≥50% growth reduction, were determined and interpreted following the CLSI M27-A3, M27-A3 S4 and M60 documents.[7–9] Results were read after 24 h of incubation.

Time-kill procedures

Before TK studies were performed, the antifungal carryover effect was determined as described by Cantón et al.[6] Studies were carried out as previously described on microtiter plates for a computer-controlled microbiological incubator (BioScreen C MBR, LabSystems, Finland) in RPMI by using an inoculum size of $1–5 \times 10^5$ CFU/ml and a final volume of 200 μl.[2,5,17,18] Antifungal drug concentrations assayed were 0.25, 2 and 8 μg/ml. Echinocandins concentrations were chosen taking into account their MICs for the studied isolates. Plates were incubated during 48 h at 36 ± 1 °C without agitation. At 0, 2, 4, 6, 24 and 48 h, aliquots were removed from each culture, including control and test solution wells. These aliquots were serially diluted in phosphate buffered saline to determine the number of CFU/ml. Volumes of 5, 10, 50 or 100 μl, depending on the dilution and concentration of the drug, were plated onto Sabouraud dextrose agar and incubated at 36 ± 1 °C for 24 to 48 h. The lower limit of accurate and detectable colony counts was 20 CFU/ml. TK studies were conducted in duplicate on two different days.

Data analysis

The killing kinetics of the fungicidal activity were analyzed by fitting the TK data at each time point to the exponential equation $N_t = N_0 \times e^{kt}$, which was transformed into a line by applying logarithms [$\log N_t = \log N_0 + Kt$]. N_t is the number of viable yeast cells at time t, N_0 is the starting inoculum, K is the killing or growing rate, and t is the incubation time. Thus, the six time points on each killing curve were reduced to one value, K. The goodness of fit for each isolate/drug was assessed by the r^2 value (>0.8). The mean time to

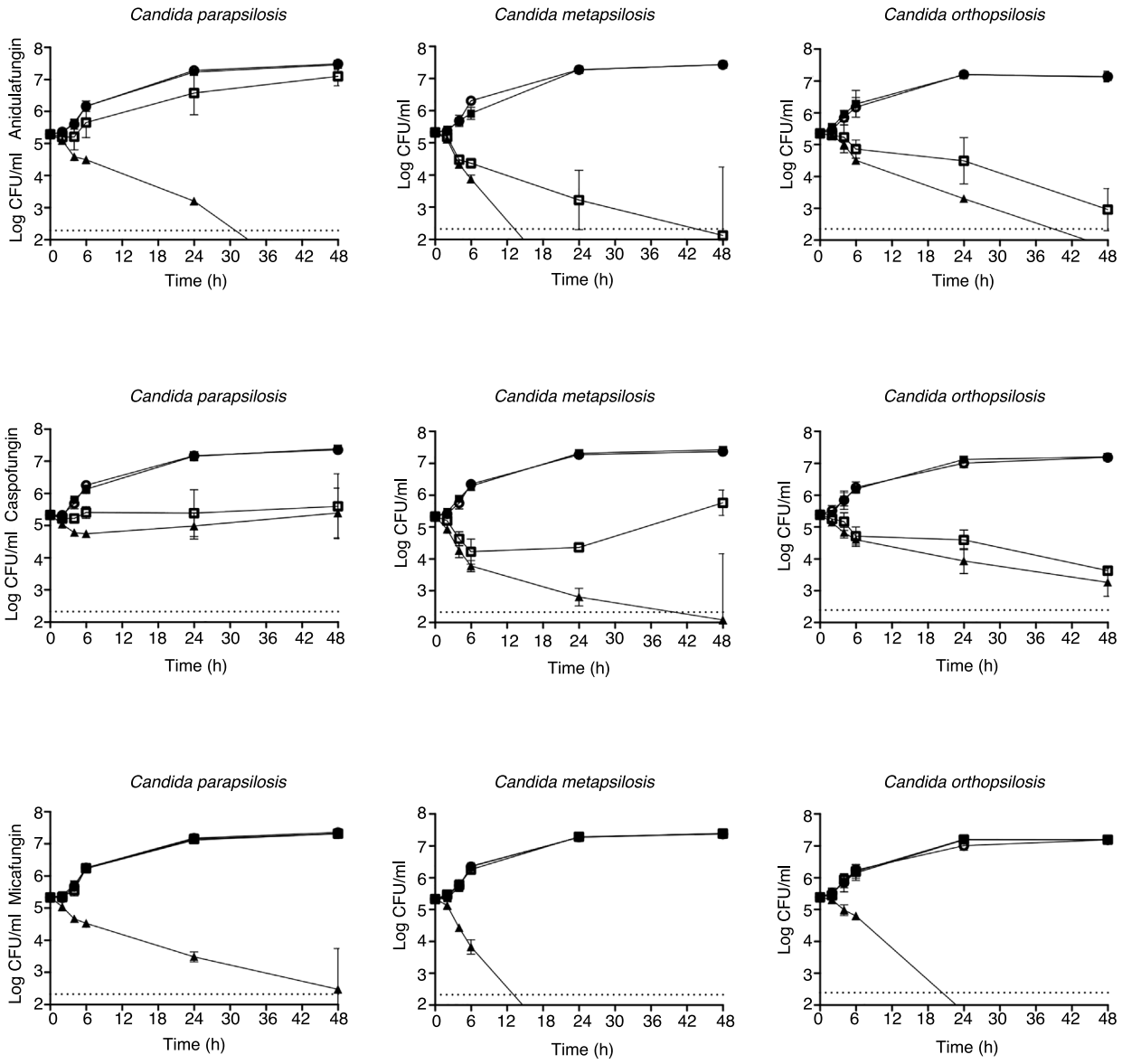

Fig. 1. Mean time-kill plots for anidulafungin, caspofungin and micafungin against 3 *C. parapsilosis*, 2 *C. metapsilosis* and 2 *C. orthopsilosis* isolates. Each data point represents the mean result ± standard deviation (error bars) for the indicated number of strains. White circles (○): control; black squares (■): 0.25 μg/ml; white squares (□): 2 μg/ml; black triangles (▲): 8 μg/ml. The broken lines represent ≥99.9% growth reduction compared with that of the initial inoculum (fungicidal effect).

achieve reductions in viable cells of 99.9%, and the time to reach the fungicidal endpoint ($t_{99.9} = 3/K$) were calculated from the K value. Fungicidal activities were compared by using K values: positive values indicate growth and negative values show killing.[4] The analysis of variance was performed to determine significant differences in killing kinetics among species, echinocandins and concentrations. A p value <0.05 was considered significant.

Results

In vitro susceptibility testing

Anidulafungin, caspofungin and micafungin showed similar activity against *C. parapsilosis* (geometric mean -GM- MIC: 1.58, 1.25 and 1.25 μg/ml, respectively), *C. metapsilosis* (GM MIC: 1, 1.41 and 2 μg/ml, respectively) and *C. orthopsilosis* (GM MIC: 1, 1.41 and 0.7 μg/ml, respectively).

Time-kill assays

No antifungal carryover effect was detected in TK studies. The mean TK curves and standard deviations of the three echinocandins against 3 *C. parapsilosis*, 2 *C. metapsilosis* and 2 *C. orthopsilosis* are depicted in Fig. 1. Killing activities of anidulafungin, caspofungin and micafungin were species- and isolate-dependent and increased with time. All echinocandins were more active against *C. metapsilosis* than against *C. orthopsilosis* or *C. parapsilosis*.

Anidulafungin reached the mean maximum log decreases of CFU (>3.5 log) with 8 μg/ml against the three species of the *C. parapsilosis* complex, except for one isolate each of *C. metapsilosis* and *C. orthopsilosis*, against which this decrease was achieved at 2 μg/ml (6 out of 7 isolates; 86%). It must be noted that against the isolate *C. orthopsilosis* UPV/EHU 07-035 no fungicidal endpoint was attained.

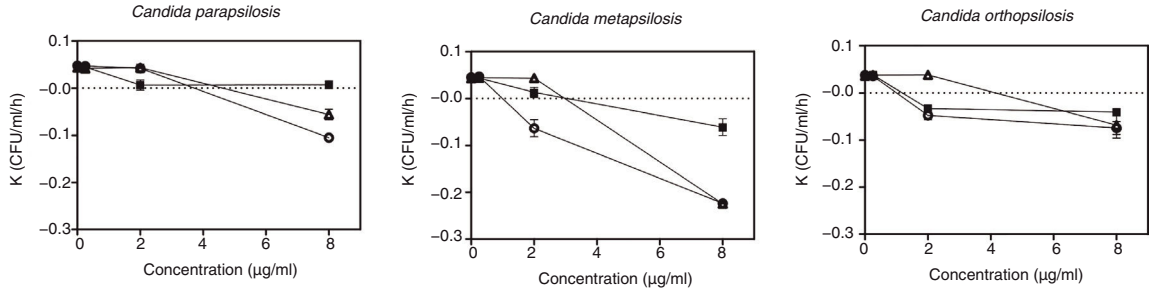

Fig. 2. Effect of anidulafungin, caspofungin and micafungin concentrations on the killing rates . White circles (○): anidulafungin; black squares (■): caspofungin; white triangles (△): micafungin. Values above the broken lines indicate growth, and values below the broken lines indicate killing.

With caspofungin, the fungicidal endpoint (99.9% killing) was only achieved against one isolate of *C. metapsilosis* (1 out of 7 isolates; 14%), with 8 μg/ml, being ≥4 log the maximum log decrease in CFU/ml. Caspofungin did not display fungicidal activity against the rest of strains and isolates studied.

A concentration of 8 μg/ml of micafungin was fungicidal for one isolate of *C. orthopsilosis*, the two isolates of *C. metapsilosis* and one isolate of *C. parapsilosis* (4 out of 7 isolates; 57%), although the log decreases of *C. metapsilosis* were higher than those reached for *C. parapsilosis* and *C. orthopsilosis* (≥4 log, 2.9 ± 2.18 log, and 3.6 ± 2.52 log, respectively). Fig. 2 shows the effect of echinocandin concentrations on the killing rates for *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*.

The highest killing rates in *C. parapsilosis* and *C. metapsilosis* were obtained with 8 μg/ml and 2 μg/ml of anidulafungin, respectively. These killing rates were significantly higher than those of micafungin and caspofungin ($p < 0.05$). Concerning *C. orthopsilosis*, anidulafungin and caspofungin showed similar killing rates with 2 μg/ml and were higher than that of micafungin. However, with 8 μg/ml similar killing rates were obtained with anidulafungin and micafungin, in both cases higher than that observed with caspofungin. The ranges of killing rates with the echinocandins against *C. parapsilosis* species complex were the following: 0.02–0.07 and 0.03 with 2 μg/ml (anidulafungin and caspofungin, respectively) and 0.07–0.22, 0.04–0.06 and 0.06–0.22 with 8 μg/ml (anidulafungin, caspofungin and micafungin, respectively) (Fig. 2).

Table 1 shows the mean times needed to reach the fungicidal endpoint for each isolate and echinocandin concentration tested. Anidulafungin required 8 μg/ml and between 13.34 and 29.67 h to reach the fungicidal endpoint against 6 out of 7 isolates (86%) of *C. parapsilosis* complex, however, the killing of the clinical isolate of *C. orthopsilosis* (UPV/EHU 07-035) required more than 48 h. By contrast, the caspofungin fungicidal endpoint was only reached in one isolate of *C. metapsilosis* after 30.12 h with 8 μg/ml (1 out of 7 isolates; 14%). Micafungin achieved the fungicidal endpoint with 8 μg/ml after 12.74–28.38 h against one isolate each of *C. parapsilosis* (UPV/EHU 09-378) and *C. orthopsilosis* (ATCC 96139) and against both isolates of *C. metapsilosis* (4 out of 7 isolates; 57%).

Discussion

C. parapsilosis is frequently involved in invasive infections worldwide, especially in neonates. In addition, recent findings suggest that *C. metapsilosis* and *C. orthopsilosis* are clinically relevant species. Lockhart et al.[26] reported that among 1929 bloodstream infections presumed to be caused by *C. parapsilosis*, 91.3% were caused by *C. parapsilosis*, 6.1% by *C. orthopsilosis*, and 1.8% by *C. metapsilosis*. Despite of this clinical importance of *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*, there are few studies on the fungicidal activities of anidulafungin, caspofungin and micafungin by TK methodology[2,15,43,47] and only two studies have head-to-head compared the activity of these echinocandins by TK curves.[2,41] Besides, echinocandins play a central role in the current prevention and treatment of invasive candidiasis. The methodology of our study and the number of isolates evaluated is based on previous TK studies. This number of isolates studied in the TK tests is significantly lower than that used in the MIC studies.[2,3,11,14,23,25,38,40,41,43,45] The greater complexity of the TK analysis, its heavier laboriousness and the complex handling of the much broader information obtained with the TK curves are some of the main reasons that justifies the lower number of isolates of our study. In the current study, anidulafungin and micafungin MIC values for *C. parapsilosis* were similar to those recently reported by Pfaller et al.[34–36] These authors observed that anidulafungin and micafungin MIC values for *C. parapsilosis* isolates ranged between 0.03–8 μg/ml and 0.03–4 μg/ml, respectively. The MIC results in our study are close to those previously reported by García-Effron

Table 1
Time (h) to achieve 99.9% ($t_{99.9}$) growth reduction from the initial inoculum.

Strains	Drug concentration (μg/ml)								
	Anidulafungin			Caspofungin			Micafungin		
	0.25	2	8	0.25	2	8	0.25	2	8
C. parapsilosis									
ATCC 22019	NA[a]	NA	29.67	NA	NA	NA	NA	NA	>48
ATCC 90018	NA	NA	27.75	NA	NA	NA	NA	NA	>48
UPV/EHU 09-378	NA	NA	28.22	NA	>48	>48	NA	NA	28.38
C. metapsilosis									
ATCC 96143	NA	27.5	13.46	NA	NA	>48	NA	NA	13.15
UPV/EHU 07-045	NA	>48	13.34	NA	NA	30.12	NA	NA	13.54
C. orthopsilosis									
ATCC 96139	NA	48	28	NA	>48	>48	NA	NA	12.74
UPV/EHU 07-035	NA	>48	>48	NA	>48	>48	NA	NA	>48

[a] NA indicates not achieved.

et al.,[16] who obtained MIC values for anidulafungin of 0.5–2 μg/ml and 0.5–2 μg/ml for micafungin against the majority of the studied isolates of *C. parapsilosis*, as well as Pfaller et al. In this sense, recently, Valentin et al.[46] evaluated the in vitro activity of anidulafungin against two isolates of *C. parapsilosis* and they found MIC values of 2 μg/ml for both isolates. The methodology used in all these articles was the same as the methodology described in our manuscript, and all tested isolates in the mentioned articles were obtained from blood cultures, as were all our isolates. Moreover, MIC values of anidulafungin, caspofungin and micafungin for *C. parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were within the MIC limits recommended by CLSI. Despite this result, caspofungin MICs should be cautiously interpreted because an important variability in modal MICs has been reported.[12]

We have compared the killing activity of the anidulafungin, caspofungin and micafungin against *C. parapsilosis* species complex up to 48 h, providing new results about killing patterns and expanding the knowledge on the activities of echinocandins against the *C. parapsilosis* species complex. Previously, the fungicidal activities of echinocandins against *C. parapsilosis*, *C. metapsilosis* and *C. orthopsilosis*, resulted in highest killing rates for *C. metapsilosis* followed by *C. orthopsilosis*.[2] Micafungin and anidulafungin showed similar killing rates, both being greater than those of caspofungin. None of the drugs reached the fungicidal endpoint, not even using a higher concentrations than in the present study, against *C. parapsilosis*.[2] However, in the current report, fungicidal activities were observed with anidulafungin and micafungin at 8 μg/ml, despite *C. parapsilosis* was the least susceptible of the three species. This discrepancy could be related to the inter-strain variability, as other authors reported differences in azole antifungal activities against these species. In this sense, Szabo et al.[42] reported that fluconazole and voriconazole, but not posaconazole, seemed to be less active in vitro against *C. orthopsilosis* and *C. metapsilosis*, than against *C. parapsilosis*. Similar to our findings, Spreghini et al.[41] did not observe a fungicidal effect of caspofungin against *C. parapsilosis* at concentrations of 2 and 32 μg/ml. In this sense, Cantón et al.[2] reported that none of the echinocandins had a fungicidal activity against *C. parapsilosis*, not even with a concentration of 32 μg/ml. Foldi et al.[15] also demonstrated the fungicidal activity of micafungin against *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* at ≥2–8 MIC concentrations after 48 h. However, the fungicidal activity of micafungin in our study was reached earlier against several isolates. Similarly, our results are consistent with the reported by Szilagyi et al.,[43] who did not observe a fungicidal effect of caspofungin against *C. parapsilosis*, not even using 16 μg/ml of this drug. In a recent study, Hall et al.[19] showed the fungicidal activity of 8 μg/ml of anidulafungin and rezafungin (formerly CD101, a novel echinocandin) against the 2 isolates studied of *C. parapsilosis*. These results are similar to ours concerning the anidulafungin.

We conclude that anidulafungin was the most active antifungal drug against *C. parapsilosis*, *C. metapsilosis* and behaved similar to micafungin against *C. orthopsilosis*. We have also previously reported this higher anidulafungin activity against *C. albicans* and the *Candida glabrata* clades, although caspofungin and micafungin also showed optimal in vitro activities.[17,18] Caspofungin demonstrated favorable efficacy and safety profiles in the treatment of invasive candidiasis caused by non-*C. albicans Candida* species, even in those regions or countries where *C. parapsilosis* is highly prevalent.[10] With respect to the studied species, *C. metapsilosis* was the most susceptible to echinocandins, followed by *C. orthopsilosis* and *C. parapsilosis*. Despite the presumed reduced susceptibility of *C. parapsilosis* to echinocandins, these antifungal drugs are currently effective in invasive candidiasis.[1,13] While the MIC values of echinocandins were similar for the three species, there were differences in the fungicidal activities against different isolates and species. These discrepant results found between MIC and TK curves may be attributed to the different methodologies used in MIC and TK studies. Information obtained from TK curves is broader than that obtained from MIC methodology. These different results between MIC methodology and TK curves methodology have also been previously reported[2,17,18,39] and for this reason it would be advisable to perform in vitro TK assays and consider the results obtained from these TK curves. Other authors that have used the TK methodology have also suggested that clinicians should consider these TK results as a tool for therapeutic decision-making.[22,24] This fact highlights the importance of a correct identification and acquaintance of the antifungal susceptibility patterns of these cryptic *Candida* species for an adequate therapeutic approach of the invasive infections caused by the *C. parapsilosis* species complex.

Conflict of interest

We have no specific conflicts of interest related to the current manuscript but declare the following: GQ has received research grants from Astellas Pharma, Pfizer, Merck Sharp & Dohme, and Scynexis. GQ has served on advisory/consultant boards for Merck, Sharp & Dohme, and Scynexis, and he has received speaker honoraria from Abbvie, Astellas Pharma, Merck Sharp & Dohme, Pfizer, and Scynexis.

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