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Evaluation of *in vivo* pathogenicity of *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* with different enzymatic profiles in a murine model of disseminated candidiasis

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Abstract

Six isolates of the *Candida parapsilosis* complex with different enzymatic profiles were used to induce systemic infection in immunocompetent BALB/c mice. Fungal tissue burden was determined on days 2, 5, 10, and 15 post challenge. The highest fungal load irrespective of post-infection day was detected in the kidney, followed by the spleen, lung, and liver, with a tendency for the fungal burden to decrease by day 15 in all groups. Significant differences among the strains were not detected, suggesting that the three species of the "psilosis" group possess a similar pathogenic potential in disseminated candidiasis regardless of their enzymatic profiles.

Key words: murine model, disseminated candidiasis, *Candida parapsilosis*, *Candida orthopsilosis*, *Candida metapsilosis*, fungal tissue burden.

Introduction

Candida parapsilosis, *C. orthopsilosis*, and *C. metapsilosis*, which comprise the "psilosis" group, are a cryptic species that frequently cause opportunistic infections. These infections are associated with high morbidity and mortality rates in hospitalized immune-compromised patients [1], principally among the pediatric population [2]. These yeasts have been the subject of an increasing number of epidemiological surveys [3,4], as well as *in vitro* studies of extracellular hydrolytic enzymes [5,6] and biofilm production capability [7,8].

To date there are limited reports that focus on the pathogenic potential of psilosis group members. Gácser et al. studied the *in vitro* behavior of the three species in oral

epithelium and epidermis and reported similar histopathological alterations due to *C. parapsilosis* and *C. orthopsilosis* in both tissues. However, *C. metapsilosis* induced minimal damage when compared with uninfected controls [9]. In a different context, Orsi et al. established that *C. metapsilosis* was more susceptible to microglia-mediated antifungal activity when compared with *C. parapsilosis* and *C. orthopsilosis* [10]. Overall, these findings led to *C. metapsilosis* being considered the least virulent member of the *C. parapsilosis* complex, as recently confirmed by Bertini et al. [11].

Our aim in this study was to evaluate the *in vivo* pathogenicity of *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* in a murine model of disseminated candidiasis. Furthermore, we investigated possible correlations of the *in vivo* pathogenicity of the tested strains with their *in vitro* aspartyl proteinase, phospholipase, esterase, and hemolysin activities.

Materials and methods

Strains

Two strains of each of the following species were included in this study: *C. parapsilosis* sensu stricto (c/c 105 and H-124), *C. orthopsilosis* (HP-179 and H-152), and *C. metapsilosis* (MEX-18 and ATCC (American Type Culture Collection)-96144). Species identification of the strains was initially performed using restriction fragment length polymorphism–*Ban*I digestion assays [12] and confirmed by sequencing the noncoding rRNA internal transcribed spacer region using the universal primers reported by White et al. [13]. The obtained sequences were submitted to GenBank with the accession numbers cited in Table 1. The strains were stored as suspensions in sterile distilled water at room temperature and cultured for 48 h on Sabouraud glucose agar (SGA) slants (Difco, Detroit, MI, USA) at 37°C before use in the investigations.

Enzymatic determinations

The *in vitro* evaluation of extracellular hydrolytic enzymes of the strains was determined using plate assays as described in our previous work [5]. Briefly, aspartyl proteinase activity was assayed using yeast carbon base–bovine serum albumin test medium reported by Chakrabarti et al. [14]; phospholipase activity was evaluated according to the methodology proposed by Price et al. [15]; Tween 80 opacity test medium was used to determine esterase activity [16]; and hemolysin activity was examined using the experimental strategy established by Luo et al. [17]. The activity was expressed according to the Pz index, that is, colony diameter/total diameter of the colony plus the precipitation or halo zone [18]. The type strain *C. albicans* ATCC 90028 was used as the quality control for all enzymatic activity determinations. The assays were conducted twice.

Inocula preparation

The strains were passed at least twice on SGA plates to check the cultures' purity and viability. After 48 h of incubation at 37°C, yeast cells were harvested, washed twice in sterile saline, their concentration quantified with a hemocytometer, and adjusted to the desired concentration. To corroborate the yeast cell counts, serial dilutions were cultured on SGA plates at 37°C for 48 h.

Animals

Male BALB/c mice aged 5 weeks (weighing 22–24 g; purchased from Harlan Mexico) were used for the *in vivo* studies. A total of 132 animals were used; the animals were

Table 1. Enzymatic profiles of strains used *in vivo* studies.

Strain	GenBank accession number	Clinical origin	Pz index[a]			
			Aspartyl proteinase	Phospholipase	Esterase	Hemolysin
Candida parapsilosis						
c/c 105	KC777378	Peritoneal fluid	1	0.77	0.53	0.73
H-124	KC777379	Blood	1	1	1	1
C. orthopsilosis						
HP-179	KC777377	Blood	1	0.68	0.56	0.75
H-152	KC777376	Blood	0.70	1	1	1
C. metapsilosis						
M-18	KC777375	Skin	0.44	0.79	1	0.77
ATCC 96144	KC777380	Skin	1	0.80	1	1

[a]Pz index: very strong, Pz < 0.69; strong, Pz = 0.70–0.79; mild, Pz = 0.80–0.89; weak, Pz = 0.90–0.99; negative, Pz = 1.

housed in cages of five mice each. All mice were given food and water *ad libitum* and were monitored daily for 15 days. Care, maintenance, and handling of the animals were in accordance with the Mexican government's license conditions for animal experimentation and the Guidelines for the Care and Use of Laboratory Animals. Experiments were conducted with the approval of the Ethics and Research Committee, Facultad de Medicina, UANL in Monterrey, Nuevo León, Mexico (registration code MB12-002).

Experimental disseminated candidiasis

Twenty animals were infected intravenously through the lateral tail vein with 1.5×10^7 CFU/mouse of each strain in 200 μl of a yeast suspension. Three uninfected mice were used as controls per experimental day. No immunosuppressive scheme was used.

Fungal tissue burden assays

Five mice per strain were sacrificed by cervical dislocation on experimental days 2, 5, 10, and 15 post infection. After sacrifice, spleen, kidneys, liver, and lungs of each mouse were immediately aseptically removed, weighed, and placed in sterile phosphate-buffered saline solution (138 mM NaCl, 3 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). The organs were mechanically homogenized (Polytron-Aggregate, Kinematica) and serially diluted 1:10 in sterile saline. Aliquots of 0.1 ml of the undiluted and diluted homogenates were then plated twice onto SGA plates, and colony counts were performed after 48 h of incubation at 37°C. The entire *in vivo* experiment was performed twice at different times.

Histopathology

After mice sacrifice, tissues were immediately removed and fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded, and sliced into 5-μm sections. The sections were stained with Grocott methenamine silver and examined by light microscopy in a blinded fashion.

Statistics

The fungal tissue burdens of the tested organs in the different experimental groups were analyzed using the Kruskal-Wallis test in SPSS (SPSS version 17.0 for Windows; SPSS Inc., Chicago, IL, USA). $P \leq 0.05$ was considered significant.

Results

The strains used in this study were chosen on the basis of their aspartyl proteinase, phospholipase, esterase, and hemolysin *in vitro* activities (Table 1). One isolate of each species was found to have phospholipase and hemolysin activities, whereas activity for just one enzyme was noted in their counterparts. However, strain H-124's enzymatic profile was negative for the four extracellular hydrolytic enzymes included in the investigation. The fungal tissue burden results are summarized in Table 2. Throughout the course of infection, mice did not lose weight or have ocular disorders and did not have motor impairment. However, slight pilo-erection episodes were sporadically noted in mice, principally during the first 5 days post challenge. In general, the highest fungal load of all six strains was detected in kidney, followed by spleen, lung, and liver tested on the 4 experimental days. The fungal burden tended to decrease by day 15 post infection in all groups, as depicted in Fig. 1. On day 2, the fungal load of the kidneys was statistically significant in comparison with liver and lung, except for strain H-152. Moreover, the fungal burden of the kidneys was significant compared with spleen, liver, and lung by day 5, except in strains H-152 and ATCC 96144.

Since their reclassification as three phylogenetically independent species, *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* have been increasingly investigated due to their possible clinical importance. Experimental models of disseminated candidiasis have been developed principally in mice, and many of them focused on the therapeutic efficacy of antifungal treatment schemes [19,20] and host immune responses against fungal infections [21,22]. However, one study of *Candida* spp. pathogenicity in a murine model of systemic infection was previously published by Arendrup et al. [23]. They reported that mice infected with 10^7 CFU of *C. parapsilosis* did not die and that yeasts were not detected in kidneys on day 7 post challenge [23]. Despite the fact that this report was our direct antecedent, the results were different from ours, likely because they used female CF1 mice and *C. parapsilosis* strains that were not confirmed by molecular approaches and because the experimental design of the their study was somewhat dissimilar from ours. Later, Gácser et al. demonstrated the important role of lipase in *C. parapsilosis* virulence using an efficient gene deletion system based on the nourseothricin resistance marker (*caSAT1*) and its subsequent deletion by FLP-mediated [24]. They found that the homozygous lipase-negative *C. parapsilosis* mutants were significantly less virulent compared with the wild-type strain when intraperitoneally inoculated in female BALB/c mice. However, they reported no differences in fungal burden or survival in the murine intravenous infection model using inocula of 10^7 fungal cells [24]. Recently, Bertini et al. tested the pathogenic potential of the *C. parapsilosis* complex in estrogen-treated BALB/c mice during a vaginal infection with 10^6 yeasts and reported significant differences in

Table 2. Fungal tissue burden results in mice intravenously infected with inocula of 1.5×10^7 CFU/mouse.

Strain (GenBank accession number)	Organ	Log CFU/g tissue (median [range]) Days post- infection 2	5	10	15
Candida parapsilosis					
c/c 105	Spleen	5.45 (5.28–5.57)	3.54 (3.45–3.91)	2.89 (0–3.08)	2.51 (0–2.83)
(KC777378)	Kidney	5.30 (5.23–5.54)	4.15 (3.89–4.28)	2.46 (0–5.04)	2.32 (2.18–3.20)
	Liver	4.72 (4.53–4.86)	2.43 (2.06–2.68)	1.40 (0–1.63)	1.67 (0–1.90)
	Lung	4.23 (4.08–4.34)	3.11 (2.65–3.20)	2.15 (0–2.30)	2.20 (0–2.53)
H-124	Spleen	5.23 (5.11–5.38)	3.93 (3.71–4.26)	3.08 (2.43–3.59)	3.04 (2.43–3.28)
(KC777379)	Kidney	5.20 (5.11–5.49)	4.28 (4.23–4.56)	4.63 (3.52–5.23)	4.83 (4.11–5.36)
	Liver	3.97 (3.86–4.26)	2.79 (2.75–3.15)	1.77 (1.34–2.00)	1.93 (1.72–2.52)
	Lung	4.36 (4.11–4.40)	3.46 (3.28–3.66)	2.72 (2.32–3.53)	3.23 (2.53–3.91)
C. orthopsilosis					
HP-179	Spleen	5.40 (5.30–5.54)	3.56 (3.18–3.72)	2.83 (2.72–3.28)	0 (0–2.36)
(KC777377)	Kidney	5.46 (5.40–5.54)	3.99 (3.72–4.08)	3.64 (3.08–4.08)	3.82 (2.67–4.54)
	Liver	4.67 (4.40–4.78)	2.53 (2.52–2.78)	1.41 (0–1.89)	0 (0–1.78)
	Lung	4.73 (4.41–4.94)	3.26 (3.11–3.38)	2.53 (2.36–2.68)	2.15 (0–2.89)
H-152	Spleen	5.43 (5.08–5.45)	3.81 (3.62–4.00)	2.57 (2.34–2.75)	2.57 (0–2.90)
(KC777376)	Kidney	5.15 (5.04–5.20)	4.52 (4.26–5.15)	4.48 (4.28–5.23)	4.38 (3.91–4.86)
	Liver	5.15 (5.04–5.20)	3.34 (3.23–3.52)	1.89 (1.32–3.04)	1.41 (0–1.51)
	Lung	4.97 (4.80–5.23)	3.76 (3.51–4.04)	2.28 (0–2.81)	2.49 (0–3.11)
C. metapsilosis					
M-18	Spleen	5.00 (4.83–5.15)	3.63 (3.34–3.72)	2.76 (2.66–3.08)	2.18 (0–2.26)
(KC777375)	Kidney	5.11 (5.04–5.18)	4.40 (4.26–4.56)	4.48 (4.34–5.11)	3.34 (0–4.71)
	Liver	4.04 (3.79–4.11)	2.62 (2.48–3.04)	2.11 (1.86–2.81)	1.30 (0–1.61)
	Lung	4.58 (4.45–5.15)	3.28 (3.23–3.59)	3.00 (2.38–3.20)	2.26 (0–2.91)
ATCC 96144	Spleen	5.11 (4.85–5.32)	3.68 (2.87–3.98)	2.58 (2.45–3.15)	2.65 (0–3.38)
(KC777380)	Kidney	5.00 (4.26–5.11)	4.11 (3.46–4.43)	4.11 (3.30–5.11)	4.36 (3.69–5.38)
	Liver	4.41 (4.11–4.46)	2.81 (2.18–3.08)	1.88 (1.83–3.23)	2.11 (1.38–3.77)
	Lung	4.34 (3.46–4.40)	3.54 (2.86–3.79)	2.68 (2.40–2.73)	2.58 (2.18–2.85)

infection kinetics among the psilosis group species [11]. Mice infected with *C. metapsilosis* displayed a reduced vaginal fungal burden, as well as spontaneous infection clearance at day 28 post challenge for all strains tested. However, given that the physiopathological basis of the local vaginal candidiasis is quite different from the disseminated disease, coupled with the fact that the strains used by Bertini et al. were not characterized according to enzymatic profiles, we cannot contrast our results with those previously reported.

Although the *in vitro* enzymatic profile of the strains showed important differences regarding their aspartyl proteinase, phospholipase, esterase, and hemolysin activities, significant differences among the psilosis group (in terms of tissue fungal burden) were not detected when assayed *in vivo*, indicating that possibly the three species of the complex have a similar pathogenic potential in disseminated infection in immunocompetent hosts, at least with the strains tested in this study and under the experimental design of our model. Further studies with more wholly characterized strains are needed in order to prove this hypothesis. To our knowledge, this is the first report looking for a correlation between the *in vivo* pathogenicity of the *C. parapsilosis* complex species with different *in vitro* enzymatic profiles.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

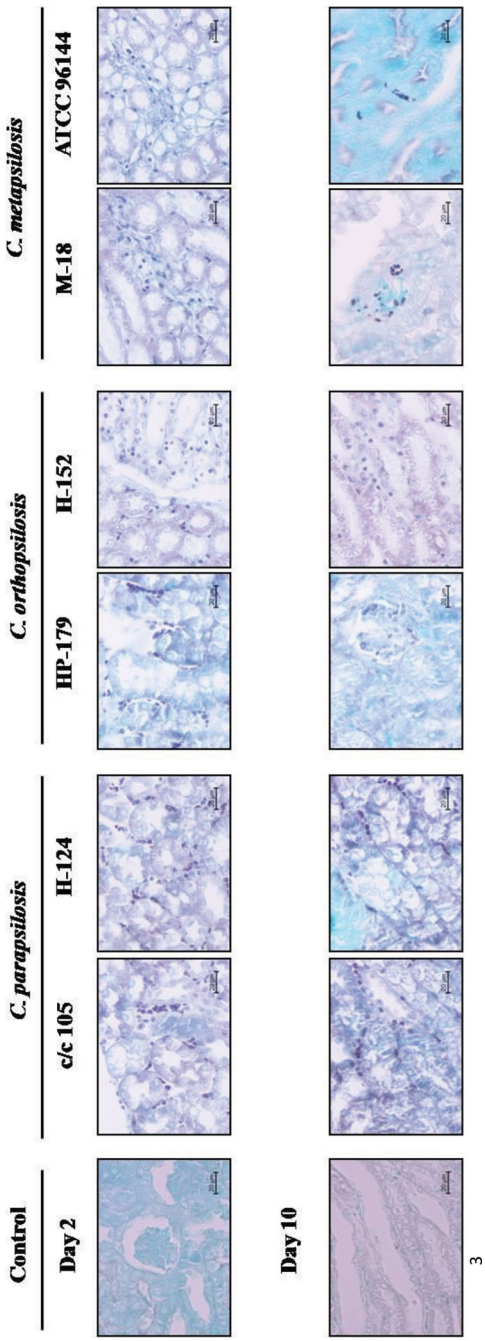

Figure 1. Representative histopathological Grocott methenamine silver–stained sections of kidneys from BALB/c mice intravenously infected with 1.5×10^7 CFU/mouse of the strains tested by day 2 and day 10 post challenge. Original magnification ×600. This Figure is reproduced in color in the online version of *Medical Mycology*.

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