RESEARCH PAPER

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The development of animal infection models and antifungal efficacy assays against clinical isolates of *Trichosporon asahii*, *T. asteroides* and *T. inkin*

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Abbreviations: AMB, Amphotericin B; CFG, Caspofungin; CLSI, Clinical Laboratory Standards Institute; FLC, Fluconazole; GMS, Gomori methenamine silver; ITZ, Itraconazole; MIC, Minimal Inhibitory Concentration; PBS, Phosphate Buffered Saline; PSC, Posaconazole; SDA, Sabouraud Dextrose Agar; VRC, Voriconazole.

The present study developed *Galleria mellonella* and murine infection models for the study of *Trichosporon* infections. The utility of the developed animal models was demonstrated through the assessment of virulence and antifungal efficacy for 7 clinical isolates of *Trichosporon asahii*, *T. asteroides* and *T. inkin*. The susceptibility of the *Trichosporon* isolates to several common antifungal drugs was tested *in vitro* using the broth microdilution and the E-test methods. The E-test method depicted a lower minimal inhibitory concentration (MIC) for amphotericin and a slightly higher MIC for caspofungin, while MICs observed for the azoles were different but comparable between both methods. All three *Trichosporon* species established infection in both the *G. mellonella* and immunosuppressed murine models. Species and strain dependent differences were observed in both the *G. mellonella* and murine models. *T. asahii* was demonstrated to be more virulent than the other 2 species in both animal hosts. Significant differences in virulence were observed between strains for *T. asteroides* in the murine model. In both animal models, fluconazole and voriconazole were able to improve the survival of the animals compared to the untreated control groups infected with any of the 3 *Trichosporon* species. In *G. mellonella*, amphotericin was not able to reduce mortality in any of the 3 species. In contrast, amphotericin was able to reduce murine mortality in the *T. asahii* or *T. inkin* models, respectively. Hence, the developed animal infection models can be directly applicable to the future deeper investigation of the molecular determinants of *Trichosporon* virulence and antifungal resistance.

Introduction

Trichosporon species are anamorphous yeast-like basidiomycetes that have a worldwide distribution and can cause a range of opportunistic infections, including superficial infections such as white piedra in immunocompetent patients, plus mucosa-associated and systemic infections in immunocompromised patients.[1] *Trichosporon* spp. can be found in soil, decomposing wood, air, rivers, lakes, seawater, cheese, scarab beetles, bird droppings, bats, pigeons, and cattle.[2] They belong to the human microbiome, as colonizers of the gastrointestinal and oral cavities, male perigenital skin (scrotal, perianal, and inguinal sites of the body) and temporarily inhabiting the respiratory tract and skin.[3-12] It is not clear how Trichosporonosis can be acquired, but the following possibilities can be envisaged: (i) poor hygiene, (ii) bathing in contaminated water, (iii) sexual transmission, (iv) hair humidity and the length of the scalp hair (more specifically in the case of acquiring white piedra), (v) gastrointestinal colonization and further translocation throughout the gut (deep-seated infections), and (vi) exogenously acquired through a percutaneously inserted intravascular catheter via colonized skin.[13-18]

In spite of the fact that *Trichosporon* spp are probably the second or third most common non-*Candida* yeast infections causing invasive disease in patients with hematological cancer, there are few reports related to virulence factors of this genus.[2,19-21] The main causes of infection by invasive *Trichosporon* spp. are central and vesical venous catheters, and peritoneal catheter-related devices. Since these organisms are skilled at adhering to these devices and apt at forming biofilms, they are able to escape from antifungal drugs and host immune responses.[2,22] It has been speculated that the secretion of proteases and phospholipases are important virulence

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factors for scavenging nutrient inside the host.[23] These pathogens are also able to produce glucuronoxylomannan (GXM) in their cell walls, similarly to *Cryptococcus neoformans*[24,25] and it is possible this polysaccharide may help to attenuate phagocytosis by host immune cells.[25] However, there is no genetic evidence about the functionality of these virulence factors.

In recent years the number of cases of *Trichosporon* infections has increased, and in the cases of systemic infections the prognosis is very poor.[26] Three of the most common species isolated from a clinical setting are *T. asahii*, *T. asteroides* and *T. inkin*. Fatal disseminated infections are predominately associated with *T. asahii*,[27,28] while superficial infections are more frequently associated with *T. asteroides*, which can also occasionally establish disseminated infections.[26,28] *T. inkin* is commonly isolated from cases of white piedra, while rarely being reported to cause deep-seated infections.[29,30] Traditional antifungal drugs commonly used to treat other fungal infections, such as amphotericin B and fluconazole, usually are not very efficient against members of the genus *Trichosporon*, including the 3 aforementioned species.[31,32] Resistance to current antifungal therapies therefore contributes to the poor survival rate of immunocompromised patients with Trichosporonosis. Subsequently, a better understanding of the virulence mechanisms deployed by *Trichosporon* species, and molecular determinants for antifungal resistance, is urgently required to improve the efficacy of current treatments. In order to develop more effective antifungal therapies, new *in vitro* and *in vivo* assays that permit large scale molecular studies are required.

The present study evaluated the *in vitro* activity of multiple antifungal agents with differing modes of action, including azoles fluconazole (FLC), itraconazole (ITZ), posaconazole (PSC) and voriconazole (VRC), one echinocandin, caspofungin (CFG) and the polyene amphotericin B (AMB). Two different *in vitro* antifungal efficacy assays were used against 7 clinical isolates of *T. asahii*, *T. asteroides* and *T. inkin*. Previous to our study, there were few studies reporting animal models for Trichosporonosis.[33-39] We extended these studies by developing *Galleria mellonella* and murine models of *Trichosporon* infection and used them to further

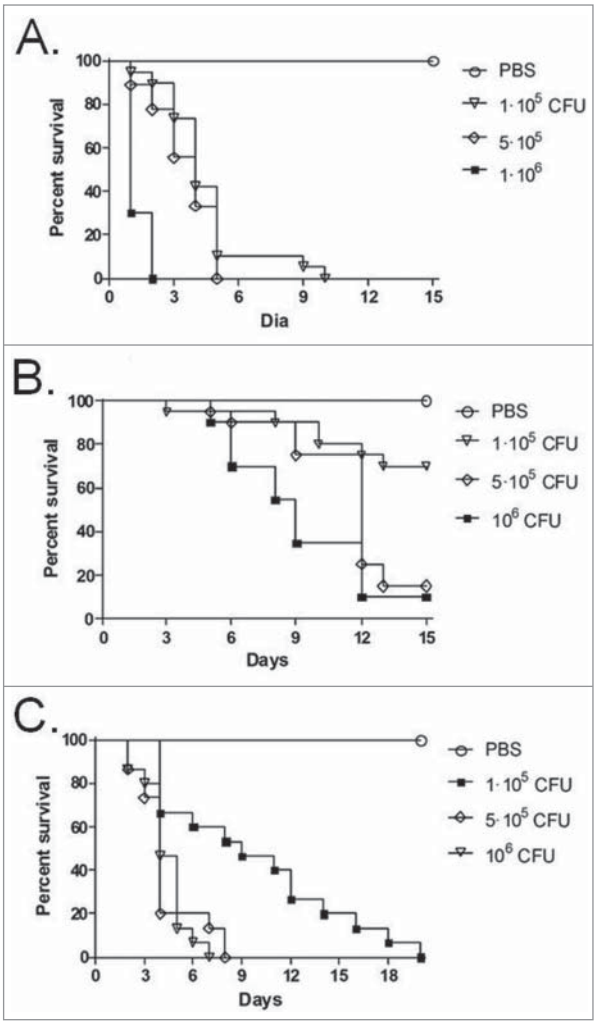

Figure 1. Determination of the cell concentrations for mortality experiments of *G. mellonella* larvae infected with *T. asahii* strain 07 (**A**), *T. asteroides* strain 13 (**B**) or *T. inkin* (**C**).

Table 1. *In vitro* antifungal activity of FLC, ITZ, PSC, VRC, AMB and CFG against all *Trichosporon* strains using the CLSI broth microdilution method

	Fluconazole MIC (μg/ml)		Itraconazole MIC (μg/ml)		Posaconazole MIC (μg/ml)		Voriconazole MIC (μg/ml)		Amphotericin B MIC[a] (μg/ml)		Caspofungin MIC (μg/ml)	
Strain	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
T. asahii 04	1.0	1.0	0.06	0.06	0.25	0.25	<0.03	0.06	1	8	8	16
*T. asahii*05	0.5	1.0	0.06	0.06	0.125	0.125	<0.03	<0.03	1	8	16	16
*T. asahii*07	1.0	1.0	0.06	0.06	0.125	0.125	<0.03	<0.03	1	1	16	16
*T. asteroides*01	0.5	0.5	0.06	0.06	0.125	0.125	<0.03	<0.03	1	2	4	8
*T. asteroides*06	0.5	0.5	0.06	0.06	0.125	0.25	<0.03	<0.03	1	8	16	16
*T. asteroides*13	0.5	0.5	0.06	0.06	0.125	0.125	<0.03	<0.03	1	4	16	16
T. inkin	1.0	2.0	<0.03	0.125	1.0	1.0	<0.03	0.06	0.5	2	8	8

[a]For amphotericin B the MIC corresponded to the concentration that produced a complete inhibition of growth (100%). For the rest of the drugs, the MIC corresponded to a growth inhibition of 50%.

Table 2. *In vitro* antifungal activity of, FLC, ITZ, PSC, VRC, AMB and CFG against the 7 strains of *Trichosporon* using the E-test method

Strain	Fluconazole MIC (μg/ml)	Itraconazole MIC (μg/ml)	Posaconazole MIC (μg/ml)	Voriconazole MIC (μg/ml)	Amphotericin B MIC[a] (μg/ml)	Caspofungin MIC (μg/ml)
T. asahii04	4.0	0.38	0.25	0.064	0.38	>32
T. asahii05	0.75	0.064	0.47	0.032	2.0	>32
T. asahii07	0.75	2.0	0.38	0.064	0.38	>32
T. asteroides01	1.5	0.047	0.47	0.032	0.25	>32
T. asteroides 06	0.75	1.5	0.25	0.047	1.0	>32
T. asteroides 13	0.5	0.5	0.064	0.023	0.19	>32
T. inkin	0.38	1.0	0.19	0.064	0.064	>32

[a]For amphotericin B the MIC corresponded to the concentration that produced a complete inhibition of growth (100%). For the rest of the drugs, the MIC corresponded to an eminent growth inhibition (80%).

assess the efficacy of the 3 antifungal therapies, AMB, FLC, and VRC, *in vivo*. The combination of these *in vitro* and *in vivo* assays represents the framework for use in large scale investigations that study the molecular determinants of virulence and antifungal resistance.

Results

In vitro antifungal efficacy evaluation

The CLSI broth microdilution and the E-test methods for determining the minimum inhibitory concentration (MIC) were used to assess the efficacy of FLC, ITZ, PSC, VRC, CFG and AMB against the 7 *Trichosporon* isolates (**Tables 1 and 2**). The MICs obtained with the CLSI method were in agreement with those obtained by Chagas-Neto *et al.*,[26] which tested the same *T. asahii* and *T. asteroides* strains. The E-test method depicted a lower MIC for AMB and a slightly higher MIC for CFG, while MICs observed for the azoles were different but comparable between both methods. These variations between methods are in accordance with the results obtained

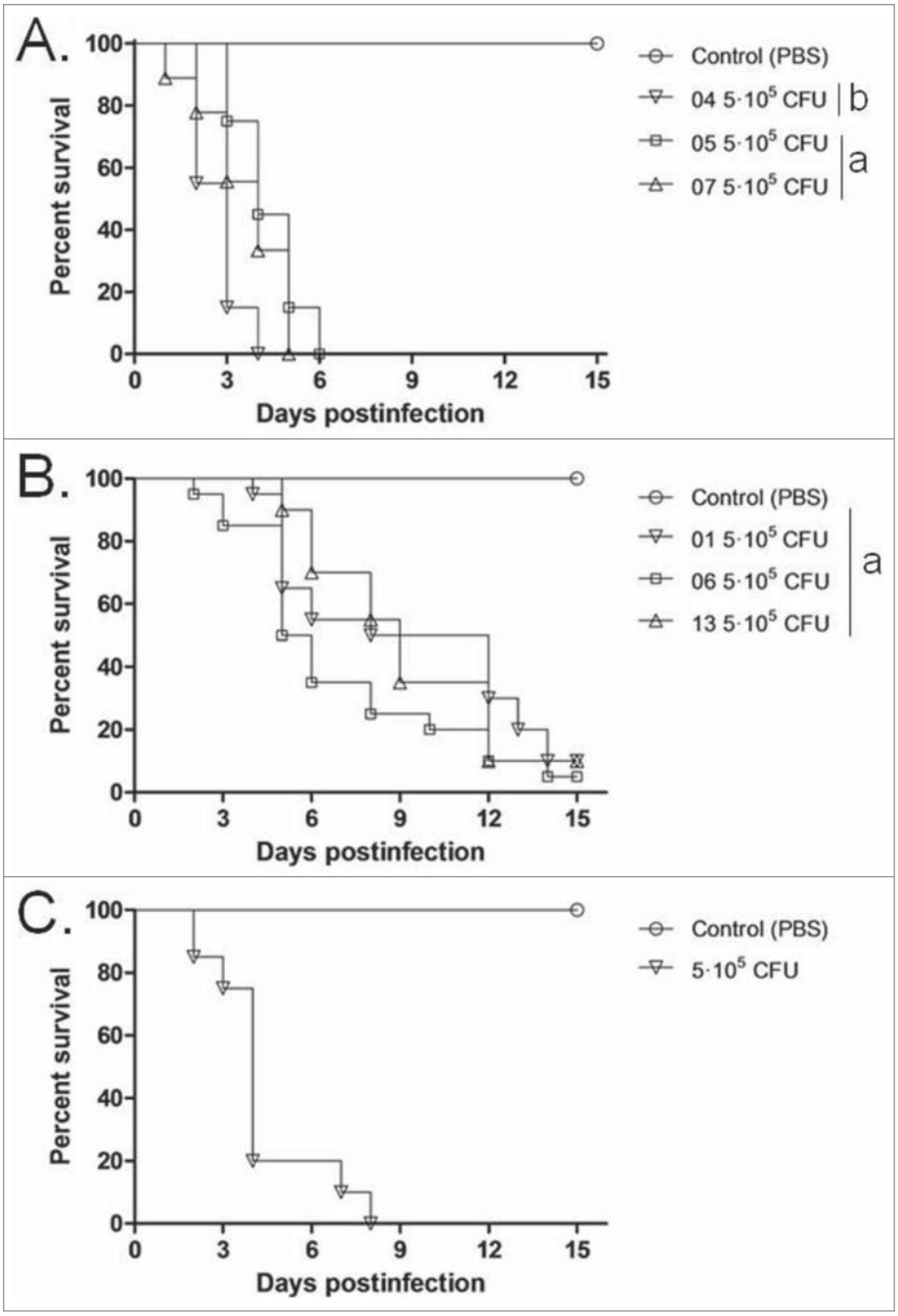

Figure 2. Cumulative mortality of *G. mellonella* larvae infected with *T. asahii* (**A**), *T. asteroides* (**B**) or *T.inkin* (**C**). The mean survival time was estimated by the Kaplan-Meier method and compared among groups using the log-rank test ([a], $P > 0.05$, and[b], $P < 0.05$). All groups are different from the control (PBS).

by Metin et al.[40] which also tested the susceptibility of *Trichosporon* isolates.

Galleria mellonella virulence model

Inocula from one strain of each species, ranging in concentration between 1×10^5 and 1×10^6 CFU/larva, were used to investigate the virulence of *Trichosporon* against *G. mellonella* (**Fig. 1**). An inoculum of 5×10^5 CFU/larva was considered to be the optimal dose for producing acute infection, with 80 to 100% of larvae dying within 15 days post-infection and was subsequently used to compare all the 7 strains (**Fig. 2**). In this model, *T. asahii* was demonstrated to be the most virulent species, with the 3 strains killing all the larvae within 6 days. Increasing the inoculum of the *T. asahii* 07 strain to 1×10^6 CFU killed all larvae within 3 d. *T. inkin* was also able to kill all the infected larvae within 8 days after infection, but no significant increase in mortality rate was observed with the larger inoculum. *T. asteroides* proved to be the least virulent species in the insect virulence model, with none of the tested strains being able to attain a mortality rate of 100% within 15 days post-infection, even when applying a larger inoculum of 1×10^6 CFU for the 01 strain (data not shown). Histopathological sections of the larvae show the high amount of fungal elements present in the larvae 2 days after infection for all 3 species (**Fig. 3**).

The effect of the AMB, FLC and VRC on *G. mellonella* mortality rate was assessed by using previously defined drug concentrations.[41,42] A high dose of FLC or VRC was able to improve the survival of the animals compared to the untreated control groups infected with any of the 3 *Trichosporon* species, while AMB was not able to reduce mortality in any of the 3 species (**Fig. 4**).

Murine virulence model

The different *Trichosporon* strains and species were evaluated in a murine virulence model. *T. asahii* once again showed greater virulence compared to the other 2 species, which both required a larger inoculum or stronger immunosuppression to attain a significant mortality rate (**Fig. 5**). The three *T. asteroides* strains demonstrated a range of virulence in the murine model, when using an inoculum concentration double that of *T. asahii*, including *T. asteroides* strain 13 which was unable to kill the immunosuppressed mice. The single *T. inkin* strain tested was unable to kill immunosuppressed mice when using the same inoculum concentrations as *T. asahii* and was only able to attain a 60% mortality rate when using the same inoculum as the one used for *T. asteroides* strains (6×10^7 CFU/ml, data not shown). A larger inoculum of *T. inkin* (1×10^8 CFU/ml) caused the death of all the mice within hours after infection, possibly due to a physical obstruction of capillaries.[43] To avoid the immediate death of the animals, a stronger immunosuppression program and the same inoculum as *T. asahii* were used (3×10^7 CFU/ml), attaining a mortality rate of approximately 70%, and in turn providing a suitable model for evaluating the efficacy of antifungal agents *in vivo*. One strain of each species was subsequently selected to evaluate the efficacy of antifungal agents *in vivo*, *T. asahii* strain 07, *T. asteroides* strain 01 and the single *T. inkin* strain. Histological studies of the 3 selected strains revealed conidial and hyphal elements for all 3 *Trichosporon* species diffusely infiltrated the kidneys of the animals (**Fig. 6**).

The effect of AMB, FLC and VRC on murine mortality rate was assessed (**Fig. 7**). A high dose of FLC was able to improve the survival of the animals compared to the untreated control groups infected with any of the 3 *Trichosporon* species, while AMB and VRC were able to reduce mortality in the *T. asahii* or *T. inkin* models, respectively.

Subsequently, the fungal burden within the kidneys and spleen of the infected mice was determined. All the 3 antifungal treatments showed some degree of reduction in fungal burden against the 3 *Trichosporon* species. These reductions were statistically significant mostly on kidneys (**Fig. 8**). All three antifungal treatments reduced the fungal burden in the kidneys of mice infected with any of the 3 species studied, but only FLC

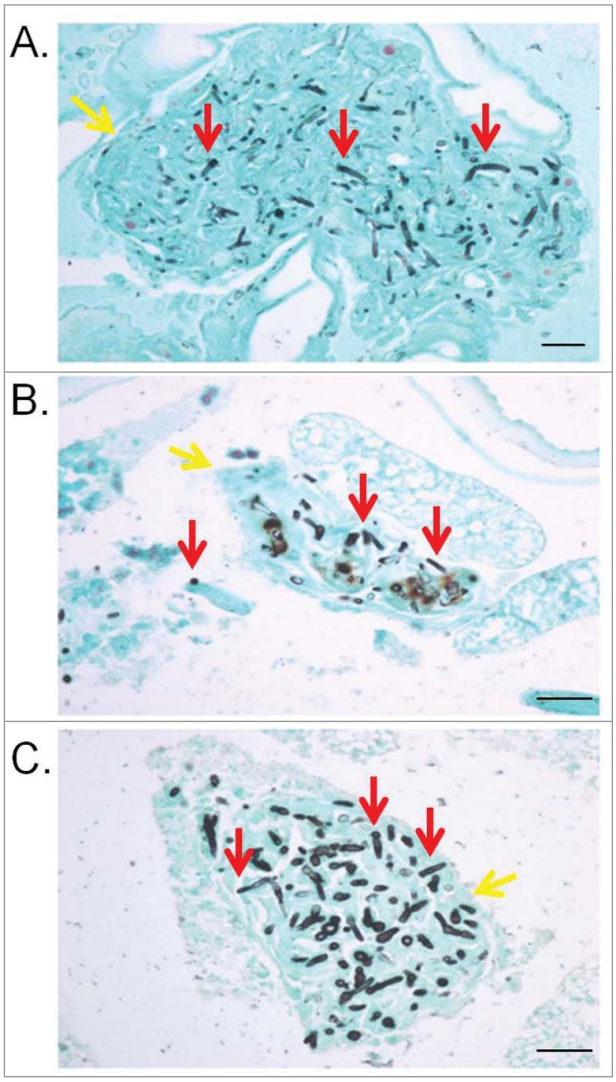

Figure 3. *G. mellonella* internal structures affected by trichorporonosis caused by *T. asahii* 07 (**A**), *T. asteroides* 01 (**B**) or *T. inkin* (**C**) 2 days postinfection. The red arrows point the fungal cells and the yellow arrows point *G. mellonella* tissues. GMS stain. Bars, 20 μm. Magnification, x400.

and VRC reduced the fungal burned in the spleens infected with *T. asahii* and *T. asteroides* respectively. FLC was more effective than VRC at reducing *T. asahii* (**Fig. 8A**) and *T. asteroides* (**Fig. 8B**) burden in the kidneys, while VRC was more effective than AMB in the case of *T. asteroides* infected spleens (**Fig. 8B**). Fungal burden reductions were more modest in the spleens of infected animals. None of 3 antifungals significantly reduced the fungal burden in spleens of mice infected with *T. inkin*.

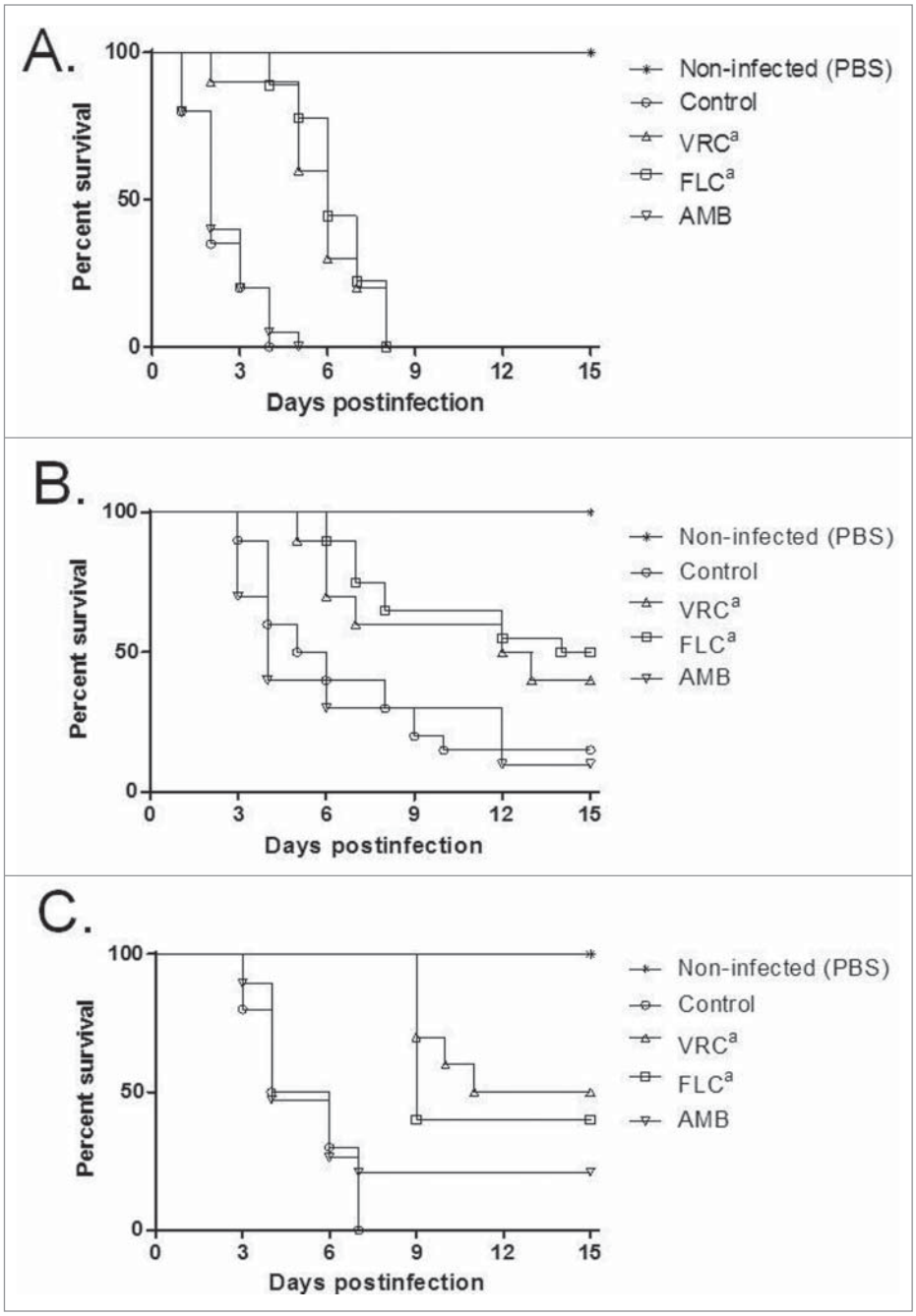

Figure 4. Effects of antifungal treatments on cumulative mortality of *G. mellonella* larvae infected with *T. asahii* strain 07 (**A**), *T. asteroides* strain 01 (**B**) or *T. inkin* (**C**). Single doses of antifungal drugs were administered 4 hours after infection in a volume of 10 μl. Control groups received 10 μl of PBS 4 hours postinfection. Drug concentrations were: AMB, amphotericin B deoxycholate at 0.5 mg/kg; FLC, Fluconazole at 20 mg/kg; and VRC, voriconazole at 15 mg/kg ([a], $P < 0.05$ versus control and amphotericin B).

Discussion

Prior to the taxonomic rearrangement of the genus in the nineties,[44] reports of the clinical importance of *Trichosporon* were scarce and inconsistent. Only recently, larger studies which utilized more accurate molecular approaches for species identification, started to shed light on the species-specific distribution, the importance of clinical manifestations and the antifungal susceptibilities, of *Trichosporon*.[26,45] The established *Candida* breakpoints have been used as a reference to describe the susceptibility results obtained with *Trichosporon*,[28,46,47] but unfortunately the availability of clinical and *in vitro* susceptibly data is not sufficient enough to clearly define guidelines for *Trichosporon* treatments or even susceptibility breakpoints. However, a common trend exists for the use of azoles, and specially VRC, rather than AMB or the echinocandins,[2] although some clinical reports have described the efficacy of CAS against *T. inkin* when used solely or in combination with AMB.[48,49]

Over recent years several non-vertebrate animal models have emerged as an alternative to the mammalian models of fungal infection. These models represent a powerful tool to study fungal pathogenesis, the efficacy of antifungal compounds, and innate antifungal immunity.[50] The advantages of these non-vertebrate models with respect to the classic mammalian models include the lower logistical and ethical constraints, plus the chance to design larger scale studies at low cost. The presented study developed a novel *G. mellonella* infection model for *Trichosporon* species, which can be used to assess the efficacy of new antifungal treatments or screening for new virulence factors using genetically modified strains. The results obtained with the *G. mellonella* model can be used to complement those obtained with the murine models. Hence, large scale non-vertebrate investigations can subsequently be used to refine the use of mammalian systems.

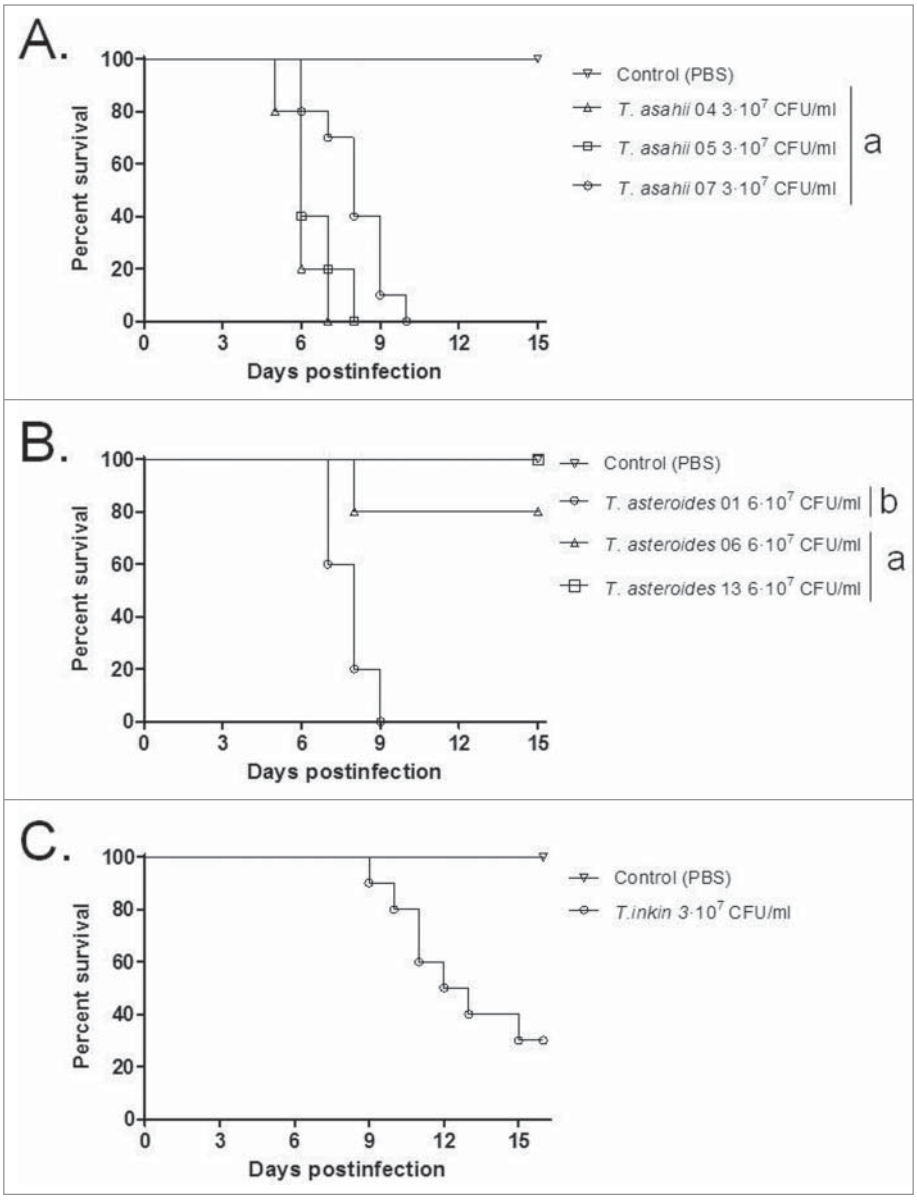

Figure 5. Cumulative mortality of mice infected with different strains of *T. asahii*, *T. asteroides* and the single one of *T. inkin*. Mice infected with *T. inkin* received an additional dose of 5-fluorouracil on day 5 after infection. The mean survival time was estimated by the Kaplan-Meier method and compared among groups using the log-rank test ([a], $P > 0.05$, and[b], $P < 0.05$).

Few mammalian models have been developed to determine antifungal efficacies or virulence for *Trichosporon* species. Older studies that used the nomenclature *T. beigelii* do not give an insight on the differences between the redefined species.[51] The only animal models currently developed are for *T. asahii* infections.[52-54] The present study, developed murine models of infection for *T. asteroides* and *T. inkin*, comparable to the already established one for *T. asahii.* The combined use of the *G. mellonella* and murine *Trichosporon* infection models will prove valuable for the evaluation of new antifungal treatments, assisting in the development of more specific therapies for the treatment of trichosporonosis.

The delayed killing rate of *G. mellonella* larvae by *T. asteroides* and *T. inkin* compared to *T. asahii* correlated with the lower virulence of these 2 species in the murine model. A remarkable observation was the strain dependent virulence of *T. asteroides*, were 2 of the 3 strains were unable to yield a high mortality rate, which was in contrast to the high fungal burden in kidneys that was similar to that of *T. asahii*. These results are in agreement with several studies where clinical data suggest that *T. asahii* is more prone to cause deep-seated infections and in the case of disseminated infections results in more fatalities than *T. asteroides* or *T. inkin*.[2,55] Interestingly, *T. asteroides* strain 06 and 13 are less virulent than *T. asteroides* strain 01. Recently, it was shown that *T. asteroides* strains 06 and 13 produce less biofilm than *T. asteroides* strain 01, and these differences could be related to the differences in virulence observed between these strains.[22] We are currently sequencing the genome of these strains and comparison among them associated to functional studies can help to reveal why they have different virulence abilities.

The antifungal susceptibility results obtained in the present study were within the range previously described for these *Trichosporon* species.[26,28] All strains were susceptible to the azole drugs and similar results were obtained using both the E-test and the microdilution methods. The *in vitro* antifungal sensitivity results correlate with the obtained reduction in mortality in the murine model, where both FLC and VRC demonstrated some degree of efficacy in the treatment of the disseminated *Trichosporon* infection. However, none of the tested antifungals completely cured the infection despite the usage of high drug doses. VRC presented lower MICs than FLC, but was not as effective at improving the survival of mice against 2 of the 3 *Trichosporon* species. This can be attributable to the inherent problems of testing VRC in the murine model where VRC levels in the serum decay rapidly even when administering grapefruit juice to the animals, which has been shown to increase the serum amounts of this drug up to therapeutic levels.[56] Accordingly, in an alternative mammalian model, the guinea pig, VRC performed better against *T. asahii* infections even at a lower dose.[57] In contrast, both VRC and FLC improved the *G. mellonella* survival infected with all 3 *Trichosporon* species. In the present study, AMB in general showed higher MICs than the azoles against all the stains tested, while

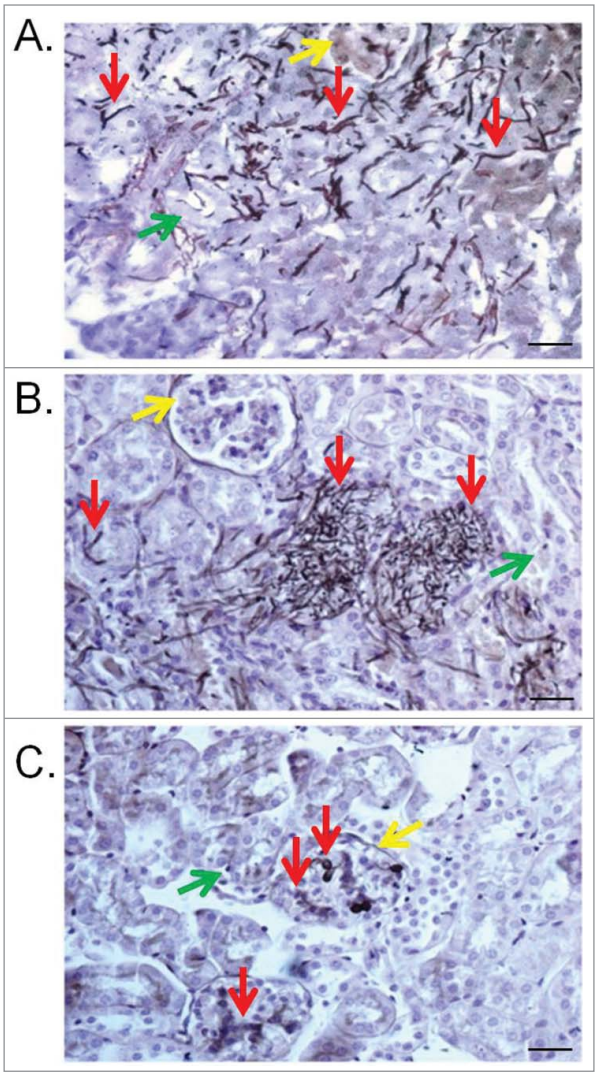

Figure 6. Kidney lesions in the murine model caused by *T. asahii* 07 (**A**), *T. asteroides* 01 (**B**) or *T. inkin* (**C**) infections 6 days after challenge. Kidney transversal sections showing hyphae, blastoconidia and arthroconidia (red arrows) in renal tubules (green arrows) and some of them in the glomerular structure (yellow arrows). GMS and hematoxylin stain. Bars, 20 μm. Magnification, x400.

overall its performance in the murine model was similar to that of VRC and slightly lower to that of FLC.

Similar to previous reports, the echinocandin CAS had almost no effect on any of the *Trichosporon* strains *in vitro* and its usage in the murine model was expected to be poorly informative. However, the usage of CAS or micafungin has yielded good results in both an experimental model and in clinical settings when used in combination with other drugs such as AMB and FLC.[49,52] In this sense, and taking in account the modest results obtained with monotherapies in our study, and their scarce efficacy in clinical setting, the usage of combined therapies appears to be the most fitting strategy to improve the outcomes of disseminated *Trichosporon* infections.[2]

The recent sequencing of *T. asahii* genome and the soon to become available genome sequences of other *Trichosporon* species (Goldman *et al.*, forthcoming) will provide a powerful tool for the dissection of the molecular virulence mechanisms deployed by *Trichosporon* species, while facilitating the identification of novel fungal targets for antifungal therapies to treat this kind of infection. Subsequently, the combination *G. mellonella* and murine assays developed in the presented study will be useful for the study of *Trichosporon* virulence and the future improvement of antifungal strategies for controlling trichosporonosis.

Materials and Methods

Strains

The three *T. asahii* strains (04,05 and 07) and 3 *T. asteroides* strains (01, 06 and 13) used in the study were all isolated from blood samples.[26] The single *T. inkin* strain used was obtained from a case of white piedra. The isolates were stored at −80°C in Yeast extract-Peptone-Dextrose with glycerol, and prior to testing they were subcultured twice on Sabouraud dextrose agar (SDA) at 37°C.

In vitro susceptibility testing

The *in vitro* susceptibility of the *Trichosporon* strains to VRC, PSC, ITZ, FLC, AMB and CFG (Sigma-Aldrich Brasil Ltda., PZ0005, 32103, I6657, F8929, Y0000005, SML0425) was determined using a microdilution method following the CLSI guidelines for yeasts.[25,51] The MICs of the echinocandins and azoles corresponded to prominent growth inhibition (approximately 50% inhibition relative to control growth) or a complete growth inhibition in the case of amphotericin B. All the strains were also tested using the E-test susceptibility method, according to the manufacturer's instructions for VRC, PSC, ITZ, FLC, AMB and CFG (bioMérieux SA, 532800, 532100, 525808, 510800, 526300, 532400).

Animal models

Experimental infection models were established for *G. mellonella* and mice. All experiments were developed in accordance with the guidelines established by the Animal Care Committee of the Universidade de São Paulo, Campus Ribeirão Preto, São Paulo, Brazil. To prepare the inocula, 24 h SDA cultures were suspended in sterile saline and filtered through sterile gauze to remove clumps of cells or hyphae. The resulting suspensions, containing ≥95% of conidial forms (arthroconidia and blastoconidia), were counted and adjusted to the desired inoculum concentration. Serial dilutions of the original suspension were cultured on SDA plates to confirm the accuracy of the counts.

Galleria model

Animals

G. mellonella larvae were obtained by cultivating crossing adult moths.[26,59,60] *G. mellonella* larvae of a similar size were selected (approximately 275–330 mg) and kept without food in petri dishes, at 37°C, in darkness for 24 h prior to use.

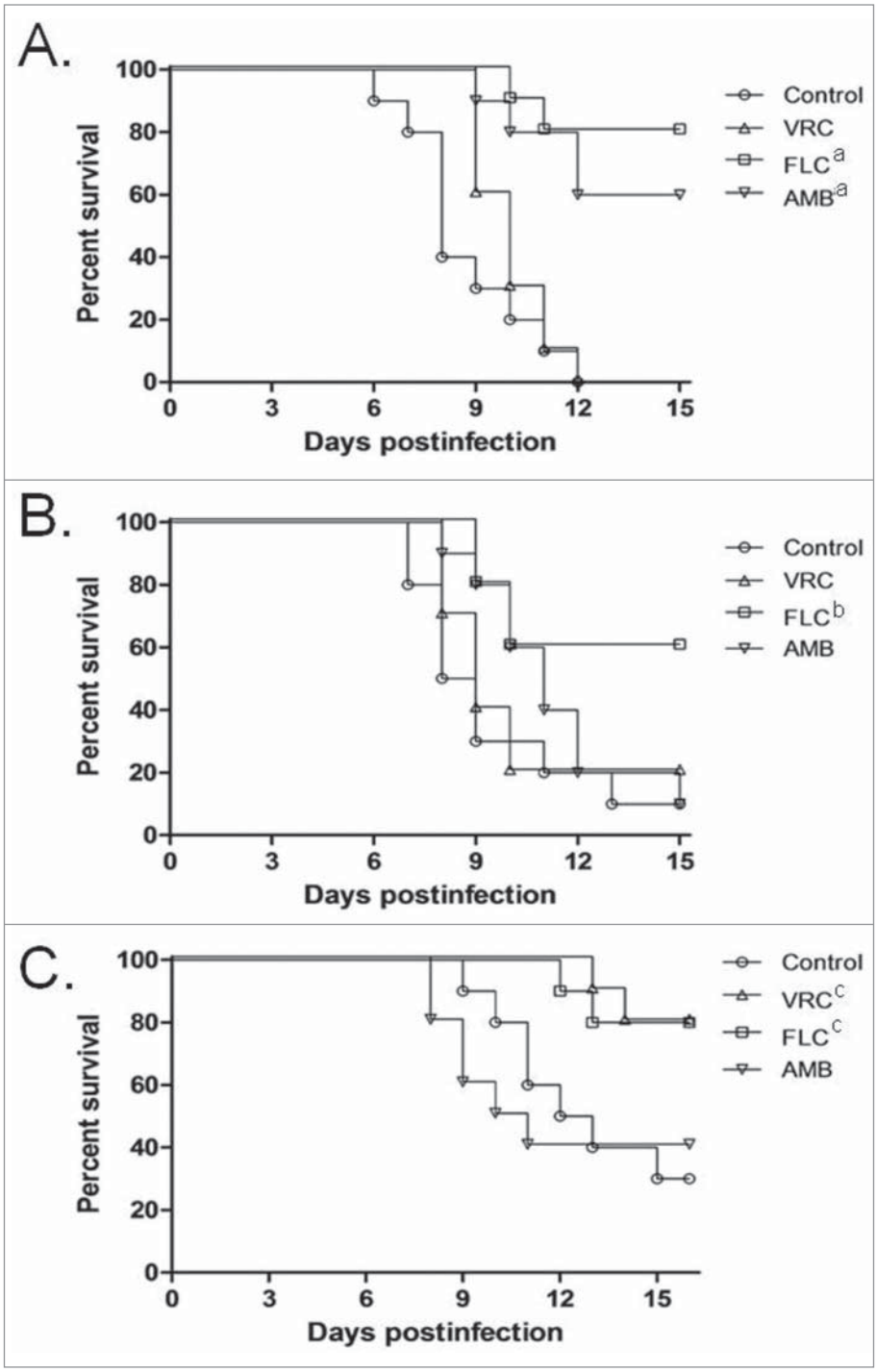

Figure 7. Effects of antifungal treatments on cumulative mortality of mice infected with *T. asahii* 07 3×10^7 CFU/ml (**A**), *T. asteroides* 01 6×10^7 CFU/ml (**B**) or *T. inkin* 3×10^7 CFU/ml (**C**). AMB, amphotericin B deoxycholate at 1.5 mg/kg/day. FLC, fluconazole at 80 mg/kg/day. VRC, voriconazole at 60 mg/kg/day. a, $P < 0.05$ vs. control. The mean survival time was estimated by the Kaplan-Meier method and compared among groups using the log-rank test ([a], $P < 0.05$, compared to the control and VRC;[b], $P < 0.05$, compared to the control and VRC;[c], $P < 0.05$, compared to the control and AMB).

Drugs

AMB deoxycholate was purchased as Unianf (União Química Farmacêutica Nacional S/A, MS 1.0497.0223.003-9), VRC was purchased as Vfend (i.v.) (Pfizer Ltda., MS 1.0216.0090.003-5), and FLC (PHR1160 Sigma).

Infection

Fungal inocula of differing concentrations were tested in order to determine the virulence of the respective strains. An inoculum of 5×10^5 CFU/animal yielded a mortality rate of 100% within 10 days after infection for *T. asahii* and *T. inkin* and >80% within 15 days for *T. asteroides*. The inoculum was prepared as described above and delivered via a Hamilton syringe to the injection site, the last left pro-leg in a volume of 5 μl.[26] After injection and recovery lapse, they were transferred to a new Petri dish and incubated at 37°C in the dark for the duration of the experiment.

Experimental design

Groups of 20 larvae were randomly chosen for each strain, while the non-infected control group was challenged with PBS. The different groups were treated as follows: AMB deoxycholate at 0.5 mg/kg of body weight; VRC 15 mg/kg and FLC at 20 mg/kg were administered in 10 μl by using a Hamilton syringe once 4 hours post-infection.[41] Control animals received 10 μl of PBS. The animals were checked daily for survival up to 15 days after infection. For survival studies, larvae were checked daily for 15 days. Larvae were sectioned in half and fixed for 24 hours in 3.7% formaldehyde–PBS. After dehydration in a series of alcohol solutions, the samples were diaphanized in xylol and embedded in paraffin. 5-μm-thick sections were collected on glass slides and stained with Gomori methenamine silver (GMS) stain following standard protocols. Briefly, sections were deparaffinized, oxidized with 4% chromic acid, stained with methenamine silver solution, and counterstained with light green. Microscopic analyses were performed using an Axioplan 2 imaging microscope (Carl Zeiss) at the stated magnifications under bright-field conditions.

Murine model

Animals

All the mice were obtained from the main bioterium from the Campus Ribeirão Preto from the Universidade de São

Paulo, Ribeirão Preto, São Paulo, Brazil. Male Swiss mice with a mean weight of 30 g were used. The animals were housed in standard boxes with wood bedding and free access to food and water. From 2 days prior to infection, the mice that received VRC were given diluted (50%) grapefruit juice (Ceres Fruit Juices Pty Ltd.) instead of water.

Drugs

AMB deoxycholate and VRC were acquired as above described and FLC as Fluconazole 150 (Prati-Donaduzzi, 7030).

Immunosuppression

For survival and tissue burden studies, mice were immunosuppressed by a single intraperitoneal (i.p.) injection of 200 mg/kg of cyclophosphamide (Genuxal, Baxter Hospitalar Ltda., MS 1.0683.0168.002-1) plus an intravenous injection of 150 mg/kg of 5-fluorouracil (Fauldfluor, Libbs Farmacêutica Ltda., MS 1.0033.0139.004-5) on the day of infection. In the case of *T. inkin* mice were immunosuppressed one day before infection and received an additional dose of 5-fluorouracil (75 mg/kg) 5 days after infection.

Infection

For survival and tissue burden studies mice were challenged with 6×10^6 CFU (*T. asahii* and *T. inkin*) or 1.2×10^7 CFU (*T. asteroides*) in 0.2 ml into the lateral tail vein. These inocula yielded a mortality rate of 90–100% within 12 days after infection, but allowing a treatment course of 5 days after infection for the strains *T. asahii* 07 and *T. asteroides* 01. For *T. inkin*, a mortality rate of 70% was attained 15 days after infection.

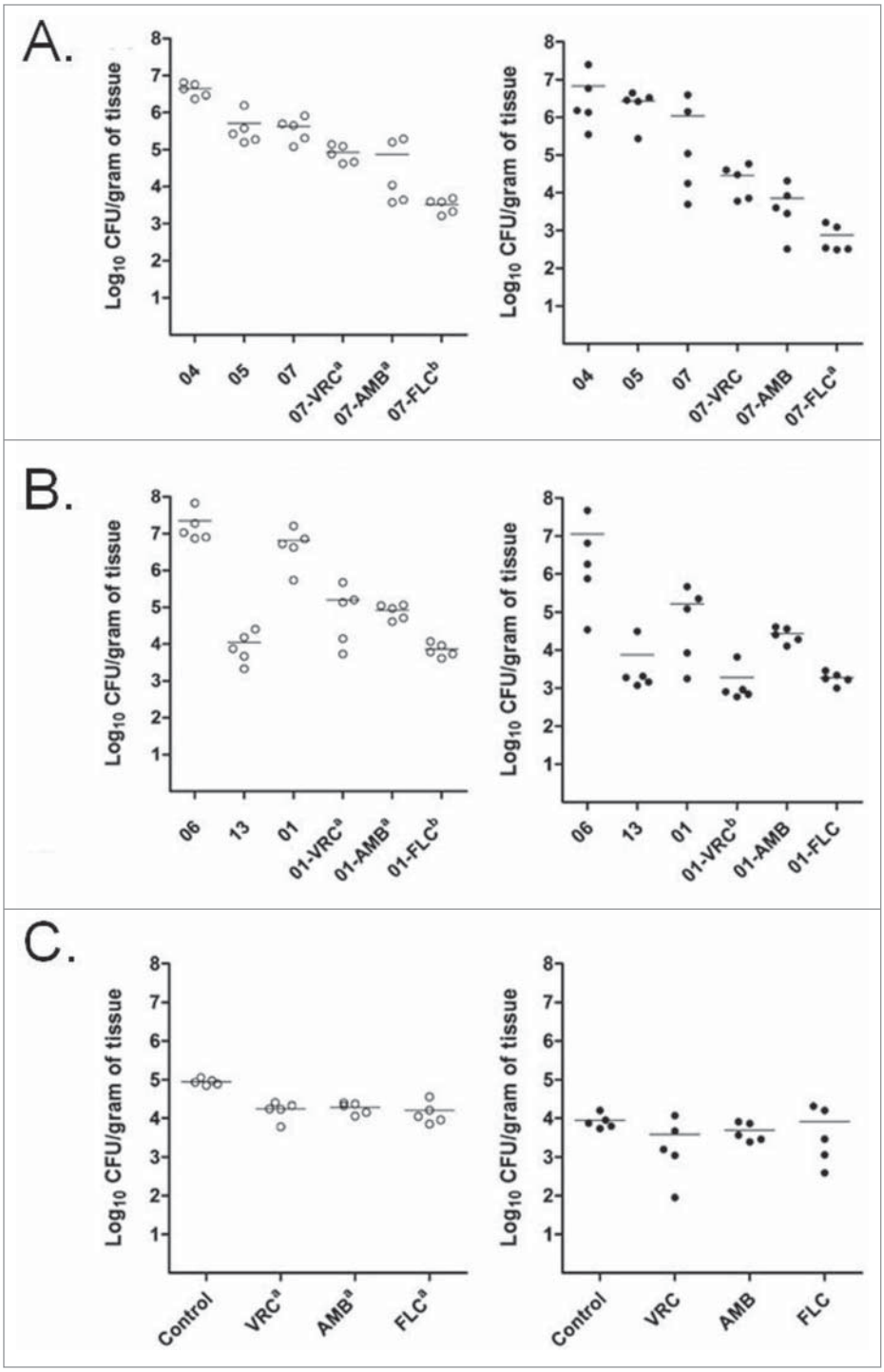

Figure 8. Effects of the antifungal treatments on tissue burden of *T. asahii* (**A**), *T. asteroides* (**B**) or *T. inkin* (**C**) in kidneys (◯) and spleen (●) of mice. AMB, amphotericin B deoxycholate at 1.5 mg/kg/day. FLC, fluconazole at 80 mg/kg/day. VRC, voriconazole at 60 mg/kg/day. a, $P < 0.05$ versus control. b, $P < 0.05$ vs. control and versus one of the other treatments [i.e., FLC vs. VRC (**A**), FLC versus AMB and VRC vs. AMB (**B**)].

Experimental design

Groups of 10 mice were randomly established for survival, and groups of 5 for tissue burden studies. The different groups were treated as follows: AMB deoxycholate at 1.5 mg/kg of body weight/dose given i.p. once daily; VRC 60 mg/kg and FLC at 80 mg/kg were given orally once daily.[60,61] Control animals received no treatment. All treatments began 24 h after challenge, and the therapy lasted for 5 days. For survival studies, mice were checked daily for 15 days. For tissue burden

and histopathology studies mice were killed one day after the completion of treatment. Spleens and kidneys were aseptically removed, and the entire organs were homogenized in 1 ml of sterile saline. Serial 10-fold dilutions of the homogenates were plated on SDA, incubated at 37°C and examined daily for 3 days. For the histopathology studies, portions of kidneys were placed in 3.7% formaldehyde–PBS and dehydrated in a series of alcohol solutions. The organs were embedded in paraffin blocks, sectioned and stained with GMS as described above. In this case the samples were counterstained with hematoxylin.

Statistics

Mean survival time was estimated by the Kaplan-Meier method and compared among groups using the log-rank test. Colony counts in tissue burden studies were analyzed using the Mann-Whitney U test. Calculations were made using Graph Pad Prism version 5.0. A P value of ≤ 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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