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

Marçal Marinê¹, Vinicius Leite Pedro Bom¹, Patricia Alves de Castro¹, Lizziane Kretli Winkelstroter¹, Leandra Naira Ramalho³, Neil Andrew Brown², and Gustavo Henrique Goldman¹,²,*

¹Faculdade de Ciências Farmacêuticas de Ribeirão Preto; Universidade de São Paulo; São Paulo, Brazil; ²Laboratório Nacional de Ciência e Tecnologia do Bioetanol – CTBE; Campinas, Brazil; ³Faculdade de Medicina de Ribeirão Preto; Universidade de São Paulo; São Paulo, Brazil

**Keywords:** antifungal, experimental infection, *Galleria mellonella*, murine model, *Trichosporon*

**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.¹²⁻¹⁹⁻²¹ 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.²²⁻²² It has been speculated that the secretion of proteases and phospholipases are important virulence
factors for scavenging nutrient inside the host. These pathogens are also able to produce glucuronoxylomannan (GXM) in their cell walls, similarly to Cryptococcus neoformans and it is possible this polysaccharide may help to attenuate phagocytosis by host immune cells. 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. 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, while superficial infections are more frequently associated with T. asteroides, which can also occasionally establish disseminated infections. T. inkin is commonly isolated from cases of white piedra, while rarely being reported to cause deep-seated infections. 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. 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. We extended these studies by developing Galleria mellonella and murine models of Trichosporon infection and used them to further

| Table 1. In vitro antifungal activity of FLC, ITZ, PSC, VRC, AMB and CFG against all Trichosporon strains using the CLSI broth microdilution method |
|---------------------------------------------------------------|
| Strain | Fluconazole (MIC, μg/ml) | Itraconazole (MIC, μg/ml) | Posaconazole (MIC, μg/ml) | Voriconazole (MIC, μg/ml) | Amphotericin B (MIC, μg/ml) | Caspofungin (MIC, μg/ml) |
|--------|---------------------------|---------------------------|---------------------------|---------------------------|-----------------------------|---------------------------|
|        | 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. asahii04 | 1.0 | 1.0 | 0.06 | 0.06 | 0.25 | 0.25 | <0.03 | 0.06 | 1 | 8 | 8 | 16 |
| T. asahii05 | 1.0 | 1.0 | 0.06 | 0.06 | 0.25 | 0.25 | <0.03 | 0.06 | 1 | 8 | 16 | 16 |
| T. asahii07 | 1.0 | 1.0 | 0.06 | 0.06 | 0.25 | 0.25 | <0.03 | 0.06 | 1 | 8 | 16 | 16 |
| T. asteroides01 | 0.5 | 0.5 | 0.06 | 0.06 | 0.125 | 0.125 | <0.03 | 0.06 | 1 | 8 | 16 | 16 |
| T. asteroides06 | 0.5 | 0.5 | 0.06 | 0.06 | 0.125 | 0.125 | <0.03 | 0.06 | 1 | 8 | 16 | 16 |
| T. inkin13 | 0.5 | 0.5 | 0.06 | 0.06 | 0.125 | 0.125 | <0.03 | 0.06 | 1 | 8 | 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 |

*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%.*
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., 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.

| Strain          | Fluconazole MIC (µg/ml) | Itraconazole MIC (µg/ml) | Posaconazole MIC (µg/ml) | Voriconazole MIC (µg/ml) | Amphotericin B MIC* (µg/ml) | Caspofungin MIC (µg/ml) |
|-----------------|-------------------------|--------------------------|--------------------------|--------------------------|-----------------------------|--------------------------|
| *T. asahii*04   | 4.0                     | 0.38                     | 0.25                     | 0.064                    | 0.38                        | >32                      |
| *T. asahii*05   | 0.75                    | 0.064                    | 0.47                     | 0.032                    | 2.0                         | >32                      |
| *T. asahii*07   | 0.75                    | 2.0                      | 0.38                     | 0.064                    | 0.38                        | >32                      |
| *T. asteroides*01 | 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                      |

*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%).*

---

**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 (*, *P > 0.05, and **, *P < 0.05). All groups are different from the control (PBS).*
by Metin et al.\textsuperscript{40} which also tested the susceptibility of \textit{Trichosporon} isolates.

**Galleria mellonella virulence model**

Inocula from one strain of each species, ranging in concentration between $1 \times 10^3$ and $1 \times 10^6$ CFU/larva, were used to investigate the virulence of \textit{Trichosporon} against \textit{G. mellonella} (Fig. 1). An inoculum of $5 \times 10^5$ 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, \textit{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 \textit{T. asahii} 07 strain to $1 \times 10^6$ CFU killed all larvae within 3 d. \textit{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. \textit{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 $1 \times 10^6$ 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 \textit{G. mellonella} mortality rate was assessed by using previously defined drug concentrations.\textsuperscript{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 \textit{Trichosporon} species, while AMB and VRC were able to reduce mortality in the \textit{T. asahii} or \textit{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 \textit{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 studies of the 3 selected strains revealed conidial and hyphal elements for all 3 \textit{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 \textit{Trichosporon} species, while AMB and VRC were able to reduce mortality in the \textit{T. asahii} or \textit{T. inkin} models, respectively.

**Murine virulence model**

The different \textit{Trichosporon} strains and species were evaluated in a murine virulence model. \textit{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 \textit{T. asteroides} strains demonstrated a range of virulence in the murine model, when using an inoculum concentration double that of \textit{T. asahii}, including \textit{T. asteroides} strain 13 which was unable to kill the immunosuppressed mice. The single \textit{T. inkin} strain tested was unable to kill immunosuppressed mice when using the same inoculum concentrations as \textit{T. asahii} and was only able to attain a 60\% mortality rate when using the same inoculum as the one used for \textit{T. asteroides} strains ($6 \times 10^7$ CFU/ml, data not shown). A larger inoculum of \textit{T. inkin} ($1 \times 10^8$ CFU/ml) caused the death of all the mice within hours after infection, possibly due to a physical obstruction of capillaries.\textsuperscript{43} To avoid the immediate death of the animals, a stronger immunosuppression program and the same inoculum as \textit{T. asahii} were used ($3 \times 10^7$ 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, \textit{T. asahii} strain 07, \textit{T. asteroides} strain 01 and the single \textit{T. inkin} strain. Histological
and VRC reduced the fungal burden 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*.

**Discussion**

Prior to the taxonomic rearrangement of the genus in the nineties, 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*. The established Candida breakpoints have been used as a reference to describe the susceptibility results obtained with *Trichosporon*, but unfortunately the availability of clinical and *in vitro* susceptibility 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, although some clinical reports have described the efficacy of CAS against *T. inkin* when used solely or in combination with AMB.

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. 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 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 (*, P < 0.05 versus control and amphotericin B).
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. The only animal models currently developed are for *T. asahii* infections. 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*. 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. 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. 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. Accordingly, in an alternative mammalian model, the guinea pig, VRC performed better against *T. asahii* infections even at a lower dose. 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...
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. In this sense, and taking into 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.
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 $5 \times 10^5$ 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. 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. 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.
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 $6 \times 10^6$ CFU (*T. asahii* and *T. inkin*) or $1.2 \times 10^7$ 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.

**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. 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 studies, mice were challenged with 6 $\times 10^6$ CFU (*T. asahii* and *T. inkin*) or 1.2 $\times 10^7$ 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)].
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 placed 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 method and compared among groups using the log-rank test. Colony counts in tissue burden studies were analyzed using the method and compared among groups using the log-rank test.

**Acknowledgments**

We would like to thank Ana Carolina Padovan and Arnaldo Lopes Colombo from the Universidade Federal de São Paulo (UNIFESP), Brazil, who kindly provided the clinical strains. We also would like to thank the editor and the 4 reviewers for their comments and suggestions.

**References**

1. Araujo Ribeiro M, Alatrace-Aizquere A, Gomez-Lopez A, Rodriguez-Tudela JL, Cuenca-Estrella M. Molecular identification and susceptibility testing of *Trichosporon* isolates from a Brazilian hospital. Rev Iberoam Micol 2008; 25:221-5; PMID:19071899; http://dx.doi.org/10.1016/S1130-4608(07)0053-6
2. Colombo AL, Padovan AG, Chaves GM. Current knowledge of *Trichosporon* spp and Trichosporonosis. Clin Microbiol Rev 2011; 24:682-709; PMID:21976044; http://dx.doi.org/10.1128/CMR.00083-11
3. Cafarchia C, Romito D, Cociocli C, Camarda A, Orranto D. Phospholipase activity of yeasts from wild birds and possible implications for human disease. Med Mycol 2008; 46:429-34; PMID:18608940; http://dx.doi.org/10.1080/13693780701885636
4. Fuenefria AM, Suh SO, Landell MF, Faganello J, Schrank A, Vainstein MH, Blackwell M, Valente P. *Trichosporon cutaneum* sp. nov., a new anamorphic biandinamunae sp. nov. Mycol Res 2008; 112:93-9; PMID:18222077; http://dx.doi.org/10.1016/j.mycres.2007.05.001
5. Gueho E, Smith MT, de Hoog GS. *Trichosporon* Behrend, p. 854-72. In C. P. Kurtzman and J. W. Fell (ed.), The Yeasts, a Taxonomic Study, 1998, 4th ed. Elsevier, Amsterdam, The Netherlands.
6. Haupt HM, Merz WG, Eicremonier WE, Vaughan WP, Saral R. Colonization and infection with *Trichosporon* species in the immunosuppressed host. J Infect Dis 1983; 147:199-203; PMID:6895994; http://dx.doi.org/10.1016/0022-1899(83)90101-3
7. Kwon-Chung KJ, Bennett JE. *Medical Mycology*, 6. Haupt HM, Merz WG, Eicremonier WE, Vaughan WP, Saral R. Colonization and infection with *Trichosporon* species in the immunosuppressed host. J Infect Dis 1983; 147:199-203; PMID:6895994; http://dx.doi.org/10.1016/0022-1899(83)90101-3
8. Kwon-Chung KJ, Bennett JE. *Medical Mycology*, 6. Haupt HM, Merz WG, Eicremonier WE, Vaughan WP, Saral R. Colonization and infection with *Trichosporon* species in the immunosuppressed host. J Infect Dis 1983; 147:199-203; PMID:6895994; http://dx.doi.org/10.1016/0022-1899(83)90101-3
9. Kwon-Chung KJ, Bennett JE. *Medical Mycology*, 6. Haupt HM, Merz WG, Eicremonier WE, Vaughan WP, Saral R. Colonization and infection with *Trichosporon* species in the immunosuppressed host. J Infect Dis 1983; 147:199-203; PMID:6895994; http://dx.doi.org/10.1016/0022-1899(83)90101-3
10. Sugita T, Kikuchi K, Makimura K, Urata K, Someya T, Martinino B, Pastore D, Picardi M, Bonini A, Chierichetti A, et al. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica 2006; 91:1068-75; PMID:16885047
11. Walsh TJ. Role of surveillance cultures in prevention and treatment of fungal infections. NCI Monogr 1990; 9:43-5; PMID:2342594
12. Walsh TJ, Groll A, Fliemens J, Fleming R, Rolides E, Anassie E. Infections due to emerging and uncommon medically important fungal pathogens. Clin Microbiol Infect 2004; 10 Suppl 1:486-66; PMID:14748880; http://dx.doi.org/10.1111/j.1476-445X.2004.00889.x
13. Benson PM, Lapins NA, Odom RB. White piedra. Arch Dermatol 1983; 119:602-4; PMID:6895990; http://dx.doi.org/10.1016/0003-6813(83)90160-3
14. Kiken DA, Sekaran A, Anaya R, Davis A, Imaeda S, Silverberg NB. White piedra in children. J Am Acad Dermatol 2006; 55:956-61; PMID:17097391; http://dx.doi.org/10.1016/j.jaad.2005.11.1033
15. Kontoyiannis DP, Torres HA, Chagau M, Hachem R, Tarrand JJ, Boderay GP, Rael II. Trichosporonosis in a tertiary care cancer center: risk factors, changing spectrum and determinants of outcome. Scand J Infect Dis 2004; 36:564-9; PMID:15370667; http://dx.doi.org/10.1080/003655404010071563
16. Roshan AS, Janaki C, Farvene B. White piedra in mothers and mother and daughter. Int J Trichology 2005; 1:140-1; PMID:20927238; http://dx.doi.org/10.1016/j.ijtrich.2004.07.006
17. Sano M, Sugitani M, Ishige T, Homma T, Tikkuchi K, Sunagawa K, Ohata T, Uehara Y, Kumasaka K, Ueno- gawa K, et al. Supplemental utility of nested PCR for the pathologic diagnosis of disseminated trichosporonosis. Virchows Arch 2007; 451:92-35; PMID:17178472; http://dx.doi.org/10.1007/s00428-007-0486-6
18. Youker SR, Andresson RJ. White piedra: further evidence of a synergistic infection. J Am Acad Dermatol 2003; 49:746-9.
19. Chagas-Neto TC, Chaves GM, Melo AS, Colombo AL. Bloodstream infections due to *Trichosporon* spp.: Species distribution, *Trichosporon asahii* genotypes determined on the basis of ribosomal DNA intergenic spacer 1 sequencing, and antifungal susceptibility testing. J Clin Microbiol 2009; 47:1074-81; PMID:19225102; http://dx.doi.org/10.1128/JCM.01614-08
20. Inoh T, Hosokawa H, Kohuda Y, Toyazaki N, Asada Y. Disseminated infection with *Trichosporon asahii*. Mycoses 1996; 39:195-8; PMID:8702074; http://dx.doi.org/10.1111/j.1365-317X.1996.tb08310.x
21. Ruan SY, Chien JY, Hsuil PR. Invasive trichosporonosis caused by *Trichosporon asahii* and other unusual *Trichosporon* species at a medical center in Taiwan. Clin Infect Dis 2009; 49:113-17; PMID:19497871; http://dx.doi.org/10.1086/599614
22. Chaumentin G, Boisier A, Piens MA, Douchet C, Burtard P, Bertrand JL, Peyramond D. *Trichosporon inkin* endocarditis: short-term evolution and clinical report. Clin Infect Dis 1996; 23:396-7; PMID:8842284; http://dx.doi.org/10.1093/clinids/23.2.396
30. Lopez JO, Alves SH, Klock C, Oliveira LT, Dal Forno NR. *Trichosporon inkin* peritonitis during continuous ambulatory peritoneal dialysis with bibliography review. Mycopathologia 1997; 139:15-8; PMID:9511232; http://dx.doi.org/10.1007/BF01103274.

31. Steinbach WJ, Perfect JR. Newer antifungal therapy for emerging fungal pathogens. Int J Infect Dis 2003; 7:525; http://dx.doi.org/10.1016/S1201-9712(03)90037-3.

32. Tokimatsu I, Kusihima H, Hashinaga K, Umecki K, Ohama M, Ishii H, Kishi K, Hiramasu K, Kadota J. The prophylactic effectiveness of various antifungal agents against the progression of trichosporonosis fungemia to disseminated disease in a neutropenic mouse model. Int J Anti Microb Agents 2007; 29:84-8; PMID:17189098; http://dx.doi.org/10.1016/j.ijantimicag.2006.09.013.

33. Yang RC, Wang WL, Ao JH, Hao ZF, Zhang J, Wang CM. Pathogenicity of *Trichosporon asahii* in a murine model of disseminated trichosporonosis. Chin Med J (Engl) 2008; 121:2557-60; PMID:19187595.

34. Serena C, Gilgado F, Mariné M, Pastor FJ, Guarro J. Efficacy of voriconazole in a guinea pig model of invasive trichosporonosis. Antimicrob Agents Chemother 2006; 50:2240-3; PMID:16723595; http://dx.doi.org/10.1128/AAC.00045-06.

35. Lacy SH, Gardner DJ, Olson LC, Ding L, Holland SM, Bryant MA. Disseminated trichosporonosis in a murine model of chronic granulomatous disease. Comp Med 2003; 53:303-8; PMID:12865877.

36. Tokimatsu I, Kusihima H, Hashinaga K, Umecki K, Ohama M, Ishii H, Kishi K, Hiramasu K, Kadota J. The prophylactic effectiveness of various antifungal agents against the progression of trichosporonosis fungemia to disseminated disease in a neutropenic mouse model. Int J Antimicrob Agents 2007; 29:84-8; PMID:17189098; http://dx.doi.org/10.1016/j.ijantimicag.2006.09.013.

37. Dismukes WE, Listemann H, Iglauer F. Invasive trichosporonosis. Antimicrob Agents Chemother 1994; 38:2541-4; PMID:7872744; http://dx.doi.org/10.1128/AAC.38.11.2541.

38. Serena C, Pastor FJ, Gilgado F, Mayayo E, Guarro J. Efficacy of micafungin in combination with other drugs in a murine model of disseminated trichosporonosis. Antimicrob Agents Chemother 2005; 49:497-2; PMID:15673724; http://dx.doi.org/10.1128/AAC.49.2.497-502.2005.

39. Serena C, Gilgado F, Mariné M, Pastor FJ, Guarro J. Efficacy of voriconazole in a guinea pig model of invasive trichosporonosis. Antimicrob Agents Chemother 2006; 50:2240-3; PMID:16723595; http://dx.doi.org/10.1128/AAC.00045-06.

40. Treviño-Rangel RD, López L, Palma-Nicolás JP, Herández-Bello R, González JC, González GM. Therapeutic efficacy of posaconazole in a murine model of disseminated trichosporonosis. J Antimicrob Chemother 2014; 69:1075-8; http://dx.doi.org/10.1093/jac/dkv466.

41. Mylonakis E, Moreno R, El Khoury JB, Idnurm A, Bassetti M, Bisio F, Di Biagio A, Pierri I, Balocco M, Soro O, Cruciani M, Bassetti D. Efficacy of voriconazole in patients with cryptococcal central nervous system infection. Antimicrob Agents Chemother 2007; 51:1319-24; PMID:17516654; http://dx.doi.org/10.1128/AAC.01477-08.

42. Arvanitis M, Fuchs BB, Mylonakis E. Nonmammalian model systems to investigate fungal biofilms. Methods Mol Biol 2012; 845:469–85; PMID:22328396; http://dx.doi.org/10.1007/978-1-61779-539-8_33.

43. Arvanitis M, Fuchs BB, Mylonakis E. Nonmammalian model systems to investigate fungal biofilms. Methods Mol Biol 2012; 845:469–85; PMID:22328396; http://dx.doi.org/10.1007/978-1-61779-539-8_33.