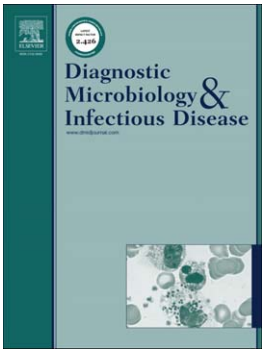

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Virulence and antifungal therapy of murine disseminated infection by *Rhodotorula mucilaginosa*.

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Abstract

Rhodotorula infections have emerged in recent years causing mainly fungemia associated to high mortality. We have evaluated the *in vitro* activity of nine antifungal drugs against four clinical strains of *Rhodotorula mucilaginosa,* being amphotericin B, voriconazole and posaconazole the most active compounds. The experimental virulence of this fungus and the efficacy of the three mentioned drugs were evaluated in disseminated infections in neutropenic mice. Infection resulted in a high fungal load in all the organs studied without evident particular tropism. All treated animals showed reduced burden respect to the control in a strain dependent manner being voriconazole slightly superior to posaconazole and amphotericin B.

Keywords: virulence, antifungal susceptibility, therapy, *Rhodotorula mucilaginosa.*

1. Introduction

The yeast *Rhodotorula* is a typical member of the Basidiomycota usually found in air, soil, continental and ocean-water, and also as contaminant of milk and fruit juices (Wirth and Goldani, 2012a). This fungus is characterized by the production of carotenoid pigments and multilateral budding cells which are subglobose, oval to elongate with or without small capsule (Andes et al., 2004). The genus contains 46 species although only three of them produce occasionally infections in humans, i.e. *R. mucilaginosa*, *R. minuta* and *R. glutinis* (De Almeida et al., 2008). *R. mucilaginosa* is the most prevalent species being recognized in the last two decades as an emerging pathogen, especially in immunocompromised patients. The bloodstream infection is the most frequent and severe affectation, especially in immunocompromised patients which is usually fatal in near 15% of cases (Miceli et al., 2011, Tuon and Costa, 2008); however, fungemia has been also reported in immunocompetent individuals (Pereira et al., 2016). Common clinical manifestations include also peritonitis, meningitis and endophthalmitis (Miceli et al., 2011).

Due to the scarce clinical experience in the management of infections by *Rhodotorula* the most appropriate treatment is still unknown. Based on clinical evidence (quality of evidence level II), ESCMID and ECMM guidelines recommend amphotericin B (AMB) with or without 5-fluorocytosine (5-FC) (strength of recommendation grade A) when *in vitro* testing shows susceptibility (Arendrup et al., 2014); however, mortality remains high despite treatment. Azoles and echinocandins are not recommended due to the high number of cases of resistance during their clinical use and the increase of infections in patients receiving such drugs as profilaxis (Arendrup et al., 2014). However, in vitro activity of posaconazole (PSC) and successful outcome in patients with fungemia

receiving voriconazole (VRC) alone or in combination have been reported, remaining uncertain the usefulness of these triazoles against this infection (Duggal et al., 2011, Zaas et al., 2003).

In the present study the *in vitro* activity of AMB, VRC, PSC, 5-FC, itraconazole (ITC), fluconazole (FLC), anidulafungin (AFG), caspofungin (CFG) and micafungin (MFG) was evaluated against four *R. mucilaginosa* strains by determining their minimal inhibitory concentration (MIC) and killing kinetics curves. Then a murine model of systemic infection by *R. mucilaginosa* was established and used for evaluating the efficacy of those drugs showing the best *in vitro* activity.

2. Materials and Methods

2.1. Strains.

Four clinical strains of *R. mucilaginosa* kindly provided by the Belgian Coordinated Collections of Microorganisms / Institute of Hygiene and Epidemiology (BCC/IHEM) were included in this study, three of them obtained from human blood cultures (IHEM 18459, IHEM 20182 and IHEM 22043), and one from nail (IHEM 1698). Identification of strains was confirmed morphologically and phenotypically by nitrate, raffinose and hexadecane assimilation (De Hoog et al., 2014) .The fungal isolates were stored lyophilized and after reconstitution were subcultured twice on potato dextrose agar (PDA) at 35 °C for 48 h.

2.2. In vitro studies.

AMB (Sigma Aldrich Quimica S.A., Madrid, Spain), CFG (Merck & Co., Rahway, EEUU), MCF (Astellas PharmaInc, Tokio, Japón), 5-FC (Sigma Aldrich, Steinheim, Germany) and ITC, FLC, PSC, VRC, and anidulafungin (AFG) (obtained from Royal Pharm, Hangzhou, China) as pure powder were tested against 4 isolates of *R.*

mucilaginosa, following the document M 27-A3 (2008). MIC determinations were performed after 48 h of incubation at 35 ºC and defined as the lowest drug concentration producing 100% inhibition of fungal growth in the case of AMB or 50 % inhibition in the case of 5-FC, azoles and echinocandins.

Time-kill curves were performed for those compounds showing higher *in vitro* activity i.e., AMB, VRC and PSC as previously described (Canton et al., 2008). In brief, dilutions of each drug consisting on 32, 8, 2, 0.5, 0.125 and 0.06 µg/ml were prepared in volumes of 9 ml and inoculated with 1 ml of a conidial suspension containing $5x10^6$ CFU/ml. At predetermined time points (0, 8, 24 and 48 h) aliquots of 100 µl were removed, serially diluted in sterile water, placed onto PDA plates and incubated at 35 °C for 24-48 h to determine CFU/ml. This procedure allowed a limit of detection of 33 CFU/ ml. All assays were carried out in duplicate and the geometric mean and standard deviation were calculated (Pereira et al., 2016). A reduction on CFU counts of ≥ 99.9 % or 3 $\log_{10}$ compared to the starting inoculum was considered indicative of fungicidal activity, while a CFU count reduction of < 99. 9 % or < or 3 $\log_{10}$ was considered fungistatic (Canton et al., 2008).

2.3. In vivo studies.

For the animal studies, four-week-old male OF1 mice weighing 28-30 g (Charles River; Criffa SA, Barcelona, Spain) were used. Mice were housed under standard conditions with food and water *ad libitum* and immunosuppressed by intraperitoneal administration of a single dose of 200 mg/kg of cyclophosphamide (Genoxal; Laboratorios Funk SA, Barcelona, Spain) beginning 2 days prior to the infection and then every 5 days. (Chiller et al., 2003).

To assess the virulence of the strains, groups of eight mice were inoculated intravenously (i.v.) via the lateral tail vein with inocula of each strain consisting of 5 x 10^6, 1 x 10^7, 2.5 x 10^7 or 5 x 10^7 CFU per animal in 0.2 ml of sterile saline solution. To evaluate the efficacy of the different treatments, 16 groups of animals consisting in 13 mice/group (5 for survival and 8 for tissue burden studies) were inoculated i.v. with a suspension of 2.5 x 10^7 CFU/animal for each strain. This inoculum was chosen based in the virulence study since it was able to cause acute infection with high tissue involvement. Treatments consisted in the use of the following drugs: AMB (Farmacia Xalabarder, Barcelona, Spain) administered i.v at 0.8 mg/kg once daily (QD), VRC (Vfend ® Pfizer S. A. Madrid. Spain) at 25 mg/kg given orally by gavage QD and PSC (Noxafil ® Merck Sharp Dohme Ltda, Spain) at 20 mg/kg given orally by gavage twice a day (BID). Mice treated with VRC received grapefruit juice instead of water from 3 days before infection until the end of the experiment (Sugar and Liu, 2000). Previous pharmacokinetic studies in experimental murine models demonstrated that these doses provided drug plasma levels above the obtained MICs (Andes et al., 2004, Andes et al., 2003). All treatments began 24 h after challenge and lasted for 7 days. Control groups received no antifungal treatment. All animals received ceftazidime at 5 mg/kg subcutaneously once daily during the experiment to prevent bacterial infection. Efficacy was assessed by survival prolongation and reduction of tissue burden. Care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare Committee.

*2.4. Determination of serum drug levels and tissue burden***.**

Mice included in the tissue burden study group i.e., control and treated groups, were anaesthetized by inhalation of sevofluorane (Sevorane; Abbott, Madrid, Spain) on day 8 post infection, 6 h after the last dose. Animals were bleed out by cardiac puncture and

then euthanized by cervical dislocation. Serum samples were obtained by blood centrifugation (3500 rpm) and stored at -20 °C for drug level determination by bioassay, as previously described (Cendejas-Bueno et al., 2012), which shows a limit of detection of 0.12 µg/ml for each drug. Kidneys, spleen, liver, lungs and brain were aseptically removed and approximately one-half of each organ was weighed and mechanically homogenized in 1 ml of sterile saline solution. Homogenates were serially diluted (1:10), placed onto PDA plates and incubated for 48 h at 35 °C for fungal load calculation (CFU/g of tissue).

2.5. Statistical analysis.

The mean survival times were estimated by Kaplan-Meier method and compared among groups using the log rank test. Results from the tissue burden studies were analysed using the Mann-Whitney *U*-test by Graph Pad Prism 6.0 for Microsoft Windows (GraphPad Software, San Diego California USA). A *P* value of ≤ 0.05 was considered statistically significant.

3. Results and Discussion

Our *in vitro* results (Table 1) have shown high MICs of all three assayed echinocandins, specially for AFG (MIC ≥ 16 µg/ml), and FLC (MIC ≥ 32 µg/ml), which have shown MIC_{90} > 8 µg/ml and > 64 µg/ml respectively, as previously reported (Gomez-Lopez et al., 2005, Posteraro et al., 2015). As expected, AMB and 5-FC were the most active *in vitro* compounds (MICs values ≤ 0.5 µg/ml and ≤ 0.06 µg/ml, respectively) reasons which have lead ESCMID and ECMM to recommend both compounds for treating disseminated infections by *Rhodotorula* spp (Arendrup et al., 2014). AMB showed fungicide activity after 14 h to 16 h of exposure at 32 µg/ml or after 29 h to 30 h at 8 µg/ml (figure 1) while fungistatic effect was achieved by VRC and PSC. Despite the

obtained as well as reported high *in vitro* activity of AMB and 5-FC, other studies including a high number of isolates of *R. mucilaginosa* have shown MICs of AMB and 5-FC as high as 8 and >64 µg/ml, respectively (Gomez-Lopez et al., 2005) which suggest the usefulness of susceptibility testing prior the election of treatment. Our results showed good *in vitro* activity of VRC with MIC ranging from 0.12 to 0.25, contrarily to previous works reporting ranges from 0.5 to 8 µg/ml (MIC90 = 8 µg/ml) (Gomez-Lopez et al., 2005), pointing that susceptibility could be strain dependent and reinforcing the usefulness of *in vitro* testing.

The establishment of animal models of fungal infections is a key tool for evaluating virulence and antifungal efficacy, especially when clinical experience with a given fungal species is scarce (Guarro, 2011). To evaluate the efficacy of those drugs showing the highest activity, we have develop to our knowledge, the first mice model of systemic infection by *R. mucilaginosa*. The experimental infection showed high mortality after challenge with any of the assayed inocula being the mean survival time (MST) correlated with the inocula size. MST ranged from 17.25- to 20.38 days after infection with the lowest inoculum (i.e., 5×10^6), while after infection with the highest inoculum (i.e., 5×10^7) MST ranged from 10.3 to 15.6 days (table 2). Our *in vivo* results provide evidence of fungal invasion in all studied organs i.e., liver, lung, kidney, brain and spleen, which were highly affected. The fungal load recovered form organs were strain and inoculum dependent without a clear tissue tropism but overall, lungs and brain were the most affected organs. Interestingly, the infection with the lowest inoculum resulted in higher brain involvement ($\log_{10}$ 5.65 to 6.32 CFU/g) than any other studied organ however; higher inocula resulted in lung as the most affected organ (Table S1). These findings differ from a previous experimental infection in a rat model which resulted in low tissue invasion, being the liver the most affected organ (Wirth and

Goldani, 2012b). These discrepancies may be due to the different animal species and inocula size used, which was this latter clearly higher (1.4 x 10^{10} CFU/animal) than those reported in the present work. Considering that the most common human infection associated to *Rhodotorula* is fungemia, our animal model mimics the acuteness and the multiorganic affectation reported in humans, including central nervous involvement (Tsiodras et al., 2014), which makes this model suitable for evaluating experimental treatments.

The efficacy study showed no statistically significant differences on mortality strain to strain, indicating a similar virulence within the assayed strains ($p \geq 0.31$). Only VRC and PSC increased the survival rates, although not against all strains tested (Figure 2). VRC increased survival in animals infected with strains IHEM 18459 ($p = 0.012$) and IHEM 22043 ($p = 0.033$). Curiously, VRC showed MICs of = 0.25 µg/ml against both strains but no efficacy was observed against both strains which MIC of VRC were 0.12 µg/ml ($p \geq 0.145$) despite that serum levels, ranged from 7.05 to 12.21 µg/ml, were above the corresponding MIC values (Table 3). PSC only prolonged the survival against the strain IHEM 20182 ($p = 0.049$) which showed a MIC of PSC (0.25 µg/ml) but no efficacy was achieved against the rest of strains which showed MICs of 0.5 µg/ml. Surprisingly, AMB was unable to increase the survival of mice even in those infected with the strain showing the lowest MIC (0.25 µg/ml) and despite that serum levels were high (7.15 to 11.60 µg/ml). Different studies seems to indicate that AMB do not cross de blood brain barrier unless inflammation increase its permeability (Nau et al., 2010). The poor penetration of AMB into CNS together to the high fungal load in such tissue could be the cause for the high mortality observed after infection with the strain most susceptible to AMB. In terms of fungal burden reduction, all treatments reduced significantly the fungal load from all studied organs in comparison to the

untreated group (Figure 3). When comparing efficacy between treatments, no drug was superior to the others although VRC and AMB shown slight higher burden reduction than PSC. Our results point out that VRC and in lesser degree PSC should be taken in consideration for treating *R. mucilaginosa* infections, especially when AMB is discouraging due to toxic adverse effects or when high MICs are obtained.

Competing interests

None declared

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TABLES

Table 1. *In vitro* activity of antifungal drugs against four isolates of *R. mucilaginosa*.

Strain	MIC (µg/ml)								
	AMB	**FLC**	**ITC**	**PSC**	**VRC**	**MFG**	**AFG**	**CFG**	**5FC**
IHEM 1698	0.5	> 32	1	0.5	0.12	4	> 16	8	0.03
IHEM 18459	0.5	> 32	1	0.5	0.25	4	> 16	1	0.03
IHEM 20182	0.5	> 32	0.5	0.25	0.12	2	> 16	1	0.03
IHEM 22043	0.25	> 32	0.5	0.5	0.25	4	> 16	8	0.06

AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; PSC, posaconazole; VRC, voriconazole; MFG, micafungin; AFG, anidulafungin; CFG, caspofungin; 5FC, 5-fluorocitosine.

Table 2. Mean survival time of infected mice with four *R. mucilaginosa* strains with four different inocula size (CFU per animal).

Strain	Mean survival time (days) ± Standard deviation			
	$5x10^6$ CFU	**$1x10^7$ CFU**	**$2.5x10^7$ CFU**	**$5x10^7$ CFU**
IHEM 1698	19.75 ± 3.54	17 ± 6.26	15.75 ± 7.49	10.25 ± 8.94
IHEM 18459	19.00 ± 3.42	17.50 ± 4.8	14.44 ± 5.30	11.38 ± 7.80
IHEM 20182	20.38 ± 1.77	19.13 ± 2.95	16.63 ± 4.98	15.63 ± 6.14
IHEM 22043	17.25 ± 4.20	14.25 ± 7.19	12 ± 8.37	10.88 ± 8.54

Table 3. Drug levels in serum (µg/ml) measured by bioassay on day 8 post infection and 4 h after last dosing.

Strain	Mean sera levels (µg/ml) ± standard deviation		
	AMB	**VRC**	**PSC**
IHEM 1698	11.60 ± 0.73	12.15 ± 1.13	9.14 ± 0.50
IHEM 18459	7.15 ± 1.47	7.05 ± 2.60	9.13 ± 0.78
IHEM 20182	7.47 ± 1.90	8.20 ± 0.94	8.37 ± 0.66
IHEM 22043	10.33 ± 2.2	12.21 ± 1.29	8.91 ± 0.25

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FIGURE LEGENDS

Fig 1. Results on time-kill kinetic assay of AMB, VRC and PSC against two strains of *R. mucilaginosa* (IHEM 1698 and IHEM 22043).

Fig 2. Survival of neutropenic mice infected i.v. with 2.5 x 10^7 CFU/animal of *R. mucilaginosa* and treated for 7 days with AMB 0.8 mg/kg QD VRC 25 mg/kg QD or PSC 20 mg/kg BID. [a] $P \leq 0.05$ versus control, [b] $P = 0.03$ versus AMB**.**

Fig 3. Effect of antifungal treatment on colony counts in neutropenic mice infected intravenously with 2.5 x 10^7 CFU/animal of four *R. mucilaginosa* strains. Scatter plot shows colony forming units (CFU) in liver, lung, kidney, brain and spleen after 7 days of treatment with amphotericin B (AMB) 0.8 mg/kg QD, voriconazole (VRC) 25 mg/kg QD or posaconazole (PSC) 20 mg/kg BID. [a] $P \leq 0.05$ versus control; [b] $P \leq 0.05$ versus AMB; [c] $P \leq 0.05$ versus VRC; [d] $P \leq 0.05$ versus PSC.

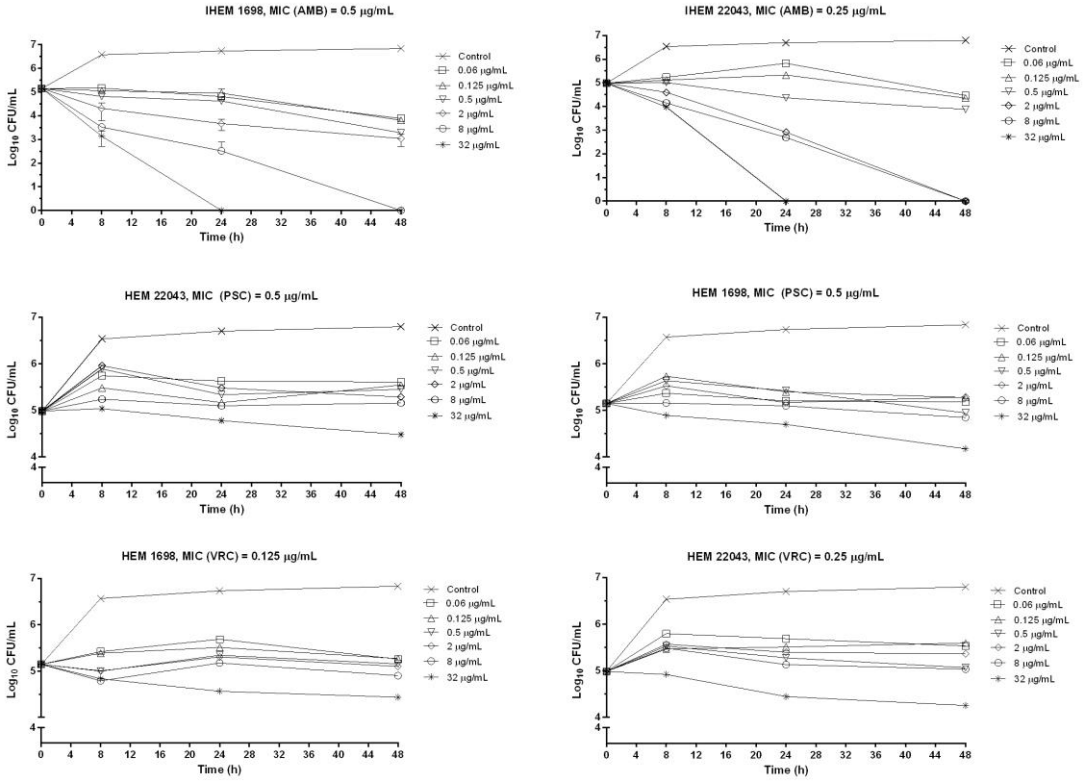

Figure 1

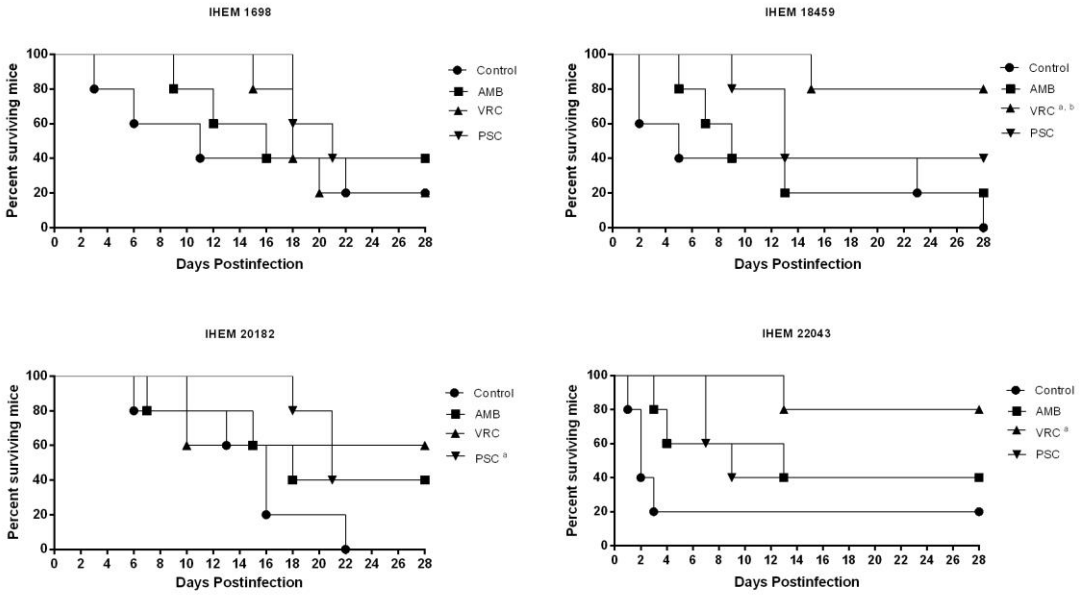

Figure 2

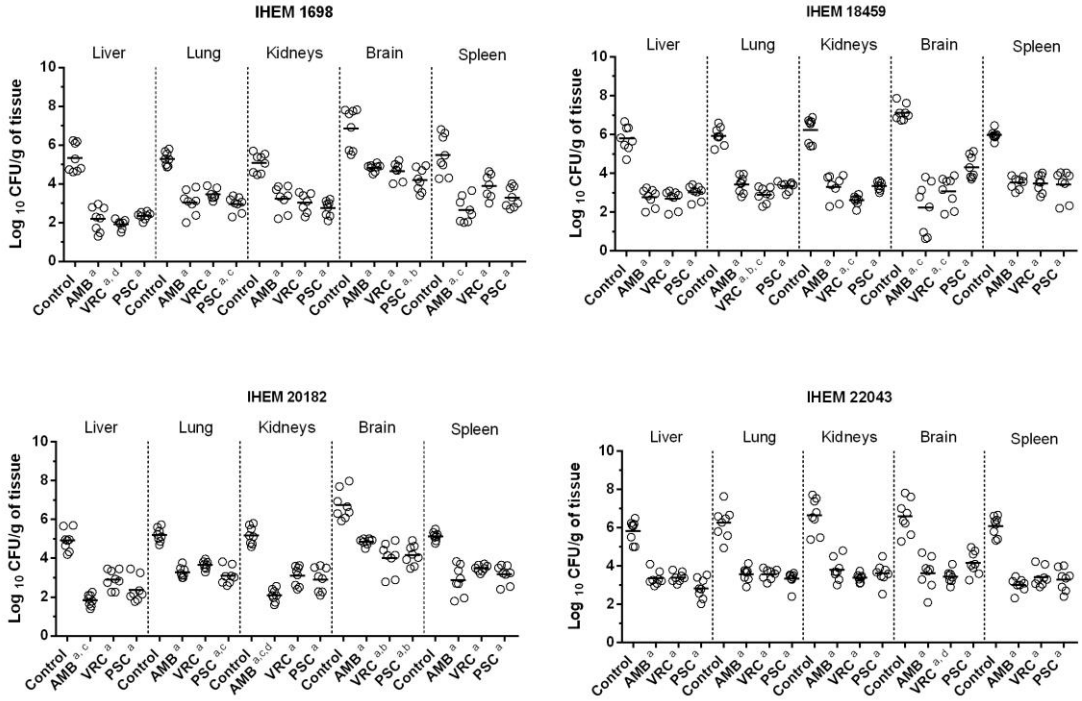

Figure 3

HIGHLIGHTS

1. A model of systemic infection by *Rhodotorula mucilaginosa* has been developed in mice
2. Systemic infection resulted in high mortality in a dose dependent manner
3. All studied organs showed high fungal load with not clear tissue tropism
4. Efficacy of voriconazole was slightly superior to amphotericin B and posaconazole