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Lack of correlation of ECV and outcome in an *in vivo* murine model of systemic fusariosis

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The increase in recent years of disseminated infections by *Fusarium* among immunocompromised patients, has driven to consider fusariosis an emerging infectious disease (Nucci et al. 2014; Tortorano et al. 2014). Approximately 70 taxonomic species, distributed into seven complexes, have been associated to human infections being *F. falciforme* and *F. keratoplasticum* from the *F. solani* species complex and *F. oxysporum* species complex the commonest (Guarro 2013; Short et al. 2013; Al-hatmi, Meis, and Hoog 2016). In general *Fusarium* spp. exhibit resistance to most azoles and echinocandins; while variable susceptibility has been reported to voriconazole and amphotericin B (Al-hatmi, Meis, and Hoog 2016), which are the recommended drugs (Tortorano et al. 2002). Despite that the outcome of the disease has been improved in the last years, the mortality remains high (García-ruiz et al. 2015; Nucci et al. 2014; Al-hatmi et al. 2017). Clinical breakpoints (CBP) have not been established but epidemiological cut-off values (ECV) for the most prevalent species have been defined. The ECVs comprising ≥97.5% of the modeled population define *F. solani* species complex and *F. oxysporum* species complex as non wild-type at MIC ≥8 µg/ml of amphotericin B, and at ≥32 and ≥16 µg/ml of voriconazole against *F. solani* species complex and *F. oxysporum* species complex, respectively (Espinel-Ingroff et al. 2016).

The aim of the present study was to test the efficacy of liposomal amphotericin B and voriconazole, in murine models of disseminated infection against clinical strains of *F. solani* and *F. oxysporum* that shown MICs corresponding to wild-type (WT) and non-WT strains in order to determine if the stablished ECVs correlate with the *in vivo* outcomes. WT and non-WT strains were selected from previous *in vitro* antifungal susceptibility studies (Guevara-suarez et al. 2016)

Two clinical strains of *F. keratoplasticum* belonging to *F. solani* species complex (LEMM-121340 and LEMM-121984), and two belonging to the Clade-3 of *F. oxysporum* species complex (LEMM-110946 and CIB-15), were used (Guevara-suarez et al. 2016). The *in vitro* antifungal testing was performed by following the CLSI guidelines (CLSI, 2008). Inocula, consisting in conidial suspensions for *in vitro and in*

vivo studies, were obtained from five days-old cultures on PDA (Pronadisa, Madrid, Spain) kept at 35°C. Inocula were adjusted to the desired concentration by hemocytometer counts and viability confirmed by placing 10-fold dilutions PDA plates. Twelve groups of male OF-1 mice (Charles River, Criffa S.A., Barcelona, Spain) weighing 30 g were used under standard conditions. Each group of animals consisted on 13 mice, 8 for survival studies and 5 for CFUs determination, and all of them were immunosuppressed 2 days prior infection by intraperitoneal administration of 200 mg/kg of cyclophosphamide and then administered every 5 days thereafter. Animals were challenged intravenously with $5x10^3$ CFU of both *F. solani* species complex strains and with $2x10^7$ CFU of *F. oxysporum* species complex strains. Inocula were selected from previous studies (data not shown) to provoke equivalent acuteness of infection. Treatments consisted on liposomal amphotericin B (AmBisome, GILEAD, Madrid, Spain), administered intravenously at 10 mg/kg once a day (QD) or voriconazole (Vfend, Pfizer, Madrid, Spain) given orally by gavage at 40 mg/kg QD. Animals treated with voriconazole received grapefruit juice during and 2 days before initiation of treatment. All treatments began 1 day after infection and lasted for 10 days. All procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Efficacy was assessed by survival rates along 22 days post infection and by fungal load in kidneys, liver and lungs 9 days after infection. Fungal load was determined by placing organ homogenates on PDA. Mean survival time was estimated by the Kaplan-Meier method, and compared among groups by the log-rank test. Tissue burden data were analyzed by the Mann-Whitney U test. All statistical analyses were performed by using GraphPad Prism 5. P values of ≤ 0.05 were considered statistically significant.

Both assayed drugs showed a poor *in vitro* activity. The MICs of amphotericin B and voriconazole were 2 and 8 µg/mL, respectively against the strain LEMM-121340, and 4 and >16 µg/ml against LEMM-121984. MICs of AMB and VRC resulted in 4 and

>16 µg/mL against LEMM-110946 and 2 and >16 µg/ml against for CIB-15, respectively.

In vivo results showed poor efficacy even against strains considered WT. No Treatment improved the survival after the infection by any of the assayed strains (Fig. 1). In the tissue burden study only liposomal amphotericin B showed some reduction against *F. solani* species complex, although only against the strain LEMM-121340, reducing CFUs in kidney ($p = 0.013$) but not in liver or lungs (Fig. 2). Liposomal amphotericin B did not show efficacy against *F. solani* species complex LEMM-121984 neither against both *F. oxysporum* species complex strains, while voriconazole was ineffective in reducing fungal load in any of the infections assayed. The lack of clinical experience, makes difficult to establish the usefulness of such parameters in predicting the infection outcome but in absence CBPs animal models are useful in correlating ECVs with therapeutic success, as it has been recently demonstrated against scedosporiosis, usually refractory to the antifungal treatment (Martin-vicente et al. 2016).

The strains selected in this study represent the most common MICs of amphotericin B and voriconazole against both complex of species, i.e., 2 and 8 µg/ml against *F. solani* species complex, and 2 and 4 µg/ml against *F. oxysporum* species complex, but MICs as low as 0.25 µg/ml and 0.5 µg/ml have been reported (Espinel-Ingroff et al. 2016). Although ECVs are not intended to predict efficacy, they can guide therapy in absence of clinical breakpoints. Our results, besides demonstrating the poor efficacy of LAMB and VRC, proved no correlation between ECVs and efficacy in our model. We are aware of the low number of species included in the present study, which limits a firm conclusion, and further studies including species showing lower MICs would be highly desirable in order to guide breakpoints for the treatment of these infections.

Conflicts of interest

None

FIGURE LEGENDS

Figure 1. Cumulative mortality of immunosuppressed mice infected with $2x10^7$ CFUs of Clade-3 (*Fusarium oxysporum* species complex) strains **A)** LEMM-110946 and **B)** CIB-15 or $5x10^3$ CFUs of *F. keratoplasticum* (*Fusarium solani* species complex) strains **C)** LEMM-121984 and **D)** LEMM-121340. Animals received no treatment (Control), intravenous liposomal amphotericin B at 10 mg/kg (LAMB 10) or oral voriconazole at 40 mg/kg (VRC 40). No statistical differences were found between treated and their respective control groups.

Figure 2. Scatter gram of fungal load in liver, lungs and kidney in immunosuppressed mice 9 days after infection with $2x10^7$ CFUs of Clade-3 (*Fusarium oxysporum* species complex) strains **A)** LEMM-110946 and **B)** CIB-15 or $5x10^3$ CFUs of *F. keratoplasticum* (*Fusarium solani* species complex) strains **C)** LEMM-121984 and **D)** LEMM-121340. Animals received no treatment (Control), intravenous liposomal amphotericin B at 10 mg/kg (LAMB 10) or oral voriconazole at 40 mg/kg (VRC 40). ^{a} $P < 0.05$ in comparison to control group.

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Highlights:

- No efficacy of LAMB and VRC was observed against murine systemic fusariosis
- Wild-type strains showed *in vivo* resistance to the antifungal treatment
- ECVs for *F. oxysporum* and *F. solani* complexes species did not guide outcome

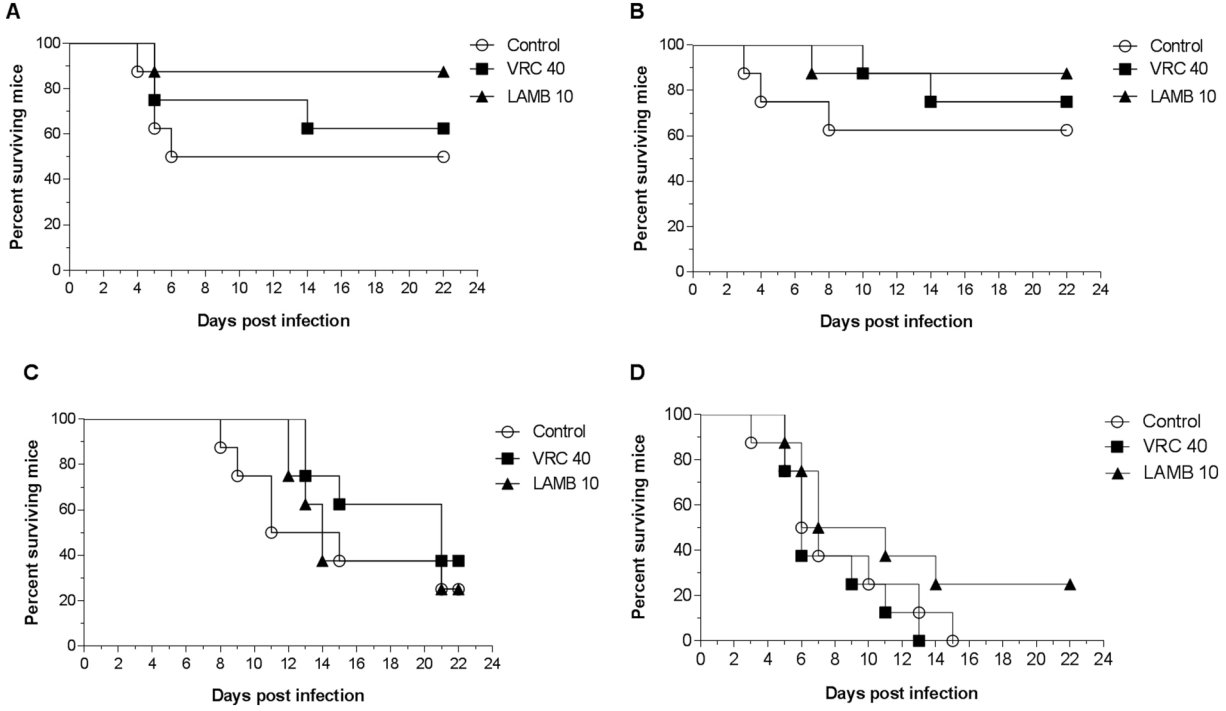
A
B
C
D
Control
VRC 40
LAMB 10
Percent surviving mice
Days post infection
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24

Figure 1

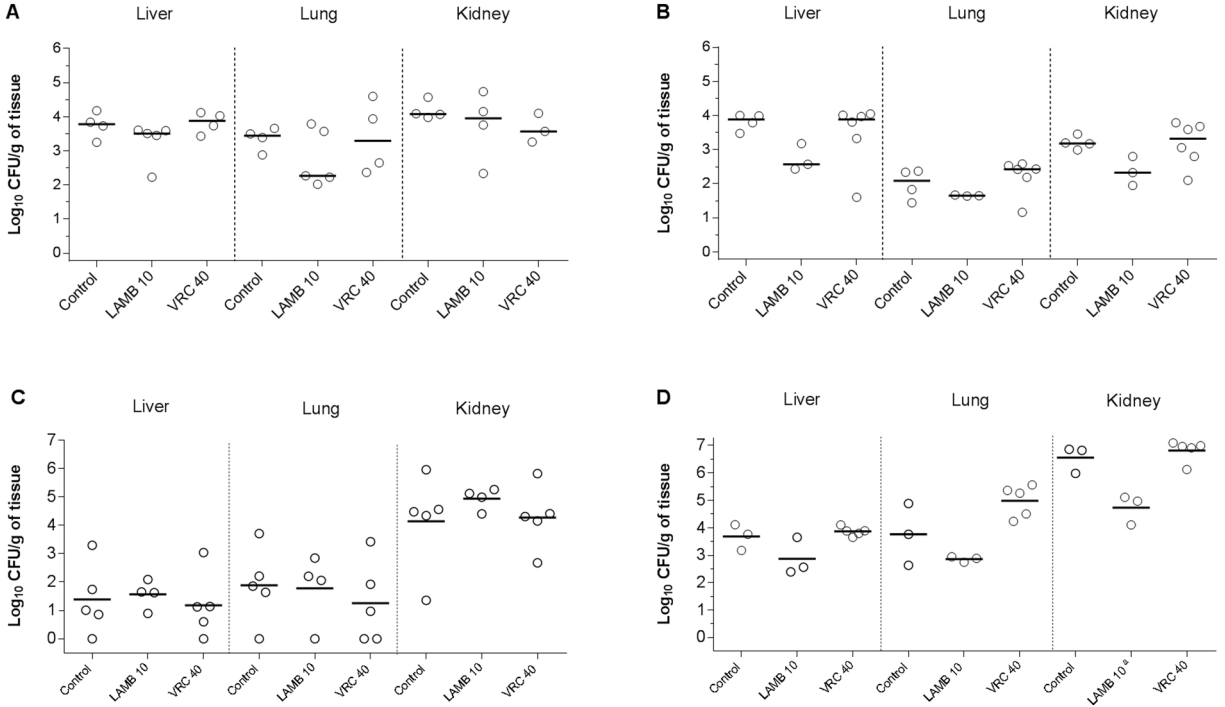
A
Liver
Lung
Kidney
Log10 CFU/g of tissue
Control
LAMB 10
VRC 40
B
C
D
LAMB 10^8

Figure 2