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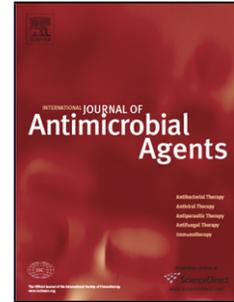
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Treatment of murine *Fusarium verticillioides* infection with liposomal amphotericin B plus terbinafine

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ABSTRACT

Using a murine model of disseminated infection by *Fusarium verticillioides*, the efficacy of liposomal amphotericin (L-AmB) B at 10 mg/kg body weight once daily and terbinafine (TRB) at 150 mg/kg body weight twice daily, alone and in combination, was evaluated. The combination of L-AmB with TRB was the only treatment able to prolong survival and to reduce fungal loads in the spleen and kidneys of mice infected with either strain of *F. verticillioides* used. The results suggest that this combination may have a role in the treatment of *F. verticillioides* infection.

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1. Introduction

Fusarium spp. are important plant pathogens able to cause a broad range of infections in humans, including superficial, locally invasive and disseminated infections with a high mortality rate, especially in haematological patients [1,2]. The most common *Fusarium* spp. involved in human infections are members of the *Fusarium solani*, *Fusarium oxysporum* and *Gibberella fujikuroi* species complexes [3]. *Fusarium verticillioides* is a member of the latter group, this species being mainly isolated from deep-seated infections [4]. However, the real incidence of this species in human infection is unknown due to confusion caused by its morphological similarity to other species of the *G. fujikuroi* species complex [5,6] and the frequent lack of identification to species level of isolates causing fusariosis. Infections caused by *Fusarium* spp. are frequently resistant to antifungal therapy in general. The recommended therapy for fusariosis is high doses of amphotericin B (AmB) (or its lipid formulation) or voriconazole; however, clinical practice has shown that these drugs have poor activity [1]. Since use of monotherapy to treat systemic fusariosis is not completely satisfactory, combined therapy could be another option.

Although in the clinical setting combined therapy has demonstrated some efficacy [7–9], there are not yet enough data available to recommend such an approach for the treatment of fusariosis. In vitro susceptibility testing has shown that terbinafine (TRB) is active against *F. verticillioides* [minimum inhibitory concentration (MIC) range 0.125–1 mg/L] [5] and its combination with other drugs has shown synergy [10] and clinical success [8,9]. For these reasons, we evaluated the efficacy of the combination of TRB with liposomal AmB (L-AmB) in a neutropenic murine model of disseminated infection by *F. verticillioides*.

2. Materials and methods

2.1. Fungi

Two clinical isolates of *F. verticillioides* (FMR 8585 and FMR 9434) were used in the study. Fungi were stored at $-80\text{ }^{\circ}\text{C}$ in potato dextrose broth with glycerol and were subcultured on potato dextrose agar (PDA) at $30\text{ }^{\circ}\text{C}$ prior to testing. Inocula were prepared by flooding the surface of the agar plates with sterile saline, scraping the sporulating mycelium with a culture loop and drawing up the resultant suspension with a sterile Pasteur pipette. The suspensions were then filtered once through sterile gauze to remove hyphae. The resulting suspensions of conidia were adjusted to the desired inoculum size by counting with a haemocytometer. Dilutions of the original suspension were subcultured on PDA plates to confirm haemocytometer counts.

2.2. In vitro studies

In vitro susceptibility of both strains was determined by the broth microdilution reference method according to Clinical and Laboratory Standards Institute (CLSI) guidelines for filamentous fungi [11]. For AmB the endpoint criterion used was 100% inhibition of growth, and for TRB the MIC was defined as the lowest concentration at which there was 80% inhibition of growth. *Paecilomyces variotii* ATCC 36257 was used as a quality control strain.

Drug interactions were assessed using the checkerboard microdilution method [12] to determine the fractional inhibitory concentration index (FICI), which is the sum of the

fractional inhibitory concentration (FIC) of each of the drugs, which in turn is defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone, i.e. $FICI = FIC \text{ drug A} + FIC \text{ drug B} = (\text{MIC drug A in combination} / \text{MIC drug A alone}) + (\text{MIC drug B in combination} / \text{MIC drug B alone})$.

2.3. *Animals*

Male OF1 mice (Charles River, Criffa S.A., Barcelona, Spain) with a mean weight of 30 g were used. Animals were housed in standard boxes with corncob bedding and had free access to food and water. All animal experiments were approved by the Animal Welfare Committee of the Universitat Rovira i Virgili (Reus, Spain).

2.4. *Drugs*

The drugs tested were L-AmB (AmBisome; Gilead Sciences S.A., Madrid, Spain) and TRB (Novartis, Basel, Switzerland).

2.5. *Immunosuppression*

Mice were immunosuppressed by a single intraperitoneal injection of 200 mg of cyclophosphamide/kg of body weight plus a single intravenous (i.v.) injection of 150 mg of 5-fluorouracil/kg on the same day of challenge [13].

2.6. *Infection and therapy*

For both strains tested, mice were challenged with 1×10^7 colony-forming units (CFU) in 0.2 mL of sterile saline injected into the lateral tail vein. Preliminary

experiments demonstrated that this inoculum was appropriate for producing an acute infection, with 90% of the animals dying within 11 days (data not shown). The efficacy of the different treatments was evaluated through prolongation of survival and reduction in fungal tissue burden in the spleen and kidneys of infected mice. Groups of 20 mice were established for each strain and each treatment. Ten mice were chosen randomly for survival studies and the other ten were used for tissue burden studies. The different groups were treated as follows: L-AmB at 10 mg/kg body weight/dose i.v. once daily; TRB at 150 mg/kg body weight/dose orally twice daily (total dose 300 mg/kg/day); and L-AmB + TRB at the abovementioned doses. Control animals received no treatment. All treatments began 1 day after challenge and the therapy lasted for 10 days. Mouse survival was evaluated daily for 30 days after challenge.

For tissue burden studies, mice were sacrificed on Day 6 after infection. The spleen and kidneys were removed aseptically and the complete organs were homogenised in 1 mL of sterile saline. Serial 10-fold dilutions of the homogenates were plated on PDA and were incubated for 48 h at 30 °C. Data are expressed as log₁₀ CFU/g of tissue.

2.7. Statistics

Mean survival time was estimated by the Kaplan–Meier method and was compared among groups using the log-rank test. In tissue burden studies, colony counts were analysed using the Mann–Whitney *U*-test. A *P*-value of ≤ 0.05 was considered statistically significant.

3. Results

TRB was the most active drug, showing MICs of 0.125 mg/L for the two strains tested. AmB showed an MIC of 2 mg/L for strain FMR 9434 and 4 mg/L for strain FMR 8585. The combination of AmB + TRB showed indifferent interactions against the two strains tested (FICI = 2 for strain FMR 9434 and 0.98 for strain FMR 8585).

All treatments significantly prolonged survival compared with that of the control group ($P < 0.05$), with the exception of TRB for strain FMR 8585 (Fig. 1).

The effects of the different treatments on tissue burden studies are shown in Fig. 2. The spleen and kidneys of untreated animals showed high fungal loads, with the spleen being the most affected organ. L-AmB given alone was able to significantly reduce fungal burden in the spleen of mice infected with strain FMR 9434 and in the kidneys of mice infected with strain FMR 8585 in comparison with that of the control group ($P = 0.005$ and $P = 0.089$, respectively). TRB given alone was not able to significantly reduce tissue burden in any of the organs tested. In all cases, the combination of L-AmB + TRB significantly reduced the fungal load in the spleen and kidneys compared with that of the control group ($P = 0.035$ and $P = 0.0001$, respectively).

4. Discussion

The high MICs of AmB against the two strains tested in this study are in agreement with in vitro data reported previously by other authors [4,14,15]. By contrast, data are controversial regarding the in vitro susceptibility of *F. verticillioides* to TRB. Whilst in

this study low MICs for TRB were obtained, others have reported high MICs for this antifungal agent [14].

In an immunocompetent murine model of disseminated infection by *F. verticillioides*, L-AmB showed efficacy [16]; however, in immunosuppressed animals, such as in this study, the drug shows poor efficacy. This correlates with clinical practice where, regardless of the antifungal treatment used, if the patient's neutropenia is not reversed [15] the mortality rate of disseminated fusariosis is near 100% [1,15]. In the clinical setting there are few, and contradictory, data on the use of L-AmB for treatment of *F. verticillioides* infection. Whilst this drug showed satisfactory results following failure of AmB in two cases of disseminated infection [17,18], in a recent case of disseminated infection by the same fungus L-AmB given alone was unable to resolve the infection, which was cured when voriconazole was added to the treatment [19]. We observed a poor correlation between in vitro results and the in vivo efficacy of TRB in a murine model. TRB was effective in prolonging the survival rate for only one strain, but not in reducing the tissue burden in any of the organs tested, despite having shown good in vitro activity. There are no described murine models for studying TRB kinetics. However, in rats, as well in humans, the high affinity of this drug for skin and adipose tissue as well as its low uptake and efflux in these tissues have been demonstrated. Maximum concentrations of TRB of 1.2 mg/L and 1.6 mg/L at 1.5 h after the administration of single doses of 500 mg and 750 mg, respectively, have been described [20]. It is possible that TRB in mice has similar kinetics, therefore large doses administered do not produced high plasma levels.

In the few clinical cases of disseminated fusariosis where combined antifungal therapy was satisfactorily used, the infection was never been caused by *F. verticillioides* [7,21]. To our knowledge, there are no data on the evaluation of combined therapy of L-AmB + TRB in experimental animal models of fusariosis. We assayed this combined treatment owing to the in vitro susceptibility of *F. verticillioides* to TRB and its common in vitro resistance to other antifungals such as AmB, triazoles and echinocandins [5]. In this study, this therapeutic approach was effective. However, this combination had already showed efficacy in a few clinical cases against other *Fusarium* spp. [8,9].

In conclusion, these results suggest that L-AmB combined with TRB is promising for the treatment of infections by *F. verticillioides*.

Funding

None.

Competing interests

None declared.

Ethical approval

Animal Welfare Committee of the Universitat Rovira i Virgili (Reus, Spain)

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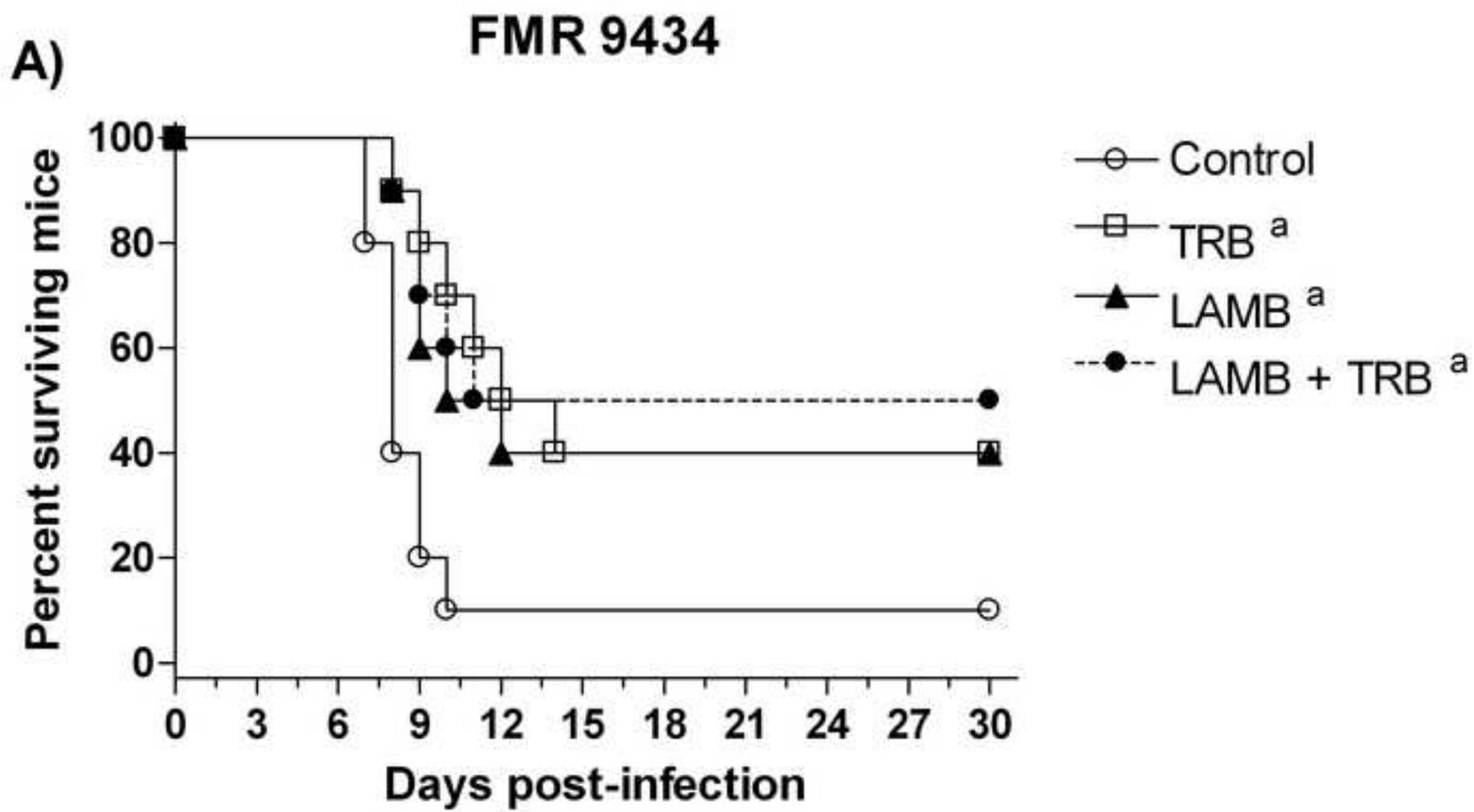
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Fig. 1. Cumulative mortality of mice infected with *Fusarium verticillioides* strains (A) FMR 9434 or (B) FMR 8585. L-AmB, liposomal amphotericin B at 10 mg/kg body weight intravenous once daily; TRB, terbinafine at 150 mg/kg body weight orally twice daily (total dose 300 mg/kg/day). Superscript ^a indicates $P < 0.05$ versus control.

Fig. 2. Effects of antifungal treatment on colony counts of *Fusarium verticillioides* strains FMR 9434 or FMR 8585 in (A) spleen and (B) kidneys of neutropenic mice. L-AmB, liposomal amphotericin B at 10 mg/kg body weight intravenous once daily; TRB, terbinafine at 150 mg/kg body weight orally twice daily (total dose 300 mg/kg/day). Superscript ^a indicates $P < 0.05$ versus control. Horizontal lines indicate mean values. CFU, colony-forming units.

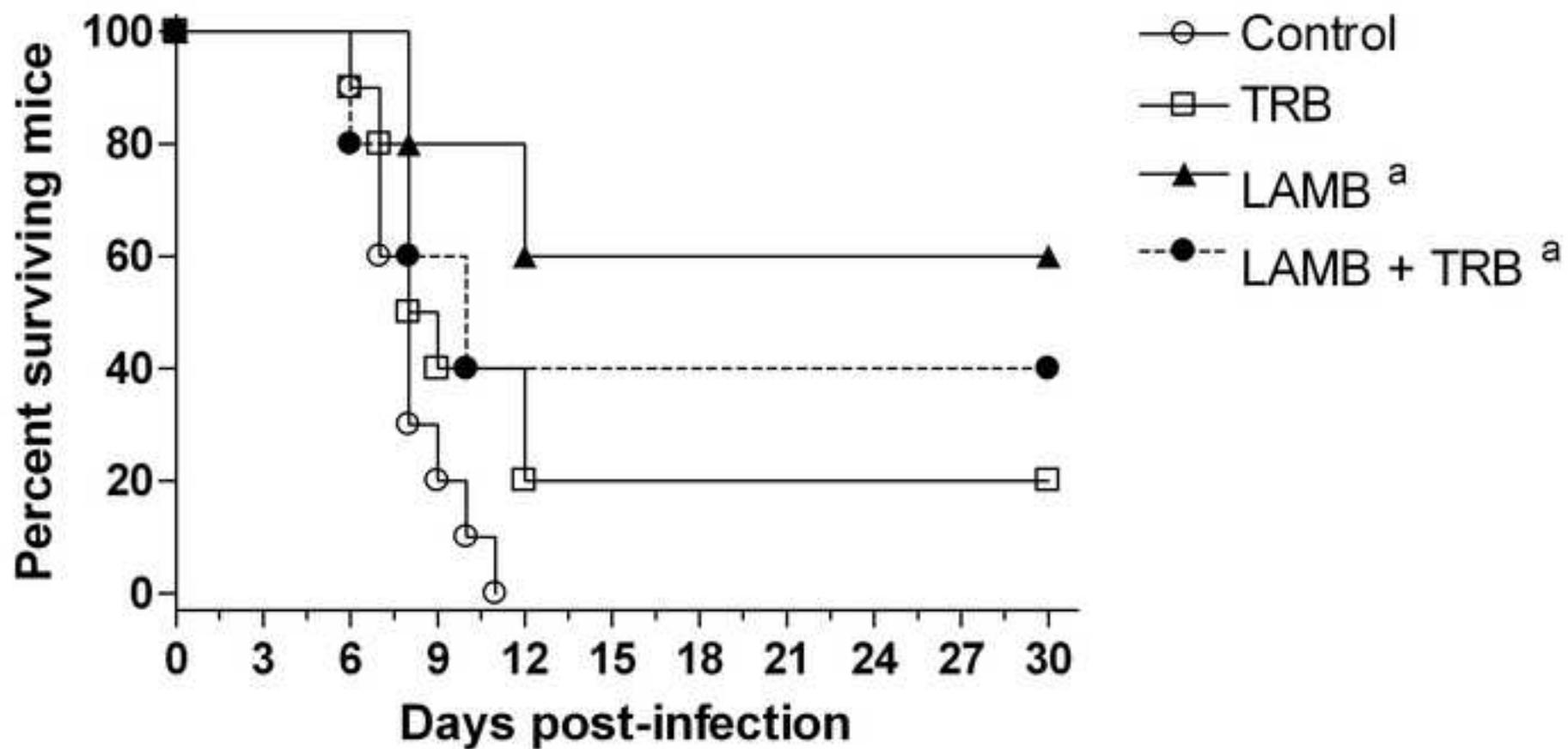
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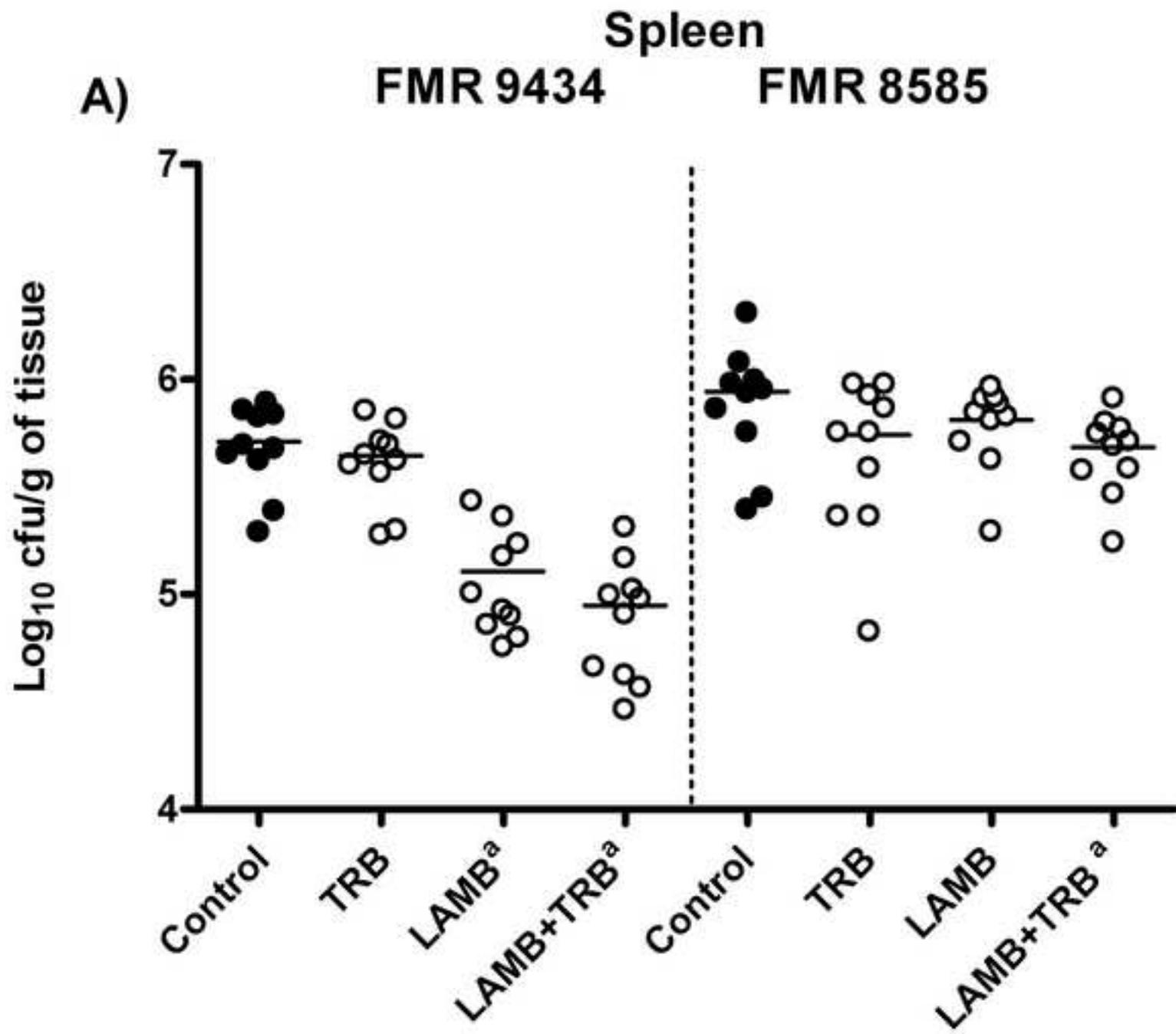


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FMR 8585

B)





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