Virulence and experimental treatment of *Trichoderma longibrachiatum,* a fungus refractory to treatment

Katihuska Paredes[1], Javier Capilla[1#], Emilio Mayayo[2] and Josep Guarro[1]

Unitat de Microbiologia[1] and Unitat d' Anatomia Patològica[2], Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Tarragona, Spain.

Running title: Infection by *T. longibrachiatum* and antifungal therapy

[#] Corresponding author. Mailing address: Unitat de Microbiologia, Facultat de Medicina, Universitat Rovira i Virgili. Carrer Sant Llorenç, 21.43201 Reus, Spain. Phone 0034-977-759350. Fax: 0034-977-759322. E-mail: javier.capilla@urv.cat

Abstract: 74 words

Body text: 1005 words

ABSTRACT:

Different inocula of *Trichoderma longibrachiatum* were tested in a murine model and only the highest one (1 x 10^7 CFU/animal) killed all the mice at day 15 post infection, being spleen and liver the most affected organs. The efficacy of amphotericin B deoxycholate, liposomal amphotericin B, voriconazole and micafungin was evaluated in the same model with very poor results. Our study demonstrated the low virulence but high resistance to antifungal compounds of this fungus.

Keywords: animal model, fungal infection, *Trichoderma,* antifungal therapy.

Trichoderma, a saprobic filamentous fungus widely distributed in nature (1) has recently emerged as human pathogen. *Trichoderma* spp. produce a wide variety of clinical manifestations (2-12), mostly attributed to *T. longibrachiatum*. In general, clinical cases have poor prognosis due to their intrinsic resistance to antifungals (13). Antifungal susceptibility data are scarce and appropriate treatment does not exist. Although amphotericin B shows poor in vitro activity is the most used drug, while voriconazole and echinocandins showed better in vitro activity (14). Our aim was to evaluate the virulence of *T. longibrachiatum* in a murine model and the efficacy of amphotericin B, liposomal amphotericin B, micafungin and voriconazole.

Two clinical strains of *T. longibrachiatum* (FMR 12626 and FMR 12643) identified using a multilocus sequence analysis (14) were used. The antifungal activity was determined following the CLSI guidelines (14, 15). For FMR 12626 MICs of amphotericin B and voriconazole were 0.13 µg/mL, 0.5 µg/mL and for FMR 12643, were 2 µg/mL, 4 µg/mL. MECs for micafungin (determined with a stereoscopic microscope) were 0.25 µg/mL; and 0.03 µg/mL, respectively.

Isolates were cultured on potato-dextrose agar at 35°C for 4 days. Suspensions were adjusted by haemocytometer counts and viability confirmed by culturing onto Dichloran Rose Bengala Chloramphenicol (DRBC) agar, which restricts the fast and invasiveness growth of *Trichoderma* (16).

For virulence studies, male OF-1 mice weighing 30 g were immunosuppressed with 200 mg/kg of cyclophosphamide, producing neutrophil counts <100 cells/mm^3 (17). Groups of 16 animals, 8 for survival and 8 for fungal burden and histopathology studies, were established. Groups were infected intravenously (i.v) into the lateral tail vein with 1 x 10^4, 1 x 10^5, 1 x 10^6 or 1 x 10^7 colony

forming units (CFU)/mouse of each strain in 0.2 mL. On day 6 post-infection, mice from tissue burden groups were euthanised by CO_2 inhalation. Lungs, kidneys, liver, spleen and brain were removed, homogenized, ten-fold diluted and placed onto DRBC agar for CFU/g calculation. All care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Treatments were evaluated in mice challenged with $1x10^7$ CFU, which produced an acute infection with all mice dying within 15 days. Treatments consisted on amphotericin B at 0.8 mg/kg i.v., liposomal amphotericin B (AmBisome) at 20 mg/kg i.v., micafungin (Mycamine) at 10 mg/kg i.p., and voriconazole (Vfend), at 25 mg/kg orally. The doses were based on previous pharmacokinetic studies (18, 19). From 3 days before infection, mice treated with voriconazole received grapefruit juice instead of water (20). Controls received no treatment. Therapy was initiated on day 1 post-infection and lasted for 5 days. To prevent bacterial infections, mice received ceftazidime 5 mg/kg/day subcutaneously. The efficacy of treatments was evaluated through prolongation of survival and reduction of fungal tissue burden.

Mean survival time (MST) was estimated by Kaplan–Meier method and compared among groups by the log-rank test. Tissue burden data were analysed by the Mann–Whitney U-test using GraphPad Prism 5 for Windows. *P* values ≤ 0.05 were considered statistically significant.

The mortality of infected mice correlated with inocula size. For both strains tested, the mortality rates of mice challenged with 1×10^4 and 1×10^5 CFU/animal were 25%, and 62.5% in those challenged with 1×10^6 CFU/animal. All mice infected with 1×10^7 CFU/animal died (MST 8.65 ± 2.8

81 days for FMR 12626 and 8.87 ± 3.35 days for FMR 12643) significantly earlier
82 with respect to smaller inocula ($P < 0.023$) (Figure 1).

83 On day 6 post-infection, mice infected with different inocula of FMR 12643
84 showed fungal loads in all organs, unlike those infected with the strain FMR
85 12626, where only the two highest inocula resulted in fungal recovery from all
86 organs. The most affected organs were liver and spleen followed by kidneys,
87 lungs and brain, regardless of the inoculum or the strain tested (Figure 2).
88 Only liposomal amphotericin B was able to prolong survival but only against the
89 strain FMR 12626 (P=0.03), which showed the highest in vitro susceptibility to
90 amphotericin B.
91 Likewise, only liposomal amphotericin B reduced the fungal load more than one
92 log in liver and spleen, of mice infected with the strain FMR 12626 ($P \leq 0.0002$)
93 whilst no statistical difference was observed with FMR 12643 (Fig. 4)
94 Some recommendations for *Trichoderma* infections include removal of
95 catheters, systemic antifungal therapy, treatment of underlying diseases and
96 surgery (11) but the best therapy is unknown. Animal models might therefore be
97 useful for evaluating antifungal therapies (21, 22). Our results demonstrate a
98 low virulence of the two strains tested. We recovered viable cells from all
99 organs only in mice infected with the highest inocula.
100 Despite its good in vitro activity, micafungin only reduced fungal load slightly.
101 The clinical use of micafungin against *Trichoderma* has not been reported,
102 although caspofungin has yielded inconclusive results (5, 23).
103 Voriconazole reduced load slightly in spleen of mice infected with the strain with
104 the greatest MIC, contrary to clinical cases that report successful treatment
105 against isolates with MICs <1 µg/mL (11, 24, 25). Here, voriconazole was

unable to reduce burden of mice challenged with the strain with the lowest MIC. This could partly be explained by the short treatment period evaluated. Other differences from successful outcomes include the use of intravenous voriconazole formulations, or prior administration of other antifungals, which might improve the effect of voriconazole.

Amphotericin B and liposomal amphotericin B, reduced tissue burden of mice infected with the strain with the lowest MIC. However, in survival studies, only liposomal amphotericin B showed efficacy in mice challenged with the strain with MIC of 2 µg/mL. In the few clinical cases reported, the use of amphotericin B or liposomal amphotericin B resulted in different outcomes, unrelated to MIC (4, 8, 26).

Clinical reports shown a low susceptibility of this fungus to available antifungals that agrees with our results that demonstrate poor efficacy of the three drugs tested and does not clarify management of this infection. Further studies are needed, testing a higher number of isolates with a wider range of MICs and a longer treatment period.

Funding: This project has been partially funded by the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. HEALTH-2013-INNOVATION-2-601963

Conflicts of interest: none

Ethical approval: Procedures were supervised and approved by L. Loriente Sanz (ID 39671243) of the Veterinary and Animal Welfare Advisory of the

130 Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

REFERENCES

1. **Hermosa R, Rubio MB, Cardoza RE, Nicolas C, Monte E, Gutierrez S.** 2013. The contribution of *Trichoderma* to balancing the costs of plant growth and defense. Int Microbiol 16:69-80.

2. **Alanio A, Brethon B, Feuilhade de Chauvin M, de Kerviler E, Leblanc T, Lacroix C, Baruchel A, Menotti J.** 2008. Invasive pulmonary infection due to *Trichoderma longibrachiatum* mimicking invasive aspergillosis in a neutropenic patient successfully treated with voriconazole combined with caspofungin. Clin Infect Dis 46:e116-118.

3. **Molnar-Gabor E, Doczi I, Hatvani L, Vagvolgyi C, Kredics L.** 2013. Isolated sinusitis sphenoidalis caused by *Trichoderma longibrachiatum* in an immunocompetent patient with headache. J Med Microbiol 62:1249-1252.

4. **Munoz FM, Demmler GJ, Travis WR, Ogden AK, Rossmann SN, Rinaldi MG.** 1997. *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. J Clin Microbiol 35:499-503.

5. **Rodriguez Peralta LI, Manas Vera MR, Garcia Delgado MJ, Perez de la Cruz AJ.** 2013. Endocarditis caused by *Trichoderma longibrachiatumin* a patient receiving home parenteral nutrition. Nutr Hosp 28:961-964.

6. **Richter S, Cormican MG, Pfaller MA, Lee CK, Gingrich R, Rinaldi MG, Sutton DA.** 1999. Fatal disseminated *Trichoderma longibrachiatum* infection in an adult bone marrow transplant patient: species identification and review of the literature. J Clin Microbiol 37:1154-1160.

7. **Gautheret A, Dromer F, Bourhis JH, Andremont A.** 1995. *Trichoderma pseudokoningii* as a cause of fatal infection in a bone marrow transplant recipient. Clin Infect Dis 20:1063-1064.

8. **Chouaki T, Lavarde V, Lachaud L, Raccurt CP, Hennequin C.** 2002. Invasive infections due to *Trichoderma* species: report of 2 cases, findings of *in vitro* susceptibility testing, and review of the literature. Clin Infect Dis 35:1360-1367.

9. **Esel D, Koc AN, Utas C, Karaca N, Bozdemir N.** 2003. Fatal peritonitis due to *Trichoderma* sp. in a patient undergoing continuous ambulatory peritoneal dialysis. Mycoses 46:71-73.

10. **Rota S, Marchesi D, Farina C, de Bievre C.** 2000. *Trichoderma pseudokoningii* peritonitis in automated peritoneal dialysis patient successfully treated by early catheter removal. Perit Dial Int 20:91-93.

11. **Festuccia M, Giaccone L, Gay F, Brunello L, Maffini E, Ferrando F, Talamo E, Boccadoro M, Serra R, Barbui A, Bruno B.** 2014. *Trichoderma* species fungemia after high-dose chemotherapy and autologous stem cell transplantation: a case report. Transpl Infect Dis 16:653-657.

12. **Kantarcioglu AS, Celkan T, Yucel A, Mikami Y, Kurugoglu S, Mitani H, Altas K.** 2009. Fatal *Trichoderma harzianum* infection in a leukemic pediatric patient. Med Mycol 47:207-215.

13. **Druzhinina IS, Komon-Zelazowska M, Kredics L, Hatvani L, Antal Z, Belayneh T, Kubicek CP.** 2008. Alternative reproductive strategies of *Hypocrea orientalis* and genetically close but clonal *Trichoderma longibrachiatum*, both capable of causing invasive mycoses of humans. Microbiology 154:3447-3459.

14. **Sandoval-Denis M, Sutton DA, Cano-Lira JF, Gene J, Fothergill AW, Wiederhold NP, Guarro J.** 2014. Phylogeny of the clinically relevant species of the emerging fungus *Trichoderma* and their antifungal susceptibilities. J Clin Microbiol 52:2112-2125.

15. **Clinical and Laboratory Standards Institute.** 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: approved standard. CLSI document M38-A2. Clinical and Laboratory Standard Institute, Wayne, PA.

16. **Hocking AD.** 2014. Foodborne Fungi: Estimation by Cultural Techniques, p 68-75. *In* Carl A. Batt, Mary Lou Tortorello (ed), Encyclopedia of Food Microbiology, 2nd ed, vol 2. Academic Press, London, UK.

17. **Chiller TM1, Luque JC, Sobel RA, Farrokhshad K, Clemons KV, Stevens DA**. 2002. Development of a murine model of cerebral aspergillosis. J Infect Dis. 186:574-577.

18. **Andes D, Safdar N, Marchillo K, Conklin R**. 2006. Pharmacokinetic-pharmacodynamic comparison of amphotericin B (AMB) and two lipid-associated AMB preparations, liposomal AMB and AMB lipid complex, in murine candidiasis models. Antimicrob Agents Chemother. 2006. 50:674-684.

19. **Warn PA, Sharp A, Mosquera J, Spickermann J, Schmitt-Hoffmann A, Heep M, Denning DW**. 2006. Comparative *in vivo* activity of BAL4815, the active component of the prodrug BAL8557, in a neutropenic murine model of disseminated *Aspergillus flavus*. J Antimicrob Chemother 58: 1198–1207.

20. **Sugar AM, Liu XP.** 2001. Efficacy of voriconazole in treatment of murine pulmonary blastomycosis. Antimicrob Agents Chemother 45:601-604.

21. **Capilla J, Clemons KV, Stevens DA.** 2007. Animal models: an important tool in mycology. Med Mycol 45:657-684.

22. **Guarro J.** 2011. Lessons from animal studies for the treatment of invasive human infections due to uncommon fungi. J Antimicrob Chemother 66:1447-1466.

23. **Santillan Salas CF, Joshi AY, Dhiman N, Banerjee R, Huskins WC, Wengenack NL, Henry NK.** 2011. Fatal post-operative *Trichoderma longibrachiatum* mediastinitis and peritonitis in a paediatric patient with complex congenital cardiac disease on peritoneal dialysis. J Med Microbiol 60:1869-1871.

24. **Trabelsi S, Hariga D, Khaled S.** 2010. First case of *Trichoderma longibrachiatum* infection in a renal transplant recipient in Tunisia and review of the literature. Tunis Med 88:52-57.

25. **Lagrange-Xelot M, Schlemmer F, Gallien S, Lacroix C, Molina JM.** 2008. *Trichoderma* fungaemia in a neutropenic patient with pulmonary cancer and human immunodeficiency virus infection. Clin Microbiol Infect 14:1190-1192.

26. **Loeppky CB, Sprouse RF, Carlson JV, Everett ED.** 1983. *Trichoderma viride* peritonitis. South Med J 76:798-799.

FIGURE LEGENDS

Figure 1. Cumulative mortality of immunosuppressed mice infected with different inocula of two strains of *T. longibrachiatum*; FMR 12626 (A) and FMR 12643 (B). [a] $P \leq 0.05$ versus $1x10^7$ CFU/animal.

Figure 2. Colony counts of *T. longibrachiatum* FMR 12626 (A) and FMR 12643 (B) in different organs of immunosuppressed mice at day 6 post-infection. Horizontal lines indicate median values.

Figure 3. Cumulative mortality of immunosuppressed mice infected with *T. longibrachiatum* FMR 12626 (A) and FMR 12643 (B) after therapy with AMB 0.8, amphotericin B at 0.8 mg/kg QD; MCF 10, micafungin at 10 mg/kg; VRC 25, voriconazole at 25 mg/kg QD; or LAMB 20, liposomal amphotericin B at 20 mg/kg QD. [a] $P \leq 0.05$ versus control.

Figure 4. Fungal load of immunosuppressed mice infected with *T. longibrachiatum* FMR 12626 (A) or FMR 12643 (B). AMB 0.8, amphotericin B at 0.8 mg/kg QD; MCF 10, micafungin at 10 mg/kg; VRC 25, voriconazole at 25 mg/kg QD; or LAMB 20, liposomal amphotericin B at 20 mg/kg QD. Horizontal lines indicate median values. [a] $P \leq 0.05$ versus control; [b] $P \leq 0.05$ versus AMB 0.8; [c] $P \leq 0.05$ versus LAMB 20; [d] $P \leq 0.05$ versus VRC 25; [e] $P \leq 0.05$ versus MCF 10.

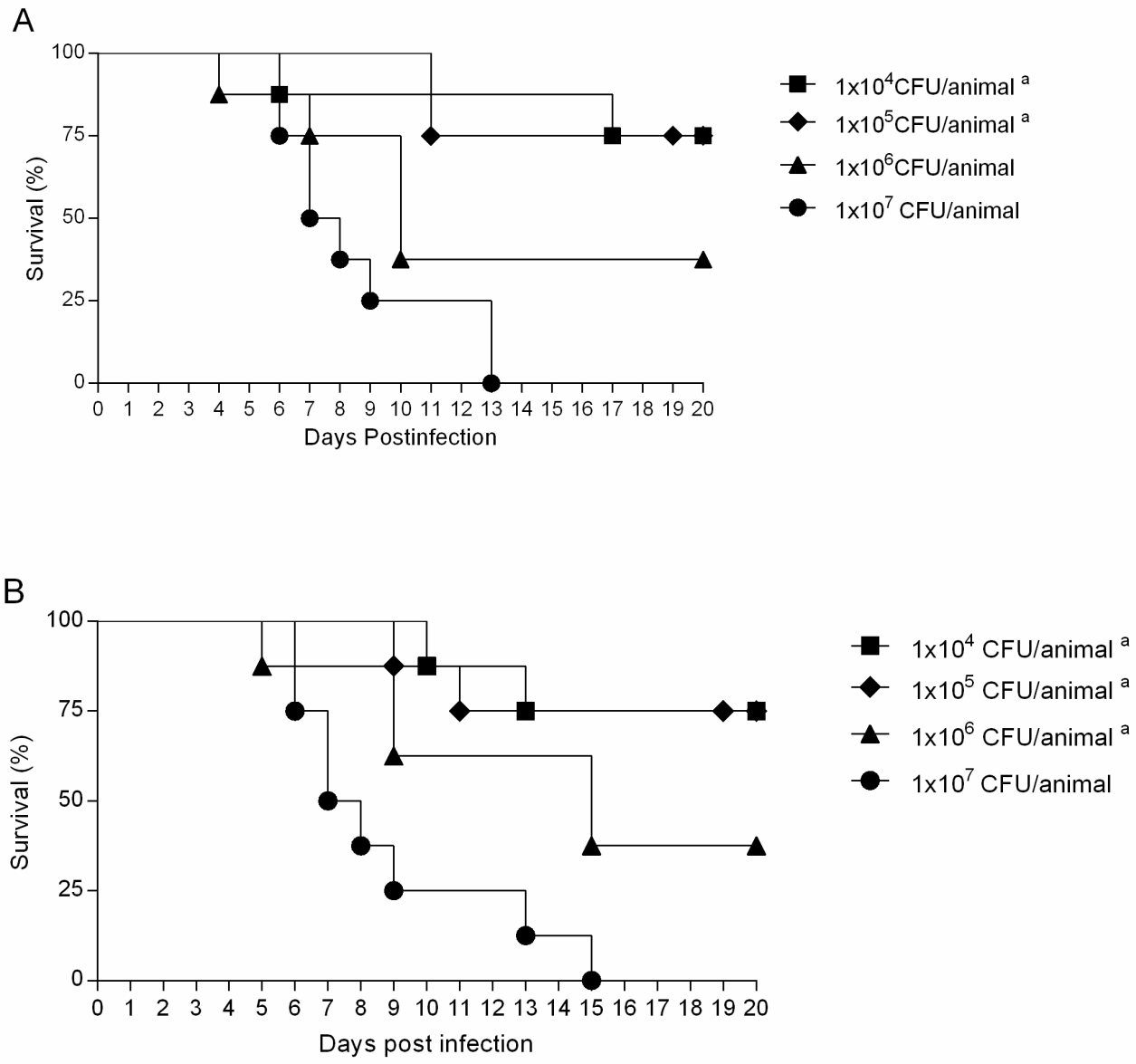

Figure 1. Cumulative mortality of immunosuppressed mice infected with different inocula of two strains of *T. longibrachiatum*; FMR 12626 (A) and FMR 12643 (B). [a] $P \leq 0.05$ versus $1x10^7$ CFU/animal.

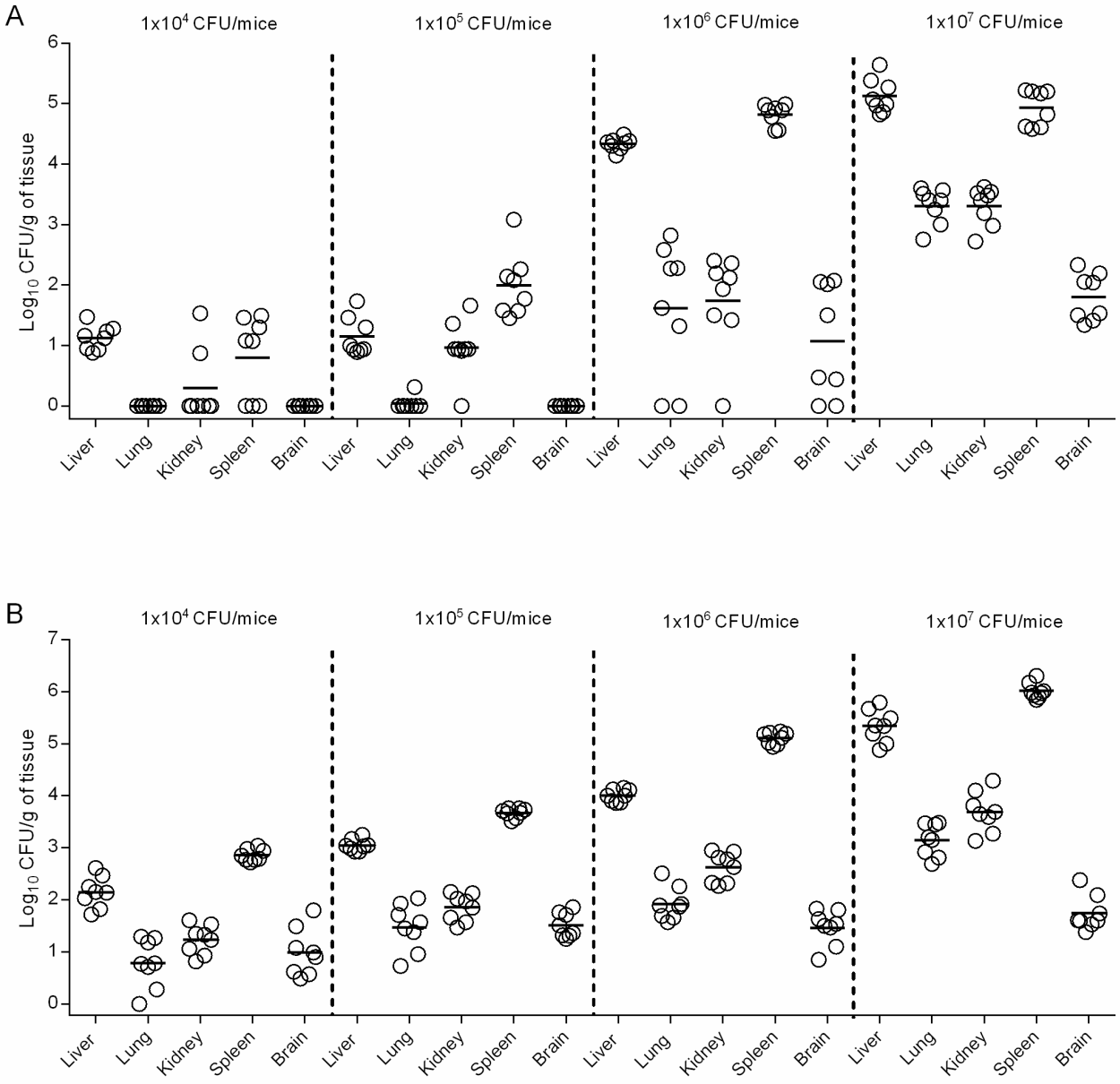

Figure 2. Colony counts of *T. longibrachiatum* FMR 12626 (A) and FMR 12643 (B) in different organs of immunosuppressed mice at day 6 post-infection. Horizontal lines indicate median values.

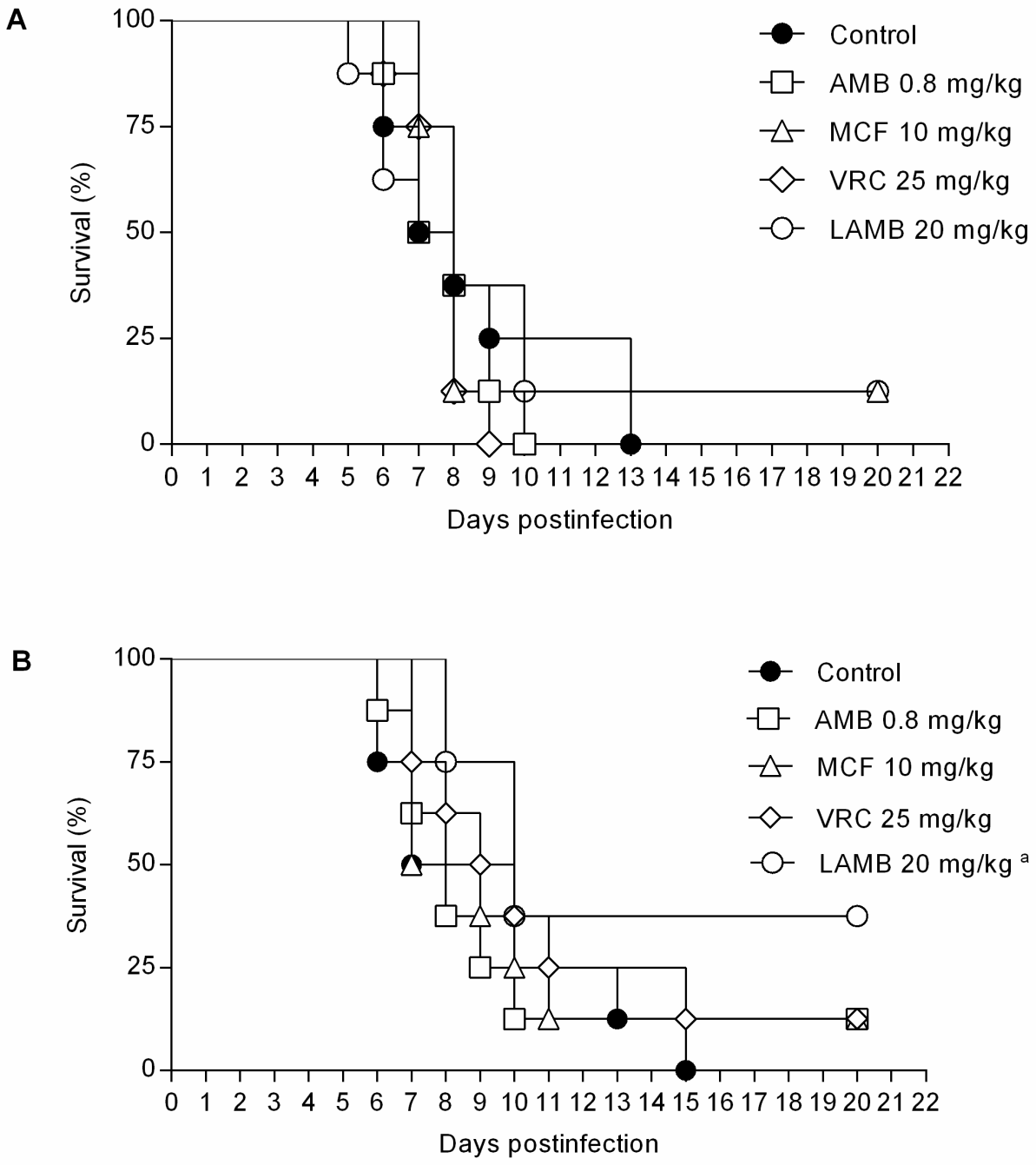

Figure 3. Cumulative mortality of immunosuppressed mice infected with *T. longibrachiatum* FMR 12626 (A) and FMR 12643 (B) after therapy with AMB 0.8, amphotericin B at 0.8 mg/kg QD; MCF 10, micafungin at 10 mg/kg; VRC 25, voriconazole at 25 mg/kg QD; or LAMB 20, liposomal amphotericin B at 20mg/kg QD. [a] $P \leq 0.05$ versus control.

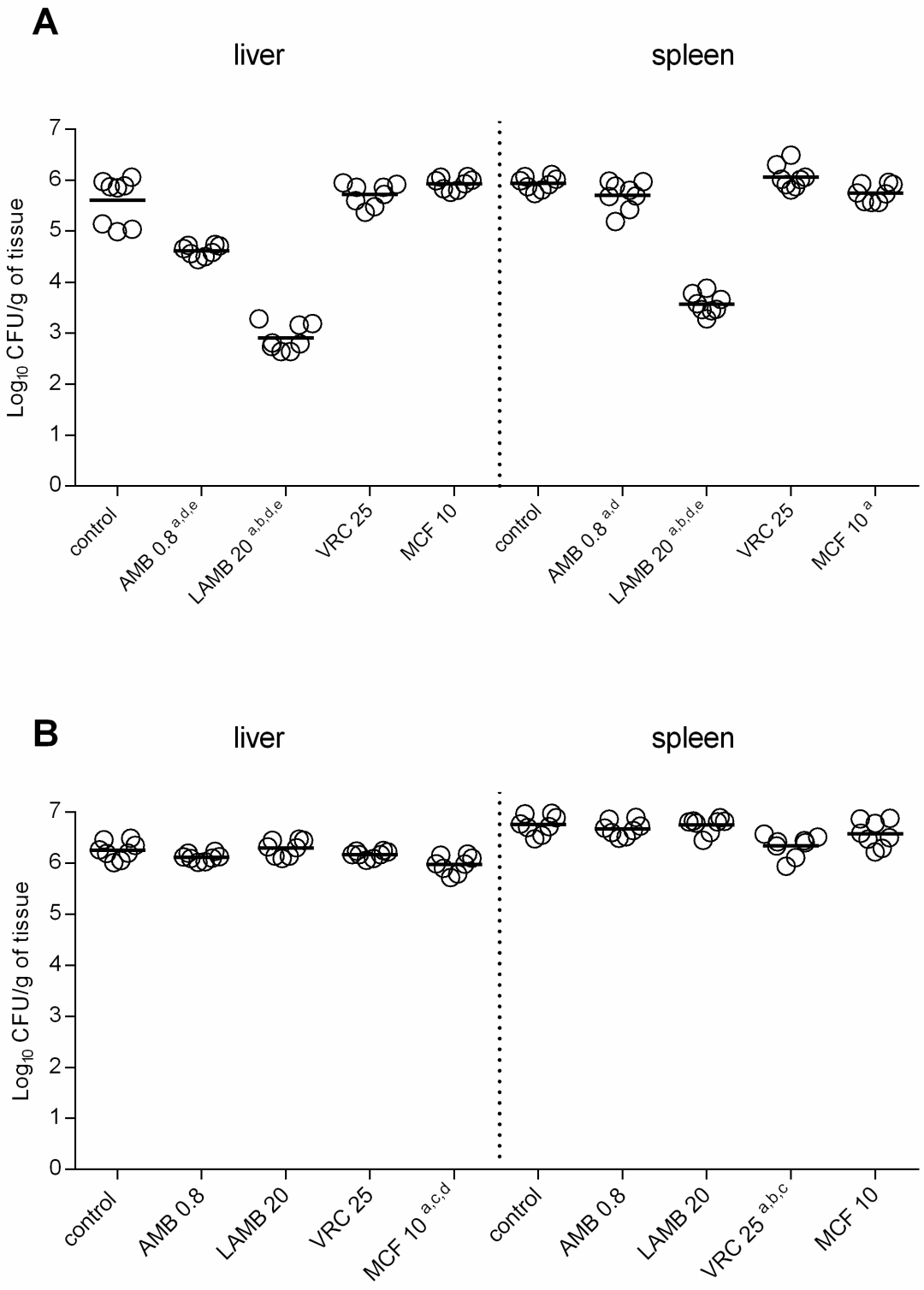

Figure 4. Fungal load of immunosuppressed mice infected with *T. longibrachiatum* FMR 12626 (A) or FMR 12643 (B). AMB 0.8, amphotericin B at 0.8 mg/kg QD; MCF 10, micafungin at 10 mg/kg; VRC 25, voriconazole at 25 mg/kg QD; or LAMB 20, liposomal amphotericin B at 20mg/kg QD. Horizontal lines indicate median values. [a] $P \leq 0.05$ versus control; [b] $P \leq 0.05$ versus AMB 0.8; [c] $P \leq 0.05$ versus LAMB 20; [d] $P \leq 0.05$ versus VRC 25; [e] $P \leq 0.05$ versus MCF 10.