Virulence and Experimental Treatment of *Trichoderma longibrachiatum*, a Fungus Refractory to Treatment

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

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, it is the most used drug, while voriconazole and echinocandins show better *in vitro* activities (14). Our aim was to evaluate the virulence of *T. longibrachiatum* in a murine model and the efficacies of amphotericin B, liposomal amphotericin B, micafungin, and voriconazole.

Two clinical strains of *T. longibrachiatum* (FMR 12626 and FMR 12643), identified by multilocus sequence analysis (14), were used. Antifungal activity was determined following the CLSI guidelines (14, 15). For FMR 12626, the MICs of amphotericin B and voriconazole were 0.13 and 0.5 $\mu\text{g/ml}$, respectively, and for FMR 12643, the MICs were 2 and 4 $\mu\text{g/ml}$, respectively. The minimum effective concentrations (MECs) of micafungin for FMR 12626 and FMR 12643 (determined with a stereoscopic microscope) were 0.25 and 0.03 $\mu\text{g/ml}$, respectively.

Isolates were cultured on potato-dextrose agar at 35°C for 4 days. Suspensions were adjusted by hemocytometer counts, and viability was confirmed by culturing onto dichloran Rose Bengala chloramphenicol (DRBC) agar, which restricts the fast and invasive 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 of <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.) in the lateral tail vein with 1×10^4 , 1×10^5 , 1×10^6 , or 1×10^7 CFU of each strain per mouse in 0.2 ml. On day 6 postinfection, mice from the tissue burden groups were euthanized by CO_2 inhalation. Lungs, kidneys, livers, spleens, and brains were removed, homogenized, 10-fold diluted, and placed onto DRBC agar for calculation of CFU per gram. All care procedures were supervised and approved by L. Lorient Sanz (ID 39671243) of the Veterinary and Animal Welfare Advisory of the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Treatments were evaluated in mice challenged with 1×10^7 CFU, which produced an acute infection with all mice dying within 15 days. Treatments consisted of amphotericin B at 0.8 mg/kg body weight (i.v.), liposomal amphotericin B (AmBisome) at 20 mg/kg i.v., micafungin (Mycamine) at 10 mg/kg intraperito-

neally (i.p.), and voriconazole (Vfend) at 25 mg/kg body weight 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 postinfection and lasted for 5 days. To prevent bacterial infections, mice received ceftazidime at 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 the Kaplan-Meier method, and MSTs were compared among groups by the log-rank test. Tissue burden data were analyzed by the Mann-Whitney U test using GraphPad Prism 5 for Windows. *P* values of ≤ 0.05 were considered statistically significant.

The mortality of infected mice correlated with inoculum size. For both strains tested, the mortality rate of mice challenged with 1×10^4 and 1×10^5 CFU/animal was 25%; the rate was 62.5% in those challenged with 1×10^6 CFU/animal. All mice infected with 1×10^7 CFU/animal died (MSTs, 8.65 ± 2.8 days for FMR 12626 and 8.87 ± 3.35 days for FMR 12643) significantly earlier than mice infected with smaller inocula ($P < 0.023$) (Fig. 1).

On day 6 postinfection, mice infected with different inocula of FMR 12643 showed fungal loads in all organs, unlike those infected with strain FMR 12626, where only the two highest inocula resulted in fungal recovery from all organs. The most affected organs were livers and spleens, followed by kidneys, lungs, and brains, regardless of the inoculum or strain tested (Fig. 2).

Only liposomal amphotericin B was able to prolong survival but only against strain FMR 12626 ($P = 0.03$), which showed the highest *in vitro* susceptibility to amphotericin B (Fig. 3).

Likewise, only liposomal amphotericin B reduced the fungal load more than 1 log in livers and spleens of mice infected with

Received 19 February 2016 Returned for modification 27 March 2016

Accepted 16 May 2016

Accepted manuscript posted online 23 May 2016

Citation Paredes K, Capilla J, Mayayo E, Guarro J. 2016. Virulence and experimental treatment of *Trichoderma longibrachiatum*, a fungus refractory to treatment. Antimicrob Agents Chemother 60:5029–5032. doi:10.1128/AAC.00373-16.

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FIG 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 1×10^7 CFU/animal.

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

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

strain FMR 12626 ($P \leq 0.0002$), while no statistical difference was observed in mice infected with FMR 12643 (Fig. 4).

Some recommendations for *Trichoderma* infections include removal of catheters, systemic antifungal therapy, treatment of underlying diseases, and surgery (11), but the best therapy is unknown. Animal models might therefore be useful for evaluating antifungal therapies (21, 22). Our results demonstrate a low virulence of the two strains tested. We recovered viable cells from all organs only in mice infected with the highest inocula.

Despite its good *in vitro* activity, micafungin only reduced fungal load slightly. The clinical use of micafungin against *Trichoderma* has not been reported, although caspofungin has yielded inconclusive results (5, 23).

Voriconazole reduced load slightly in spleens of mice infected with the strain with the greatest MIC, contrary to clinical cases that report successful treatment against isolates with MICs of $<1 \mu\text{g/ml}$ (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 the 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 a MIC of $2 \mu\text{g/ml}$. In the few clinical cases reported, the use of amphotericin

FIG 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 once a day (QD); MCF 10, micafungin at 10 mg/kg; VRC 25, voriconazole at 25 mg/kg QD; 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.

B or liposomal amphotericin B resulted in different outcomes unrelated to MIC (4, 8, 26).

Clinical reports have shown a low susceptibility of this fungus to available antifungals, which agrees with our results that demonstrate poor efficacies of the three drugs tested and which 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.

ACKNOWLEDGMENT

We report that we have no conflicts of interest.

FUNDING INFORMATION

This work, including the efforts of Josep Guarro, Javier Capilla, Emilio Mayayo, and Katihuska Paredes, was funded by EC | Seventh Framework Programme (FP7) (HEALTH-2013-INNOVATION-2-601963).

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