Veterinary Microbiology 157 (2012) 137–142

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

In vitro and *in vivo* susceptibility of two-drug and three-drug combinations of terbinafine, itraconazole, caspofungin, ibuprofen and fluvastatin against *Pythium insidiosum*

Juliana S. Argenta [a], Sydney H. Alves [b], Flávio Silveira [b], Grazieli Maboni [b], Régis A. Zanette [a], Ayrton S. Cavalheiro [b], Patrique L. Pereira [b], Daniela I.B. Pereira [c], Elisa S.V. Sallis [c], Luciana Pötter [d], Janio M. Santurio [b,*], Laerte Ferreiro [a]

[a] *Programa de Pós Graduação em Ciências Veterinárias, Setor de Micologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*
[b] *Laboratório de Pesquisas Micológicas (LAPEMI), Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil*
[c] *Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Pelotas, RS, Brazil*
[d] *Departamento de Zootecnia, Universidade Federal de Santa Maria (UFSM-CESNORS), Palmeira da Missões, RS, Brazil*

ARTICLE INFO

Article history:
Received 2 August 2011
Received in revised form 1 December 2011
Accepted 5 December 2011

Keywords:
Pythium insidiosum
Terbinafine
Itraconazole
Caspofungin
Ibuprofen
Fluvastatin
Rabbits

ABSTRACT

The present study investigated the *in vitro* inhibitory activity of terbinafine, itraconazole, caspofungin, fluvastatin and ibuprofen against 15 isolates of *Pythium insidiosum* in double and triple combinations and determined *in vivo* correlations using rabbits with experimental pythiosis. The minimal inhibitory concentration (MIC) was determined in accordance with the Clinical and Laboratory Standards Institute M 38-A2 protocol (2008), and the *in vitro* interactions were evaluated using a checkerboard microdilution method. For the *in vivo* study, 20 rabbits inoculated with *P. insidiosum* zoospores were divided into four groups: group 1 was treated with terbinafine and itraconazole; group 2 was treated with terbinafine, itraconazole and fluvastatin; group 3 was treated with terbinafine and caspofungin; and group 4 was the control group. Combinations of terbinafine with caspofungin or ibuprofen were synergistic for 47% of the isolates, and antagonism was not observed in any of the double combinations. The triple combinations were mostly indifferent, but synergism and antagonism were also observed. In the *in vivo* study, the histological aspect of the lesions was similar among the groups, but group 2 showed the lowest amount of hyphae and differed significantly from the other groups.

1. Introduction

Pythium insidiosum is pathogenic to mammals when zoospores invade the host tissue (De Cock et al., 1987; Mendoza et al., 1993). *P. insidiosum* causes pythiosis, which is a chronic infectious disease that is primarily diagnosed in horses, dogs and humans; however, it has also been described in cattle, sheep, cats and wild species (Gaastra et al., 2010). A unique characteristic of *P. insidiosum* is the lack of ergosterol in the cytoplasmic membrane (Grooters, 2003), which reduces the effectiveness of antifungal therapies in different species. Nevertheless, a pharmacological cure is occasionally achieved (Shenep et al., 1998).

Studies of *P. insidiosum* susceptibility have been developed to find treatments for this disease through chemotherapy; however, the present study was the first study *in vitro* and *in vivo* to use a triple combination of antifungal and nonantifungal drugs. The lack of effective therapeutic alternatives for pythiosis has increased the interest in investigating the activity of nonantifungal agents and their associations with other agents, including antifungal drugs (Cavalheiro et al., 2009b). The association

* Corresponding author. Tel.: +55 55 32208906; fax: +55 55 32208906.
E-mail address: janio.santurio@gmail.com (J.M. Santurio).

0378-1135/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2011.12.003

of antifungal agents, mainly azoles, with ibuprofen or statins to treat *Candida* spp., *Cryptococcus neoformans* and *Aspergillus* spp. has demonstrated synergistic effects *in vitro* (Chin et al., 1997; Pina-Vaz et al., 2005; Qiao et al., 2007).

The aims of the present study were to investigate the *in vitro* inhibitory activity of two-drug and three-drug combinations of terbinafine (TRB), itraconazole (ITC), caspofungin (CAS), fluvastatin (FVT) and ibuprofen (IBP) against 15 isolates of *P. insidiosum* and determine *in vivo* correlations of the same *in vitro* associations in rabbits with experimental pythiosis.

2. Materials and methods

2.1. In vitro susceptibility tests

The present study included 15 Brazilian *P. insidiosum* strains that were isolated from horses (13 strains) with pythiosis and rabbits (2 strains) with experimental pythiosis. The identities of these isolates were confirmed by a PCR-based assay (Rodrigues et al., 2006). The inocula prepared for susceptibility tests consisted of *P. insidiosum* zoospores that were obtained following a zoosporogenesis technique. Zoospores were counted in a Neubauer hemacytometer, diluted in RPMI 1640 broth containing L-glutamine and buffered to pH 7.0 with 0.165 M MOPS. The final concentration ranged from 2 to 3×10^3 zoospores/ml (Pereira et al., 2007; Argenta et al., 2008) for all susceptibilities tests.

The drugs tested included TRB (Novartis, 0.25–32 mg/l), ITC (Sigma Pharma, 0.25–64 mg/l), CAS (Merck, 0.5–128 mg/l), FVT (Novartis, 1–64 mg/l) and IBP (Whitehall, 4–1024 mg/l). The minimal inhibitory concentrations (MICs) were determined by the Clinical and Laboratory Standards Institute M-38A2 protocol (microdilution method) (CLSI, 2008). For the double combinations, a two-dimensional checkerboard with twofold dilutions of each drug (Cuenca-Estrella, 2004) was used for the study of TRB with ITC, CAS, FVT or IBP and ITC with FVT or IBP. The triple combinations (TRB–ITC–FVT, TRB–ITC–IBP, TRB–ITC–CAS and TRB–FVT–CAS) were tested by a three-dimensional checkerboard technique where the third drug was added at a single concentration on each plate. Each well of the microtiter plates contained 100 μl of the diluted drug concentrations (150 μl for the triple combinations) and was inoculated with 100 μl of the inoculum suspension (50 μl for the triple combinations). This method resulted in a final volume of 200 μl. The MIC readings were visual and assessed the presence (i.e., growth) or absence of hyphae after a 24-h incubation at 37 °C. The MIC-0 was used as the reading criteria for this study, which was the lowest drug concentration at which no growth was evident compared with the positive control (hyphae under optimal growth conditions).

Based on the fractional inhibitory concentration index (FICI), interactions were interpreted as synergistic ($FICI \leq 0.5$), indifferent ($0.5 < FICI \leq 4$) or antagonistic ($FICI > 4$) (Johnson et al., 2004) using the formula: FICI = (MIC A in combination/MIC A alone) + (MIC B in combination/MIC B alone) + (MIC C in combination/MIC C alone), where MIC A, MIC B and MIC C indicate the MICs of drugs A, B and C, respectively (Dannaoui et al., 2004). For double combinations, the third term (C) of the equation was omitted. Off-scale MICs were converted to the next higher dilution for calculation purposes.

2.2. Animal model

The *in vivo* study included twenty three-month-old males New Zealand rabbits that were experimentally infected with *P. insidiosum* strain from horse to evaluate the efficacy of three different treatments for pythiosis. The inoculum consisted of *P. insidiosum* zoospores that were obtained following a zoosporogenesis technique. Zoospores were counted in a Neubauer hemacytometer and diluted in sterile distilled water. All animals received 1 ml of inoculum, which contained approximately 20,000 viable *P. insidiosum* zoospores, subcutaneously in the right costal region as previously described (Santurio et al., 2003; Pereira et al., 2007). Rabbits inoculated with *P. insidiosum* zoospores were divided into four groups with five animals in each: group 1 was treated with TRB + ITC, group 2 was treated with TRB + ITC + FVT, group 3 was treated with TRB + CAS and group 4 was the control group. Treatment started on the 30th day after inoculation. Rabbits in group 1 were treated with 125 mg/day terbinafine and 5 mg/kg/day itraconazole by gavage for 60 days. Rabbits in group 2 were treated with 125 mg/day terbinafine, 5 mg/kg/day itraconazole and 1 mg/kg/day fluvastatin by gavage for 60 days. The rabbits in group 3 were treated with 125 mg/day terbinafine by gavage and 1 mg/kg/day caspofungin by intraperitoneal injection for 28 days. The drugs were dissolved in sterile distilled water. Group 4 (control) did not receive any treatment. Inoculated rabbits were checked every seven days by measuring the subcutaneous nodular area (cm^2) using a sliding calliper, and blood samples were obtained every 15 days by intracardiac puncture to examine the plasma concentration of TRB and ITC (groups 1 and 2) (Hurtado et al., 2009).

The rabbits were necropsied at the end of the treatments. Representative fragments of the subcutaneous lesions were fixed in 10% buffered formalin, routinely processed for histopathologic evaluation, and stained with hematoxylin and eosin (H&E) or Grocott's stain. An Olympus Oly-200 color video camera coupled to a BX51/BX52 Olympus binocular microscope was used to capture images, as described by Pereira et al. (2008). Images were acquired at 400× magnification. Hyphae were quantified using Alpha Ease FC software (Alpha Innotech Corporation version 4.0), and five microscopic fields were used for each slide. The images were converted into 255 greyscale intensities. A dark intensity range (values 0–175), which corresponded to hyphae stained by silver in Grocott's stain, was selected using a selection tool. The data obtained were submitted to statistical analysis. The procedure was approved by the Animal Welfare Committee of the Federal University of Santa Maria.

2.3. Statistical analysis

The lesional areas of each group were measured every 7 days. After each measurement was made, the average was calculated for each group, and the areas were