Caspofungin in vitro and in vivo activity against Brazilian Pythium insidiosum strains isolated from animals

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Received 11 May 2007; returned 5 July 2007; revised 3 August 2007; accepted 6 August 2007

Objectives: The present study evaluated the susceptibility of 27 clinical isolates of Pythium insidiosum to caspofungin in vitro and correlated the results with the therapeutic response in vivo in rabbits with experimental pythiosis.

Methods: The macrodilution method was performed in accordance with the CLSI document M38-A technique. Three reading criteria for MICs were adopted: MIC0, MIC1 and MIC2 (100%, 90% and 50% growth inhibition, respectively). The minimum fungicidal concentration was also determined. Ten rabbits inoculated with viable P. insidiosum zoospores were divided into two groups: group 1 (control) and group 2 (treated with caspofungin at a dosage of 1 mg/kg/day for 20 consecutive days).

Results: Of the isolates 51.8% had an MIC0 of 64 mg/L, 88.8% of isolates had an MIC1 between 8 and 64 mg/L and 62.9% of isolates had a minimum fungicidal concentration of 64 mg/L. In the in vivo assay, growth of subcutaneous lesions reduced during treatment, but rapidly resumed when treatment was stopped.

Conclusions: The results showed that caspofungin has limited fungistatic activity against P. insidiosum. This work is the first study to analyse the susceptibility of this oomycete to inhibitors of β-glucans of the cellular wall.

Keywords: pythiosis, oomycete, susceptibility

Introduction

Pythium insidiosum is an aquatic oomycete, classified in the Kingdom Stramenopila. The genus Pythium comprises more than 200 species, and the majority of them are soil saprobes and plant pathogens. Only P. insidiosum is pathogenic to mammals. It causes pythiosis, a chronic pyogranulomatous disease that affects mainly horses, but can also affect dogs, cats, cattle, sheep and humans that inhabit tropical and subtropical regions.

The composition of the cell wall and the lack of ergosterol in the cytoplasmic membrane of oomycetes explain the difficulties of antifungal therapy against pythiosis, since ergosterol is the target of action for the majority of the antifungal drugs available. Various attempts to treat animals as well as humans with antifungal drugs have presented variable and sometimes contradictory results as compared with results of in vitro assays. Nevertheless, Shenep et al. affirm that a pharmacological cure for pythiosis is possible; however, it must be guided by tests of in vitro susceptibility.

Caspofungin (L-743, 872, MK-0991) is a semi-synthetic derivative of the pneumocandin Bo, whose mechanism of action consists of blocking the synthesis of β(1,3)-d-glucan of the fungal cell wall through the non-competitive inhibition of the enzyme β(1,3)-d-glucan synthase. It is believed that this drug can be efficient against P. insidiosum, since this oomycete contains a great amount of β-glucan in the cell wall.

The present study aimed at evaluating the in vitro susceptibility of P. insidiosum to caspofungin using the CLSI
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(formerly NCCLS) technique of macrodilution in broth as well as relating the results obtained in vitro with the response of in vivo therapy in rabbits with experimental pythiosis.

Materials and methods

Culture of P. insidiosum
Twenty-six samples of P. insidiosum isolated from pythiosis lesions from animals in Brazil (24 horses, 1 dog and 1 sheep) and one ATCC isolate (58637) were utilized.

Inoculum preparation for the antifungal susceptibility test
Each inoculum consisted of P. insidiosum zoospores obtained according to a previously described zoosporogenesis technique.\(^5\)

Antifungal agent
Caspofungin (Cancidas\(^6\), MSD SHARP & DOHME GmbH, Haar, Germany) in the form of 50 mg was commercially acquired. It was dissolved in sterile distilled water and serially diluted in assay medium to yield final concentrations of 0.25–128 mg/L.

In vitro susceptibility test
The tests were performed using the macrodilution method in broth following the CLSI M38-A protocol.\(^6\) After zoosporogenesis, the zoospores were counted using a Neubauer chamber. The induction medium containing the zoospores was diluted 1:10 in RPMI 1640 broth with L-glutamine, glucose and buffered to pH 7.0 with 0.165 M MOPS (assay medium), yielding an inoculum with a final concentration of 2–3 \( \times 10^3 \) zoospores/mL. All the assays were performed in duplicate. The MICs were read after 24 h of incubation at 37°C. The reading was visual and assessed the growth or absence of growth of hyphae. Three MIC reading criteria were adopted: MIC0, MIC1 and MIC2 (growth inhibition of 100%, 90% and 50%, respectively). The concentration above MIC0 was utilized to determine the minimum fungicidal concentration.

Animals
Ten 3-month-old New Zealand rabbits, including males and females, were divided into two groups of five animals. All the animals were inoculated with P. insidiosum zoospores (isolate with MIC of 64 mg/L) by the subcutaneous route according to the methodology described by Santurio et al.\(^5\) Group 1 (control) did not receive treatment. Animals in group 2 were treated with 1 mg/kg/day caspofungin by the intraperitoneal route for 20 days, with treatment commencing on the 25th day after inoculation. Inoculated rabbits were checked every 3 days by measuring the subcutaneous nodular area (cm\(^2\)) using a sliding calliper. The procedure was approved by the Animal Welfare Committee of the Federal University of Santa Maria.

Statistical analysis
The areas of the lesions (cm\(^2\)) were transformed into percentage and submitted to analysis of variance and F-test using a significance level of 5%. The Tukey test was carried out when differences between the treatments were detected. Regression analysis of the dates of the measurements was also performed, with adjustments in the polynomial model made up to the third order.

Results
The in vitro susceptibility of 27 isolates of P. insidiosum against caspofungin is listed in Table 1. The results indicated that 51.8% of the isolates had an MIC0 of 64 mg/L. Readings using MIC1 showed that 88.8% of the isolates required MICs that varied from \( \geq 8 \) to 64 mg/L. The partial readings of 50% inhibition (MIC2) demonstrated that 55.5% of the isolates had MICs between 2 and 4 mg/L, and 44.4% had MICs between 8 and 16 mg/L. The minimum fungicidal concentration was 64 mg/L for 62.9% of the samples.

The animals developed subcutaneous nodules 25 days after the inoculation of the zoospores. The subcutaneous lesion areas exhibited a reduction in their progression during the treatment with caspofungin. However, lesions resumed growth after the end of the treatment, even though they were smaller (\( P = 0.0004 \)) than the lesions in the control group (Figure 1). One animal of the treated group died on the 23rd day after the end of the treatment and had pythiosis lesions in the lungs and kidneys.

Discussion
Susceptibility studies on P. insidiosum are rare and there is no standardized technique for in vitro tests for this oomycete. Only Shenep et al.\(^3\) and Sekhon et al.\(^7\) have performed tests in vitro with P. insidiosum to evaluate its susceptibility to amphotericin B, 5-fluorocytosine, terbinafine and azoles without, however,

\begin{table}
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\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{Reading criteria} & 2 & 4 & 8 & 16 & 32 & 64 & 128 & \textbf{MIC range (mg/L)} & \textbf{GM MIC (mg/L)} \\
\hline
MIC0 & 0 & 0 & 3 (11.1) & 3 (11.1) & 7 (25.9) & 14 (51.8) & 0 & 8–64 & 36.38 \\
MIC1 & 0 & 3 (11.1) & 9 (33.3) & 7 (25.9) & 8 (29.6) & 0 & 0 & 4–32 & 13.36 \\
MIC2 & 6 (22.2) & 9 (33.3) & 6 (22.2) & 6 (22.2) & 0 & 0 & 0 & 2–16 & 5.44 \\
MFC & 0 & 0 & 2 (7.4) & 3 (11.1) & 1 (3.7) & 17 (62.9) & 4 (14.8) & 8–128 & 50.79 \\
\hline
\end{tabular}
\caption{Distribution of caspofungin MICs for 27 P. insidiosum isolates using three reading criteria}
\end{table}

MIC0, 100% growth inhibition; MIC1, 90% growth inhibition; MIC2, 50% growth inhibition; MFC, minimum fungicidal concentration; GM, geometric mean.
following a standard methodology. The present study employed the parameters recommended by the document M38-A to test the susceptibility of *P. insidiosum* isolates to caspofungin.

The standardized inoculum obtained by counting zoospores led to reproducible results *in vitro*. In our opinion, this procedure provides the most reliable methodology for susceptibility tests with this oomycete. Adjustment of the inoculum through the spectrophotometric procedure cannot be applied to the zoospore suspensions because it does not produce turbidity in the medium and Espinel-Ingroff et al. emphasize that the preparation of the inoculum with a suspension of hyphae is not reliable. However, other authors have utilized spectrophotometrically adjusted suspensions of *P. insidiosum* hyphae as inocula.

The lack of a clear standard to estimate caspofungin MIC, in addition to the absence of data concerning standardization of *in vitro* tests for *P. insidiosum*, justifies the employment of MIC readings considering the three criteria. Likewise, other *in vitro* assays with filamentous fungi that evaluated susceptibility to caspofungin also utilized different reading criteria for the MICs. The detection of morphological alterations in the hyphae, defined as minimum effective concentration (MEC), may constitute a more appropriate method for evaluating the susceptibility to echinocandins when compared with the conventional MIC. Nevertheless, it was not possible to determine the MEC for *P. insidiosum* isolates in the present work because morphological alterations were not detected on hyphae.

The high MICs and lack of fungicidal activity seen in this study suggest that *P. insidiosum* is poorly susceptible to caspofungin. Studies demonstrate that caspofungin displays *in vitro* activity against species of *Candida* and *Aspergillus* spp. with MICs and MECs of 1 mg/L. However, it shows little activity against *Cryptococcus neoformans*, *Trichosporon* spp., *zygomycetes* and *Fusarium* spp., generally with MICs just above 16 mg/L.

The low susceptibility of *P. insidiosum* to caspofungin was also established by the *in vivo* assay. The results indicated that caspofungin activity against *P. insidiosum* was only fungistatic. Further studies using doses >1 mg/kg/day may yield better results. Alternative dosing protocols may also improve efficacy since variations in the efficacy of caspofungin *in vivo* according to the dosage schedule employed were observed in neutropenic rats with invasive pulmonary aspergillosis.

This work is the first study to analyse the susceptibility of this oomycete to inhibitors of β-glucans of the cell wall. It should be noted that we have only tested Brazilian isolates and more detailed studies using geographically and genetically diverse isolates of *P. insidiosum* will be required before making any general conclusions regarding the susceptibility of this oomycete to caspofungin.

**Funding**

Financial support was provided by CNPq (National Council for Scientific and Technological Development of Brazil). This manuscript is based on the thesis submitted by the first author in partial fulfilment of requirements for the obtainment of the degree of Doctor in Veterinary Science, PPGC/FAVET, UFRGS, RS, Brazil.

**Transparency declarations**

None to declare.

**References**

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