In vitro susceptibility of zoospores and hyphae of Pythium insidiosum to antifungals

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Objectives: The purpose of this study was to compare the in vitro susceptibilities of 22 Brazilian isolates of Pythium insidiosum to antifungals using a standardized inoculum of zoospores and a proposed novel inoculum prepared from cultured mycelia (hyphae) of P. insidiosum.

Methods: A zoospore suspension of P. insidiosum was obtained by the zoosporogenesis technique. The hyphal inoculum was prepared from a suspension of P. insidiosum mycelium. Susceptibility to each drug was evaluated using the CLSI M38-A2 method.

Results: Of the 88 MIC comparisons performed, 36 (41%) showed the same MIC value for the two inocula. The agreement (differences not greater than one dilution) between MICs obtained with both types of inocula was 39.8% (35/88). In other MIC comparisons analysed, 17 (19.3%) showed differences of two or three dilutions.

Conclusions: We conclude that the use of hyphal inocula of P. insidiosum for in vitro susceptibility tests could be a suitable method for evaluating antimicrobial susceptibility, particularly when it is not possible to obtain a standardized zoospore inoculum.

Keywords: oomycetes, pythiosis, azoles, terbinafine

Introduction

Members of the genus Pythium are fungal-like microorganisms in the kingdom Stramenopila. Although several species of this genus have been described, only Pythium insidiosum has the ability to cause disease in animals, including humans.1

Infections caused by P. insidiosum respond poorly to commonly used therapeutic methods, such as surgery, immunotherapy and antifungal drugs. Although these strategies are occasionally successful, therapeutic failure is more often observed.1 Thus, the growing number of studies evaluating the susceptibility of P. insidiosum to various antimicrobials in recent years is well warranted.1–7 In light of the fact that the zoospore is the infectious form of P. insidiosum, Pereira et al.3 utilized this form to prepare an inoculum for reproducible in vitro and in vivo studies. However, the preparation of the inoculum from zoospores has several disadvantages, which include the time required for obtaining zoospores (~6 days), a lack of zoosporogenesis in some situations and difficulties in obtaining the number of zoospores needed for experimental studies.9

Because P. insidiosum infections are characterized by the presence of sparsely septated hyphae within eosinophilic granulomatous lesions in the tissues of infected animals,8 we believe that using oomycete hyphae could serve as an alternative method of preparing inocula to be used for in vitro susceptibility studies.

The purpose of this study was to compare the in vitro susceptibilities of 22 Brazilian isolates of P. insidiosum to antifungals using standardized inocula of fungal zoospores and our proposed novel inocula prepared from cultured mycelia (hyphae) of P. insidiosum.

Materials and methods

Isolates of P. insidiosum

Twenty-one isolates of Brazilian P. insidiosum derived from horses (n = 19) and domestic dogs (n = 2) with pythiosis and one standard strain (CBS 101555) were used for these studies. The identities of the isolates were confirmed by PCR and sequencing.7

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Preparation of inocula

Zoospore inocula

Each zoospore inoculum suspension contained 20,000–30,000 zoospores/mL of *P. insidiosum* obtained by the zoosporogenesis method previously described and standardized by Pereira *et al.*

Mycelial inoculum culture (hyphae)

These inocula were prepared from suspensions of *P. insidiosum* mycelium. These suspensions were obtained by cultivation of the microorganism in yeast agar consisting of agar (20 g/1000 mL; Merck) and yeast extract (1 g/1000 mL; Difco) with incubation for 96 h at 37°C. The cultures were covered with 10 mL of sterile distilled water and the mycelium was scraped with a sterile scalpel blade. Subsequently, this solution was transferred to a test tube, the optical density was determined using a spectrophotometer and the inoculum was adjusted to a transmittance of 80%–85% at 530 nm. To test the viability of this suspension, an aliquot of 100 μL was added to 900 μL of Sabouraud broth and incubated at 37°C for 48 h. The homogeneity of the suspension was evaluated by microscopy. Then, 10 μL of the suspension was placed on a glass microscope slide and subsequently covered with a cover slip and analysed under a microscope (×20 objective).

Antifungals

The drugs tested included miconazole (Pharma Nostra, Rio de Janeiro, Rio de Janeiro, Brazil; 128–0.125 mg/L), ketoconazole (All Chemistry, São Paulo, São Paulo, Brazil; 128–0.125 mg/L), terbinafine (All Chemistry; 128–0.125 mg/L) and itraconazole (EMS Sigma Pharma São Bernardo do Campo, São Paulo, Brazil; 256–0.25 mg/L). The drugs were diluted in DMSO according to protocol M38-A2 of the CLSI.

In vitro tests

The MIC of each antifungal agent was determined by broth microdilution testing according to the CLSI M38-A2 method. After preparation, both types of inoculum were diluted 1:10 in RPMI 1640 glucose and buffered to pH 7.0 with 0.165 M MOPS. The plates were incubated at 37°C for 48 h.

The tests were read by visually observing the growth or lack of hyphae, and the MIC was taken to be the lowest concentration at which no hyphal growth occurred (100% inhibition). All tests were performed in triplicate.

Statistical analysis

MIC values for both inocula were submitted to a normality test regardless of the antifungal drug used. As the response variable did not show normality, data were subjected to the non-parametric χ² test and frequency distribution analysis. The analyses were performed using the SAS software package, version 8.2, assuming a 5% probability.

Results

The method used for preparing the hyphal inoculum in this study yielded highly purified, viable and homogeneous suspensions free of mycelial aggregates.

The in vitro susceptibilities of 22 isolates of *P. insidiosum* to individual antimicrobials are listed in Table 1. Of the 88 MIC comparisons performed, 36 (41%) showed the same MIC value for the two inocula. The agreement (differences not greater than one dilution) between MICs obtained with the two types of inocula

| Table 1. In vitro susceptibility of *P. insidiosum* (n = 22) to ketoconazole, miconazole, terbinafine and itraconazole determined using hyphae and zoospores as inocula |
|---|---|---|---|
| MIC (mg/L), number of isolates (%) | HZ | H | Z | H |
| Ketoconazole | 0 | 0 | 0 | 0 | 2 (9.1) |
| | 0 | 0 | 0 | 0 | 5 (22.7) |
| | 1 (4.5) | 7 (31.8) | 2 (9.1) | 0 |
| Miconazole | 0 | 0 | 0 | 0 | 6 (27.3) |
| | 0 | 0 | 4 (18.2) | 2 (9.1) |
| | 1 (4.5) | 6 (27.3) | 8 (36.4) | 4 |
| Itraconazole | 7 (31.8) | 7 (31.8) | 0 | 0 | 0 |
| Terbinafine | 0 | 0 | 1 (4.5) | 8 (36.4) | 7 (31.8) |
| | 0 | 0 | 13 (59.1) | 7 (31.8) | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 1 (4.5) | 0 | 0 |
| | 0 | 0 | 8 (36.4) | 16 | 16 |
| | 0 | 0 | 16 | 16 | 16 |
| | 0 | 0 | 16 | 16 | 16 |

H, hyphae; Z, zoospores. χ² minimal concentration to inhibit the growth of 50% of isolates. χ² minimal concentration to inhibit the growth of 90% of isolates.

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was 39.8% (35/88). In other MIC comparisons analysed, 17 (19.3%) showed differences of two or three dilutions (Figure 1).

The statistical analyses demonstrated that, regardless of the antifungal evaluated, the two inocula had similar MIC values \((P=0.17)\). The MIC frequency distribution was also similar for the two inocula \((P=0.45)\). Regardless of antifungal and inoculum, more than 60% of isolates of Pythium insidiosum had an MIC between 4 and 16 mg/L.

Discussion

Because the oomycete Pythium insidiosum is the causative agent of pythiosis, an emerging disease that is very difficult to treat, there has been an increase in the number of studies regarding the in vitro susceptibility of Pythium insidiosum in recent years.\(^2\)\(^-\)\(^7\)\(^,\)\(^11\)

Although the CLSI has not standardized susceptibility testing for this oomycete, Pereira et al.\(^3\) and Argenta et al.\(^4\) proposed the standardization of in vitro testing using Pythium insidiosum zoospores as the inoculum. However, Argenta et al.\(^4\) mention several disadvantages of this methodology, including the difficulty of obtaining the required amounts of zoospores and the fact that the zoospore is not a pathological form. Because of the difficulties and the time required to perform the tests using zoospores in vitro, the present study proposes the use of a hyphal inoculum of Pythium insidiosum.

The method using hyphal inoculum was less labour intensive and time consuming than the zoosporogenesis method proposed by Pereira et al.\(^3\) for testing in vitro susceptibility of Pythium insidiosum.

Among previous studies that evaluated the susceptibility of Pythium insidiosum, only Sekhon et al.\(^11\) used hyphal inocula to test eight oomycete isolates. Our results are comparable to those of Sekhon et al.\(^11\) in some respects because the MICs of itraconazole and ketoconazole were similar in both studies. However, the results for miconazole differed between the two studies. These differences may be due to the Sabouraud broth macrodilution (pH 6.3) method used by Sekhon et al.\(^11\) for the development of in vitro assays. In addition, they used a hyphal suspension adjusted to a transmittance of 90%, but in our preliminary tests such inoculum densities resulted in non-uniform growth (data not presented). When we performed susceptibility tests using the standard inoculum prepared with zoospores of Pythium insidiosum, the results were similar to those of studies by Cavalheiro et al.,\(^5\) which reported MIC ranges of 8–32 mg/L for terbinafine, 16–64 mg/L for ketoconazole and 4–32 mg/L for miconazole, and by Argenta et al.,\(^6\)\(^,\)\(^7\) which found MICs of 16 to >32 mg/L for itraconazole and 0.5–16 mg/L for terbinafine.

Nevertheless, when comparing the MICs obtained with the two inocula tested, it was shown that although 80.7% of MIC comparisons performed had the same MIC value or differed by one dilution, 19.3% of MIC comparisons had a value that differed by two or three dilutions for the two types of inoculum. These findings agree with other studies that reported differences in MIC values when comparing inocula based on hyphae and zoospores in antifungal susceptibility testing of Aspergillus, Cladosporium, Cladophialaphora, Paecilomyces, Fusarium and dermatophytes.\(^12\)\(^-\)\(^20\) In some of these studies it was shown that hyphae were more susceptible than conidia.\(^13\)\(^,\)\(^14\)\(^,\)\(^16\)\(^-\)\(^20\) However, in other studies the susceptibilities of conidia and hyphae were similar,\(^15\)\(^,\)\(^18\) and in yet others conidia were more susceptible.\(^12\)\(^,\)\(^19\)

Our results show that it is feasible to test the in vitro susceptibility of Pythium insidiosum using as an inoculum a suspension of hyphae prepared from a mycelial culture of Pythium insidiosum. Furthermore, considering that the hyphae of this microorganism colonize cutaneous and subcutaneous tissues, produce intestinal lesions, invade blood vessels and proliferate within bone,\(^8\) the development of susceptibility tests using hyphae could better mimic the characteristics of Pythium insidiosum in infected tissue and could better demonstrate the therapeutic potential of drugs against pythiosis.

We conclude that the use of hyphal inocula of Pythium insidiosum for the development of in vitro susceptibility tests could be a suitable method for evaluating antimicrobial susceptibility, particularly when obtaining standardized inocula of zoospores is not possible.

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Transparency declarations

Conflicts of interest: none to declare.

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