mycoses
Diagnosis, Therapy and Prophylaxis of Fungal Diseases

Original article

Antifungal susceptibility profile *in vitro* of *Sporothrix schenckii* in two growth phases and by two methods: microdilution and E-test

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Summary

The susceptibility profile of 91 *Sporothrix schenckii* isolates in both growth phases was determined by microdilution test (Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing; AFST-EUCAST). Amphotericin B (AMB), itraconazole (ITC), posaconazole, ravuconazole and terbinafine were found active *in vitro* against both phases but minimum inhibitory concentrations values for mycelial phase were significantly higher. Fluconazole (FLC) and voriconazole (VRC) were inactive *in vitro* against both phases. The E-test technique was also performed with 41 representative isolates for AMB, FLC, ITC and VRC. Average agreement rates between yeast phase microdilution results and E-test results were high for AMB (77.5%) and FLC (87.8%), but low for ITC and VRC with rates of 56.4% and 54.5%, respectively. AFST-EUCAST is not the most recommended test to perform drug susceptibility testing of *S. schenckii* in clinical laboratories, and E-test could be an alternative methodology for this purpose, mainly when the activity *in vitro* of antifungal agents of AMB and FLC are evaluated.

Key words: E-test, growth phases, amphotericin B, itraconazole.

Introduction

Sporotrichosis is a subacute or chronic subcutaneous infection caused by the dimorphic fungus *Sporothrix schenckii*. Itraconazole (ITC) is used in the treatment of sporotrichosis, while amphotericin B (AMB) may be indicated for extensive involvement of the disease.[1]

Most studies to determine the susceptibility profile of *S. schenckii* to antifungal agents have used the mycelial form because colonies grow easily within a few days to 2 weeks at 25–28 °C.[2,3] However, which growth phase should be used in those methodologies is still an open question for dimorphic fungi. Based on the biological cycle of this class of fungi where filamentous phase is found in the environment, and the infective yeast phase into the host, it has been suggested, therefore, that susceptibility testing for those species such as *Histoplasma capsulatum* should be performed with the yeast forms.[4]

Broth dilution methods seem to be the reference methodologies for those species,[2,3,5] but the yeast phase is difficult to test in this format. The E-test appears to be a viable and practical alternative to the reference methods for susceptibility testing of fastidious or slow growing fungus like the yeast phase of *Sporothrix*. However, the E-test technique has been hardly ever evaluated to test the susceptibility profile of those species.

This is the largest study reported describing the antifungal susceptibilities *in vitro* of *S. schenckii* in both phases and comparing results obtained by microdilution reference methodologies with those achieved by the commercial E-test technique.

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Accepted for publication 15 January 2009

  doi:10.1111/j.1439-0507.2009.01701.x

Materials and methods

Isolates

A total of 91 clinical isolates of *S. schenckii* were included in this study. Organisms were obtained from the collection of the Mycology Laboratory of Instituto de Pesquisa Clínica Evandro Chagas (FIOCRUZ) and from the Mycelial Collection of the Spanish National Center for Microbiology. Isolates were retrieved from storage in distilled water at a controlled temperature of 4 °C, subcultured on Potato dextrose agar (PDA) plates and incubated at 25 °C until adequate growth was obtained. All these isolates were identified as *S. schenckii* by typical colony morphology on PDA and microscopical appearance (septate hyaline hyphae, conidiophores and typical conidia). For the conversion to the yeast form, mycelial cultures grown on PDA were subcultured on brain–heart infusion agar (BHI) at 35 °C, and microscopical appearance of growth on culture was observed as oval to cigar-shaped yeast cells.

Antifungal susceptibility testing

Microdilution test

The susceptibility testing was performed on the 91 isolates in both yeast and mould forms following the recommendations of the European Subcommittee for Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing (AFST-EUCAST) with minor modifications.[6] Briefly, susceptibility testing included RPMI medium supplemented with 2% glucose as the assay medium and an inoculum size of 1×10^5–5×10^5 CFU ml^{-1}. The mycelial inoculum was adjusted by microscopical enumeration with a cell-counting haematocytometer. The yeast inoculum was adjusted by a spectrophotometer at 530 nm and turbidity was measured and adjusted to match a 0.5 Mc Farland density. The microplates were incubated for 72 h (the exponential phases of *S. schenckii*) at 30 °C for mycelial phase and at 35 °C for yeast phase. Mycelial phase was tested in static incubation the preferential condition for good growth of fungi. However, yeast phase was incubated in a shaker because the aeration improve the growth of *S. schenckii*. Microplates were wrapped with a film sealer to prevent the medium from evaporating and agitated at 16.4 g.

Visual readings were performed with the help of a mirror. Minimum inhibitory concentrations (MIC) were defined as the lowest concentration showing complete inhibition of growth. Susceptibility test was performed in duplicate on two different days. The antifungal agents used were AMB (Sigma-Aldrich Quimica SA, Madrid, Spain), fluconazole (FLC; Pfizer SA, Madrid, Spain), ITC (Janssen Pharmaceutica, Madrid, Spain), voriconazole (VRC; Pfizer, Ltd, Sandwich, UK), ravuconazole (Bristol-Myers Squibb, Princeton, NJ, USA), posaconazole (Schering-Plough, Kenilworth, NJ, USA) and terbinafine (TRB; Novartis, Basel, Switzerland). *Aspergillus fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were included as control isolates for the mycelial phase and *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 for the yeast phase.

E-test for yeast phase

A total of 41 representative isolates converted to the yeast phase were tested. Mould phase were converted by culturing isolates on BHI agar (0.77% calf brain, 0.98% beef heart; Difco, Oxoid, Madrid, Spain) at 37 °C. E-test strips with AMB, FLC, ITC and VRC were obtained from AB-Biodisk (Stockolmon, Sweden). The E-test was also performed on BHI agar and in accordance with the manufacturer's instructions.[7] The MIC was read after 72 h of incubation at 35 °C. Because of the continuous gradient antifungal agents, the MIC endpoint was elevated to the next twofold dilutions concentration, which matched the dilution schema of the microdilution technique. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as control strains.

Analysis of results

The reproducibility between MIC values obtained against both phases and between microdilution and E-test results were calculated by determining the percentage of agreement between MIC values. Agreement was defined as discrepancies in MIC results of no more than twofold dilutions. In addition, the correlation between the results was evaluated by using the intraclass correlation coefficient (ICC), which was expressed to a maximum value of 1 and with a confidence interval of 95%. The ICC is a reverse measurement of the variability of the counting values. To approximate a normal distribution, the MICs were transformed to $\log_2$ values. A $P < 0.01$ was considered with statistical significance. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS version 15.0; SPSS S.L, Madrid, Spain).

Results

Overall, the antifungal agents tested for microdilution reference method (AFST-EUCAST) were active *in vitro*

Table 1 Susceptibility testing results in μg ml^{-1} of *Sporothrix schenckii* isolates according to methods of susceptibility testing and their form.

	AMB	FLC	ITC	VRC	RVC	POS	TRB
Method/form (no tested)	GM (Range)	GM (Range)	GM (Range)	GM (Range)	GM (Range)	GM (Range)	GM (Range)
Microdilution/Mycelial form (91)	0.91 (0.12 to 4.0)	>64.0 (>64.0)	0.78 (0.12 to >8.0)	12.19 (1.0 to >8.0)	2.05 (0.12 to >8.0)	0.71 (0.06 to >8.0)	0.18 (0.01 to >16.0)
Microdilution/Yeast form (91)	0.43 (0.03 to 4.0)	>64.0 (16.0 to >64.0)	0.65 (0.03 to >8.0)	2.69 (0.06 to >8.0)	1.08 (0.01 to >8.0)	0.39 (0.03 to >8.0)	0.29 (0.03 to >16.0)
E-test/Yeast form (41)	0.51 (0.02 to 2.0)	>64.0 (4.0 to >64.0)	0.31 (0.03 to 4.0)	0.84 (0.01 to 4.0)	–	–	–

AMB, amphotericin B; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; RVC, ravuconazole; POS, posaconazole, TRB, terbinafine; GM, geometric mean; –, not done.

Table 2 Per cent agreement of microdilution MIC results of yeast with mycelial phases within 2+ dilutions.

Antifungal agents	Isolates	% Agreement
Amphotericin B	89	88.7.6
Fluconazole	82	87.8
Itraconazole	87	88.5
Voriconazole	84	47.6.2
Ravuconazole	86	82.5
Posaconazole	81	76.5
Terbinafine	87	88.5

against *S. schenckii* (Table 1). AMB and TRB were the most active compounds *in vitro* and only FCL were inactive for the both phases. VRC also was inactive against a significant rate of antifungal agents. When MIC values obtained by microdilution were compared per phase, higher values were found against the mycelial phase for all antifungal agents tested apart from TRB that was more active *in vitro* against the mycelial than against the yeast form. Difference in MIC values between phases was up to two or three twofold dilutions and thus, agreements were high (>85%) and ICCs were statistical significant for antifungal compounds with the exception of VRC. Table 2 shows the per cent agreement rates between the MICs of the two phase within +2 dilutions.

The correlation between microdilution yeast phase and E-test results is given in Table 3. Average agreements rates were high for AMB (77.5%) and FLC (87.8%), but low for ITC and VRC with rates of 56.4% and 54.5% respectively. MIC values of both ITC and VRC by E-test were invariably lower than those obtained by microdilution (Table 1 and Table 3). The ICCs between results by microdilution and E-test MIC values were significant for AMB, FLC and ITC. Minimum inhibitory concentrations values of quality control strains were in the expected quality control ranges.

Discussion

The results of this study conform to those of others showing the good susceptibility of *S. schenckii* to most of the antifungal agents apart form FLC and VRC.[2,3,5,8–10] In general, higher MIC values were found against the mycelial phase for all antifungal agents as demonstrated in other studies.[8–10] A better understanding of what leads to the difference between the yeast and mycelial antifungal susceptibility of *S. schenckii*, and also which of them better reflects the therapeutic response should

Table 3 Correlation coefficients between results obtained by microdilution for both phases of *Sporothrix schenckii* and the E-test technique classified per antifungal agent.

Antifungal agents	Microdilution yeast phase/microdilution mycelial phase ICC (95% CI)	Microdilution yeast phase/E-test ICC (95% CI)	Microdilution mycelial phase/E-test ICC (95% CI)
Amphotericin B	0.917* (0.873 to 0.945)	0.711* (0.453 to 0.847)	0.853* (0.722 to 0.922)
Fluconazole	0.612* (0.249 to 0.839)	0.623* (0.504 to 0.856)	0.601* (0.509 to 0.824)
Itraconazole	0.724* (0.577 to 0.819)	0.710* (0.448 to 0.848)	0.688* (0.406 to 0.837)
Voriconazole	0.074 (–0.428 to 0.399)	0.410 (0.126 to 0.554)	0.259 (–0.467 to 0.626)
Ravuconazole	0.652* (0.468 to 0.773)	–	–
Posaconazole	0.534* (0.275 to 0.700)	–	–
Terbinafine	0.668* (0.186 to 0.652)	–	–

ICC, intraclass correlation coefficient; 95% CI, confidence interval of 95%; –, not performed.
*Values with statistical significance, $P < 0.01$.

be investigated. However, in this study, only VRC presented different results between both phases if we look at ranges and geometric means. Kohler *et al.* [10] suggested that one of the possibilities for the higher MIC values of mycelial phase could be because of inoculum size. However, a same size of the inoculum (10^5) was utilised by us for both phases. Some other factors such as continuous shaking of *S. schenckii* yeast phase during 3 days because of the slow growth of fungi may have favoured media evaporation and this small change in the initial conditions could interfere in our results.

Nowadays, it is not possible to determine the isolates, which are resistant or susceptible to the drugs tested because the MIC breakpoints have not been well established yet for *S. schenckii.* However, 'susceptible' isolate with low MIC and 'resistant' strain with relatively high MIC of determined antifungal were observed in this study. The correlation between methods to classify the strains as 'susceptible' was very good for AMB. Also, the E-test was able to detect 'resistance' to FLC, as described in the microdilution testing as also previously reported elsewhere.[2,8,9] However, the use of the E-test to study the activity of ITC and VRC was not good and needs further studies. It should be noted that BHI was needed to do susceptibility testing in solid format with the yeast phase of *S. schenckii* and that assay medium is not completely defined in the E-test what could have significant influence on our results.

In summary, our results suggest that the conditions of growth used in the antifungal susceptibility testing for *S. schenckii* can influence the MIC. Mycelial phase, the non-parasitic form, showed higher MIC in *vitro* than the yeast phase. Susceptibility testing with *S. schenckii* by EUCAST method in the mycelial form is laborious and reproducible; in the yeast form, is hardly affordable for clinical laboratories because of the slow growth of the fungus. On the other hand, the E-test of *S. schenckii* in the yeast form could be an alternative method to determine the susceptibility of those organisms to AMB and FLC.

Acknowledgments

We express our gratitude to Monica Vasquez, Laura Jimenez and Rodrigo de Almeida Paes for the technical support. Part of financial support for this work was provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Proc. 200953/2004-4).

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