

Comparison between two culture media for *in vitro* evaluation of antifungal susceptibility of the *Sporothrix schenckii* complex*

Comparação entre dois meios de cultura para avaliação *in vitro* da suscetibilidade a antifúngicos do complexo *Sporothrix schenckii*

Cheila Denise Ottonelli Stopiglia¹

Daiane Heidrich³

Fabiane Jamono Vieira⁴

Daiane Péres Marchese²

Julia Medeiros Sorrentino⁴

Maria Lúcia Scroferneker⁵

Abstract: BACKGROUND: The standard methodology for determining the antifungal sensitivity against the *Sporothrix schenckii* complex recommends the use of the 1640 Roswell Park Memorial Institute culture medium (RPMI) buffered with morpholinepropanolsulfonic acid (MOPS). However, while this is a high-cost medium which requires a laborious implementation and sterilization by filtration, the Sabouraud dextrose broth is a low-cost medium, widely used in mycology, sterilized by autoclave.

OBJECTIVE: To evaluate the performance of the Sabouraud dextrose broth culture medium as a substitute for the RPMI 1640-MOPS in determining the antifungal sensitivity of *S. schenckii*.

METHODS: Forty-eight clinical isolates were evaluated against five antifungal agents: itraconazole, ketoconazole, fluconazole, amphotericin B and terbinafine, using the method of broth microdilution advocated by the M38-A2 protocol of the Clinical and Laboratory Standards Institute.

RESULTS: There were no significant differences between the Minimum Inhibitory Concentrations obtained in the two culture media for all the antifungals, with the exception of the amphotericin B. Regarding this drug, the Minimum Inhibitory Concentration range obtained were wider for the Sabouraud dextrose broth than for the Roswell Park Memorial Institute - morpholinepropanolsulfonic acid.

CONCLUSIONS: The Sabouraud dextrose broth showed potential to be used in the *in vitro* evaluation of the *S. schenckii* complex antifungal activity.

Keywords: Antifungal agents; Microbial sensitivity tests; Mycoses; *Sporothrix*; Sporotrichosis

RESUMO: FUNDAMENTOS: A metodologia padronizada para a determinação da sensibilidade aos antifúngicos frente ao complexo *Sporothrix schenckii* preconiza a utilização do meio de cultura *Roswell Park Memorial Institute* (RPMI) 1640 tamponado com ácido morfolinopropanosulfônico (MOPS). No entanto, este meio possui custo elevado, execução trabalhosa e esterilização por filtração. Já o caldo Sabouraud-dextrose é amplamente utilizado em micologia, de baixo custo e pode ser esterilizado por autoclavagem.

OBJETIVO: Avaliar o desempenho do meio de cultura caldo Sabouraud-dextrose em substituição ao RPMI 1640-MOPS na determinação da sensibilidade de *S. schenckii* a antifúngicos.

MÉTODO: Foram avaliados 48 isolados clínicos frente a cinco antifúngicos: itraconazol, cetoconazol, fluconazol, anfotericina B e terbinafina, utilizando a metodologia da microdiluição em caldo preconizada pelo protocolo M38-A2 do *Clinical and Laboratory Standards Institute*.

RESULTADOS: Não houve diferenças significativas nas Concentrações Inibitórias Mínimas obtidas nos dois meios de cultura para todos os antifúngicos, com exceção da anfotericina B. Para este fármaco, foram obtidas faixas mais amplas de Concentrações Inibitórias Mínimas para caldo Sabouraud-dextrose do que para Roswell Park Memorial Institute-morfolinopropanosulfônico.

CONCLUSÕES: O caldo Sabouraud-dextrose mostrou potencial para ser utilizado na avaliação *in vitro* da atividade antifúngica do complexo *S. schenckii*.

Palavras-chave: Antimicóticos; Esporotricose; Micoses; *Sporothrix*; Testes de sensibilidade microbiana

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¹ Pharmacist-Biochemist – PhD Candidate at the Post-Graduation Program in Medicine, Medical Sciences of the Universidade Federal do Rio Grande do Sul (UFRGS) – Porto Alegre (RS), Brazil.

² Pharmacist-Biochemist by the Universidade Federal do Rio Grande do Sul (UFRGS) – Porto Alegre (RS), Brazil.

³ Pharmacist – Masters Candidate by the Post-Graduation Program in Medicine, Medical Sciences of the Universidade Federal do Rio Grande do Sul (UFRGS) – Porto Alegre (RS), Brazil.

⁴ Student at the Pharmacy School of the Universidade Federal do Rio Grande do Sul (UFRGS) – Porto Alegre (RS), Brazil.

⁵ Post-Doc - Associate Professor of the Microbiology, Immunology and Parasitology Department of the Universidade Federal do Rio Grande do Sul (UFRGS) – Porto Alegre (RS), Brazil.

INTRODUCTION

Sporotrichosis is a subacute or chronic infection caused by dimorphic fungi of the genus *Sporothrix*. Nowadays, *Sporothrix schenckii* is considered as a complex of species composed of *S. brasiliensis*, *S. mexicana*, *S. globosa*, *S. schenckii stricto sensu* and *S. schenckii* var. *luriei*.¹ Sporotrichosis predominates in tropical and temperate zones and Brazil is one of main endemic areas.² An epidemic of sporotrichosis was reported in Rio de Janeiro in which cases of human infection were related to exposure to cats.³

The most frequently observed clinical forms of sporotrichosis are the nodular-lymphangitic and the fixed ones, which usually affect immunocompetent individuals, unlike the disseminated cutaneous forms with or without visceral involvement, and the osteoarticular and the pulmonary forms, which are less frequent and affect patients with changes in their adaptive immunity mediated by cells such as in acquired immune deficiency syndrome (AIDS), lymphoma, cirrhosis, etc.⁴

Systemic chemotherapy with potassium iodide or sodium iodide is the treatment of choice for the cutaneous lesions of sporotrichosis.⁵ Amphotericin B has been used with different therapeutic results in the disseminated cutaneous, the recurrent lymphocutaneous and in the extracutaneous forms.^{4,5} However, the frequency of intolerance to iodine and the high toxicity of amphotericin B may often represent limiting factors to their use. In the last few decades, there has been an increase in the use of azole derivatives such as ketoconazole, itraconazole and fluconazole as a therapeutic alternative to the classic schemes, and itraconazole came as the most effective of them.⁶ On the other hand, by virtue of its excellent *in vitro* and *in vivo* activity, terbinafine has been used in various fungal infections.^{7,8,9}

With the increase in the incidence of systemic fungal infections and with the growing number of antifungal agents, the interest in laboratory methods to guide the selection of antifungal therapy has also increased. In 2002, the M38-A norm of the Clinical and Laboratory Standards Institute (CLSI), a reference method for *in vitro* tests of broth microdilution for determination of the antifungal sensitivity of filamentous fungi, including *S. schenckii*.¹⁰

This methodology recommends the use of the 1640 Roswell Park Memorial Institute (RPMI) culture medium buffered with morpholinepropanesulfonic acid (MOPS). However, while this is a high-cost medium which requires a laborious implementation, the use of buffer and sterilization by filtration, the Sabouraud dextrose broth is a low-cost medium, widely used in mycology, sterilized by autoclave. In

addition, the RPMI broth has been described as an obstacle for the detection of isolates resistant to amphotericin B.¹¹

From the evidence above, the objective of this study was to evaluate the performance of the Sabouraud dextrose broth culture medium as a substitute for the 1640-RPMI culture medium buffered with MOPS to evaluation of the *in vitro* antifungal susceptibility of *Sporothrix schenckii* complex against the antifungals, with the purpose of cost reduction of the *in vitro* test, so that helpfully it can be widely used in clinical practices.

MATERIALS AND METHODS

Microorganisms

Forty-eight clinical isolates of *Sporothrix schenckii* complex were used, namely 13 *S. brasiliensis*, 22 *S. schenckii* and 13 *S. mexicana*.

Antifungal activity *in vitro*

The antifungal sensitivity test was developed according to the technique of broth microdilution advocated by the M38-A2 protocol of the Clinical and Laboratory Standards Institute.¹² Five antifungal drugs commercially available for the treatment of sporotrichosis were used: ketoconazole (Pharmaceutical Chemistry, Bayer, Barcelona, Spain), fluconazole (Sigma, St. Louis, MO, USA), itraconazole (Janssen-Cilag, São Paulo, Brazil), terbinafine (Novartis Research Institute, Vienna, Austria) and amphotericin B (Sigma-Aldrich Co. St. Louis, USA).

The culture media used were the Sabouraud dextrose broth (Difco Laboratories, USA) and the 1640-RPMI (Gibco-BRL, USA) buffered at pH 7.0 with 165mM of morpholinepropanesulfonic acid - MOPS (Sigma-Aldrich, Spain).

A stock solution of the antifungal agents was prepared in dimethyl sulfoxide (DMSO; Vetec, Brazil) and, subsequently, dilutions in both culture media were held to obtain final concentrations ranges of 0.25 to 128µg/ml for fluconazole, of 0.03 to 16µg/ml for itraconazole, ketoconazole, and amphotericin B and of 0.01 to 8 µg/ml for terbinafine.

The clinical isolates were cultured in potato dextrose agar (Biobrás S.A., Brazil) at 35°C for 7 days. After the growth, the spores were suspended in a 0.85% sterile saline solution, obtaining optical density from 0.09 to 0.13, standardized in the spectrophotometer at 530 nm. The suspension was diluted 1:50 on each of the culture media.

The test was carried out on sterile plates with 96 U-wells, where 100 µl of each concentration of the antifungal agent to be tested were added. Subsequently, aliquots of 100 µl of the inoculums at

1:50 dilution were inoculated into each of the wells. The final concentration of microorganisms reached 10^4 CFU/ml.

A free control of antifungal drugs (growth control) and a free control of microorganism (sterility control) were included in those tests. The plates were incubated at 35 °C, for 46-50 hours. The determination of the minimum inhibitory concentration (MIC) was performed visually by comparison with the growth of the drug free control. The MIC was defined as the lowest concentration of the drug able to inhibit the fungal growth completely for itraconazole and amphotericin B, 80% of the fungal growth for terbinafine and 50% of the growth for fluconazole and ketoconazole.¹² All analyzes were performed in triplicate.

The statistical analysis was assessed by the t Test from the SPSS program for Windows, version 17.0.

RESULTS

The resulting values of the *in vitro* susceptibility for the 48 isolates of *S. schenckii* complex are shown in table 1. The statistical analysis showed that the MICs obtained for amphotericin B were statistically different for the two utilized culture media ($p < 0.05$). However, for the other antifungal agents, there were no significant differences between the MICs obtained in the two culture media.

Terbinafine was the most active of the drugs, followed by ketoconazole, itraconazole, amphotericin B and fluconazole.

DISCUSSION

The increase in the incidence of opportunistic fungal infections, as well as the use of invasive surgical procedures, and even the appearance of new diseases and immunosuppressive drugs, have aroused interest in the search of new alternatives to drugs for the treatment of these infections and, also, alternatives to methodologies for the analysis of the antifungal sensitivity.¹³

Methods for the analysis of antifungal activity amongst laboratories, in order to determine the sensitivity of the antifungal agents, were recommended. The Clinical and Laboratory Standards Institute standardized the method of broth microdilution with the aim of analyzing the antifungal susceptibility of filamentous fungi in a consensual way to facilitate the interpretation of the results by the laboratories. In addition, this methodology provides a basic pattern from which other methods may be developed.¹⁰

The effect of the culture media upon the antifungal activity *in vitro* for filamentous fungi has shown contradictory results.¹⁴⁻¹⁶ For example, using the method of microdilution to evaluate caspofungin against the species of *Aspergillus*, *Fusarium*, Arikian *et al.* (2000)¹⁴ obtained lower MICs with the Antibiotic Medium 3 (AM3) culture medium than with the RPMI. In the same way, Llop *et al.* (1999)¹⁷ observed that the five culture media evaluated had influenced the MIC values of amphotericin B, ketoconazole and flucytosine for dematiaceos fungi. However, Tortorano *et al.*

TABLE 1: Comparison of the susceptibility *in vitro* of 48 isolates of the *S. schenckii* complex against five antifungal agents using two culture media

	Itraconazole		Ketoconazole		Fluconazole		Terbinafine		Amphotericin B	
	SDB*	RPMI†	SDB*	RPMI†	SDB*	RPMI†	SDB*	RPMI†	SDB*	RPMI†
<i>S. brasiliensis</i> (n=13)										
Range	0.03-2.00	0.06-2.00	0.06-0.5	0.12-0.50	32->128	32->128	0.01-0.25	0.01-0.25	0.50-8.00	0.50-2.00
GM**	0.28	0.33	0.19	0.22	71.20	71.20	0.07	0.07	1.79	1.00
<i>S. schenckii</i> (n=22)										
Range	0.06-2.00	0.06-2.00	0.03-1.00	0.03-1.00	32->128	32->128	0.01-0.50	0.01-0.50	0.25-16.00	0.50-2.00
GM**	0.32	0.32	0.18	0.17	60.09	62.01	0.06	0.07	2.41	1.24
<i>S. mexicana</i> (n=13)										
Range	0.03-0.50	0.03-0.50	0.03-1.00	0.03-1.00	32->128	32->128	0.01-0.50	0.01-0.50	0.01-8.00	0.50-4.00
GM**	0.15	0.17	0.08	0.09	67.50	71.20	0.05	0.06	1.44	1.45
All strains (n=48)										
Range	0.03-2.00	0.03-2.00	0.03-1.00	0.03-1.00	32->128	32->128	0.01-0.50	0.01-0.50	0.01-16	0.50-4.00
GM**	0.25	0.27	0.15	0.15	64.93	66.83	0.06	0.07	1.94	1.22

*SDB: Sabouraud-dextrose broth; †RPMI: Roswell Park Memorial Institute broth buffered with 165mM of morpholinepropanesulfonic acid (MOPS).

** Geometric mean

(2000)¹⁸ and Manavathu *et al.* (2000)¹⁹, did not observe differences between AM3 and RPMI when comparing them to evaluate amphotericin B and itraconazole against *Aspergillus fumigatus*. The only exception was for amphotericin B in peptone-yeast extract, which gave higher MICs than those obtained with RPMI and AM3. Fernández-Torres *et al.* (2006), demonstrated that the differences in MICs are influenced by the culture medium used and the microorganism analyzed.²⁰

In addition, the RPMI broth has been described as an obstacle for the detection of isolates resistant to amphotericin B.¹¹ However, failures to therapies with amphotericin B continue to be reported, with isolates showing, mostly, *in vitro* sensitivity to this drug.^{21,22} The inadequacy of RPMI determined that in the document M27-A2 the CLSI suggested the evaluation of susceptibility using other means of culture such as the AM3.²³

Boff *et al.* (2008) showed that the AM3 media allowed better differentiation of susceptibility among groups of *Candida*.¹¹ The capacity of this media to generate wide ranges of MICs by the RPMI broth has been observed, but as it is a complex medium, without a completely known chemical composition, there are problems of reproducibility of the results when the same samples are tested with different batches of AM3.²⁰

Our results show that the maximum and minimum values, as well as the geometric mean (GM) of MICs were equal to or very similar between the two culture media for all the antifungal agents evaluated, with the exception of amphotericin B. For this, the range of MICs obtained for the Sabouraud dextrose broth (0.01 - 16 µg/ml) was wider than for the RPMI-MOPS (0.50 - 4 µg/ml), and the GM for the Sabouraud dextrose broth was superior to the GM for the RPMI-MOPS.

The study conducted by Espinel -Ingroff (1997) suggests that the 1640 RPMI medium does not offer the necessary conditions for the proper growth of *C. neoformans*, thus making the detection of resistance to amphotericin B difficult.²⁴

Throughout this work, terbinafine showed high activity against all isolates tested, which is in agreement with other studies.^{1,25} However, the therapeutic potential of terbinafine was confirmed only for the cutaneous and lymphocutaneous sporotrichosis.^{9,26} On the other hand, this drug did not demonstrate efficacy in the treatment of systemic sporotrichosis in murine model.²⁷ Studies *in vivo* are necessary to confirm this activity.

Ketoconazole was the second more active drug. However, the detection of *in vitro* susceptibility to this antifungal agent should be interpreted with caution because it does not often correlate with the therapeutic activity, mainly for the extracutaneous forms.²⁸

Despite the absence of comparative clinical studies (randomized, double-blind), itraconazole is considered the first choice in the treatment of cutaneous, lymphocutaneous and osteoarticular sporotrichosis.²⁸ Our results showed a good *in vitro* performance of this drug, which can be correlated with the therapeutic success of the treatment obtained in 94.6% of the cases of lymphocutaneous and fixed sporotrichosis reported in another study.²⁹

For fluconazole, the values are compatible with the results from other studies.^{1,9} In spite of its low sensitivity profile, fluconazole is considered a possible therapeutic option in cases of lymphocutaneous or cutaneous sporotrichosis, when high doses should be used.³⁰

CONCLUSION

The results obtained indicate that the Sabouraud dextrose broth has the potential to be used in the assessment of the antifungal activity of the *Sporothrix schenckii* complex using the CLSI method of broth microdilution, since the results were very similar to the RPMI-MOPS results for all tested antifungal agents, with the exception of amphotericin B. For this drug, the MICs range obtained was wider for the Sabouraud dextrose broth than for the RPMI-MOPS. However, *in vivo* studies are necessary to assess the clinical correlation between these results, as well as the reproducibility with different batches of culture media. □

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MAILING ADDRESS:

Cbeila D. O. Stopiglia
Rua Sarmiento Leite, 500 - laboratório 210
90050-170 Porto Alegre, RS
E-mail: cbeila.dos@gmail.com / scrofern@ufrgs.br

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