In vitro antifungal drug susceptibilities of dermatophytes microconidia and arthroconidia

Luciene M. Coelho¹, Roseli Aquino-Ferreira¹, Cláudia M. Leite Maffei² and Nilce M. Martinez-Rossi¹*

¹Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900 Ribeirão Preto, São Paulo, Brazil; ²Departamento de Biologia Molecular, Celular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900 Ribeirão Preto, São Paulo, Brazil

Received 11 March 2008; returned 11 April 2008; revised 19 May 2008; accepted 27 May 2008

Objectives: Arthroconidia have been considered as the primary cause of infection by dermatophytes. However, the in vitro antifungal testing evaluates the responses mainly of microconidia or hyphae, and dermatophytes in vivo often produce arthroconidia, a cellular structure presumably more resistant to antifungals. The aim of this study was to compare the in vitro susceptibility of microconidia and arthroconidia of Trichophyton rubrum, Trichophyton tonsurans and Trichophyton equinum to griseofulvin, itraconazole, terbinafine, fluconazole, amphotericin B and hygromycin B.

Methods: Microconidia and arthroconidia were produced in vitro, and their susceptibility to each drug was evaluated by assessing the CLSI M38-A broth microdilution method.

Results: Arthroconidia of all strains analysed appeared to be more resistant to fluconazole, griseofulvin and itraconazole than microconidia. The MIC of terbinafine was the same for microconidia and arthroconidia for all strains, and the MIC of amphotericin B for microconidia and arthroconidia was the same for isolates of T. equinum and T. tonsurans, but differed for T. rubrum. Finally, the level of resistance of microconidia for all strains towards the antibiotic hygromycin B was from 25 to 400 mg/L.

Conclusions: The difference in the susceptibility between microconidia and arthroconidia depends on the drug and on the strain, and may be one of the causes of therapeutic failure. Also, the level of resistance to the antibiotic hygromycin B presented by microconidia of these isolates will allow the use of hygromycin resistance as a dominant marker in fungal transformation procedures in future studies of gene function.

Keywords: antifungal susceptibility, dermatophytoses, MICs, Trichophyton

Introduction

Dermatophytes are pathogenic fungi that have a high affinity for keratinized structures like nails, skin or hair, causing superficial infections known as dermatophytoses in both humans and animals. They belong to the genera Trichophyton, Microsporum and Epidermophyton, and based on their natural habitats, they are classified into three different groups: geophilic, zoophilic and anthropophilic species.¹ These fungi annually affect millions of individuals establishing an important public health problem because of prolonged treatment of the disease and its refractoriness to therapy.² Some forms of dermatomycoses are subjects of concern, particularly in patients undergoing organ transplants, suffering from AIDS and receiving anticancer chemotherapy. Tinea unguium (onychomycosis) is, perhaps, the most refractory to treatment and represents most of the cases of dermatophytoses, affecting 20% of the world population over 40 years of age.³ Some reasons for failure of dermatophytosis treatment in clinical practice are peripheral vascular disease, resistant structures such as subungual dermatophytomas and the presence of dormant fungal spores (arthroconidia).³,⁴ In many filamentous fungi, the formation of microconidia and arthrospores occurs regularly during the asexual cycle and can be distinguished due to their morphology and development. Microconidia appear...
from the ends of conidiophores, which are formed laterally from hyphae, whereas arthrospores are formed by the fragmentation of hyphae during prolonged cultivation.\(^5\) Arthroconidia are defined as a genetically programmed disarticulation of septate hyphae\(^6\) forming both short-chained or spherical or oval single arthrospores\(^1\) and is considered the primary cause of infections in the host.\(^8\) They are metabolically active cells enriched with lipid-containing vacuoles and intracellular organelles, being resistant to some antifungal agents and adverse environmental conditions.\(^4\)

The aim of this study was to determine the \textit{in vitro} susceptibility of microconidia and arthroconidia of \textit{Trichophyton rubrum}, \textit{Trichophyton tonsurans} and \textit{Trichophyton equinum} isolates to griseofulvin, itraconazole, terbinafine, fluconazole, amphotericin B and hygromycin B. Considering that these three species of dermatophytes are included in the sequencing project by the Fungal Genome Initiative (The Broad Institute of Harvard and MIT/National Institute of Health), it will be very useful to: (i) standardize the condition to produce \textit{in vitro} arthroconidia in these species; (ii) compare the susceptibility of isolates of these three species to antifungal drugs used in clinical practice; (iii) compare the susceptibility of microconidia versus arthroconidia; and (iv) verify the susceptibility of these isolates to hygromycin B. This information is relevant because hygromycin resistance is a dominant marker used in fungal transformation.

\section*{Materials and methods}

\subsection*{Fungal strains}

The strains of dermatophytes used in this study were obtained from the culture collection of the CBS Fungal Biodiversity Centre (Utrecht, The Netherlands): \textit{T. rubrum} CBS118892; \textit{T. equinum} CBS12797 and \textit{T. tonsurans} CBS112818. The genomes of these strains are in the process of sequencing. The strain H6 of \textit{T. rubrum} (ATCC MYA-3108), obtained from the clinical routine of University Hospital of Ribeirão Preto, SP, Brazil,\(^9\) was also used as a reference.

\subsection*{Production in vitro of microconidia and arthroconidia}

For the preparation of the microconidia suspensions, cultures were grown on malt extract agar (MEA), pH 5.5, at 28°C for 10 days. For arthroconidial formation, a standard pH 7.5 was employed for MEA and cultures were grown for 15 days on 5% CO\(_2\) at 37°C in a water-jacketed CO\(_2\) incubator (Forma Scientific, USA). The microscopic examination of arthroconidial suspensions obtained under these conditions usually showed the presence of microconidia, but the arthroconidia were greatly predominant components in all preparations made with all isolates (80% to 90%).

\subsection*{Susceptibility testing}

Susceptibility of microconidia and arthroconidia of each strain to fluconazole (Zolstatin, BioChimico), griseofulvin (Sigma), terbinafine (Lamisil, Sandoz), itraconazole (Janssen Pharmaceuticals), amphotericin B (Fungizone) and hygromycin B (Invitrogen) was tested by assessing the MICs of these drugs according to the M38-A microdilution technique for filamentous fungi, proposed by the CLSI (formerly the NCCLS).\(^10\) Fluconazole and hygromycin B were used in their commercial formulations. Terbinafine was dissolved in sterile water. Griseofulvin and itraconazole were dissolved in DMSO. The final concentration of DMSO never exceeded 1%. Briefly, colonies were covered with sterile saline containing 1% Tween 80, microconidia or arthroconidia were harvested by sterile scraping and the solution was filtered through glass wool to remove residual hyphae or long chains of arthrospores. Arthroconidia were defined as any hyphal compartment or spherical cell representing a disarticulated arthroconidium that was not obviously a microconidium. The number of microconidia or arthroconidia was estimated microscopically and the conidial suspensions were diluted in RPMI 1640 (Sigma) buffered with MOPS. The tests were performed in poly-styrene microlitre plates with 96-well flat-bottomed wells. Each microdilution well containing 100 mL of the diluted (twice) drug concentrations was inoculated with 100 mL of the diluted (twice) microconidial or arthroconidial inoculum suspensions (final volume in each well was 200 mL) to bring the dilutions of the inoculum from \(0.5 \times 10^4\) to \(5 \times 10^7\) cfu/mL. Growth and sterility controls were included for each isolate tested. Microtite trays were incubated at 28°C and MICs were recorded after 5 days of incubation. The susceptibility endpoint was recorded for each strain and for each drug. MIC was defined as the lowest drug concentration resulting in total inhibition of visible growth. Tests were performed in duplicate and all experiments were repeated three times.

\section*{Results and discussion}

The arthroconidial production, in the conditions described here, was very efficient for \textit{T. rubrum}, \textit{T. tonsurans} and \textit{T. equinum} isolates. Although most infections caused by filamentous fungi are characterized by the presence of hyphal elements in tissue, arthroconidia constitute the primary mode of transmitting dermatophytic infections in humans and animals.\(^8\) Hyperkeratosis, which often occurs in onychomycosis, may lead to a decrease in a local O\(_2\) concentration and an increase in CO\(_2\) concentration,\(^11\) a condition similar to that used for the induction of arthroconidia \textit{in vitro}.\(^12\) There are discrepancies between data obtained by \textit{in vitro} antifungal testing and those gathered from clinical experience, mainly on onychomycosis.\(^13\) In fact, the \textit{in vitro} testing evaluates the responses of microconidia or hyphae, and dermatophytes \textit{in vivo} often produce arthroconidia, a cellular structure presumably more resistant to antifungals.\(^4,13\) In this work, we compare the susceptibility of microconidia and arthroconidia of \textit{Trichophyton} isolates to griseofulvin, itraconazole, terbinafine, fluconazole, amphotericin B and hygromycin B. Griseofulvin, itraconazole, terbinafine and fluconazole are currently used to treat dermatophytes. Although amphotericin B did not appear to be effective in treating dermatophyte infections,\(^13\) this drug is active against \textit{T. rubrum} \textit{in vitro}.\(^15\) Our results show that the difference in the susceptibility between microconidia and arthroconidia depends on the drug and on the isolate/species (Table 1). The MIC of terbinafine, for example, is the same for microconidia and arthroconidia for all strains. The MIC of amphotericin B for microconidia and arthroconidia is the same for \textit{T. equinum} and \textit{T. tonsurans}, and differs only 2-fold for both strains of \textit{T. rubrum}. However, in general, arthroconidia appear to be more resistant to the essayed drugs than microconidia (Table 1) and may be one of the causes of therapeutic failure, mainly in patients whose lesions contain abundant arthrospores.\(^16\) Arthroconidia formation in \textit{Trichophyton mentagrophytes} has been reported to be triggered by an inhibition of mycelial growth.\(^17\) Subinhibitory concentrations of
some antifungal agents, including amphotericin B and griseofulvin, stimulate the arthroconidial formation in *Trichophyton rubrum in vitro*, enhancing the conversion of hyphae into arthroconidia. These data could account for the fact that exacerbation or intermittent recrudescence of infections takes place in some patients when the administration of the drugs is discontinued.¹⁸

Another aspect shown in Table 1 is the level of resistance of all strains towards antibiotic hygromycin B. This piece of information is useful because it allows the selection and maintenance of cells expressing the hygromycin-resistance gene, present in vectors used for gene disruption. Heterologous dominant drug resistance cassettes are powerful tools used to study gene function in many organisms, including the H6 strain of *Trichophyton rubrum*.¹⁹,²⁰ Preliminary results in our laboratory show that all strains presented in Table 1 are likely to form proplastos, which is the first step for them to be submitted to transformation procedures and gene disruption.

In conclusion, these results may be relevant to the comparative and functional genomic analysis of these species, which may help the development of more accurate diagnosis and new strategies for antifungal therapy.

### Funding

This research was supported by grants from the Brazilian agencies FAPESP, CAPES, CNPq and FAPEA.

### Transparency declarations

None to declare.

### References


Antifungal drugs against dermatophytes


