Characterization of the antimicrobial susceptibility of fungi responsible for onychomycosis in Spain

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Due to the increase of choices relative to antifungals, there is a need to improve the standardization of in vitro methods used to determine the antifungal susceptibility of fungal pathogens. Our study evaluated the in vitro susceptibility of filamentous fungi isolated from patients with toenail onychomycosis against itraconazole, ciclopirox, eberconazole, fluconazole and terbinafine. The minimum inhibitory concentration (MIC) of these antifungal agents was determined with 100 isolates, including dermatophytes (70 strains) and non-dermatophyte molds (30 strains). The susceptibility of fungal isolates was measured by using a technique modified for dermatophytes (0.5 x 10^-3 – 0.5 x 10^-4 conidia/ml as inocula) which followed the procedures described by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST) and the Clinical and Laboratory Standard Institute (CLSI M38-A). MIC ranges were 0.016–8.0 μg/ml for itraconazole, ciclopirox and eberconazole, 0.063–32.0 μg/ml for fluconazole, and 0.004–2.0 μg/ml for terbinafine. In vitro susceptibility tests indicated that eberconazole has a broad antimicrobial profile, including dermatophytes, as well as other filamentous fungi. Terbinafine was active (0.016–0.250 μg/ml) against dermatophytes.

**Keywords** antifungal agents, in vitro susceptibility testing, onychomycosis

**Introduction**

Onychomycosis, a chronic nail infection caused by dermatophytes, yeasts or non-dermatophyte molds, is one of the most common dermatological diseases worldwide [1,2]. This infection is the most frequent nail disorder in adults, accounting for up to 50% of all nail diseases [3].

Despite recent advances in the development of antifungal drugs and therapy, onychomycosis is still difficult to treat [4]. Conventional treatments frequently fail and it has been proposed that many factors, such as age, peripheral vascular disease, nail growth, poor drug penetration into the nail, extent of nail lesions and fungal growth patterns (i.e., presence of vegetative cells as well as spores) adversely affect clinical outcomes [5].

The Clinical and Laboratory Standard Institute (CLSI) and the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST) have established methods for in vitro antifungal susceptibility testing for filamentous molds and yeast [6].

While the AFST-EUCAST has proposed a broth microdilution method to test Candida susceptibility, there is no standard method for susceptibility testing of dermatophytes.

Antifungal agents used to treat onychomycosis, such as terbinafine (an allylamine), itraconazole (an azole) and ciclopirox (a hydroxypyridone), show marked activity against dermatophytes [7,8]. Since several variables, such as inoculum size, temperature and time of incubation, culture medium, and cut-off point determination, may all greatly affect MIC values they should be taken into account. A significant proportion of patients with onychomycosis do not respond satisfactorily to treatment with current antifungal drugs [9]. Thus, new treatments, as well as alternative methods to evaluate their efficacy are required.
Here we determined the MICs of five antifungal agents against 100 clinical isolates (70 dermatophytes and 30 non-dermatophyte molds) using CLSI M38-A2 for filamentous fungi and Eucast Document 7.1 for yeasts [10].

Materials and methods

Isolates

A total of 100 clinical strains were studied including *Trichophyton rubrum* (n = 35), *T. mentagrophytes* (n = 29), *Microsporum canis* (n = 3), *Epidermophyton floccosum* (n = 3); and non-dermatophyte molds, i.e., *Scopulariopsis brevicaulis* (n = 14), *Acremonium* spp. (n = 7), and *Aspergillus* spp. (n = 9). Each isolate was recovered from specimens of patients with toenail onychomycosis that were attended at the Podiatric Clinic of the University of Barcelona. Nail samples were inoculated onto Sabouraud dextrose agar (SDA; Scharlau, Barcelona, Spain) supplemented with 20 mg/ml of chloramphenicol to inhibit bacterial growth. A replica was done on plates of dermatophyte test medium (DTM) prepared in house by adding 500 μg/ml cycloheximide (Sigma-Aldrich, Spain) and 200 μg/ml phenol red (Sigma-Aldrich, Spain) to the base SDA. Plates were observed each week and the fungi recovered were identified using standard microscopic methods and urease production tests [11]. Isolated fungi were subcultured onto antimicrobial-free potato dextrose agar (PDA; Scharlau, Barcelona, Spain) and incubated for 3 weeks at 28°C to prepare inocula. Fungi were stored at room temperature (23–25°C) on PDA slants [12]. The results for the reference QC strain *C. albicans* ATCC 90028 were within the acceptable ranges recommended by the Clinical and Laboratory Standards Institute (CLSI) [13].

The medium used for broth microdilution susceptibility testing was RPMI 1640 (Sigma-Aldrich, Spain) with L-glutamine but without sodium bicarbonate, and buffered with HEPES 25mM (Sigma-Aldrich, Spain). The medium was sterilized by filtration.

Materials for antimicrobial susceptibility testing

For *in vitro* testing, we used the following antifungal agents, provided by the manufacturers as assay powders; ciclopiroxolamine (Laboratorios Ferrer, Spain), eberconazole (Laboratorios Salvat, Spain), fluconazole, itraconazole and terbinafine (Sigma-Aldrich, Spain). All drugs were dissolved in 100% dimethyl sulfoxide, followed by further dilutions in RPMI 1640 medium to yield twice the final strength required for the test. For itraconazole, ciclopirox and eberconazol, final concentrations ranged from 0.016–8,000 μg/ml, for fluconazole from 0.063–32,000 μg/ml and for terbinafine from 0.004–2,000 μg/ml.

Drug dilution plates were stored at −70°C for up to a maximum of 3 months.

Inoculum preparation

To prepare inocula, the fungi were subcultured onto potato dextrose agar (PDA, Scharlau, Barcelona, Spain) and incubated at 28°C for 3 weeks. The plates were then covered with 10 ml of sterile distilled water and conidia were collected by scraping the colonies with the tip of a sterile Pasteur pipette. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. Heavy particles were allowed to settle for 5–15 min, and the upper suspension was collected and homogenized.

The suspension obtained was then filtered once through sterile gauze folded three times to remove hyphal fragments [15] and transferred to a sterile tube. We compared two methods of inoculum preparation, namely hemocytometer counts and spectrophotometric readings. We obtained a mixture of conidia and adjusted it to 0.5 × 10^5–5 × 10^6 spores/ml spectrophotometrically at 546 nm wavelength. Quantitative cultures were prepared by plating 10 μl of the adjusted inoculum on SDA- chloramphenicol agar. The plates were incubated at 30°C and observed daily for the presence of growth.

The volume of the inoculum was diluted in RPMI 1640 to the desired concentration of 0.5 × 10^5–0.5 × 10^6 ufc/ml.

Test procedure

All tests were performed in round-bottomed 96-well microplates. Aliquots of 100 μl of the two-fold drug dilutions were transferred into the microplate wells by means of a multichannel pipette. Each well was then inoculated with 100 μl of the diluted inoculum suspension. For each test plate, two drug-free growth controls were included, one with the media alone (growth control) and the other with media containing an equivalent amount of solvent used to dissolve the drugs (solvent control).

Growth and sterility controls were included for each assay and tests were performed in triplicate. The plates were incubated at 30°C and read after 3–5 days of incubation. Growth in the control and test wells was checked every day. The incubation was prolonged until growth was detected [17].

Reading and interpretation of MICs

Endpoint values were determined by visual comparison of wells and controls and were established as the lowest concentrations giving 50% growth inhibition. Geometric mean and minimum inhibitory concentration (MIC) ranges were determined to allow comparisons of drug activities.
In addition, MIC values at which 50% and 90% of isolates were inhibited (MIC_{50} and MIC_{90} respectively) were recorded. All isolates when cultivated in the absence of antifungals, produced clearly detectable growth after 5 days of incubation.

**Results**

We determined the *in vitro* susceptibility of the dermatophytes most commonly isolated from our patients to five antifungal agents. While in general, all drugs except fluconazole showed activity, eberconazole gave the lowest geometric mean MIC (0.113 μg/ml) of the drugs tested and was active against most of the test isolates assayed.

The activity of both terbinafine and eberconazole was significantly higher than that of the other drugs, especially against *T. mentagrophytes* and *T. rubrum*. The highest MIC value (≥32 μg/ml) for isolates of these two species was found with fluconazole (Table 1).

Similar results were obtained by other authors independently of the experimental parameters used. These data confirm the resistance of dermatophyte species to fluconazole and contradict the cure rates achieved in patients with dermatophytosis treated with this drug [18].

No significant differences in susceptibility were observed between *M. canis* and *E. floccosum* for any of the tested drugs.

**Discussion**

*In vitro* antifungal susceptibility testing of dermatophytes is still technically difficult to conduct as a result of their growth and spore formation characteristics. Repeated subcultures often make it extremely hard to prepare conidial inocula [19], although in this study we determined the fungal MICs immediately after the isolate in PDA cultures.

The parameters employed in this investigation for evaluating the *in vitro* susceptibility of dermatophytes to antifungal agents proved to be suitable and reliable. Furthermore, they may provide insights for the development of a standard assay. While the MIC endpoints and clinical significance of the susceptibility testing of the remaining fungi and antifungal drugs remain unclear, intense efforts to overcome the present drawbacks and limitations are currently focused on modifications of available methods, as well as the development of alternative techniques [20].

The predictive value of the MICs obtained using these techniques is unknown due to the fact that the limited number of studies of *in vivo*–*in vitro* correlation have involved only a few species and a few antifungal drugs. Consequently, concise endpoints are not available. Several collaborative studies involving the testing of filamentous fungi have demonstrated thatazole MICs are extremely variable as they are affected by the testing conditions [18,20]. The strong antifungal activity of terbinafine against dermatophytes has been reported by other authors [16,21,22].

Eberconazole is a novel topical imidazole with a mode of action similar to that of otherazole antifungal agents and has shown potent *in vivo* and *in vitro* activity against dermatophytes.

Although the association of non-dermatophyte filamentous fungi with onychomycosis is unclear, we cannot rule out that the clinical evolution of this infection may worsen depending on the species involved.

Fernández-Torres *et al.* evaluated an agar dilution method in studies of 100 strains of *T. rubrum*. They used

![Table 1 In vitro susceptibility of 100 isolates to the five drugs tested by a microdilution method.](https://academic.oup.com/mmy/article-abstract/49/5/495/973189)
four antifungal drugs commonly administered to treat dermatophytes (i.e., clotrimazole, ketoconazole, itraconazole and terbinafine) and also compared the results with the MICs obtained by a microdilution method [18,22]. Their results revealed higher MICs for the assayed azoles with the agar dilution method while MIC values remained low in tests with the microdilution technique. Thus, the latter appeared to be more predictive of clinical outcome. Many species and antifungal drugs have been assayed using this method. In addition, it is technically less cumbersome than other approaches.

We observed high in vitro activity of itraconazole and eberconazole while terbinafine and ciclopirox showed slightly higher activity than that reported by other authors. Among the group of azole derivatives, fluconazole was found to have the lowest antifungal activity. This observation is consistent with the high MICs described by other authors when comparing different methods. These results could reflect the unsuccessful therapeutic effect that we obtained with this antifungal agent (data not shown).

Certain factors, such as temperature, incubation period, MIC endpoints, culture medium or kind (microconidial or hyphae) and size of inocula, influence the determination of the susceptibility of yeasts and filamentous fungi to antifungal agents. A multicentric study developed to determine the optimal parameters for the use of the microdilution assay with dermatophytes concluded that the optimal reproducibility conditions were as follows; incubation temperature 28°C, 7 days of incubation, inocula of 10⁴ microconidia/ml and endpoint at 100% of growth inhibition. Other variables such as the color of the spores can also influence the OD values [23]. The comparison of hematocytometer counts and spectrophotometric readings in the preparation of inocula indicate that there is no significant difference between the two approaches. Counting with a hematocytometer is a universal procedure that is not affected by the color of the strain or by spore size. Moreover, both the CLSI and the EUAST-AFST recommend that inocula standardization be based on the determination of cell density by spectrophotometry [24].

In summary, our study confirms previous work on in vitro antifungal susceptibility patterns and reports a novel modified method for testing dermatophytes. However, because MIC endpoints have not yet been established for onychomycosis, it remains unclear whether the in vitro activity of antifungal drugs is predictive of the clinical outcome.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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