Antimycotic effectiveness against dermatophytes: comparison of two in vitro tests

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Abstract Seven antimycotic drugs (econazole, enilconazole, fluconazole, griseofulvin, itraconazole, ketoconazole, and miconazole) were tested against 36 dermatophyte strains (19 *M. canis*, 7 *T. mentagrophytes*, 5 *M. gypseum*, 2 *M. cookei*, 1 *T. rubrum*, 1 *T. ajelloi*, and 1 *T. terrestre*) isolated from animals, humans, and the environment. Two in vitro methods were compared: a micro-dilution test based on the CLSI M38-A method, and a disk-diffusion test. Fluconazole was not effective in vitro against the dermatophytes. Econazole and enilconazole were the most effective. Thirteen strains were griseofulvin-resistant. The correlation between the two methods was statistically significant for enilconazole, griseofulvin, itraconazole, and miconazole.

Keywords Antimycotic activity · Dermatophytes · In vitro tests

Abbreviations

MD	Microdilution method
DD	Disc diffusion method
DMSO	dimethylsulfoxide
PDA	potato dextrose agar
MIC	minimum inhibitory concentration

Introduction

A reference method for the antifungal susceptibility testing of dermatophytes is not available. Some microdilution or disk diffusion systems are commercially available,

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suitable mainly for yeasts and some filamentous fungi, such as *Aspergillus fumigatus*, *Fusarium* spp., and *Rhizopus* spp. The application of these tests to dermatophytes is problematic, due to, for example, the standardization of the inoculum and the final interpretation. The aim of this study was to evaluate the in vitro activity of certain antifungal drugs against different species of dermatophytes, using and comparing two methods: a microdilution method (MD) based on the document of the Clinical Laboratory Standard Institute CLSI M38-A, and a disk diffusion procedure (DD). The purpose was to establish a routine method to answer the demand of veterinary practitioners who want to verify whether the lack of response to treatment is linked to the resistance of dermatophytes involved rather than to host predisposing factors.

Materials and methods

Seven antifungal drugs (Econazole, Enilconazole, Fluconazole, Griseofulvin, Itraconazole, Ketoconazole, and Miconazole - Sigma Aldrich, St. Louis, MO, USA), commonly present in products used in the veterinary clinic, were tested against 36 strains of dermatophytes (19 *Microsporum canis*, 7 *Trichophyton mentagrophytes*, 5 *M. gypseum*, 2 *M. cookei*, 1 *T. rubrum*, 1 *T. ajelloi*, and 1 *T. terrestre*) isolated 1) from the skin of animals with or without lesions, 2) from the environment, and 3) from man (one case). The strains were spread on PDA (DIFCO) incubated at $30 \pm 2^\circ\text{C}$ for 14 days; for *M. canis*, three plates for each strain were prepared. The inocula were prepared by covering the PDA culture dishes with sterile saline solution, and the colonies were gently scraped with the tip of a Pasteur pipette. The resulting mixture of conidia and hyphal fragments was drawn and transferred to tubes containing five sterile glass balls, vortexed for 2–3 min, and kept at room temperature for 10 min (Singh et al. 2007). The optical density of the homogeneous supernatant suspensions was adjusted to level 2 of the McFarland scale. For each antifungal drug, a stock solution was prepared by dissolving the antimycotic powder in 100% DMSO, except for Fluconazole, which was dissolved in sterile water (Jessup et al. 2000).

For the DD test, blank paper disks (Biolife) with a diameter of 6.0 mm were impregnated with 20 μL of each concentration of stock solution (Table 1) or with 20 μL DMSO. The disks were allowed to dry at room temperature. Petri dishes containing RPMI 1640 medium with L-glutamine (Sigma) buffered with 0.165 M MOPS (Sigma) and 15 g/L Bacto agar (DIFCO) (RPMI agar) were inoculated by dipping a sterile cotton swab into the inoculum suspensions and streaming the swab in four directions over the entire agar surface. The dishes were allowed to dry for 5 min before the disks loaded with drugs were applied. The

Table 1 Dilutions of the different antifungal drugs tested

Antifungal drugs	Stock solution concentrations ($\mu\text{g}/20 \mu\text{L}$)	Concentrations used for MD test ($\mu\text{g}/\text{mL}$)
Enilconazole	2 (Fernandez-Torres et al. 2005)	2 to 0.0019 (Esteban et al. 2005)
Econazole	2 (Fernandez-Torres et al. 2005)	2 to 0.0019 (Esteban et al. 2005)
Miconazole	10 (Karaca and Koc 2004)	4 to 0.008 (Karaca and Koc 2004)
Ketoconazole	15 (Karaca and Koc 2004)	4 to 0.008 (Karaca and Koc 2004)
Fluconazole	25 (Singh et al. 2007)	64 to 0.125 (Singh et al. 2007)
Griseofulvin	25 (Karaca and Koc 2004)	16 to 0.03 (Singh et al. 2007)
Itraconazole	10 (Singh et al. 2007)	8 to 0.05 (Singh et al. 2007)
DMSO	whole	250 to 0.48

Table 2 In vitro activity of six drugs tested against 36 strains of dermatophytes determined by the DD and MD methods

Antifungal drug	MD		DD	
	MIC ($\mu\text{g/mL}$)		Inhibition zone diameter (mm)	
	Geometric mean	Range	Mean $\pm$ std. dev.	Range
Econazole	0.21	0.03–1.0	43.69 $\pm$ 3.011	0–80
Enilconazole	0.18	0.03–1.0	50.47 $\pm$ 3.842	0–82
Griseofulvin	2.20	0.50–8.0	41.19 $\pm$ 3.815	0–82
Itraconazole	0.96	0.06–4.0	17.43 $\pm$ 1.788	0–38
Ketoconazole	0.76	0.06–1.0	24.19 $\pm$ 3.507	0–62
Miconazole	0.29	0.03–1.0	23.22 $\pm$ 2.76	0–50

100% inhibition zone diameter was measured in mm using a ruler after 14 days of incubation at 30°C. For the MD test, a 96-well microtiter plate (Sterilin) was used for each strain. RPMI broth (as above but without agar) was used. A column of the plates was used as a negative control (the culture medium only); another column was used as a positive control (100 μL medium and 100 μL inoculum suspension). Ten different 2-fold dilutions of each drug and of DMSO were distributed in eight rows, and 100 μL inoculum were added to each well. The plates were incubated at 30°C for 14 days. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that produced 100% inhibition of growth. The correlation between the MD and DD tests were analyzed with the Pearson test, while the Mann-Whitney U test was used to evaluate the differences between the MIC of the geophylic vs. antropophylic/zoophylic dermatophytes.

Results

Fluconazole caused total inhibition of dermatophyte growth at the maximum concentration tested (64 $\mu\text{g/mL}$) only on three strains (one *M. canis* and two *T. mentagrophytes*) but was

Fig. 1 Enilconazole and Griseofulvin disk zone diameters plotted against the MICs determined by the MD method

ineffective against all other strains. In the DD test, these data were confirmed by the lack of an inhibition zone, except for one *T. mentagrophytes* strain, showing an inhibition halo of 18 mm. Therefore, this drug was not considered further. The results obtained for the other antifungal drugs are shown in Table 2.

In the MD method, DMSO inhibited fungal growth up to the third dilution; for this reason, the first three dilutions of the drugs could be not indicative of real drug effects. As a whole, Econazole and Enilconazole were more effective than the other antimycotics tested, with MICs ranging from 0.03 to 1 µg/mL. The geometric means of the MICs appear higher in geophylic vs. antropophylic/zoophylic strains: the Mann-Whitney U test showed statistically significant differences for Griseofulvin and Enilconazole. In the DD method, Econazole and Enilconazole resulted in greater inhibition zone diameters than the other drugs, despite their lowest concentrations (2 µg) on the disk. When comparing the inhibition zone diameter and the MICs with the Pearson's test, statistically significant results were observed for Enilconazole, Griseofulvin, Itraconazole, and Miconazole.

Considering the regression lines obtained, we can affirm that for routine DD antimycogram, we considered, as effective, an inhibition zone diameter greater than or equal to 4 mm for Griseofulvin and greater than or equal to 18 mm for Enilconazole (Fig. 1).

Discussion

Fluconazole was considered ineffective against dermatophytes in accordance with other authors (Barros et al. 2007); indeed, the strains with MICs greater than or equal to 64 µg/mL are considered resistant (Gonçalves Krakhecke et al. 2005; Santos and Hamdan 2007). According to Randhawa (2006), high concentrations of DMSO, usually used as a drug diluent in antifungal in vitro tests, can inhibit fungal growth. For Griseofulvin, a MIC of 3 µg/mL was considered a limit of effectiveness (Scholz and Meinhof 1991). The mean MIC value was within the efficacy range of this drug (2.2 µg/mL), but 13 strains (1 *M. canis*, 2 *T. mentagrophytes*, 5 *M. gypseum*, 2 *M. cookie*, and the *T. rubrum*, *T. terrestre* and *T. ajelloi* strains) were resistant, with MICs ranging from 4 to 8 µg/mL. In the literature, cases of dermatophyte resistance to Griseofulvin are described for *T. rubrum* (Korthing et al. 1995). With the DD method, it was not possible to evaluate the minimum inhibition diameter for Miconazole and Itraconazole, as the MIC described by some authors as indicative of drug resistance in different mycetes are higher than the concentrations of the drug in the paper disk. Nevertheless, we conclude that, with some adjustment, the DD method could be used as a screening procedure to evaluate the in vitro sensitivity of dermatophytes to commonly used antimycotics in clinical practice.

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