

CLSI BROTH MICRODILUTION METHOD FOR TESTING SUSCEPTIBILITY OF *MALASSEZIA PACHYDERMATIS* TO THIABENDAZOLE

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ABSTRACT

Thiabendazole, classified as antiparasitic and also used as an antifungal drug, can be found as otological solution indicated for treatment of parasitic and fungal external otitis in small animals. *Malassezia pachydermatis* is a yeast recognized as a normal inhabitant on the skin and mucous membranes of dogs and cats. However, it is considered an opportunistic agent that causes external otitis and dermatitis in these animals. The aim of this study was to evaluate the *in vitro* effect of thiabendazole against 51 isolates of *M. pachydermatis* using the CLSI Broth Microdilution method that has been adapted for this yeast species (NCCLS, 2002). Based on this test, the Minimum Inhibitory Concentrations (MIC) of thiabendazole was calculated. Subsequently, the susceptibility of each isolate against this antifungal was determined. It was observed that the MIC of thiabendazole against *M. pachydermatis* ranged from 0.03 to > 4 µg/mL. A total of 13.7% of the isolates were found to be resistant, 47.1% were intermediate and 39.2% were sensitive to the drug. The rate of resistance of the yeasts against thiabendazole was similar to the results previously obtained with other antifungals, while the adapted broth microdilution technique used in this study proved to be efficient.

Key words: *Malassezia pachydermatis*, Minimum Inhibitory Concentration, thiabendazole

INTRODUCTION

The need for an antihelmintic, with a broad spectrum of antiparasitic effect, highly efficient, with good safety limits and that would also be versatile in its administration, promoted a study of hundreds of benzimidazole compounds. Thiabendazole was the first compound that fulfilled all of those requirements with an extraordinary performance (26,27). Its *in vitro* antifungal activity was observed against *Blastomyces* spp, *Aspergillus* spp, *Fusarium* spp, *Penicillium* spp and *Trichophyton* spp, although there have been controversies regarding its effect against some yeasts, such as *Candida* sp. (26,30).

Malassezia pachydermatis is a yeast that is considered a normal inhabitant and an opportunistic pathogen of the external

ear canal of dogs and cats, and it can also be found on the skin and in the rectum, anal sacs and vagina of these animals (2,3,4,5). This yeast is one of the most frequent microorganisms associated with external otitis in dogs (9,19). In recent years, studies have also reported *M. pachydermatis* as the cause of canine dermatitis (4,6,21,24,28).

The appearance of the clinical manifestations of malasseziosis indicates an alteration in the balance between the commensal yeast and its host. The onset of clinical disease occurs when there is excessive multiplication of *M. pachydermatis* due to alterations in the defense mechanisms of the host. An increase in the number of *M. pachydermatis* cells is favored when there is a modification in the microenvironment, such as excessive production of cerumen, after therapy with antibiotics or corticoids (17,24) and also with

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concomitant primary diseases, such as hypersensitivity to food, flea bites and/or atopy, pyodermitis, demodicosis and endocrine problems (hyperadrenocorticism, hyperthyreodism, diabetes mellitus). Therefore, malasseziosis is an infection frequently associated with concomitant primary diseases (3,17).

Only one study of the *in vitro* activity of thiabendazole against *M. pachydermatis* has been carried out (18). This study used the agar diffusion method for testing this and other antifungal drugs against *M. pachydermatis*. Thiabendazole had its *in vitro* inhibitory activity confirmed by this technique, without the calculation of the Minimum Inhibitory Concentration (MIC).

This drug has been used in suspension as a fungicide for poultry breeding and livestock farming, mainly in the prevention of aspergilosis, without showing any incompatibility. Currently, an otological solution of thiabendazole is recommended for the treatment of mycoses caused by *Microsporum canis*, *M. gypseum*, *Trichophyton mentagrophytes* and *T. equinum*. In Brazil, the otological solution of this drug is found in products such as Otoden plus® (Vetbrands) and Otoneodexa® (Usinas Quimicas Brasileiras) for the treatment of canine external otitis. This drug is used in association with neomycin and dexametazone, which amplify the spectrum of action of Thiabendazole, enabling it to be effective for the treatment of bacterial, fungal and inflammatory canine external otitis (12), including those infections caused by *M. pachydermatis*, which is recognized as a normal inhabitant and is also considered an opportunistic agent that causes external otitis and dermatitis (1,5,16,23).

In vitro testing of antimicrobial susceptibility is of great importance. Some authors recommend the evaluation of antifungal sensitivity through the use of antimycograms in special clinical conditions, such as cases of therapeutic failure, which is quite common in cases of canine external otitis and dermatitis exacerbated by *M. pachydermatis* (1,8,14).

The aim of this study was to use the broth microdilution method (22) to test the activity of thiabendazole against 51 isolates of *M. pachydermatis* obtained from clinical cases of canine external otitis.

MATERIAL AND METHODS

All strains, collected with sterile swabs from canine ear canals, were cultured in Sabouraud dextrose agar medium with chloramphenicol and cycloheximide, kept at 32°C for ten days, and their macroscopic characteristics were observed daily. Cell morphology was observed through the use of colony smears, which were stained with Gram stains and then characterized (15).

The *National Committee for Clinical Laboratory Standards* (22), currently known as the *Clinical and Laboratory Standards Institute* (CLSI) recently published two standardized methods

for broth microdilution for use in antifungal testing, the M27A2 and the M38P. The NCCLS Broth Microdilution (BM) method adapted for *M. pachydermatis* was performed by Eichenberg *et al.* and Nascente *et al.* (9,19) according to the M27A2 document (22) and thiabendazole recommendations. This methodology was used to evaluate the susceptibility of 51 isolates of *M. pachydermatis* against thiabendazole, with a few modifications in the method for this specific antifungal. The antifungal susceptibility test by BM was carried out in sterile microdilution plates (Nuclon®) with 96 flat bottomed wells, composed of eight series identified from A to H, each one with twelve wells.

The *M. pachydermatis* isolates were suspended in saline solution with the turbidity adjusted to level 1 of the McFarland scale. After that, they were diluted in Sabouraud broth medium and a volume of 100 µl was dispensed into the wells, which contained 100 µl of twice the final concentrations of the antifungal. Thus, the final dilution of the starting inoculum was 1:10.

From the stock-solution of the drug (1.6 mg/mL), diluted according to the methodology recommended by the NCCLS/CLSI (22), ten successive dilutions were prepared with a concentration 10 times greater than the final solution of thiabendazole. The dilutions were further diluted to 1:5 in Sabouraud dextrose broth with chloranphenicol and 100 µl aliquots were sequentially dispensed into the microdilution plates, filling the wells in the columns numbered from 1 to 10.

The columns 11 and 12 were reserved as controls. The positive control was composed of 100 µl of Sabouraud dextrose agar and 100 µl of the half-inoculum solution. The negative control was composed of 200 µl of the same culture medium. The plates were incubated at 37°C for 72h. The readings were made visually, comparing the growth of the yeast in the wells numbered one to ten (with varying concentrations of antifungal) with the wells that had the positive control (cavity 11). The lowest concentration that produced a significant inhibition (around 50%) of the growth of the yeast in comparison with the positive control was identified as the MIC.

RESULTS AND DISCUSSION

The MIC of thiabendazole, observed through the BM method against 51 isolates of *M. pachydermatis* ranged from 0.03 to >4 µg/mL (Fig. 1) with a mean MIC of 3.67 µg/mL and a mode of 4 mg/mL. In the current literature there are no studies of thiabendazole and the adapted BM method to compare these results.

After observation of the MIC, *M. pachydermatis* isolates were classified as sensitive (S), intermediate (I) and resistant (R), according to the criteria proposed by Colombo *et al.* (7) and the results of the MIC₅₀ and MIC₉₀: S = MIC-strain < MIC-50%; I = MIC-50% > MIC-strain < MIC-90%; R = MIC-

strain > MIC-90%. Resistance was verified in seven (13.7%) isolates of the yeast, intermediate sensitivity in 24 (47.1%) isolates and sensitivity in 20 (39.2%) strains (Table 1).

However, when comparing the susceptibility of *M. pachydermatis* against thiabendazole with other antifungals, the MIC has different values and the percentage of resistant isolates to this drug.

The NCCLS/CLSI methods are indicated for some yeasts and filamentous fungi against the antifungals ketoconazole, fluconazole, itraconazole, amphotericine and flucitosine. The NCCLS (22) recommends the use of RPMI 1640 in antifungal tests with yeasts (*Candida* spp. and *Cryptococcus* spp.), although it has not yet been used with the *Malassezia* species (25). Other authors (9,19) used Sabouraud dextrose liquid instead of RPMI 1640 in the BM method, and reported a better growth of the yeasts.

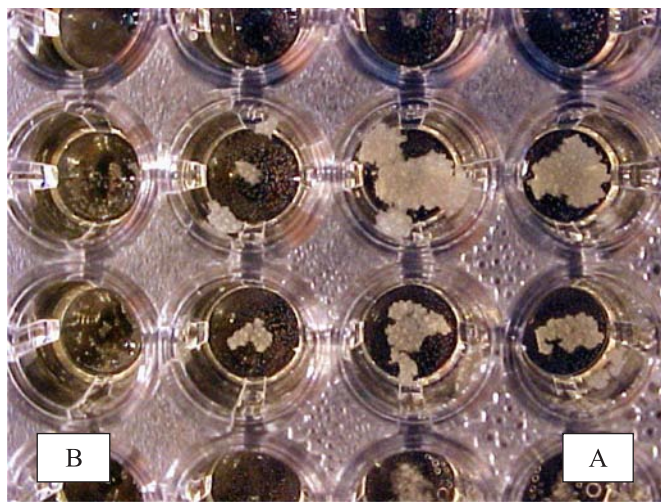


Figure 1. Microdilution plate used for Broth Microdilution method with thiabendazole against *Malassezia pachydermatis* isolated from canine external otitis, showing the wells with growth (A) and the ones without growth (B) of the yeast (negative control).

Table 1. Minimum Inhibitory Concentration (MIC) of thiabendazole against 51 isolates of *M. pachydermatis* obtained from canine external otitis.

Antifungal	Susceptibility (mg/mL)		
	S*	I*	R*
Thiabendazole	<3.055	3.055 to 7.154	>7.154

*S = Susceptible; I = Intermediate susceptible; R = Resistant

The M27A2 is a reference protocol that was written to provide consensus on the evaluation of the susceptibility of yeasts against antifungals. However, the method includes invasive yeasts, such as *Candida* sp. and *C. neoformans* var. *neoformans*, and does not standardize the evaluation of other yeasts. Several developed countries have Committees of Standardization. The NCCLS (U.S.A.) is one of the most active. The M27A2 document (22) is not appropriate for *Malassezia*, because it is lipodependent yeast. RPMI1640 is not an appropriate culture medium for *Malassezia* because it is not supplemented with lipids (13). Therefore, methods described in the literature need to be frequently adapted. The methodology described by Eichenberg *et al.* (9) and Nascente *et al.* (19) was followed in the current study. Eichenberg *et al.* (9) compared the RPMI1640 medium with Sabouraud dextrose medium and observed a better isolation of *M. pachydermatis* in RPMI1640 (31).

The antifungals reported in the CLSI document (22) included the azoles (itraconazole, fluconazole and ketoconazole), 5-flucitosine and amphotericine. Thiabendazole is not mentioned in that document. Therefore, the methodology had to be adapted for the current work.

For the BM method, a dilution of the yeast in a proportion of 1:10, from a suspension of approximately 1-5 x 10⁶UFC/mL, was used according to the studies carried out by Eichenberg *et al.* (9) and Nascente *et al.* (19). Excellent results for the growth of *Malassezia* sp. were obtained. Other authors successfully used the same dilution in an experiment with *M. pachydermatis* isolates and the broth macrodilution method (29).

The interval used for reading the test was 72 h, in accordance with the intervals suggested other authors, which recommend 24 h for *Candida* spp. and 48-72 h for *Cryptococcus neoformans* (11,22). The same methodology used by Nascente *et al.* (19) was also followed, as they observed a significant difference when the reading was carried out after 72 h, when the best fungal growth was observed. The results observed after 72 h was used for the calculation of the mean MIC.

Broth microdilution testing of *Malassezia* is under development; therefore, there is controversy as to what particular broth medium will support sufficient growth to reliably and reproducibly give MICs. In the single previous study examining thiabendazole and *M. pachydermatis*, Lorenzini *et al.* (17) reported that the substances were effective in inhibiting the growth of these organisms, but that there was no relation between *in vitro* activity and commercial formulations of the antimicrobials. The fact that this drug is not used by the veterinary community as a sole drug for the treatment of disease underscores its insufficiency. Furthermore, there was no attempt to experimentally compare the current results with those from methods used and published in internationally peer reviewed journals or compare with drugs having MICs defined by these methods.

The M27A2 method (22), adapted and used for the MIC evaluation of thiabendazole against *M. pachydermatis*, was satisfactory, showing similar results to the ones obtained by other tests carried out with other antifungal drugs regarding susceptibility. The antifungal thiabendazole showed MIC values compatible with those observed with other drugs, with similar rate of resistant yeasts.

RESUMO

CLSI método de Microdiluição em Caldo para teste de suscetibilidade da *Malassezia pachydermatis* frente ao tiabendazol

Tiabendazol, um fármaco classificado como antiparasitário e também usado como antifúngico, pode ser encontrado como solução otológica indicada no tratamento da otite externa parasitária e fungica em pequenos animais. *Malassezia pachydermatis* é uma levedura considerada habitante normal da pele e das mucosas de cães e gatos. Entretanto, considera-se um agente do oportunista causador de otite externa e dermatite nestes animais. A finalidade deste estudo foi avaliar o efeito *in vitro* do tiabendazol frente a 51 amostras de *M. pachydermatis* através do método CLSI de Microdiluição em Caldo adaptado para esta espécie de levedura (NCCLS, 2002). Baseado neste teste calculou-se as Concentrações Inibitórias Mínimas (CIM) do tiabendazol e, subseqüentemente, foi detectada a suscetibilidade de cada amostra frente a este antifúngico. Observou-se que a CIM do tiabendazol frente a *M. pachydermatis* variou de 0,03 a $> 4 \mu\text{g/mL}$. Estas amostras foram classificadas em resistente (13,7%), sensibilidade intermediária (47,1%) e sensível (39,2%). A resistência da levedura frente ao tiabendazol mostrou similaridade com resultados anteriormente observados com outros antifúngicos e a adaptação da técnica de Microdiluição em Caldo utilizada mostrou-se eficiente.

Palavras-chave: *Malassezia pachydermatis*, Concentração Inibitória Mínima, tiabendazol.

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