# Comparison of the broth microdilution technique and ETEST to ketoconazole front *Malassezia pachydermatis*

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## Abstract

*Malassezia pachydermatis* is recognized as a normal inhabitant and an opportunistic pathogen of the external ear canal and skin of dogs and cats. In special clinical conditions, and mainly in the cases of therapeutic failure related to external otitis and dermatitis complicated by this yeast, is recommended testing susceptibility to antifungal drugs. Different approaches of evaluating the susceptibility of yeasts faced to antifungals in laboratory exist, some of them are commercial approaches and others previously standardized by the CLSI (NCCLS, 2002). The purpose of this study was to evaluate the susceptibility of 17 samples of *M. pachydermatis* from canine external otitis using two different *in vitro* antifungal susceptibility methods: the *Etest*® and the broth Microdilution Method (MD) with ketoconazole. The mean MIC observed between the 17 samples were 0.103mg/mL to ETEST and 0.0012mg/mL to MD ranging from 0.004 to 0.75mg/mL in ETEST and 0.0019 and 0.03mg/mL in MD using the same samples. By ETEST, two (11.8%) samples were resistant, eight (47.1%) susceptible and seven (41.1%) showed intermediate susceptibility. Through the MD it was observed four (23.5%) resistant samples, seven (41.2%) susceptible and six (35.3%) samples with intermediate susceptibility. Despite of the percentages being equivalent in each rank of susceptibility through the two techniques, the results do not correspond to the same sample. These results showed that there is an urgent need to standardize those values considered as parameters for growth inhibition of this yeast. Then a simple and efficient method could be used routinely in the laboratory practice.

**Key words:**
*Malassezia pachydermatis*.
CLSI.
ETEST.
Broth microdilution.

## Introduction

The interest by the stardandization of the susceptibility tests to antifungals is relatively recent. The development of susceptibility tests to antimicrobials has its history linked to the advancements obtained in the antibacterial therapy. With the advent of new chemotherapics, antibiotics and the recognition of resistant bacteria to the penicillin, microbiology laboratories passed it carry out tests of sensitivity.[1] There are two decades, new therapeutic options have being developed and there are fungi resistance, mainly in the yeasts of the genus *Candida* to the drugs commercially available.[2,3] Due the tests used for *in vitro* evaluation of the antifungal activity was the same used in the evaluation of antibacterial activity, usually, the same techniques are developed: broth dilution, agar dilution and agar diffusion. The final reading of the tests of dilution in liquid and/or solid is going to identify the smaller concentration of the drug that inhibits the

growth of the microorganism studied. Recently the National Committee Clinical Laboratory Standards[4], nowadays known as Clinical and Laboratory Standards Institute (CLSI), defined two standardized approaches of broth Microdilution as antifungal tests, the M27A2 and the M38P, to some yeasts and fungi filamentous respectively. The NCCLS[4] recommends the use of the RPMI 1640 in the achievement of antifungal susceptibility testing with yeasts (*Candida* spp. and *Cryptococcus* spp.), despite of not to have been used in yeasts of the genus *Malassezia*[5]. Others authors[6,7] used to the same technique of broth Microdilution, but with Sabouraud dextrose, obtaining better growth of the yeast. However, this is not a routine procedure technique in the laboratory.[8] The ETEST method (agar diffusion) was introduced in 1988 as an alternative for determination of the MIC, being used mainly to confirm the resistance to the antimicrobials. In Brazil, only in 1997, the first study was carried out about antifungal susceptibility testing of *M. pachydermatis*, with the ETEST method.[9,10]

The ketoconazole is the imidazol more used until now and it presents elevated efficacy against agents of systemic and superficial mycoses.[11,12] It includes *Malassezia* and others dermatophytes, resistant or not resistant to griseofulvina, to several agents of systemic mycoses, detaching their purpose in the paracoccidioidomicosis and histoplasmosis treatmen.[11,13] It was the first antifungal substance used orally in the systemic mycoses treatments and up to date, is used in non and dermatological mycoses in the veterinary small animals, mainly due to the short value.[13,14]

*Malassezia pachydermatis* is recognized as a normal inhabitant and an opportunistic pathogen from the external ear channel and skin of dogs and cats, being considered one of the most frequent microorganisms associated with external otitis in dogs by several authors. This yeast also can be found in the rectum, interdigital, anal sacs and vagina. In the last years, some investigations also pointed out this yeast as cause of canine dermatitis.[15,16, 17,18,19,20,21,22,23,24,25,26,27]

The purpose of this study was to evaluate the antifungal susceptibility of 17 isolates of *M. pachydermatis* to ketoconazole using two antifungal susceptibility methods.

## Material and Method

The NCCLS' broth microdilution method (CLSI)[4] recommendations were adapted to *M. pachydermatis* by Eichenberg et al.[6] and Nascente et al.[7]. Ten dilutions of the drug storage solution were prepared obtaining 10 solutions with a gradient 10 times greater than the final solution of each drug used. The ten solutions obtained this way had the drug concentration from 0.0000574 to 0.03mg/mL to ketoconazole and they were put into the first 10 contiguous wells of a sterile microtiter plate. The solution containing each sample of yest was transferred in aliquots of 100μL into each of the sterile plates that previously had 100μL of the solution containing the antifungal drug tested. The wells 11 and 12 had the positive control (100μL of Sabouraud dextrose agar and 100μL of the half-inoculum solution) and the negative control (200μl of the same culture medium), respectively. The plates were incubated at 37°C for 72 hours. The readings were made by visual comparison of the yeast growth into the wells one to ten with wells that had the positive control (wells 11). The lowest concentration that produced a relative significant inhibition (around 50%) of the yeast growth compared to the positive control was identified as the MIC (Minimum Inhibitory Concentrations) of the drug (10, 11).

The strips of the *Etest*® have a defined and consistent gradient of the antifungal drug (0.002 to 32.0μg/mL of ketoconazole allowing a quantitative reading. The culture medium used was Sabouraud dextrose agar with chloramphenicol according to the *Etest*® manufacturer´s recommendations. *M. pachydermatis* samples were suspended in saline with turbidity adjusted to level 1 of the McFarland scale and cultivated by

spreading the sample in the culture medium. After 15 minutes, the strips were distributed over the medium and incubated at 37°C. The readings were performed at 48hours and the the lowest concentration (MICs) of the drug was determined by the *Etest®* strip pattern.

The comparison of the results was carried out with the 17 samples of *M. pachydermatis* tested. The MICs were calculated according to Coutinho and Paula[9] for the *Etest®* and Eichenberg et al.[6] for the broth microdilution method. *M. pachydermatis* was classified as susceptible (S), intermediary susceptible (I) and resistant (R) using the criteria: S = MIC sample ≤ MIC50, I = MIC50 < MIC sample ≤ MIC90, R = MIC sample > MIC90.

## Results and Discussion

The MICs averages observed between the 17 samples evaluated simultaneously with ketoconazole and using the two methodologies were 0.103mg/mL to ETEST and 0.0012mg/mL to Broth Microdilution. The mean MIC presented difference between the two methods, ranging from 0.004 to 0,75mg/mL in the ETEST and from 0.0019 to 0.03mg/mL in BM, using the same *M pachydermatis* samples.

Comparing the results obtained with the two methodologies, it can be possible to observe that by the ETEST, two (11.8%) samples were resistant, eight (47.1%) susceptible and seven (41.1%) samples presented intermediate susceptibility. Using the BM method, it was observed four (23.5%) resistant samples, seven (41.2%) susceptible ones and six (35.3%) samples with intermediate susceptibility front the same drug. However, despite the percentages being similar in each rank of susceptibility based on the two methods studied, the results do not correspond to the same for each sample, as can be observed in the table 1.

Considering the samples with similar results (S, I, R) in the both methodologies tested, it was observed an agreement in six (35.3%) samples. Four samples with corroborating results were susceptible and two presented intermediate susceptibility (Table 1).

Independently of the method chosen for the determination of the antifungal MIC, different conditions of achievement of the test alter the final results[23]. Galgiane et al.[28] and Calhoum et al.[29] showed that the variability of MIC results obtained with anphotericin B, flucitosin and ketoconazole for yeasts studied in different conditions, should not be accepted; such difference was attributed to the absence of standardization of the method and the factors recognized as responsible for this variability of the results obtained in different laboratories such as: composition of the cultivation medium, pH, time and temperature of inoculation, size of the inoculum and reading criteria[4]. The culture medium employed in methods of susceptibility to antimicrobials should be capable of support an adequate growth of the microorganisms without, however, because any interaction with the activities of the drugs used in the study.[30]

**Table 1** - Susceptibility to ketoconazole in Microdilution broth technique and ETEST front 17 *M. pachydermatis* isolates from ear canal of dogs obtained in the Faculdade de Veterinária (UFPel) in 2007

| | | **Microdilution broth technique** | | | |
|---|---|---|---|---|---|
| | | **I** | **R** | **S** | **Total** |
| **ETEST** | **I** | 2 | 3 | 2 | 7 |
| | **R** | 1 | 0 | 1 | 2 |
| | **S** | 3 | 1 | 4 | 8 |
| | **Total** | **6** | **4** | **7** | **17 (100%)** |

I – Intermediary susceptibility; R – Resistant; S - Susceptible

Nascente et al.[7] observed that KTZ MICs for *Etest®* ranged from 0.002 to 0.25mg/mL and the MIC average was 0.057mg/mL. The broth microdilution method showed a MIC ranging from 0.03 to 8mg/mL and a MIC average of 1.28mg/mL. The MIC results of the *Etest®* obtained in this study had a wide range of higher values and an average MIC higher than that reported by Coutinho and Paula[9]. These authors[9] found a MIC average of 0.08mg/mL (0.015 to 0.25 mg/mL) and MIC values lower than the data obtained by Uchida, Nakade and Kitazawa[31] that ranged from 0.002mg/mL to 10mg/mL, but similar to the values obtained by Lorenzini, Mercantini and Bernardis[32] and Mickelsen et al.[33].

The concentrations of the antifungal drugs tested were different for each of the two techniques and for this reason it was not possible to estimate whether there was a coincidence in the MICs found in each of the tests alone. It was possible to establish relations between the values just after classifying the yeast isolates in susceptible, intermediary susceptible, and resistant using the susceptibility calculation (Table 1).

The differences between the methods did not allow an analysis-in-depth of the results because the MIC test is highly dependent of factors such as the inoculum concentration, chemical composition of the medium, pH, temperature, and incubation time.[6,7,16,28,30,34] The results found in this work showed that it is possible to compare the degree of susceptibility to each antifungal drug but without compare the MICs. To compare the MIC values, it is necessary an agreement for the drug concentrations to be tested in both methods because the *Etest®* have a broader range for drug concentrations than the broth Microdilution test.

## Conclusion

The aspects related to the different methods indicate the necessity of a standardized method that could be widely used in research and in mycology laboratories as in the same manner as in bacteriology. More studies about the MICs of *M. pachydermatis* are necessary to standardize the values for growth inhibition of the yeast in both methods, allowing comparisons of results obtained from different laboratories.

## Comparação da técnica de microdiluição em caldo e ETEST para o cetoconazol frente à *Malassezia pachydermatis*

## Resumo

*Malassezia pachydermatis* é reconhecida como um habitante normal e patógeno oportunista do meato acústico externo e da pele de cães e gatos. Em condições clínicas especiais e em casos de falha terapêutica relatada em otite externa e dermatite complicada por esta levedura, é recomendado o teste de suscetibilidade antifúngica. Existem diferentes métodos de avaliação da suscetibilidade da levedura frente a antifúngicos em laboratório, alguns métodos comerciais e outros previamente padronizados pelo CLSI (NCCLS, 2002). O objetivo deste estudo foi o de avaliar a suscetibilidade de 17 amostras de M. pachydermatis proveniente de otite externa canina por meio de duas técnicas *in vitro* de antifungigramas: o E*test®* e a microdiluição em caldo (MC) com o cetoconazol. A media da Concentração Inibitória Mínima (CIM) observada entre as 17 amostras foram 0.103mg/mL para o ETEST e 0.0012mg/mL para a MC variando de 0.004 a 0.75mg/mL no ETEST e entre 0.0019 e 0.03mg/mL na MC usando as mesmas amostras. Pelo ETEST, duas (11.8%) amostras foram resistentes, oito (47.1%) sensíveis e sete (41.1%) mostraram

**Palavras-chave:**
*Malassezia pachydermatis*.
CLSI.
ETEST.
Microdiluição em caldo.

sensibilidade intermediária. Na MC foram observadas quatro (23.5%) amostras resistentes, sete (41.2%) sensíveis e seis (35.3%) amostras com sensibilidade intermediária. Apesar das porcentagens de sensibilidade serem semelhantes pelas duas técnicas, os resultados de CIM não correspondem na uma mesma amostra. Estes resultados mostraram que há uma urgente necessidade de padronização dos valores considerados como parâmetros para inibição do crescimento da levedura. Portanto, um método simples e eficiente deveria ser usado rotina na prática de laboratório.

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