

EVALUATION OF *MALASSEZIA PACHYDERMATIS* ANTIFUNGAL SUSCEPTIBILITY USING TWO DIFFERENT METHODS

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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 especial clinical conditions, and mainly in the cases of therapeutic failure related to external otitis and dermatitis complicated by this yeast, it is recommended test susceptibility to antifungal drugs. The purpose of this work was to evaluate the susceptibility of 44 isolates of *M. pachydermatis* from the external ear canal and skin of dogs and cats using two different *in vitro* antifungal susceptibility methods: the *Etest*® and the broth microdilution method. Thirty-five samples were tested using the *Etest*®, twenty-four samples were tested using the broth microdilution method and fifteen samples were tested using both tests. The antifungal agents used were ketoconazole (KTZ), fluconazole (FLZ) and itraconazole (ITZ). In the broth microdilution method the yeast was less susceptible to ITZ while KTZ had the strongest activity. On the other hand, the *Etest*® showed that *M. pachydermatis* was more susceptible to ITZ while the less active drug was FLZ. The simultaneous evaluation using both methods identified FLZ as the antifungal drug with the highest activity (64.3%), followed by KTZ (57.1%) and ITZ (28.6%). These results showed that there is an urgent need to standardize of the values considered as parameters for growth inhibition of this yeast so a simple and efficient method can be used routinely in the laboratory practice.

Key words: *Malassezia pachydermatis*, *Etest*®, broth microdilution method, ketoconazole, itraconazole, fluconazole.

INTRODUCTION

Malassezia pachydermatis is recognized as a normal inhabitant and an opportunistic pathogen of the external ear canal and skin of dogs and cats, also can be found in the rectum, interdigital, anal sacs and vagina (3,22,24). The identification of this yeast as a common agent of mycosis of dogs and cats (4,9,14,22) make it necessary to test the antifungal susceptibility of the isolates to the drugs currently used for its treatment (1,5,21,22) which are itraconazole, fluconazole and, mainly ketoconazole.

The broth microdilution method, and more recently, the *Etest*® are recommended for testing antifungal susceptibility of yeasts like *Candida* sp. and *Cryptococcus* sp., but there are few studies dealing with susceptibility of isolates of *M.*

pachydermatis commonly used with broth macrodilution and agar gel diffusion methods (2,6,16,17,18,25). *Etest*® (13) and broth microdilution methods (23) were already used for testing *M. pachydermatis* but there are no data comparing the performance of both methods.

The purpose of this work was to evaluate the antifungal susceptibility of 44 isolates of *M. pachydermatis* to ketoconazole, fluconazole and itraconazole using *Etest*® (13) and broth microdilution methods (23).

MATERIALS AND METHODS

The isolates were obtained from the external acoustic meatus and skin of dogs and cats and were cultivated in

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Sabouraud's agar-dextrose with chloramphenicol and cycloheximide. The tests were performed at the mycology laboratory of the Faculdade de Veterinária da Universidade Federal de Pelotas, Pelotas, Brazil. Antifungal susceptibility of *M. pachydermatis* against ketoconazole (KTZ), fluconazole (FLZ) and itraconazole (ITZ) was determined by *Etest*® (35 isolates), broth microdilution (24 isolates) and both methods (15 isolates).

The strips of the *Etest*® have a defined and consistent gradient of the antifungal drug of 0.002 to 32.0 µg/mL to KTZ and ITZ and 0.016 to 256.0 µg/mL to FLZ allowing a quantitative reading. The culture medium used was Sabouraud dextrose agar with chloramphenicol according to the *Etest*® manufacturer recommendations. *M. pachydermatis* isolates 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 min, the strips were distributed over the medium and incubated at 37°C. The readings were performed at 48, 72 and 96 h and the MICs (Minimum Inhibitory Concentrations) the lowest concentration of the drug was determined by the *Etest*® strip pattern.

The NCCLS' broth microdilution method (23) recommendations were adapted by Eichenberg (10,11) for *M. pachydermatis*. Ten dilutions of each 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 64 to 0.125 µg/mL to FLZ, 4 to 0.078 µg/mL to ITZ and 8 to 0.015 µg/mL to KTZ and they were put into the first 10 contiguous wells of a sterile microtiter plate. The solution containing each isolate was transferred in aliquots of 100 µL into each well of the sterile plates that already had 100 µL of the solution containing the antifungal drug tested. The wells 11 and 12 had the positive control (100 µL of Sabouraud dextrose agar and 100 µL of the half-inoculum solution) and the negative control (200 µL of the same culture medium). The plates were incubated at 37°C for 96 h. The readings were made by visually comparing the growth of the yeast on 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 growth of the yeast observed for the positive control was identified as the MIC of the drug (10,11).

The MICs were calculated according Coutinho (7,8) for the *Etest*® and Eichenberg (10,11) for the broth microdilution method. *M. pachydermatis* was classified as susceptible (S), intermediary susceptible (I) and resistant (R) using the criteria established by Colombo *et al.* (7) such as: S = MIC sample ≤ MIC₅₀, I = MIC₅₀ < MIC sample ≤ MIC₉₀, R = MIC sample > MIC₉₀. The results were codified and analyzed using the software Statistix 7.0 (27). The frequency distribution and the chi-square test were applied to evaluate the two *in vitro* antifungal susceptibility methods.

RESULTS AND DISCUSSION

The range for *Etest*® MICs to KTZ was 0.002 to 0.25 µg/mL and the mean MIC was 0.057 µg/mL while the broth microdilution method showed a MIC ranging from 0.03 to 8 µg/mL and the mean MIC of 1.28 µg/mL. The results of the *Etest*® obtained in this work for the MICs had a broader range, higher values and an average MIC higher than the values reported by Coutinho (7,8). This author found a mean MIC 0.08 µg/mL (0.015 to 0.25 µg/mL) and MIC values lower than the data obtained by Uchida *et al.* (28) that ranged from 0.002 µg/mL to 10 µg/mL, but similar to the values obtained by Lorenzini *et al.* (17), Mickelsen *et al.* (20).

Regarding to FLZ, the *Etest*® had a mean MIC of 21.24 µg/mL (0.016 to 256 µg/mL) and the broth microdilution method showed a mean MIC of 9.22 µg/mL (0.5 to 64 µg/mL). Using the *Etest*®, the values obtained were lower than the values of Coutinho (7,8) that found a mean MIC of 37.47 µg/mL (8 to 256 µg/mL). In addition, the broth microdilution method provided average MIC values higher than the value of 9.22 µg/mL (1 to 32 µg/mL) obtained by Eichenberg (10,11).

ITZ tested by *Etest*® presented mean MIC = 0.073 µg/mL (0.002 to 2 µg/mL) and using the broth microdilution method the mean MIC = 0.62 µg/mL (0.03 to 4 µg/mL). Those values were higher than average MIC reported by Coutinho (7,8) that used the *Etest*® and found 0.004 µg/mL (0.002 to 0.008 µg/mL). However, Eichenberg (10,11) found an average MIC of 0.05 µg/mL (0.007 to 0.125 µg/mL).

The concentrations of the antifungal drugs used 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 described above (Table 1).

Comparing the MICs obtained in this work with the results of Coutinho (7,8) and Eichenberg (10,11) there is little similarities among the results. At this moment, it is only possible to evaluate what drug is more efficient for an isolate in a specific method but it is not possible to compare susceptibilities (MICs) using different methods.

In the *Etest*®, ITZ had the highest number of susceptible isolates (82.9%) followed by KTZ (71.4%) and FLZ (60%). According to the broth microdilution test, the most efficacious drug was KTZ (70.8%), followed by FLZ (62.5%) and ITZ (41.7%). The percentage of susceptible samples to FLZ and KTZ using both methods were similar, but there was a great divergence among those results to ITZ. Using this approach, the fungus *Malassezia pachydermatis* was more susceptible ($p < 0.05$) to ITZ using the *Etest*® while there was no difference ($p > 0.005$) between the two different methods to FLZ and KTZ.

Table 1. MICs to ketoconazole (KTZ), fluconazole (FLZ) and itraconazole (ITZ) to evaluate the susceptibility of *M. pachydermatis* isolated from the external ear canal and from skin of dogs and cats using two different *in vitro* antifungal susceptibility methods: the *Etest*® and the broth microdilution method (BM).

Antifungal	ETEST (µg/mL)			T	BM (µg/mL)			T
	S	I	R		S	I	R	
	MIC n (%)	MIC n (%)	MIC n (%)		MIC n (%)	MIC n (%)	MIC n (%)	
KTZ	≤ 0.064 25 (71.4)	0.094 3 (8.6)	> 0.094 7 (20)	35	≤ 0.125 17 (70.8)	0.25 - 0.50 3 (12.5)	> 0.5 4 (16.7)	24
FLZ	≤ 16 21 (60)	24 - 64 6 (17.1)	> 64 8 (22.9)	35	≤ 2 15 (62.5)	4 - 8 6 (25)	> 8 3 (12.5)	24
ITZ	≤ 0.003 29* (82.9)	0.004 - 0.016 4 (11.4)	> 0.016 2 (5.71)	35	≤ 0.125 10* (41.7)	0.25 - 0.50 10 (41.7)	> 0.5 4 (16.7)	24

S = susceptible, I = intermediary susceptible, R = resistant; n = absolute number, (%) = percentage, T = number of samples; > = larger than, ≤ = smaller than and/or equal to; * Statistically different (P<0,05).

Also, there was no difference (p>0.005) among the activity of the three different drugs used when they were evaluated using the same method. In opposition to it, Lorenzini *et al.* (17) and Uchida (28), who tested respectively 5 and 10 antifungal drugs against this yeast, concluded that KTZ is the most active antifungal drug.

In the 15 isolates evaluated by both methods, it was possible to observe the percentage of results that were susceptible or resistant in the same sample (Table 2). Nonetheless, it was not possible to compare the MIC of the isolates in each test due to the difference in the pattern of the interval of the concentrations. The mean MIC to KTZ, FLZ and ITZ observed in these 15 isolates were respectively 0.059 µg/mL, 16.99 µg/mL and 0.0027 µg/mL using the *Etest*® and 0.70 µg/mL, 2.43 µg/mL and 0.38 µg/mL using the broth microdilution method (MC). The means MIC had different values to the same drug when comparing the two methods although there was agreement for the increasing values for MICs following the order ITZ, KTZ and FLZ. The results obtained to KTZ agreed in 57.1% (8) of the samples, 64.3% (9) to FLZ and, just 28.6% (4) to ITZ. In the reviewed literature, there no any study comparing two different *in vitro* antifungal susceptibility methods for *Malassezia pachydermatis*. However, a similar study applying the same two methods was reported by Martin-Mazuelos *et al.* (19) using *Candida spp* and testing FLZ and ITZ, whit FLZ having highest agreement (74.5% of the samples).

The differences between the methods did not allow a deep analysis of the results because the MIC test is highly dependent of factors such as the inoculum concentrations, chemical composition of the medium, pH, temperature, and incubation time (7,8,12,15,26). The results found in this work showed that it is possible to compare the degree of susceptibility to each antifungal

Table 2. Susceptibility of *Malassezia pachydermatis* isolated from the external ear canal and from skin of dogs and cats tested simultaneously against ketoconazole (KTZ), fluconazole (FLZ), and itraconazole (ITZ) using *Etest*® and broth microdilution method (BM).

Antifungal	ETEST			BM		
	S n (%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)
KTZ	11 (73.3)	0	4 (26.7)	12 (80)	2 (13.3)	1 (6.7)
FLZ	10 (66.7)	1 (6.7)	4 (26.7)	13 (86.7)	2 (13.3)	0
ITZ	12* (80)	3 (20)	0	3* (20)	10 (66.7)	2 (13.3)

S = susceptible, I = intermediary susceptible, R = resistant; n = absolute number, (%) = percentage.

drug but without comparing the MICs. To compare the MICs, it is necessary an agreement for the drug concentrations to be used 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, to allow comparisons of results obtained from various laboratories.

RESUMO

Avaliação da sensibilidade da *Malassezia pachydermatis* frente a antifúngicos através de duas técnicas

Malassezia pachydermatis é considerada um habitante normal e patógeno oportunista do meato acústico externo e tegumento cutâneo de cães e gatos. Em condições clínicas especiais e nos casos de fracasso terapêutico, comum em casos de otite externa ou dermatite complicadas por esta levedura, é recomendado testar a sensibilidade aos antifúngicos. O objetivo do trabalho foi avaliar a sensibilidade de 44 isolados de *M. pachydermatis* do meato acústico externo e do tegumento cutâneo de cães e gatos, através de duas técnicas de antifungograma: ETEST e Microdiluição em Caldo (MC). Foram testadas 35 amostras pelo método ETEST (1994) e 24 pelo método de MC e 15 pelos dois testes. Os antifúngicos utilizados foram: cetoconazol (KTZ), fluconazol (FLZ) e itraconazol (ITZ). Através da MC, a levedura foi menos sensível ao ITZ, enquanto que o antifúngico com maior atividade foi o KTZ. *M. pachydermatis* foi mais sensível ao ITZ pelo ETEST, enquanto a droga menos ativa foi o FLZ. A avaliação simultânea através das duas técnicas aponta o FLZ como o antifúngico com maior concordância entre os resultados (64,3%), seguido pelo KTZ (57,1%) e ITZ (28,6%). Os resultados indicam que há uma necessidade urgente de padronização dos valores de inibição do crescimento desta levedura para que um método, simples e eficaz, possa ser amplamente utilizado na rotina laboratorial.

Palavras-chave: *Malassezia pachydermatis*, ETEST, antifungograma, Microdiluição em caldo, cetoconazol, itraconazol, fluconazol.

REFERENCES

- Alves, S.H.; Lopes, J.O.; Cury, A.E. Teste de suscetibilidade aos antifúngicos: por que, quando e como realizar. <http://www.newslab.com.br/antifung.htm>, 1999.
- Andrews, J.M.; Wise, R. Comparison of the Etest with a conventional agar dilution method in evaluating the *in vitro* activity of moxifloxacin. *J. Antimicrob. Chemother.*, 45:257-258, 2000.
- Bond, R.; Saijonmaa-Koulimies, L.E.M.; Lloyd, D.H. Population sizes and frequency of *Malassezia pachydermatis* at skin and mucosal sites on healthy dogs. *J. Small Anim. Pract.*, 36:147-150, 1995.
- Carlotti, D.N. Canine and feline superficial fungal skin infections. *Vet. Q.*, 19(Suppl.1):45-46, 1997.
- Casali, A.K. *Composição lipídica e susceptibilidade a drogas antifúngicas de diferentes amostras de Sporothrix schenckii* Belo Horizonte, 1995 (Tese de Mestrado. Faculdade de Veterinária. Universidade Federal de Minas Gerais).
- Colombo, A.L.; Barchiesi, F.; McCough, D.A.; Rinaldi, M.G. Comparison of Etest and National Committee for clinical laboratory standards broth microdilution method for azole antifungal susceptibility testing. *J. Clin. Microbiol.*, 33:535-540, 1995.
- Coutinho, S.D.A. *Malassezia pachydermatis: caracterização fenotípica de amostras isoladas de pelame e meato acústico externo de cães*. São Paulo, 1997, 108p. (Tese de Doutorado. Instituto de Ciências Biomédicas da Universidade de São Paulo).
- Coutinho, S.D.; Paula, C.R. Suscetibility to antifungal agents of *Malassezia pachydermatis* isolates from dogs. *Pol. J. Vet. Sci.*, 4(3):77-81, 2001.
- Duffait, R. Über die Bedeutung von *Ptyrosporium canis* bei otitis externa und Dermatitis des Hundes. *Kleintierpraxis*, 23:29-32, 1978.
- Eichenberg, M.L. *Susceptibilidade antifúngica da Malassezia pachydermatis isolada de cães com otite externa através do método de microdiluição em caldo*, Porto Alegre, 2000. (Dissertação de Mestrado. Universidade Federal do Rio Grande do Sul).
- Eichenberg, M.L.; Berg, V.; Appelt, C.E.; Muschner, A.C.; Nobre, M.O.; Matta, D.; Alves, S.H.; Ferreira, L. Susceptibility of *Malassezia pachydermatis* to azole antifungal agents evaluated by a new Microdilution method. *Acta Scientiae Veterinariae*, 31(2):000-000, 2003.
- Espinell-Ingroff, A.; Pfaller, M.; Erwin, M.E.; Jones, R.N. Interlaboratory evaluation of ETEST method for testing antifungal susceptibilities of pathogenic yeasts to five antifungal agents by using casitone agar and solidified RPMI 1640 medium with 2% glucose. *J. Clin. Microbiol.*, 848-852, 1996.
- ETEST Technical Guide 4b, *Antifungal susceptibility testing of yeasts*. AB BIODISK, June 1994, Solna, Sweden.
- Guillot, J.; Bond, R. *Malassezia pachydermatis*: a review. *J. Mycol. Med.*, 37:295-306, 1999.
- Hammer, K.A.; Carson, C.F.; Riley, T.V. *In vitro* activities of ketoconazole, econazole, miconazole and *Melaleuca alternifolia* (Tea tree) oil against *Malassezia* species. *Antimicrob. Agents Chemother.*, 44:467-469, 2000.
- Gupta, A.K.; Kohli, Y.; Faergemann, J.; Summerbell, R.C. *In vitro* susceptibility of seven *Malassezia* species to ketoconazole, voriconazole, itraconazole and terbinafine. *Br. J. Dermatol.*, 142(4):758-765, 2000.
- Lorenzini, R.; Mercantini, R.; Bernardis, F. *In vitro* sensitivity of *Malassezia* spp to various antimycotics. *Drugs Exptl. Clin. Res.*, 11:393-395, 1985.
- Maestrone, G.; Thompson, E.; Yeisley, H.; Mitrovic, M. *In vitro* activity of antimicrobial agents against *Pityrosporium canis*. *Vet. Med. Small Anim. Clin.*, 1681-1683, 1976.
- Martin-Mazuelos, E.; Gutierrez, M.J.; Aller, A.I.; Bernal, S.; Martinez, N.A.; Monteiro, O.; Quindos, G. A comparative evaluation of ETEST and broth microdilution methods for fluconazole and itraconazole susceptibility testing of *Candida* spp. *J. Antimicrobiol. Chemother.*, 43:477-481, 1999.
- Mickelson, P.A.; Viano-Paulson, M.C.; Stevend, D.A.; Diaz, P.S. Clinical and microbiological features of infection with *Malassezia pachydermatis* in high-risk infants. *J. Infect. Dis.*, 157:1163-1168, 1988.
- Moriello, K.A. Ketoconazole: clinical pharmacologic and therapeutic recommendations. *J. Am. Vet. Med. Assoc.*, 188:303, 1986.
- Nascente, P.S. *Malassezia pachydermatis em cães e gatos: estudo da frequência e avaliação da sensibilidade aos antifúngicos cetoconazol, fluconazol e itraconazol*. Pelotas, 2001, 84p. (Dissertação de Mestrado. Faculdade de Veterinária. Universidade Federal de Pelotas).
- NCCLS - *National Committee for Clinical Laboratory Standards*. Reference method for broth dilution antifungal susceptibility testing of yeasts: proposed standard. M27, 1997.
- Nobre, M.O.; Meireles, M.C.A.; Gaspar, L.F.; Pereira, D.; Schramm, R.; Schuch, L.F.; Souza, L.; Souza, L. *Malassezia pachydermatis* e outros agentes infecciosos nas otites externas e dermatites em cães. *Ciência Rural*, 28(3):447-452, 1998.
- Pereira, S. A. *Avaliação da sensibilidade de Malassezia pachydermatis aos antifúngicos cetoconazol, miconazol e nistatina*. Itaguaí, 2000. 70p. (Dissertação de Mestrado. Instituto de Veterinária. Universidade Federal Rural do Rio de Janeiro).
- Richardson, M.D.; Warnock, D.W. Fungal infection – Diagnosis and management. *Antarct. Sci.*, 3:17-43, 1993.
- STATISTIX. Statistix 7 User's manual. Analytical software. 359, 2000.
- Uchida, Y.; Nakade, T.; Kitazawa, K. *In vitro* activity of five antifungal agents against *Malassezia pachydermatis*. *Nippon Juigaku Zasshi.*, 52(4):851-853, 1990.