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# Susceptibility of dermatophytic fungi to commonly used disinfectants*

## Suscetibilidade de fungos dermatófitos a desinfetantes comumente utilizados

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### Abstract

This study aimed evaluate the antidermatophytic activity of three commercial disinfectants commonly used for environmental control of microorganisms in veterinary medicine. Sodium hypochlorite at 40 µL/mL, chloro-phenol derived at 30 µL/mL and chlorhexidine digluconate at 66.7 µL/mL were tested against 14 strains of dermatophytes, identified as *Microsporum canis* (*n:* 3) and *Microsporum gypseum* (*n:* 11). The tests was performed in accordance with guidelines of the Clinical and Laboratory Standards Institute (CLSI), documents M38-A2 and M51-A, adapted to disinfectants. In the microdilution broth test, chlorhexidine digluconate had MIC values (Minimum Inhibitory Concentration) of 4.16 µL/mL and MCF (Minimum Fungicidal Concentration) from 4.16 to 8.33 µL/mL, while chloro-phenol derived obtained MIC and MCF of 1.87 µL/mL, and both disinfectants had fungicidal activity at concentrations below the recommended. Sodium hypochlorite obtained MIC from 10 to 80 µL/mL and MFC of 40 to 80 µL/mL, requiring at most isolates twice the recommended concentration to achieve same activity. In the disc diffusion test, the mean inhibition zones for chlorhexidine digluconate was 10.53 mm, for chloro-phenol of 9.9 mm and for sodium hypochlorite was 6.2 mm. Chlorhexidine digluconate and chloro-phenol presented a significant reduction in the growth of dermatophytes, while sodium hypochlorite in concentration recommended showed a low antifungal activity against tested isolates.

*Keywords*: Antidermatophytic activity, Disinfection, Microdilution broth technique, Disc diffusion.

### Resumo

O objetivo do estudo foi avaliar a atividade antidermatofítica de três desinfetantes comerciais frequentemente utilizados no controle ambiental de micro-organismos em medicina veterinária. Hipoclorito de sódio a 40 µL/mL, derivado de clorofenol a 30 µL/mL e digluconato de clorexidine a 66.7 µL/mL foram testados contra 14 cepas de dermatófitos, identificados como *Microsporum canis* (*n*: 3) e *Microsporum gypseum* (*n*: 11). Foram utilizadas as diretrizes do Clinical and Laboratory Standard Institute (CLSI), documentos M38-A2 e M51-A, com adaptações para desinfetantes. Na microdiluição em caldo, digluconato de clorexidine apresentou valores de CIM (Concentração Inibitória Mínima) de 4.16 µL/mL e CFM (Concentração Fungicida Mínima) entre 4.16 a 8.33 µL/mL; derivado de clorofenol obteve CIM e CFM de 1.87 µL/mL, demonstrando que ambos os desinfetantes apresentaram atividade fungicida em concentrações inferiores às recomendadas. O hipoclorito de sódio demonstrou CIM entre 10 a 80 µL/mL e CFM de 40 a 80 µL/mL, requerendo duas vezes a concentração recomendada pelo fabricante para obter atividade fungicida frente a maioria dos isolados fúngicos testados. No teste de disco-difusão, a média das zonas de inibição do digluconato de clorexidine foi de 10.53 mm; do derivado clorofenol 9.9 mm e do hipoclorito de sódio 6.2 mm. O digluconato de clorexidine e o derivado cloro-fenol apresentaram redução significante no crescimento dos dermatófitos testados, enquanto o hipoclorito de sódio, na concentração recomendada, demonstrou baixa atividade antifúngica contra os dermatófitos testados.

*Palavras-chave*: Atividade antidermatofítica, desinfecção, técnica de microdiluição em caldo, disco-difusão.

### Introduction

Annually millions of superficial mycoses are diagnosed in humans and animals, most caused by *Trichopohyton* sp., *Epidermophyton* sp. and *Microsporum* sp. dermatophytes, a group of filamentous fungi that infect keratinized tissue (Grumbt et al., 2011). Dermatophytosis is a contagious and zoonotic dermatomycosis which leads to costly treatment, requiring control measures and prevention (Cafarchia et al., 2006; Chermette et al., 2008).

The control of this mycosis is hampered by the presence of arthroconidia in the environment for a long period of time (Rycroft and McLary, 1991; Chermette et al., 2008). Additionaly to treatment with antifungal medication, one of the most important

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measures in controlling dermatophytosis in veterinary cases is the environmental disinfection, failures in disinfection can result in chronic disease, infection and reinfection (Chermette et al., 2008).

However, there are few informations regarding the use of disinfectants in controlling dermatophytosis, and the dilution of 1:10 household bleach on nonporous surfaces is recommended (Moriello and Newbury, 2006). Chlorine at 1% had a high level disinfectant bringing about a rapid inactivation of *Trichophyton* species, followed by 5% phenol and 0,5% quartenary ammonium compounds (Gupta et al., 2001).

In Brazil, the analysis of the antifungal activity of chemicals disinfectants is not standardized, using different methods (Estrela et al., 2003; Menezes et al., 2008; Madrid et al., 2012) in accordance with the standards of Association of Official Analytical Chemists (AOAC) (Horwitz and Latmer Jr., 2010) and the National Health Surveillance Agency (ANVISA).

The objective of this study was evaluate the antidermatophytic susceptibility to three commercial disinfectants – sodium hypochlorite, chlorhexidine gluconate and chlorophenol derivated – commonly used for environmental control of dermatophytes in veterinary medicine.

## Materials and methods

Fourteen dermatophytes isolates of *Microsporum canis* (*n:* 3) and *Microsporum gypseum* (*n:* 11) previously isolated from clinical cases of feline and canine dermatophytosis were subcultured on Potato Dextrose Agar (PDA; Difco Laboratories, Detroit, MI, USA) and incubated at 25°C for seven days. Inoculum suspensions were obtained by scraping the agar surface with 1% Tween 20 in saline solution. The inoculum consisting of conidia and fungal hyphae was standardized at McFarland scale 1, at a final cell density of approximately 1 to 5 x $10^6$ CFU $ml^{-1}$.

For disinfectant tests, sodium hypochlorite solution at 4% (40 µL/mL) (QBoa® Indústria Anhembi S/A, São Paulo, Brazil), chlorhexidine digluconate at 6,6% (66,7 µL/mL) (Clorexidina-Cetrimida Chemitec®, Chemitec Agro-veterinária, São Paulo, Brazil) and chloro-phenol derivate at 3% (30 µL/mL) (Pinho Sol®, orto-benzil p-clorofenol 0.25%, Colgate-Palmolive Indústria e Comércio Ltd, São Paulo, Brazil) according to the manufacturer›s recommendations were evaluated using the disc diffusion method. Broth microdilution method was also utilized for test six dilutions in $\log_2$, varying from 2 to 0.062 times the concentrations recommended by the manufacturers.

The broth microdilution method was performed in accordance with Clinical and Laboratory Standard Institute (CLSI) guidelines, document M38-A2 (CLSI, 2008), with modifications adapted to disinfectants as follows. A fungal inoculum aliquot diluted in RPMI-1640 (Roswell Park Memorial Institute - Sigma Chemical Co., Steinheim, Germany) (1:50) buffered to a pH 7.0 with MOPS (3-morpholin-4-yl-propane-1-sulfonic acid) was added to each microdilution well containing the disinfectant previously diluted in RPMI-1640. The microplates were incubated at 25ºC for seven days. The Minimal Inhibitory Concentration (MIC) was defined as the lowest disinfectant concentration at which no growth could be seen. To determinate Minimal Fungicidal Concentration (MFC), 10 µl aliquots from each well were spread on SDA (SDA, Neogen Acumedia®, Michigan, USA) Petri dishes and incubated at 25°C until thirty days.

Disc diffusion assays were performed according to the guidelines provided by the CLSI document M51-A (CLSI, 2010), adapted to disinfectants as follows. Briefly, sterile filter papers discs (Whatman® Whatman International Ltd., number 1 with 5 mm diameter) were impregnated with the disinfectant solution and sterile distilled water (control). The discs were placed in duplicates on SDA added with chloramphenicol (Neogen Acumedia®, Michigan, USA) previously inoculated with 0.1 mL of each fungal isolate. The plate was incubated at 25ºC for seven days. The antifungal activity of each disinfectant was assessed by measuring the inhibition growth around the discs and compared to the control.

For MIC the average of duplicates from the reciprocal of the lowest dilution where there was inhibition of microbial growth was performed. The diffusion test result was obtained from the average of duplicates of each microorganism. The comparative analyses of the data were performed by the Student's t- test.

## Results and Discussion

The culture microdilution of the disinfectants chlorhexidine digluconate and chloro-phenol derivated showed MIC and MFC values below the concentration recommended by the manufacturer. Chlorhexidine digluconate reached 0.41% (4.16 µL/mL) of MIC and MFC varied from 0.41% to 3.3% (4.16-8.33 µL/mL) for all isolates, presenting fungicidal action at half the recommended concentration. Chloro-phenol derivated presented MIC value 0.18% (1.87 µL/mL) for all isolates and MFC 0.18% (1.87 µL/mL) having fungicidal activity with 1/8 of the recommended concentration.

Sodium hypochlorite had the worst performance, requiring twice the recommended concentration to have antifungal activity. It presented MIC ranging from 1 to 8% (10- 80 µL/mL) remaining above the recommended concentration in 40% (*n:* 6) of the isolates tested, the MFC ranged from 4 to 8% (40-80 µL/mL) in 66% (*n:* 10) of the isolates (Table 1).

**Table 1**: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) in µL/mL of chlorhexidine digluconate, sodium hypochlorite and chloro-phenol derivated in dermatophytes isolates

| Species | Chlorhexidine Digluconate at 66.7 µL/mL* | | Sodium Hypochlorite at 40 µL/mL* | | Chloro-phenol Derivated at 30 µL/mL* | |
|---|---|---|---|---|---|---|
| | MIC | MFC | MIC | MFC | MIC | MFC |
| *M. canis* | ≤4.16 | 4.16-8.33 | 10-40 | 40-80 | 1,87 | 1,87 |
| *M. gypseum* | ≤4.16 | 4.16-16.67 | 10 ≥80 | 40 ≥80 | 1,87 | 1,87 |

*Concentration recommended by the manufacturer.

In disc diffusion significant difference was observed ($p<0.05$) in fungicidal activity between the disinfectants. The highest inhibition zones means were chlorhexidine digluconate (10.53 mm) and chloro-phenol derivated (9.9 mm), while sodium hypochlorite had the lowest (2.06 mm). No zones of inhibition were observed on the control discs (Table 2).

**Table 2**: Average diameter (mm) of the inhibition zone of fungal growth in the disc diffusion test

| Species | Chlorhexidine Digluconate mean±SD | Sodium Hypochlorite mean±SD | Chloro-phenol Derivated mean±SD | Control |
|---|---|---|---|---|
| *Microsporum canis* | 10 ± 0.6 | 7 ± 4.0 | 12 ± 0 | 0 |
| *Microsporum gypseum* | 14.3 ± 5.5 | 8 ± 2.0 | 11.4 ± 1 | 0 |

SD: standard deviation.

In isolates of *M. gypseum*, highly significant differences were found in antifungal activity of chlorhexidine digluconate and chloro-phenol derivated in relation to sodium hypochlorite. In isolates of *M. canis*, only chloro-phenol derivated and sodium hypochlorite differed significantly. However, chlorhexidine digluconate and chloro-phenol derivated had equal antifungal activity in all fungal species evaluated (Table 3).

**Table 3**: Comparative analysis of the disinfectants in the disc diffusion among the species *Microsporum canis* and *Microsporum gypseum*, by Student's t test

| Disinfectants | *Microsporum canis* | *Microsporum gypseum* |
|---|---|---|
| /CD-SH/ | 5.67 (p= 0.0740)[2] | 12.81 (p=0.0001)[1] |
| /CD-CPD/ | 1.17 (p=0.0572)[2] | 3.12 (p=0.1526)[1] |
| /SH-CPD/ | 6.83 (p= 0.0037)[1] | 9.68 (p= 0.0000)[2] |

CD: chlorhexidine digluconate, SH: sodium hypochlorite, CPD: chloro-phenol derivate, / /: Value of the difference between disinfectants expressed in module. [1] : Heterogeneous Variance by F test at 5% probability, [2] : Homogeneous variance by F test at 5% probability.

The microdilution culture and disc diffusion techniques showed similar results regarding the fungicidal activity of the three disinfectants tested. In both used techniques the sodium hypochlorite demonstrated the worst performance as a fungicidal agent.

Studies on the effect of disinfectants on fungal organisms are limited and most often involve yeast (McDonnell and Russel, 1999; Bambace et al., 2003) with limited information on filamentous fungi (Xavier et al., 2007; Menezes et al., 2008). It may still be considered the occurrence of response variations of micro-organisms to disinfectants and that filamentous fungi are more resistant to disinfectants than yeasts and considerably more resistant than non sporulated bacteria (Russel, 2003).

Chlorhexidine digluconate was effective against dermatophytes in lower values of MIC and MFC (4.16 µL/mL). Similar research using this disinfectant at 66.7 µL/mL against clinical and environmental isolates of *Sporothrix schenckii*, dimorphic fungus, obtained inhibitory effect at concentrations below 0.8% (Madrid et al., 2012). The efficacy of chlorhexidine at 4.16 µL/mL was previously demonstrated in filamentous fungi of the genera *Aspergillus* (Xavier et al., 2007; Xavier et al., 2008) and using a 1% aqueous solution of chlorhexidine against yeasts for the disinfection of surfaces using the technique spray wipe spray (Bambace et al., 2003).

The best performance of the chlorhexidine digluconate in relation to sodium hypochlorite in the broth microdilution is in accordance with the study (Menezes et al., 2008) that used sodium hypochlorite at 5.25% e chlorhexidine digluconate at 2% against *Candida albicans*. However, there is a report (Estrela et al., 2003) that both disinfectants at 2% were effective against *C. albicans*, with better performance in the sodium hypochlorite direct exposure test and of the chlorhexidine digluconate in the disc diffusion test.

The poor performance of the sodium hypochlorite in broth microdilution and disk diffusion tests was similar to that found in a study that evaluated the performance of the chlorhexidine digluconate and sodium hypochlorite in isolates of *S. schenckii* (Madrid et al., 2012). In a trial evaluating the efficacy of disinfectants in bacteria using the disk diffusion technique, the 0.5% chlorhexidine was effective, while sodium hypochlorite only had bactericidal activity at concentrations of 2% or more (Pedrini and Margatho, 2003).

However, it is considered that the growth inhibition zone on the disk diffusion test depends on the solubility and diffusivity of the tested substances, which may not express its full fungicide potential (Estrela et al., 2003). Thus, underperforming of the sodium hypochlorite can be partly explained by the acidity of the medium and instability of chlorine. However we take into account the ability of dermatophytes to raise the pH of the culture medium (Weitzman and Summerbel, 1995). Thus, sodium hypochlorite used in environment should have greater concentrations than that used for in vitro tests, due to the influences of external factors such as temperature, pH and the presence of organic matter (McDonnell and Russel, 1999).

Contrary to the findings, the solution of sodium hypochlorite at 1% associated with solution spray of enilconazole at 0.6% applied daily for 10 minutes was effective in controlling dermatophytosis in animal shelters (Carlotti et al., 2010). However, enilconazole is not available in Brazil. The same way, the 5% sodium hypochlorite solution was 100% fungicidal at a final dilution of 1:80, while chlorhexidine-diacetate at 2% was ineffective even when used at four times the manufacturer's recommended dilution (1:100) (Moriello et al., 2004). However, these results were obtained with techniques different from those used in this present study.

Despite the diversity of techniques and results on the fungicidal activity of chloro-phenol derivate, in our study, this disinfectant achieved satisfactory results, in accordance with previous published data which demonstrated that phenolic solution at 2% had fungicidal activity after 15 minutes interaction with the dermatophytes (Terleckyj and Axler, 1993). However, in an experiment using feline hair naturally infected by *M. canis*, chloro-phenol derivate had a low performance, whereas hypochlorite sodiumachieved better results (Rycroft and McLay, 1991). Similarly, 1% chlorine had a better performance against T. mentagrophytes than 5% phenol (Gupta et al., 2001).

## Conclusion

Chlorhexidine digluconate and chloro-phenol derivate had fungicidal activity against dermatophytes at concentrations below those recommended by the manufacturer. However, the most used and prescribed disinfectant in Brazil is hypoclorite sodium, that was ineffective against most dermatophytes tested, requiring concentrations two times above the recommended to achieve fungicidal activity. Techniques of disc diffusion and broth microdilution presented similar results. Therefore, it demonstrates the need for reassessment of the recommended concentrations as fungicide of the main disinfectants used in Brazil.

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