

A pilot study of the efficacy of wipes containing chlorhexidine 0.3%, climbazole 0.5% and Tris-EDTA to reduce *Malassezia pachydermatis* populations on canine skin

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Background – Wipes containing chlorhexidine and azole derivatives have been recommended for veterinary use. No study has been published about their activity against *Malassezia pachydermatis*.

Hypothesis/Objectives – To evaluate the *in vivo* and *in vitro* activity of wipes soaked in a chlorhexidine, climbazole and Tris-EDTA solution against *Malassezia pachydermatis*.

Animals – Five research colony shar-pei dogs.

Methods – Wipes were applied once daily onto the left axilla, left groin and perianal area (protocol A), and twice daily on the right axilla, right groin and umbilical region (protocol B) for 3 days. *In vivo* activity was evaluated by quantifying *Malassezia* colonies through contact plates on the selected body areas before and after wipe application. The activity of the solution in which the wipes were soaked was assessed *in vitro* by contact tests following the European Standard UNI EN 1275 guidelines.

Results – Samples collected after wipe application showed a significant and rapid reduction of *Malassezia* yeast CFU. No significant difference in the *Malassezia* reduction was found between protocols A and B. *In vitro* assay showed 100% activity against *Malassezia* yeasts after a 15 min contact time with the wipe solution.

Conclusions and clinical importance – Wipes containing chlorhexidine, climbazole and Tris-EDTA substantially reduced the *M. pachydermatis* population on the skin of dogs. The results, although this was an uncontrolled study performed on a small number of dogs, suggest that these wipes may be useful for topical therapy of *Malassezia* dermatitis involving the lips, paws, perianal area and skin folds.

Introduction

Malassezia pachydermatis is a lipophilic yeast that is part of the normal cutaneous microflora of many warm-blooded vertebrates. Alterations in the skin surface microclimate or host defence promote *Malassezia* proliferation.^{1,2} Given that *M. pachydermatis* is located on the stratum corneum, topical therapy may be sufficient to resolve clinical signs of infection.² Wipes soaked in a solu-

tion with antiseptic and antifungal agents have been recommended for veterinary use. To the best of the authors' knowledge no study has been published about their efficacy.

The aim of this study was to assess the *in vivo* and *in vitro* activity of commercial cotton wipes (CLX® Wipes, ICF; Cremona, Italy) against *M. pachydermatis* from naturally infected dogs. The wipes are soaked in a solution containing chlorhexidine digluconate 0.3%, climbazole 0.5%, zinc gluconate 1%, ethylene diamine tetra acetic acid-tromethamine (Tris-EDTA) with benzoyl alcohol, propylene glycol, ethoxylated isotridecanol and glycerin as excipients.

Material and Methods

Dogs

Five shar-pei dogs living in the kennel facility research, two males and three females, aged between 4 and 6 years were used. They showed an average ≥ 4 *Malassezia* yeasts in 10 microscopic fields, at

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×1000 magnification, using the tape strip technique on left and right axilla, left and right groin, umbilical region and the perianal area. The study was performed according to institutional animal welfare regulations. The Ecole Nationale Veterinaire d'Alfort Ethics Committee was consulted and the methods used in the present study were considered to cause neither discomfort nor pain to the dogs.

In vivo wipes activity

The wipes were applied once daily (09.00 h) on the left axilla, left groin and perianal area (protocol A), and twice daily (09.00 and 21.00 h) on the right axilla, right groin and umbilical region (protocol B) for three consecutive days. One wipe (21 cm × 29 cm) was scrubbed on each area for 30 s. The population size of *M. pachydermatis* was estimated using contact plates containing modified Dixon's medium.³ They were pressed on each site for 10 s before the first morning application and subsequently after 30 min, 3 h and 12 h. The same selected areas were sampled once daily for the following 3 days and 7 days after the last wipe application. Plates were incubated at 30°C for 3 days. *Malassezia* yeasts were identified by microscopic examination, using lactophenol cotton blue stain. *Malassezia* colonies were counted up to a maximum of 900 and results were reported as *Malassezia* colony forming units (CFU) values; if there were >900 CFU/plate, the presence of 1,000 UFC was considered.³

In vitro assay

The activity of the wipe solution (WS) in which the wipes are soaked and its dilutions 1/10, 1/100 and 1/1000 in sterile distilled water, against *M. pachydermatis* was evaluated, following the guidelines of the European regulation UNI EN 1275.⁴ A reference strain (*M. pachydermatis* CBS1879) and five isolates of yeast from the left axilla of dogs used in the *in vivo* study were tested. These isolates were cultured on Sabouraud's dextrose agar (Biolife; Milan, Italy) for 3 days at 30°C. After two subcultures the yeast colonies were diluted in distilled water with Tween 80 0.1% (Sigma-Aldrich; Milan, Italy). These test suspensions (TS) were standardized to $1.5\text{--}5.0 \times 10^7$ CFU/mL by a spectrophotometer at 630 nm (Ultraspec2000, Pharmacia Biotech; Milan, Italy). Two mL of the TS were added, respectively, to 8 mL of the WS and to 8 mL of sterile physiological solution used as a growth control. After fixed contact times (1, 5, 15 and 30 min), 1 mL of the TS/WS mixture was added to a neutralizing solution (lecithin 3 g/L; Tween 80.3%, Sigma-Aldrich) to suppress the fungicidal activity.⁴ Then 100 µL of the resulting suspension and 100 µL of the TS/WS mixture, without being neutralized, were placed onto Sabouraud's dextrose agar plates. After incubation at 30°C for 3 days the number of CFU per single plate was evaluated. According to the UNI EN 1275 guidelines, the WS and its dilutions were considered fungicidal if at least a four decimal log (i.e. 99.99%) reduction of the *Malassezia* yeast after 15 min contact time was observed.⁴ Two tests for each *Malassezia* strain were performed.

Data analysis

The percentage of CFU reduction between day one, T0, and different fixed times (FT) after wipe application was calculated as follows: % reduction of *Malassezia* count = [(Count at T0 – Count at FT)/Count at T0] × 100. The normality of the data was assessed by the Shapiro-Wilk test. The *post hoc* test after ANOVA with repeated measures was employed to evaluate the CFU reduction in protocols A and B. Wilcoxon rank-sum test with continuity correction was used to compare the CFU reduction in both protocols. All of the analyses were performed with R Core Team software (2014) (<http://www.R-project.org/>). A *P*-value < 0.05 was considered significant.

Results

In vivo wipes activity

The percentage of CFU reduction after wipe application is shown in Table 1. In both protocols, 30 min after the

Table 1. *In vivo* activity of the wipes: percentage of *Malassezia* CFU reduction: 30 min and 3 h after the application on the 3 days of wipe application (first, second & third day); 12, 24, 36, 48 and 60 h after the first wipe application; 1, 2, 3 and 7 days after the last wipe application. A = protocol A (once a day wipe application); B = protocol B (twice a day wipe application); WA = wipe application.

	After morning WA		After the first WA		After the last WA			
	A	B	A	B	A	B		
First day			12 h	71%	78%	1 day	96%	99%
30 min	66%	74%	24 h	83%	96%	2 days	97%	99%
3 h	70%	76%	36 h	98%	98%	3 days	98%	99%
Second day			48 h	98%	99%	7 days	94%	97%
30 min	88%	87%	60 h	99%	99%			
3 h	93%	78%						
Third day								
30 min	82%	79%						
3 h	79%	42%						

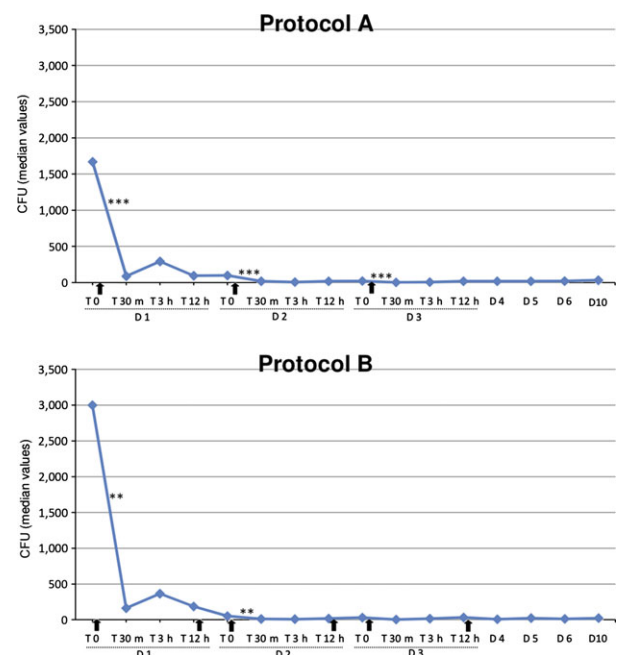


Figure 1. *Malassezia* CFU (median values) before and after wipes application at different sampling times. Protocol A: once a day wipe application. Protocol B: twice a day wipe application. D = days; h = hours; arrows = wipe applications. Stars indicate significant decreases of CFU compared with sampling immediately previous (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). CFU at all time points are significantly lower than CFU count at T0. Protocol A: all samples *P* < 0.001, except the sample collected after 3 h on D1, *P* < 0.05. Protocol B: all samples *P* < 0.001, except the samples collected after 30 min and 3 h on D1, *P* < 0.01 (*post hoc* test after ANOVA with repeated measures).

first wipe application, *Malassezia* CFU reduction was statistically significant compared with the initial value (Figure 1). *Malassezia* CFU values from all samples collected at different times during the wipe application days and from the samples collected within 3 days and 7 days after the last wipe application remained significantly lower than initial CFU values (Figure 1). No significant difference in the *Malassezia* reduction was found between protocols A and B (Wilcoxon rank-sum test:

$W = 3309.5$, $P = 0.71$). No adverse effects were noted except mild and transient erythema and pruritus at the sites of wipe application in one dog.

In vitro assay

The undiluted WS reduced viable *Malassezia* cells with a linear trend (Table 2). After one minute contact time the yeast reduction was between 25% and 53%, while after five minute contact time the percentage of decrease was >95%. After 15 min contact time the WS activity was complete with 100% reduction of all yeast strains. All dilutions of the WS showed poor efficacy in reducing *Malassezia* strains when the fungicidal activity was suppressed by the neutralizing solution at fixed contact times. Conversely, the 1/10 and 1/100 WS dilutions showed >99% reduction of all yeast isolates with prolonged contact time, i.e. when the fungicidal activity was not suppressed by the neutralizing solution.

Discussion

The present study demonstrated that once or twice daily applications of wipes soaked in antiseptic and antifungal agents are effective in reducing *M. pachydermatis* populations on canine skin. The *in vivo* activity of wipes was supported by *in vitro* tests. Wipes were quick and effective in reducing *Malassezia* yeasts on the skin of all naturally infected dogs. In both protocols, 30 min after wipe application there was already >60% *Malassezia* reduction. Both protocols resulted in a 99% reduction of *Malassezia* CFU: as soon as the third day under protocol A and as soon as the second day after application under protocol B, respectively.

Malassezia CFU decrease was observed during the 12 h following each wipe application and significantly reduced *Malassezia* populations were found within 3 and 7 days after the last wipe application. Residual antifungal activity may be suspected to explain this finding because residual antimicrobial activity of hair shafts after application of chlorhexidine shampoos and conditioner was previously demonstrated.⁵ Our *in vitro* data support this hypothesis because the WS, even after 1/10 and 1/100 dilutions, prevented the growth of *Malassezia* yeast when these were kept in prolonged contact with the active solution.

In the present study, fungal culture was chosen to assess the cutaneous *Malassezia* yeast population because it has higher sensitivity than cytological examination.⁶ Contact plates have been used to quantify *Malassezia* on skin areas.^{3,6} *In vitro* WS activity has been evaluated by contact tests, previously used to assess the efficacy of solutions against bacteria and yeasts.⁴ This approach takes into account the main factors which influence the efficacy of antimicrobial topical products, namely the product formulation effects and the duration of contact.⁷

Only six *M. pachydermatis* isolates were tested *in vitro*. This *in vitro* assay was performed to simulate the *in vivo* behaviour of *Malassezia* in contact with wipes on the cutaneous sites. There was no intention to perform an epidemiological study on *Malassezia* susceptibility to WS.

The wipes' activity is likely to be due to chlorhexidine, climbazole and Tris-EDTA. *In vitro* and *in vivo* 2%–4.5% chlorhexidine showed efficacy against *Malassezia* yeasts.^{1,2} *In vivo*, 3% chlorhexidine and 0.5% climbazole shampoo and, *in vitro*, a combination of Tris-EDTA and

Table 2. *In vitro* activity of the wipe solution and its dilutions against *Malassezia pachydermatis* (1–5: strains from the dogs used for *in vivo* assay; reference strain: CBS1879). Numbers indicate the percentage of CFU reduction (mean of two tests for each strain).

Strain	Wipe solution	Contact times					Prolonged
		1 min	5 min	15 min	30 min		
1	Undiluted	37.8	99.0	100	100	100	
	1/10	15.9	28.6	29.0	30.2	100	
	1/100	8.6	9.0	20.7	28.3	99.9	
	1/1000	1.2	3.4	9.4	10.1	11.2	
2	Undiluted	53.3	96.8	100	100	100	
	1/10	12.4	31.9	28.7	31.3	100	
	1/100	7.5	10.9	19.0	27.1	99.9	
	1/1000	2.4	4.5	7.3	5.3	10.4	
3	Undiluted	42.8	97.6	100	100	100	
	1/10	10.3	29.2	30.2	37.9	100	
	1/100	7.5	10.9	19.0	27.1	99.9	
	1/1000	1.3	4.0	4.3	4.9	9.0	
4	Undiluted	35.6	95.7	100	100	100	
	1/10	12.3	25.6	30.0	31.9	100	
	1/100	7.5	10.9	19.0	27.1	99.9	
	1/1000	2.3	4.9	3.4	5.8	8.9	
5	Undiluted	40.8	93.4	100	100	100	
	1/10	8.9	23.3	35.0	32.4	100	
	1/100	7.5	10.9	19.0	27.1	99.9	
	1/1000	1.2	3.4	4.6	5.8	10.1	
Reference strain	Undiluted	24.8	96.8	100	100	100	
	1/10	7.1	20.2	25.4	27.3	100	
	1/100	6.4	8.3	21.2	35.6	99.8	
	1/1000	2.3	4.6	3.3	4.3	9.3	

0.15% chlorhexidine demonstrated anti-*Malassezia* activity.^{8,9} Climbazole was effective *in vitro* against *M. pachydermatis* showing a 0.06 µg/mL minimal inhibitory concentration.¹⁰

In conclusion, once or twice daily applications of wipes soaked in a chlorhexidine, climbazole and Tris-EDTA solution are effective in reducing the numbers of *M. pachydermatis* yeast on canine skin. These wipes may be useful for treating lips, interdigital spaces, the perianal area and skin folds frequently affected by *Malassezia* overgrowth.^{1,2} It must be stated that this was an uncontrolled study performed on a small number of dogs. A controlled study using placebo wipes on a large number of dogs should follow this pilot study.

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Résumé

Contexte – Des lingettes contenant de la chlorhexidine et des dérivés azolés ont été recommandés en médecine vétérinaire. Aucune étude n'a été publiée sur leur activité contre *Malassezia pachydermatis*.

Hypothèses/Objectifs – Évaluer l'activité *in vivo* et *in vitro* de lingettes imprégnées d'une solution de chlorhexidine, climbazole et Tris-EDTA contre *Malassezia pachydermatis*.

Sujets – Cinq colonies de shar-pei de recherche.

Méthodes – Les lingettes ont été appliquées une fois par jour au niveau du pli axillaire gauche, le pli inguinal droit et de la zone périanale (protocole A) et deux fois par jour sur le pli axillaire droit, le inguinal droit et l'ombilic (protocole B) pendant 3 jours. L'activité *in vivo* a été évaluée par quantification des colonies de *Malassezia* par disques de contact sur les zones corporelles choisies avant et après application des lingettes. L'activité de la solution d'imprégnation des lingettes a été testée *in vitro* par tests de contact suivant les recommandations de l'European Standard UNI EN 1275.

Résultats – Les échantillons prélevés après application ont montré une diminution importante et rapide des CFU des levures *Malassezia*. Aucune différence significative dans la diminution des *Malassezia* n'a été mise en évidence entre les protocoles A et B. Des tests *in vitro* ont montré 100% d'activité contre les *Malassezia* après un temps de contact de 15 minutes avec la solution des lingettes.

Conclusions et importance Clinique – Les lingettes contenant la chlorhexidine, le climbazole et le Tris-EDTA réduisent substantiellement la population de *M. pachydermatis* sur la peau des chiens. Les résultats, bien qu'il s'agisse d'une étude non contrôlée réalisée sur un faible nombre de chiens, suggère que ces lingettes peuvent être utiles en traitement local de la dermatite à *Malassezia* des lèvres, des pattes, du périnée et des plis cutanés.

Resumen

Introducción – las gasas que contienen clorhexidina y derivados de azol son recomendadas para uso veterinario. No se ha publicado ningún estudio acerca su actividad contra *Malassezia paquidermatis*.

Hipótesis/Objetivos – evaluar la actividad *in vivo* e *in vitro* de las gasas con clorhexidina, climbazol y solución Tris-EDTA frente a *Malassezia pachydermatis*

Animales – cinco colonias de investigación de perros Sharpei.

Métodos – las gasas se aplicaron una vez al día en la axila izquierda, zona inguinal izquierda, y zona perianal (protocolo A), y dos veces al día en la axila derecha, zona inguinal derecha, y región umbilical (protocolo B) durante tres días. La actividad *in vivo* se evaluó mediante cuantificación de las colonias de *Malassezia* en

placas de contacto de zonas seleccionadas del cuerpo antes y después de la aplicación de las gasas. La actividad de la solución en las que las gasas se empaparon se evaluó *in vitro* mediante pruebas de contacto siguiendo los estándares europeos UNI EN 1275.

Resultados – las muestras recogidas tras la aplicación de las gasas mostraron una reacción rápida y significativa de las colonias de *Malassezia*. No hubo significativas en la reducción de *Malassezia* en los dos protocolos. El ensayo *in vitro* demostró una actividad del 100% frente a *Malassezia* tras 15 minutos de contacto con la solución de las gasas.

Conclusiones e importancia clínica – las gasas que contienen clorhexidina, climbazol y Tris-EDTA reducen sustancialmente la población de *Malassezia pachydermatis* en la piel de perros. Aunque este era un estudio no controlado y desarrollado en un pequeño número de perros, los resultados sugieren que estas gasas pueden ser útiles para la terapia tópica de dermatitis producida por *Malassezia* en zonas de los labios, almohadillas plantares, y zonas perianales y pliegues de la piel.

Zusammenfassung

Hintergrund – Feuchttücher, die Chlorhexidin und Azolderivate beinhalten, werden für den veterinärmedizinischen Gebrauch empfohlen. Es gibt keine Studie über ihre Wirkung im Einsatz gegen *Malasseziapachydermatis*.

Hypothese/Ziele – Eine Evaluierung der *in vivo* und der *in vitro* Aktivität dieser Feuchttücher in einer Chlorhexidin, Climbazol und Tris-EDTA Lösung gegen *Malasseziapachydermatis*.

Tiere – Fünf Shar-Peis aus einer Versuchstierkolonie.

Methoden – Die Feuchttücher wurden einmal täglich in der linken Achsel, in der linken Inguinalgegend und perianal (Protokol A) angewendet, und zweimal täglich in der rechten Achsel, in der rechten Inguinalgegend und umbilikal (Protokol B); beide Protokolle wurden 3 Tage lang durchgeführt. Die *in vivo* Aktivität wurde durch die Quantifizierung von *Malassezien*kolonien durch Kontaktplatten an den ausgewählten Körperstellen vor und nach Verwendung der Feuchttücher beurteilt. Die Aktivität der Lösung, in der die Feuchttücher getaucht waren, wurde *in vitro* mittels Kontakttest, den European Standard UNI EN 1275 Richtlinien folgend, beurteilt.

Ergebnisse – Die Proben, die nach Anwendung der Feuchttücher genommen wurden, zeigten eine signifikante und rasche Abnahme der *Malassezien*hefen CFU. Zwischen den Protokollen A und B wurden keine signifikanten Unterschiede bei der Reduktion der *Malassezien* gefunden. Der *in vitro* Test zeigte nach einer 15 minütigen Kontaktzeit mit der Feuchtlösung eine 100%ige Wirkung gegen *Malassezien*.

Schlussfolgerungen und klinische Bedeutung – Feuchttücher, die Chlorhexidin, Climbazol und Tris-EDTA enthalten, reduzierten die *M. pachydermatis* Population auf der Haut der Hunde signifikant. Obwohl es sich um eine unkontrollierte Studie handelte, die an einer kleinen Zahl von Hunden durchgeführt wurde, weisen die Ergebnisse darauf hin, dass diese Feuchttücher für eine topische Behandlung der *Malasseziendermatitis* der Lippen, der Pfoten, der Perianalgegend und der Hautfaltennützlich sein könnten.

要約

背景 – クロルヘキシジンとアゾール誘導体を含んだ拭き取りシートが獣医学領域の使用に推奨されている。それらのマラセチアに対する活性は発表されていない。

仮説/目的 – クロルヘキシジン、クリンバゾールならびにTris-EDTA溶液に浸した拭き取りシートの*Malasseziapachydermatis*に対する*in vivo*および*in vitro*の活性を評価すること。

供与動物 – 研究所で飼育するシャーペイ犬5頭。

方法 – 拭き取りシートで3日間1日1回左腋窩、左鼠径部、および肛門周囲を拭き取った(プロトコルA)、および1日2回右腋窩、右鼠径部、および臍部を拭き取った(プロトコルB)。In vivo活性を評価するために選択した体の部位に拭き取る前後で平板培地を押し付けることによってマラセチアコロニーを定量化した。拭き取りシートを浸した溶液の活性を*in vitro*でヨーロッパの標準UNI EN 1275ガイドラインに従った接触試験によって評価した。

結果 – 拭き取りシートを使用した後、回収した材料はマラセチア酵母CFUの有意で迅速な減少を示した。プロトコルAとBの間でマラセチアの減少に有意差はみられなかった。In vitro分析は拭き取りシートの溶液と15分の接触時間後にマラセチア酵母に対し、100%の活性を示した。

結論および臨床的な重要性 – クロルヘキシジン、クリンバゾールならびにTris-EDTAを含んだ拭き取りシートはイヌの皮膚において*M. pachydermatis*母集団を大幅に減少させた。これは少数のイヌで実施した対照群を設定しない試験だが、結果はこれらの拭き取りシートは口唇、四肢、肛門周囲ならびに皮膚の皺壁に関連したマラセチア皮膚炎の外用治療に有益な可能性を示唆していた。

摘要

背景 – 推荐兽医使用含有氯己定和唑类衍生物的湿巾。没有发表的文献表明其对马拉色菌性皮炎的作用。

假设/目的 – 用体内和体外实验评估,浸有氯己定、氯咪巴唑和Tris-EDTA的湿巾对马拉色菌性皮炎的作用。

动物 – 5个来自沙皮犬的菌落。

方法 — 每日一次用湿巾涂抹左侧腋下、左侧腹股沟和肛周(A组),每日两次用湿巾涂抹右侧腋下、右侧腹股沟和脐部(B组),分别进行三天。通过接触使用湿巾前后选中的身体部位,量化马拉色菌菌落来评估其体内活性。通过欧洲标准UNI EN 1275指导接触试验,评估湿巾所浸溶液体外活性。

结果 — 涂抹湿巾后的样本,马拉色菌菌落数显著和迅速下降。A、B两组菌落下降程度没有显著差异。体外实验显示,接触15分钟湿巾溶液,抗马拉色菌活力为100%。

结果和临床意义 — 含氯己定、氯咪巴唑和Tris-EDTA的湿巾确实能够减少犬皮肤上的厚皮马拉色菌数量。尽管本实验研究的犬只数量少,而且没有对照组,实验结果认为,对于唇、爪、肛周和皮褶的马拉色菌皮炎,这种湿巾可以用于其外部治疗。