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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Abstract

Background – Wipes containing chlorhexidine and azole derivates 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. pachyderm atis* 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 solution 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 X1000 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 X 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 1.5–5.0 x 10^7 CFU/mL by a spectrophotometer at 630 nm (Ultrospec2000, 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] x100. 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 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 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.

References

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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%						

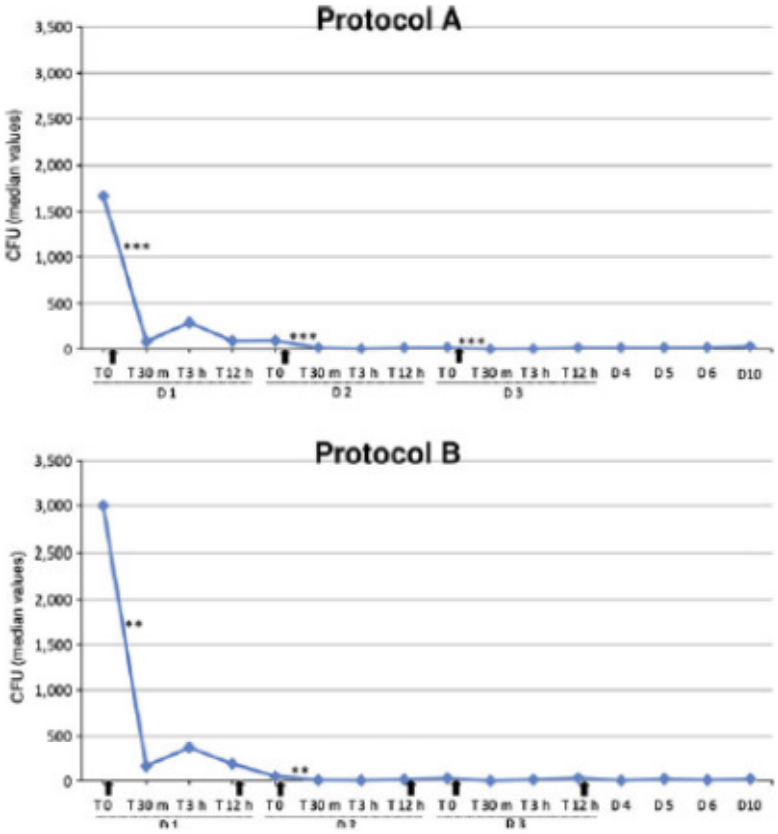

Figure 1. *Malassezia* CFU (median values) before and after wipes application at different sampling times. Protocol A: once a day wipe application. Protocol B: twice a day wipe application. D = days; h = hours; arrows = wipe applications. Stars indicate significant decreases of CFU compared with sampling 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).

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				
		1 min	5 min	15 min	30 min	Prolonged
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