A clinical and histopathological comparison of the effectiveness of salicylic acid to a compound of inorganic acids for the treatment of digital dermatitis in cattle

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ABSTRACT

Bovine digital dermatitis (DD) is a painful infectious disease, causing lameness, reduced animal welfare, and production losses in dairy herds. The main factors contributing to DD are an infection with *Treponema* spp. and poor hygiene. Topical treatment has primarily consisted of antibiotics; however, the demand for effective nonantibiotic alternatives is increasing. The objective was to evaluate the performance of 3 nonantibiotic topical treatments (salicylic acid and a compound of inorganic acids in a 20% solution and in a dry form) on DD in a commercial dairy herd. Within the 30-d test period, 42 DD lesions on 33 Holstein cows were assigned to receive 1 of the 3 treatments. Lesions were biopsied before and after treatment and were clinically evaluated 5 times. Improved lesions were clinically defined as either healed (regeneration of the skin) or healing (dry lesions covered by a scab). Unhealed lesions were defined as either active [with a raw, moist, strawberry-like (granulating) surface] or mature (with a raised papillomatous appearance). The effectiveness of treatment was evaluated histopathologically using the following scores: 0 (no spirochetes present), 1 (small number of spirochetes present in the epidermis), 2 (moderate number of spirochetes present and reaching an intermediary level in the epidermis), and 3 (large number of spirochetes present and reaching the deepest part of the epidermis or the superficial dermis). The improvement rate was 10/14 (71%) for salicylic acid, 11/15 (73%) for the inorganic acid solution, and 8/13 (62%) for the inorganic acid powder. The analysis showed no difference among treatments. The association between clinical score and histopathological score

was determined by an odds ratio. The odds ratio of a healed lesion having spirochetes in the epidermis was 0.58 and that of an active DD lesion having spirochetes in the epidermis was 26.5.

Key words: digital dermatitis, clinical score, histopathology, nonantibiotic treatment

INTRODUCTION

Bovine digital dermatitis (DD) has become a major problem in countries with intensive dairy industries (Laven and Logue, 2006; Capión et al., 2008). Digital dermatitis is an infectious disease causing pain and lameness, which results in significantly reduced animal welfare and leads to production losses in dairy herds (Bruijnij et al., 2010; Ettema et al., 2010). The exact pathogenesis of DD is not fully understood, but the etiology is considered multifactorial. Spirochetes, especially *Treponema* spp., which are obligate anaerobic bacteria, are considered to be the predominant infectious agents (Read and Walker, 1998; Evans et al., 2008; Klitgaard et al., 2008) and have been shown to account for more than 90% of the bacteria found in DD lesions (Klitgaard et al., 2008). Spirochetes are only isolated in lesions and not in the adjacent healthy skin (Döpfer et al., 1997; Evans et al., 2009). Spirochetes can be visualized using different histopathological evaluation techniques, including silver staining (Demirkan et al., 1998; Cruz et al., 2001).

Treponema spp. are able to produce both ammonia and hydrogen sulfide (Chu et al., 2003). Ammonia is a skin irritant and even a short exposure can cause injury to the skin (Miller, 1981). Hydrogen sulfide can cause injury or death to epithelial cells (Beauchamp et al., 1984). The production of hydrogen sulfide is necessary for *Treponema* spp. to survive in oxygen-free atmospheres (Lai and Chu, 2008). In addition to infection with *Treponema* spp., poor hygiene is considered

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a main contributing factor to the development of DD (Holzhauer et al., 2006; Cramer et al., 2009; Relun et al., 2013). Cattle slurry has been found to have a pH of 8.4 (Mohaibes et al., 2011), and the pH of normal skin in cattle is 6.8 (Meyer and Neurand, 1991).

Antibiotic treatments have been found to be effective but the risk of antibiotic residues in milk and meat, as well as soil contamination, and concern regarding the development of antibiotic resistance have resulted in a growing interest in nonantibiotic treatment alternatives (Britt et al., 1999; Laven and Logue, 2006; Relun et al., 2012).

When evaluating the effectiveness of treatment and control strategies, several methods can be used, the most common of which is clinical evaluation of skin and lesions (Hernandez and Shearer, 2000; Higginson Cutler et al., 2013). However, the absence of pathogens in the deeper layer of epidermis is required for complete recovery, because clinical improvement or healing of the skin can sometimes be apparent even when bacteria are still present, resulting in a possible recurrence of the lesion within a short time (Döpfer et al., 2011; Gomez et al., 2012). Therefore, clinical and histopathological evaluation must be combined to evaluate the true effect of treatment.

Salicylic acid (SA), or 2-hydroxybenzoic acid, has been used for several decades in Denmark as a nonantibiotic topical treatment for DD lesions. It is a keratolytic, anti-inflammatory powder with bactericidal and antiseptic effects (Vane, 1971). A recent study has shown SA to be more effective in terms of healing and improvement of DD lesions than chlortetracycline spray (Schultz and Capion, 2013); however, it must be applied in a bandage, which is labor intensive, and effective nonantibiotic treatments that are easy to apply in a herd setting are therefore needed.

A nonantibiotic product that is a compound of inorganic acids (Agron, Alfafarm, Solrød Strand, Denmark) has been used for the treatment and prevention of DD in Danish dairy herds. The effectiveness of the product has not previously been clinically tested. The compound has a pH of 2.5 in a 20% solution and contains sulfates that form ammonium sulfate in reaction with ammonium, iron that forms iron sulfide in reaction with hydrogen sulfide, as well as astringents and coagulants. The product is not considered a disinfectant because its main action is to lower the pH on the skin; the effect is probably not impaired by manure and lesions do not need to be cleaned for effect. The product is said to have a drying effect and can promote scab formation (Alfafarm; <http://www.alfafarm.com/>).

The objective of this study was to perform a clinical and histopathological evaluation of the effectiveness of

DD treatments using salicylic acid, a solution of the inorganic acids compound, and the same product in a dry powder, and to assess the correlation between the clinical stage of DD and a histopathological evaluation of the presence of spirochetes.

MATERIALS AND METHODS

Cows were randomly allocated to 3 treatment groups, and the effectiveness of the treatment was evaluated over a 30-d period. All procedures were carried out in agreement with the Danish Council for Animal Experimentation (Fødevarestyrelsen, Glostrup, Denmark).

Study Population

The study population consisted of 34 lactating Danish Holstein cows from a commercial dairy herd of 120 cows in Denmark. The population included 15 first-lactation cows, 13 second-lactation cows, 3 third-lactation cows, and 3 fourth-lactation cows. The farm had mattresses in the cubicles and a concrete slatted floor. The cows were milked twice a day in a herringbone milking parlor. The farm did not use footbaths or any other control measures for DD, and claw lesions were managed by the professional claw trimmer over 4 annual visits.

Clinical Scoring of DD

Lesions were scored clinically on observation days 0, 6, 15, 24, and 30. Lesions scored as active (acute and chronic) were round or oval; level with the surface of the skin or concave; raw, moist, red to gray; and had granular to strawberry-like surfaces (Hernandez and Shearer, 2000; Higginson Cutler et al., 2013). Mature lesions (chronic) were raised above the level of the normal skin and covered by filiform papillae (Hernandez and Shearer, 2000). Healing lesions were dry, covered by a scab, and displayed normal skin features (Higginson Cutler et al., 2013). If lesions had more than one clinical score; for example, a small part covered by a scab and another part with an active or mature lesion, the lesions were scored according to the active or mature lesion.

On observation days 6, 15, 20, 24, and 30, skin changes were scored for pain by palpation. Assessment was performed by the first author on cows standing or lying in cubicles according to the method previously described by Capion et al. (2012). In brief, the skin adjacent to the horn capsule and the interdigital cleft was inspected and palpated. The scores were as follows: 0 = skin looks normal, no pain or soreness, normal

smooth, soft skin; 1 = skin looks irritated, no pain or soreness, skin is rough and hard on palpation; 2 = skin looks irritated and changes are visible, with a marked line between normal and changed skin, pain or soreness on palpation, skin feels moist or rough on the surface.

Experimental Procedure

On d 0, during routine claw trimming of all cows on the farm, 34 cows were selected in the order they came in to the trimming chute, on the basis that they had at least one DD lesion clinically scored as either active or mature, and disregarding age, parity, or affected leg. Following clinical evaluation of the lesions, skin was biopsied (**B1**). Before sampling, the skin was washed with water or wiped with paper and anesthetized using ice spray (Prof Care ice spray; Select Sport A/S, Glostrup, Denmark); then, a 6-mm punch biopsy (Kruuse, Odense, Denmark) was taken from the center of the lesion to the level of the dermis perpendicular to the skin surface. Biopsies were randomly numbered to ensure a blinded reading and fixed in 10% neutral buffered formalin for 5 to 30 d before processing.

After biopsy sampling, the lesions were randomly allocated, using global randomization in an alternating scheme to 1 of 3 treatment groups: inorganic acid in a 20% solution (**IAS**), inorganic acid powder (**IAP**), and SA powder. The first DD lesion recognized and included in the study (regardless of whether it was an active or mature lesion) received IAS, the second lesion received SA, the third lesion received IAP, the fourth lesion received IAS, and so on.

The inorganic acid compound was used both as the powder (IAP) applied locally on the lesion and as a solution prepared by mixing the powder with water to a 20% solution (IAS) and sprayed onto the lesion. Salicylic acid (Salicylsyre, Polyvet, Vejle, Denmark) was used as a 100% concentration powder applied to the skin. The IAP and SA treatments were given only once; they were applied in a bandage using cotton wool and wrap (Eickewrap, Eickemeyer, Haderslev, Denmark) on d 0 and left on for 2 d. For lesions treated with IAS, the treatment was repeated by the farmer or a technician once every third (3 ± 1) day from d 0 until d 15. After d 15, the IAS treatments was repeated every day. The IAS was applied with a hand-operated compression sprayer on an unwashed lesion while cows were standing in the milking parlor or at the feed bunk.

The effectiveness of treatment (lesion score and signs of pain by palpation) was evaluated on site and from photographs. The person examining the animals was blinded to the type of treatment and previous findings on the cows.

A second biopsy (**B2**) was taken when the lesion caused no pain on palpation and the skin appeared normal, or at d 30 regardless of the clinical findings. If a lesion was still present, the biopsy was taken adjacent to but not overlapping the B1 biopsy site. The B2 biopsy was taken from completely healed skin in the area that had been infected. The animals were examined in a trimming chute and biopsies were collected following the same procedure as on d 0. After B2, all lesions were treated with SA as a topical treatment packed in a bandage for 2 d; SA was considered standard treatment of DD lesions on the farm.

Histopathological Evaluation

Biopsies were removed from the formalin container, divided into 2 equal halves by a perpendicular cut to the skin surface, dehydrated, and embedded in paraffin wax. All biopsy samples were stained with hematoxylin and eosin (**H&E**) and modified Warthin-Starry stain (Luna, 1968). The H&E stain was used as a reference for the location and orientation of the skin sample, and the modified Warthin-Starry stain was used to demonstrate spirochetes.

The number and the depth of spirochetes within the epidermis were recorded and combined into the following scores (Figure 1): 0 = no spirochetes present; 1 = small number of spirochetes present in the epidermis; 2 = moderate number of spirochetes present and reaching an intermediary level in the epidermis; 3 = large number of spirochetes present and reaching the deepest part of epidermis or the superficial dermis.

Statistical Analysis

The effectiveness of IAS and IAP was compared with that of SA. Using Fisher's exact test (R, version 2.14.2; <https://www.r-project.org/>), the following comparisons were assessed: the effect of treatment on the total number of lesions; the effect of treatment on active lesions; and the effect of treatment on mature lesions. Statistical significance was defined as $P \leq 0.05$. Data on biopsy scores from B1 (d 0) and B2 (either when no pain was recorded from palpation or d 30) were compared using Fisher's exact test.

The association between the clinical and the histopathological scores of DD lesions was evaluated by calculating the odds ratio (**OR**) of confirming the clinical diagnosis by histopathology. Only lesions that had a clearly defined clinical score (healed, healing, active lesion, and mature lesion) were included in the analysis. The 2 independent observers who scored all histology samples were blinded to the identity of the cow, treat-

Figure 1. Histopathological scoring of digital dermatitis. Panels A and B = score 0: no spirochetes present (bar = 200 μ m); panels C and D = score 1: spirochetes present in epidermis (bar = 400 μ m) with obvious tropism for tubular horn (insert; bar = 30 μ m); panels E and F = score 3: the front of the spirochetes reaches deep into a necrotic and inflamed epidermis encroaching on the superficial tips of the dermal papillae (T; bar = 500 μ m). Panels A/B, C/D, and E/F are pairs of parallel sections, with A, C, and E being stained with modified Warthin-Starry stain, and B, D, and F with hematoxylin and eosin.

ment, and test day, and interobserver variability was evaluated using a weighted kappa analysis.

RESULTS

Study Population

On d 0, 46 DD lesions (26 mature lesions and 20 active lesions) from 34 cows were included in the study. Some animals had lesions on more than one leg and, because treatment was assigned per lesion, one cow could be exposed to different treatments. Out of these 46 lesions, 2 were excluded from the study due to culling before B2 was obtained, and 2 were excluded because of incomplete sampling or loss of the sample. In total, 42 lesions from 33 cows were used in the statistical analysis. Of these 42 lesions, 14 were treated with SA, 15 were treated with IAS, and 13 were treated with IAP. Table 1 shows the distribution of lesions at d 0 among the different treatments.

Clinical Scoring of DD at Termination of the Trial

At termination of the trial, 2 originally active lesions had diminished in size and were covered by a scab, and 6 originally mature lesions had an area that was healed and an area that was covered by a small scab. These 8

lesions were classified as “healing,” and were therefore improved but not healed (Table 1).

The total percentage of lesions that improved after treatment was 71% (10/14) with SA, 67% (10/15) with IAS, and 54% (7/13) with IAP. The total number of healed lesions at termination of the trial was 8/14 treated with SA, 7/15 treated with the IAS, and 4/13 treated with the IAP, indicating cure rates of 57, 47, and 31%, respectively. Fisher’s exact test was used to test the difference in improvement rate among treatments. We detected no significant difference in the improvement rate between treatment with SA or IAS ($P = 0.69$). This applied to the analysis of total number of lesions ($P = 0.69$) and to active ($P = 1$) and mature ($P = 0.35$) lesions separately. When testing the difference in improvement rate between SA and IAP, the test showed no significant differences in the total number of lesions ($P = 1$), active lesions ($P = 0.27$), or mature lesions ($P = 0.59$) tested separately.

Time Required for a Lesion to Heal

We observed a difference in the time for lesions to heal after each treatment; for example, treatment effects seemed to occur earlier for SA than for the other treatments. By d 15, 6 of 8 lesions treated with SA, 1 of 7 lesions treated with IAS, and 1 of 4 lesions treated

Table 1. The distribution of clinical scores for digital dermatitis lesions among treatments at d 0 and at termination of the trial (when pain was relieved or at d 30)

Treatment	Clinical scores at d 0	No. of lesions	Clinical scores at termination of the trial	No. of lesions	Proportion of improved lesions
Salicylic acid	Active	7	Healed	6	7/7
			Healing	1	
			Active	0	
	Mature	7	Mature	0	3/7
			Healed	2	
			Healing	1	
Inorganic acid solution	Active	5	Active	1	3/5
			Mature	3	
			Healed	3	
	Mature	10	Healing	0	7/10
			Active	2	
			Mature	0	
Inorganic acid powder	Active	6	Healed	4	2/6
			Healing	3	
			Active	1	
	Mature	7	Mature	2	5/7
			Healed	2	
			Healing	2	
			Active	0	
			Mature	2	

Table 2. The number of spirochetes identified by modified Warthin-Starry stain, as evaluated by a histopathological score from samples of digital dermatitis lesions collected on d 0 [biopsy (B)1] before treatment and at termination of the trial (B2), either when pain was relieved or at d 30

Treatment	Sample day	No. of spirochetes present on histopathology			
		None	Small	Moderate	Large
Salicylic acid (SA)	B1	0	1	7	6
	B2	6	5	2	1
Inorganic acid solution (IAS)	B1	1	2	7	5
	B2	7	4	3	1
Inorganic acid powder (IAP)	B1	0	4	5	4
	B2	2	5	5	1

with IAP had healed. By d 24, the remaining SA-treated and IAP-treated lesions had healed, in addition to 5 of 7 IAS-treated lesions. By d 30, the remaining IAS-treated lesions had healed. Statistical analysis of this observation was not performed.

Histopathology of DD Lesions

A total of 86 biopsies were taken during the study period. One of the biopsies could not be evaluated correctly because it included only the superficial part of the epidermis, and the stratum basale could not be identified. In addition, one biopsy was lost. Therefore, 84 biopsies were evaluated (42 from B1 and 42 from B2).

The number of spirochetes in the B1 and B2 samples are presented in Table 2. At B1, the number of spirochetes was generally moderate to large and found in 34/42 biopsies. One sample from a lesion in the IAS group had no spirochetes visible in the biopsy despite it being clinically recognized as active. At B2, 3/42 samples contained large numbers of spirochetes and 15/42 showed no recognizable spirochetes.

The association between clinical lesion scores and histopathological scores was evaluated by estimating the OR. The OR of the clinical score “healed” (no spirochetes found by histopathology) was 0.58. The OR of the clinical scores “active” and “mature” (with small, moderate, or large numbers of spirochetes found by histopathology) was 26.5. The distribution of clinical

lesion score and number of spirochetes is presented in Table 3.

Two observers blinded to the cow identity, treatment type, and test-day performed the histopathological evaluation of the Warthin-Starry stained biopsies, and inter-observer agreement was estimated using weighted kappa. Kappa was 0.7 with a standard error of 0.07, which corresponds to substantial agreement (Viera and Garrett, 2005).

DISCUSSION

All lesions improved irrespective of the treatment used. The proportion of lesions that healed varied across the 3 treatments. The SA treatment resulted in a slightly higher number of healed lesions compared with the other 2 treatments, but the difference was not statistically significant. A higher proportion of mature lesions compared with active lesions were treated with IAS, which might explain why fewer lesions healed with this treatment, as mature lesions are more difficult to treat (Nielsen et al., 2012; Schultz and Capion, 2013) due to severe hyperkeratosis, compared with active lesions, which are erosive and ulcerative. The keratolytic potential of IAS is not known but the keratolytic effect of SA is superficial, and it is expected to have difficulty reaching the deeper layers of the epidermis, particularly if the stratum corneum or stratum spinosum is severely proliferated. This study was performed in a herd that had a high prevalence of DD, which might explain why

Table 3. Distribution of the number of spirochetes identified by modified Warthin-Starry staining of the 84 biopsies taken from different stages of digital dermatitis lesions throughout the study and across all treatments

Clinical lesion score	No. of spirochetes present on histopathology			
	None	Small	Moderate	Large
Healed	12	3	4	0
Healing	1	3	4	0
Active	1	5	4	14
Mature	1	3	13	16

many proliferative mature lesions were present. We recommend that randomization should ensure an even distribution of active and mature lesions between treatment groups in future studies.

The lesions appeared to heal 1 to 2 wk earlier with SA than with IAP treatments. Both SA and IAP require a bandage for application, which is time consuming, but only one application is required. In contrast, IAS treatment does not require a bandage for application but requires continual treatment several times per week to produce the same results. Applying bandages to young stock and heifers can be challenging, and spray treatment with IAS may be an easier and effective alternative.

The legs and lesions were not washed before IAS was applied. The product was used according to the manufacturer's instructions. It is not known what the effect would have been if lesions had been cleaned before application.

The IAS treatment can be easily applied by the farmer to a cow standing in the stable or during milking, and might therefore be a preferred choice of treatment. It might be interesting to perform a similar study of the efficacy of IAS on equally sized groups with active and mature lesions and over a longer period.

In this study, we chose to focus on the effect of treatment of a single lesion by performing frequent repeated observations at short intervals and not to consider reinfection within a longer period. We did not evaluate the preventive effect of treatment.

The clinical scoring and pain evaluation of the lesions on cows standing or lying was performed by the same observer, and there is a risk of missing some changes or misinterpreting movement of the leg as a sign of pain rather than a sign of avoidance. The clinical scoring method was preferred because it causes less stress to the cows and reduces disturbance in the herd routine. All lesions were re-evaluated in the trimming chute when the lesion appeared to be healed or at the end of the study (d 30).

Further limitations to this study were the lack of baseline comparison before the start of the study, the possibility of mutual interaction of treatments (as individual cows could be exposed to more than one treatment), and a possible effect of ice spray used for local anesthesia. The ice spray seemed effective, because the cows did not respond to the biopsy procedure. The effect of ice spray on development and healing of the lesions is considered minimal (Capion et al., 2012), but a larger sample size might be desirable to achieve more reliable results. Ideally, a control group receiving a placebo should also be included, although this could make it difficult to convince farmers to participate in such a study and to obtain approval from the Danish Council

of Animal Experimentation. Without treatment, some DD lesions may self-resolve, but the number depends on cow- and herd-specific risk factors (Barker et al., 2009; Nielsen et al., 2012).

Treponema spp. have been found to persist in the deeper layers of the epidermis after different topical treatments with oxytetracycline and nonantibiotic compounds, which suggests that topical treatments have a limited capacity for completely inactivating bacteria deeper in the lesions (Döpfer et al., 2011). Our results support this finding of bacteria in the deeper layers in the active and mature lesions, where we found a large number of spirochetes targeted toward the more profound parts of the epidermis, with tropism for the tubular heel horn and for the dermal papillae.

The clinical scores of active and mature lesions generally matched the histopathological evaluation of the biopsies obtained on both d 0 and d 30 (end of the study), where we found moderate-to-large numbers of spirochetes present. In 2 cases clinically classified as active and mature, we recorded an absence of spirochetes by histopathology. Because the biopsy was taken in the center of the lesion rather than on the edge, we might have missed the most infected area. However, 7/19 lesions clinically scored as healed were positive for spirochetes by histopathology at the termination of the study. In 2 of the healed lesions, we found a moderate number of spirochetes in the stratum spinosum, indicating that these lesions were wrongly classified as healed when they actually had active DD. In 5 biopsies, we found a small number of spirochetes located at the surface or outermost layers of the stratum corneum, which might suggest that these lesions had been resolved in the deeper layers of epidermis, and that what we observed might have been contamination from spirochetes in the environment (Klitgaard et al., 2014; Zinicola et al., 2015). When a high prevalence of DD in the herd is combined with concurrent poor hygiene, the risk of reinfection might be high. There is also the risk that these cows are chronically infected (Capion et al., 2012).

The sampling technique used in this study could introduce bias in the histopathological evaluation, because a 6-mm punch biopsy taken from the center of the clinically scored DD lesion represents only a minor part of the entire lesion, and lesions often present with different scores simultaneously. Lesions classified as healed or healing may contain different numbers of spirochetes depending on the exact location of the biopsy, and the association (OR) between the clinical and histopathological scores may be affected. Therefore, it might be desirable to obtain several biopsy samples from the same lesion to evaluate the correlation between clinical appearance and histopathology more ac-

curately. We had originally planned to take the second biopsy next to the location of the first biopsy. However, in many cases, the lesions had changed appearance and were often smaller, so the second biopsy was taken from an area within the remaining lesion. We could speculate that taking biopsies induces inflammation that in itself could influence healing and therefore our interpretation of effectiveness of treatment. Our results suggest, however, that the best way to evaluate the effectiveness of treatment is to include histopathology rather than rely solely on clinical evaluation, despite the risk of an inflammatory response elicited by the punch biopsy and affecting the healing process. A previous study showed that the risk of inducing lesions with biopsies was minimal (Capion et al., 2013).

CONCLUSIONS

Compared with the standard nonantibiotic therapy used in Denmark—salicylic acid—the compound of inorganic acids was almost equally good, with a positive effect on pain management and improvement and healing of DD lesions. Histopathology can be helpful in correctly classifying healed lesions but should be investigated further. When using the histopathology score described in this study, we detected high inter-observer agreement.

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