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Evaluation of two topical therapeutic protocols for udder lesions positive to stephanofilariasis in Holstein cows[1]

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ABSTRACT.- Moura RBR, Mendonça APA, Teodoro PHM, Renato R, Lisbôa JAN, Headley SA, Rodrigues CA. **Evaluation of two topical therapeutic protocols for udder lesions positive to stephanofilariasis in Holstein cows.** *Pesquisa Veterinária Brasileira 47:e07857, 2027*. Departamento de Cirurgia Veterinária e Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Doutor Walter Mauricio Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. E-mail: celso.a.rodrigues@unesp.br

The present study aimed to evaluate the effectiveness of a topical treatment with HoofCare® on udder lesions in lactating Holstein cows and to compare it with a formulated paste containing trichlorfon as a positive control. The study was conducted on a dairy farm with more than 2,000 lactating cows housed in a free-stall system, with a history of ulcerative udder lesions. Before starting treatment, 36 cows presenting ulcerative udder lesions were selected and subjected to biopsy for a diagnostic confirmation by histopathology and direct sediment examination. *Stephanofilaria* spp. was detected in 100% of the samples by direct sediment examination. Thirty cows were randomly allocated into two treatment groups: HC (n=15), treated with HoofCare®, and FPT (n=15), treated with a formulated paste with trichlorfon. A second biopsy was performed on these 30 cows at the end of 60-day treatment period. Treatments were applied topically every 48 hours until complete healing or for up to 60 days. The area and temperature of the lesion were measured before the start of the therapy, with follow-up assessments every 30 days for lesion area and every 15 days for thermography. Histopathological examination revealed infiltration of inflammatory cells, however, the parasite was not detected in any tissue samples. In contrast, direct sediment examination confirmed the presence of the parasite in all samples before and after treatment, demonstrating its effectiveness as a diagnostic method. There were no treatment differences, however, there was a significant reduction in lesion area between the D0 and D60 in the FPT group. There was no significant difference within HC group regarding lesion area reduction index across D0, D30, and D60, whereas differences were observed over time in the FPT group (P = 0.016) and between groups at D60 (P = 0.033). Although a reduction in lesion area occurred in both groups over the 60-day period, complete healing was observed in only two cows (one from each group). Furthermore, all the cows remained positive for the parasite by direct sediment examination at D60, including those with healed lesions. In conclusion, the topical application of both treatments every 48 hours was ineffective in eliminating the parasite or promoting complete lesion healing.

INDEX TERMS: *Stephanofilaria* spp., direct sediment examination, udder dermatitis, filarial dermatitis, dairy cattle.

Online First

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RESUMO.- [**Avaliação de dois protocolos terapêuticos tópicos para lesões de úbere positivas para estefanofilariose em vacas da raça Holandesa.**] O presente estudo teve como objetivo avaliar a eficácia do tratamento tópico com HoofCare® em lesões cutâneas no úbere de vacas da raça Holandesa e compará-lo com uma pasta formulada contendo triclorfon, como um controle positivo. O estudo foi conduzido em uma fazenda leiteira com mais de 2.000 vacas em lactação alojadas em sistema *free-stall*, com histórico de lesões ulcerativas no úbere. Antes do início do tratamento, 36 vacas com lesões ulcerativas no úbere foram selecionadas e submetidas à biópsia para confirmação diagnóstica por histopatologia e exame direto do sedimento. *Stephanofilaria* spp. foi detectada em 100% das amostras no exame direto do sedimento. As vacas foram aleatoriamente alocadas em dois grupos de tratamento: HC (n=15), tratado com HoofCare®, e FPT (n=15), tratado com uma pasta formulada com triclorfon. Uma segunda biópsia foi realizada nessas 30 vacas ao final dos 60 dias de tratamento. Os tratamentos foram aplicados topicamente a cada 48 horas até a completa cicatrização ou por um período máximo de 60 dias. A área e temperatura da lesão foram medidas antes do início da terapia, com avaliações de acompanhamento a cada 30 dias para área da lesão e a cada 15 dias para a termografia. O exame histopatológico revelou infiltração de células inflamatórias, porém, o parasita não foi detectado em nenhuma das amostras de tecido. Em contrapartida, o exame direto do sedimento confirmou a presença do parasita em todas as amostras, antes e depois do tratamento, demonstrando sua eficácia como método diagnóstico. Não houve diferença significativa entre os tratamentos, no entanto, observou-se uma redução significativa na área da lesão entre os dias D0 e D60 no FPT. Não houve diferença significativa no grupo HC quanto ao índice de redução da área da lesão entre D0, D30 e D60, enquanto observou-se diferenças ao longo do tempo no grupo FPT (P = 0,016) e entre os grupos no D60 (P = 0,033). Embora uma redução na área da lesão tenha ocorrido em ambos os grupos ao longo dos 60 dias, a cicatrização completa foi observada em apenas duas vacas (uma de cada grupo). Além disso, todas as vacas permaneceram positivas para o parasita no exame direto do sedimento no D60, incluindo aquelas com lesões cicatrizadas. Conclui-se que a aplicação tópica de ambos os tratamentos a cada 48 horas foi ineficaz na eliminação do parasita ou na promoção da cicatrização completa das lesões.

TERMO DE INDEXAÇÃO**:** *Stephanofilaria* spp., exame direto do sedimento, dermatite mamária, dermatite filarial, vaca leiteira.

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INTRODUCTION

Stephanofilariasis is a disease with worldwide distribution, characterized by ulcerative skin lesions caused by the nematode *Stephanofilaria* spp. It is more prevalent during the summer, a period associated with increased proliferation of flies that act as vectors and intermediate hosts of the parasite, such as *Musca conducens* and *Haematobia irritans.* The disease affects various animal species, with dairy cattle being the most affected (Johnson et al. 1981). In these animals, it manifests as chronic dermatitis with papular eruptions that may progress to alopecia in the affected area, nodule formation, and ulcerative lesions with crusting (Novaes et al. 2006).

In Brazil, although the first reported cases occurred in the state of São Paulo (Oba et al. 1977), recent reports describe lesions in various regions of the country, such as Goiás, Rio Grande do Sul, and Paraná (Miyakawa et al. 2010; Silva et al. 2010). Lesions have been observed in different anatomical locations, including the head, scapular region, udder, hock, scrotal skin, chest (Oba et al. 1977; Silva et al. 2010), as well as near the tail, rump, thigh (Novaes et al. 1988), and pastern (Novaes et al. 1990). In dairy cows, lesions are most frequently located in the anterior portion of the udder and are more commonly observed during lactation. These lesions may be single or multiple, typically circular, with an initial diameter of 1cm, which may increase to more than 25cm (Miyakawa et al. 2010).

A presumptive diagnosis is based on clinical history and evaluation of the lesion characteristics. Definitive diagnostic is achieved through techniques that allow visualization of the parasite, such as histopathological examination of tissues collected from the edges of the lesions (Oduye 1971), and scraping of the lesion or smears stained with Giemsa or Congo red to identify the nematode (Novaes 2005). Ueno and Chibana (1977) described a simple and rapid method called direct sediment examination, which is based on the migration of parasites from biopsied tissue into isotonic saline solution (0.9% NaCl). After centrifugation of the sediment, it is possible to visualize the nematode in its larval and/or adult form.

Stephanofilaria spp. are considered the primary etiological agent of ulcerative skin lesions in cattle, especially on udder, however, recent studies suggests that bacteria may also contribute to lesion development and persistence. Frequent isolation of *Staphylococcus agnetis* and *Staphylococcus hyicus* from ulcerative and exudative lesions associated with *Haematobia* spp. infestation and from the flies themselves, suggesting potential transmission during blood feeding.

Both species harbor genes encoding exfoliative toxins capable of disrupting epidermal cell adhesion and promoting skin damage. *Staphylococcus aureus* has also been implicated in the pathogenesis of skin lesions in cattle, whereas other bacterial species associated with bovine skin and soft-tissue infections, including *Trueperella*

pyogenes, *Fusobacterium necrophorum*, *Streptococcus dysgalactiae*, and *Actinomyces bovis*, may colonize ulcerated areas and contribute to inflammation and lesion chronicity. Therefore, although *Stephanofilaria* spp. remain the main etiological agents, bacterial involvement should also be considered, particularly in chronic or treatment-refractory lesions (Mala et al. 2021; Naseem et al. 2022a; Naseem et al. 2022b).

Therapeutic protocols commonly include the topical use of organophosphates, such as 2% coumaphos and 6% trichlorfon (Taylor et al. 2004), as well as antiparasitic agents, such as ivermectin paste at concentrations of 1% and 2% (Miyakawa et al. 2012). Furthermore, topical application of zinc oxide, due to its antiseptic and astringent properties, may act as an adjunct to the healing process in stephanofilariasis lesions (Rai et al. 1994; Silva et al. 2010). The effectiveness of the treatment depends on several factors, including the disease duration, lesion size and stage of lactation, since some of these therapeutic options have restrictions for use in lactating cows, given the risk of drug residues in milk (Silva et al. 2010; Miyakawa et al. 2012).

The commercial product HoofCare® containing copper citrate, zinc chloride, tea tree essential oil and citric acid, has recently been evaluated for the treatment of digital dermatitis lesions (Mendonça et al. 2024; 2026). Although it has been used empirically on dairy farms to treat ulcerative udder lesions suggestive of stephanofilariasis, there is currently no scientific evidence regarding its true efficacy for these specific lesions.

Therefore, the aim of this study was to determine the effectiveness of HoofCare® for treatment of cows naturally affected by ulcerative udder lesions positive for *Stephanofilaria* spp. on direct sediment examination, in comparison with a formulated paste containing trichlorfon.

MATERIALS AND METHODS

Ethical approval. This study was approved by the Ethics Committee on the Use of Animals (Approval number: 0181/2022) of the "Faculdade de Medicina Veterinária e Zootecnia" (FMVZ) of the "Universidade Estadual Paulista Júlio de Mesquita Filho" (UNESP) in Botucatu, São Paulo, Brazil.

Contextualization of the farm. The study was conducted on a commercial dairy farm located in Araras, São Paulo State, Brazil, with an estimated lactating herd of 2,200 Holstein cows housed in a free-stall system. The facility consisted of a single barn with sand bedding, slatted concrete floors, and a cross-ventilation system, maintaining an average temperature of approximately 21°C. Barn aisles were cleaned by spelling and flushing once per milking, and cows were milked three times daily using a rotary system with capacity of 72 cows per session.

Lactating cows were divided into eight groups according to productivity and category. As no changes were made to the farm management during the experimental period, except for the footbath protocol (evaluated in a concurrent study), some cows included in the study moving between different groups throughout lactation. Nevertheless, all cows were kept under standardized management, nutritional, and environmental conditions, minimizing the potential effects of these factors on the parameters evaluated.

Study design. Thirty-six lactating cows presenting ulcerative udder lesions with clinical characteristics suggestive of stephanofilariasis (Miyakawa et al. 2009), such as crusts and serosanguineous exudate, were selected. Before starting the treatment, the 36 cows were subjected to biopsy for diagnostic confirmation of *Stephanofilariasis spp*. using two methods: histopathology and direct sediment examination. All animals remained housed in the barn throughout the experimental period, and no historical information was available regarding the onset of the lesions.

The cows were restrained in a headlock without chemical sedation, and the lesions were initially cleaned with dry paper towels. Subsequently, the area was aseptically prepared using iodized alcohol, and local infiltrative anesthesia was administered with 0.5 to 1 mL of 2% lidocaine without vasoconstrictor, depending on the size of the lesion. Three tissue samples were collected using a 6-mm punch biopsy: one from the center of the lesion for direct sediment examination, and two from the edge of the lesions, including both affected and adjacent healthy tissue, for histopathology analysis, following previously described methods (Miyakawa et al. 2009; 2012).

For direct sediment examination, the tissue sample was immersed in a container with isotonic saline solution (0.9% NaCl) at a ratio of 10 :1 (solution to tissue) for at least three hours at room temperature. The tissue fragment was then removed, and the remaining solution was centrifuged at 1,500 rpm for five minutes. Drops of the sediment were subsequently placed between a glass slide and coverslip and examined under a light microscope (400x magnification) for parasite visualization. Adut forms of *Stephanofilaria* spp. were observed in 100% of the samples, including live parasites in some cases, consistent with the findings of Miyakawa et al. (2010). The remaining two samples were placed in 80ml vials containing 10% buffered formalin solution for subsequent histopathological examination at the Veterinary Pathology Laboratory of the State University of Londrina.

Following and diagnostic confirmation, the 36 cows were randomly allocated into two topical treatment groups: HC (n=18) and FPT (n=18). The HC group received application of HoofCare® (Salmix Indústria e Comércio Ltda). A positive control with trichlorfon was chosen for the standard recommendation for treating these lesions (Novaes et al. 1988; Miyakawa et al. 2010; 2012). For this, a paste formulated by 500 g of Triclorsil® (Vansil Saúde Animal; each 100 g contains 98 g of trichlorfon) with 1 kg of Unguento® (permethrin, zinc oxide, and piperonyl butoxide), achieving a final trichlorfon concentration of 32.7%. Furthermore, the base ointment containing permethrin and zinc oxide, commonly used by the farm staff, was intended to provide an adjuvant effect on the

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lesions. Both treatment protocols consisted of topical applications by brushing over the lesion every 48 hours, following prior cleaning with dry paper towels, until complete healing of the lesions or up to 60 days**.**

Healing lesion evaluation. Lesion healing was evaluated using morphometric analysis and infrared thermography at five time points: D0 (before treatment), D15 (15th day of treatment), D30, D45, and D60, as illustrated in Fig.1. Thermographic images were collected immediately after restraining the cows in the headlock and before any handling procedures, to avoid temperature alterations. Images were obtained using a FLIR SC660 Thermal camera (FLIR Systems Inc®.), with a temperature range of 26ºC to 38ºC, at 1 meter from the udder lesion. As environmental conditions can influence thermographic measurements, ambient temperature and relative humidity were recorded in accordance with Harris-Bridge et al. (2018). Image analysis was performed using FLIR Thermal Studio software, with adjustments for ambient temperature, relative humidity and emissivity (0.98). To account for potential variation during the thermography exam, maximum temperature values were mathematically corrected based on the standardized methodology described by Basile et al. (2010), as previously applied in study by our research group (Mendonça et al. 2024).

Quantitative evaluation of lesion healing was performed following thermographic images and cleaning of the lesions. Clean lesions were photographed using a smartphone featuring a high-resolution 64 MP main camera (f/1.8), alongside a 20 cm long ruler subdivided in millimeters to enable morphometric analysis.

The area of each lesion was measured three times using ImageJ® software, and the mean value (cm^2) was used for comparisons across time points and between treatment groups. Changes in lesion area were evaluated at D15, D30, D45, and D60 relative to the initial baseline area (D0). The absolute reduction in lesion area was first determined by the difference between D0 and each subsequent evaluation, and the percentage reduction (lesion regression index) was then calculated relative to D0. Positive values indicated a reduction in lesion area, whereas negative values represented an increase in area relative to baseline.

Efficacy criteria. Treatment efficacy was defined as both lesion healing and parasite elimination. At the end of the 60-day period (D60), all remaining cows were subjected to a second biopsy following the same procedures described for the initial diagnosis.

Statistical analysis. Data were tabulated in Excel® and tested for normality using the Shapiro-Wilk test. Lesion area, thermographic data, and lesion regression index were compared to cross time points within each group and between treatment groups using the Kruskal-Wallis and Wilcoxon tests. The significance level was set at $P < 0.05$.

RESULTS

Thirty-six cows were initially included in the study and underwent biopsy for diagnostic confirmation of stephanofilariasis prior to treatment initiation (n = 18 per group). However, only 30 completed the full 60-day study period, as six cows were excluded due to farm due sale or drying off. Consequently, 30 cows underwent a second biopsy, and all statistical analyses were performed using data from these cows (15 per treatment group).

Direct sediment examination confirmed the presence of the adult form of parasite in all 36 samples analyzed (Fig. 2), with a high number of filariae found per sample (3 to 4 per slide) before treatment (D0). The method was simple and allowed visualization of *Stephanofilaria* spp. motility on the slide in same samples. However, the parasite was not detected in any tissue sample analyzed by histopathology.

Histopathological examination revealed granulation tissue with vascular proliferation and the presence of perivascular inflammatory cells in the dermis (Fig.3), predominantly composed of mononuclear cells (histiocytes, plasma cells and lymphocytes) and eosinophils.

Lesion healing was evaluated using thermography at 15-day intervals, with subsequent temperature correction, as illustrated in Fig.4, which show lesions from each treatment group at three different time points along with measurement sites. The Kruskal-Wallis and Wilcoxon tests showed no significant differences in thermographic temperature either between time points within each group or between treatment groups (Table 1).

Area measurements of the lesion showed no significant differences between treatment groups at any time point, according to the Kruskal-Wallis test. However, a significant reduction in lesion area between D0 and D60 was observed in the PC group ($P = 0.003$), as shown in Table 2.

Regarding the percentage of lesion area regression index, no statistical difference was observed across time points in the HC group. In contrast, the FPT group show a significant difference between D0 and the subsequent evaluation time points ($P = 0.016$). As the regression index was calculated based on the initial lesion area, with D0 defined as 100%, Table 3 presents the evaluation at D30 and D60. Furthermore, when comparing the two treatments within each time point, a statistical difference was observed at D60 ($P = 0.033$).

The lesion regression index was calculated after measuring lesion areas in both groups, as illustrated in Fig.5.

In addition, Fig.6 presents representative examples of lesion regression throughout the treatment period. In the HC group (green line), the initial mean area was 42.7 cm^2, with a reduction of 4.3 cm^2 after 60 days, resulting in a final are of 38.4 cm^2. In the FPT group (yellow line), the lesions had an initial area of 45.5 cm^2 and showed a reduction of 22.9 cm^2 over 60 days, reaching a final area of 22.6 cm^2.

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Complete lesion healing was observed in only two animals (one from each group) within the first 30 days. However, in both cases, healing occurred less than 30 days after the interruption of treatment.

At the end of the 60-day treatment period, all 30 cows from both groups underwent to biopsy of the lesion center for direct sediment examination, and all remained positive for *Stephanofilaria* spp., including the two cows that showed complete healing**.**

DISCUSSION

The experimental period was conducted from January to March, and during which there was accumulated rainfall of approximately 300mm and high temperatures (Inmet, 2022). These seasonal conditions favor the proliferation of flies, which are recognized vectors of the disease (Miyakawa et al. 2009). The period of disease occurrence observed in this study is consistent with previous reports indicating that the months of December to March, characterized by high rainfall, temperature, and humidity, promote the proliferation of insect vectors of stephanofilariasis (Miyakawa et al. 2009; Singh et al. 2014; Silva et al. 2010; Fonteque et al. 2017).

In the present study, all animals were housed within a barn environment with controlled temperature and ventilation, and cleaning management three times a day, which would be expected to limited vector proliferation. However, the presence of flies inside the barn was observed, although they not identified at the genus or species level. Possibly, the opening of the gates for cows displacement (fresh and dry cows), as well as circulation of machinery for cleaning and feeding, facilitates the entry of flies and contributes to the spread of the disease (Silva et al. 2010; Schade et al. 2018). Additionally, during the approximately 30-day dry period, cows are housed in a semi-extensive system, where greater accumulation of organic matter and higher fly presence are observed. Thus, the combination and disease transmission (Miyakawa et al. 2009; Silva et al. 2010).

Another important consideration is that the farm did not fly control measures implemented in the barns for lactating and dry cows, only for calf facilities. All lesions were located in the udder region and adjacent tissues, and were characterized by serosanguineous discharge, circular in shape, and presence of adherent scab. These findings are consistent with lesions caused by *Stephanofilaria stilesi*, one of the four species reported in the Americas to be associated with udder lesions in cattle (Suthers et al. 2006; Miyakawa et al. 2009).

Biopsy of the ulcerative udder lesions was performed to confirm the diagnosis prior to initiating treatment in the 30 lactating cows included in the study, using both the recommended method (histopathology) and direct sediment examination. Direct sediment examination was positive in 100% of the analyzed samples, confirming the effectiveness and practicality of this method, as previously described in studies of stephanofilariasis lesions (Miyakawa et al. 2008; Miyakawa et al. 2009; Miyakawa et al. 2010). Since this diagnostic technique requires only a light microscope and provides rapid results under field conditions, treatment could be initiated immediately without awaiting histopathology analysis, as conducted in this study.

It should be emphasized that this examination aimed only at parasite detection based on the diagnostic approach previously described for bovine stephanofilariasis (Miyakawa et al. 2008), rather than performing a detailed morphological characterization or species-level identification of the nematodes as performed by Saparov et al. (2014). However, the lack of characterization information for both the observed nematodes and the flies present on the farm represents a limitation of this study. Morphological similarities among known species, which makes species-level classification more challenging. Thereby, further research is needed to better characterize the morphological differences among species of *Stephanofilaria* spp. (Miyakawa et al. 2009; 2010).

No larval or adult forms of *Stephanofilaria* spp. were detected in histopathological analysis, which contrasts with the concept that this technique is an effective method for confirming the diagnosis based on parasite visualization (White & Evans, 2002; Scott 2007; Matos et al. 2022) and corroborates the non-detection previously reported in 24 cows (Miyakawa et al. 2007). It is important to note that direct sediment examination relies on the migration of parasites from the tissue into a 0.9% NaCl solution (Ueno & Chibana, 1977). Thus, a possible explanation for the absence of parasites in histopathological sections is their migration into the formaldehyde solution, similar to what occurs during direct sediment examination, a finding recently reported by Matos et al. (2022).

The histopathological examination revealed perivascular inflammatory infiltrates composed of mononuclear cells and eosinophils, together with granulation tissue formation. These findings are consistent with chronic active dermatitis and an ongoing tissue-repair response. Similar changes were described by Matos et al. (2022), who reported intense eosinophilic infiltration in the superficial dermis, moderate perivascular lymphocytic infiltration, and proliferation of fibrous connective tissue in lesions associated with Stephanofilaria spp. Those authors also observed orthokeratotic hyperkeratosis, papillary acanthosis, spongiosis, and ulceration, although parasitic structures were not identified in the histological sections (Matos et al. 2022; Naseem et al. 2023).

Eosinophilic inflammation appears to be an important histopathological feature of lesions associated with *Stephanofilaria* spp. by Naseem et al. (2023) demonstrated that Stephanofilaria positive lesions had significantly greater inflammatory infiltration and more severe epidermal, dermal, and adnexal damage than negative lesions. Eosinophils, macrophages, and lymphocytes were significantly more abundant in positive lesions, with eosinophils

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particularly concentrated around adult nematodes and microfilariae. The presence of eosinophils has also been reported in cytological smears obtained from lesions containing *Stephanofilaria* spp., supporting the association between eosinophilic inflammation and parasitic infection (Silva et al. 2010; Hargis & Ginn 2013; Matos et al. 2022; Naseem et al. 2023).

A comparable tissue response has been described in equine cutaneous habronemiasis, in which the presence of *Habronema* spp. larvae or parasitic remnants in the tissue induces a marked eosinophilic and granulomatous inflammatory response, frequently associated with excessive granulation tissue formation and delayed healing (Traversa et al. 2007; Hargis & Ginn 2013; Mauldin & Kennedy 2016). Although stephanofilariasis and habronemiasis are etiologically distinct diseases affecting different host species, this comparison highlights that the presence of parasites in the tissue can contribute to chronic inflammation and impair lesion resolution. Therefore, considering the macroscopic characteristics of the lesions, their anatomical location, and the histopathological findings, the observations are consistent with stephanofilariasis. In this context, direct examination appears to be the simplest and fastest method for confirming the diagnosis (Miyakawa et al. 2009; Schade et al. 2018).

The treatment of granulomatous lesions of parasitic origin aims to reduce lesion size, control the associated inflammation response, and eliminate the agent (Pugh et al. 2014). Therefore, parasite elimination is a key factor for treatment success. Previous studies indicate that effective treatment strategies for these types of ulcerative lesions involve a combination of local and systemic approaches, including the use of healing ointments, antiseptics, and administration of anthelmintics, and in some cases, surgical or chemical debridement (Merlo et al. 2023; Pliego et al. 2023).

Prior to the topical application of both treatments, during the cleansing of the lesions with dry paper towels, the cows showed signs of discomfort and irritation upon contact, responding with kicking and agitation. However, throughout the treatment, an apparent improvement in the lesion condition was observed in the HC group, including a reduction in serosanguineous secretion, decreased accumulation of debris, and fewer adhered scabs. Farm staff also reported a decrease in foul odor from the lesions in both therapeutic protocols. This observation is consistent with findings reported by Deola Filho et al. (2024).

Treatment with FPT resulted in a significant reduction in lesion area between D0 and D60, as well as a higher regression index at D60 compared to the HC treatment. This effect may be primarily related to the antiparasitic activity of trichlorfon, an organophosphate that inhibits acetylcholinesterase, leading to paralysis and death of the parasite. Favorable results have also been reported following the topical application of 6% trichlorfon to stephanofilariasis lesions (Miyakawa et al. 2012; Pazinato et al. 2013; Schade et al. 2018). In addition, the permethrin present in the commercial ointment (Unguento®) may have contributed to the observed response due to its insecticidal properties and potential repellent effect against flies (Al-Eissa et al. 2008). Considering that the formulation was applied every 48 h, repeated applications may have reduced fly contact with the lesions during the treatment period. Piperonyl butoxide, in turn, acts as a synergist and may enhance the insecticidal activity of permethrin (Pap et al. 2001). Finally, zinc oxide has been associated with the promotion of re-epithelialization and cutaneous wound repair (Ågren et al. 1991; Kietzmann 1999).

These components, therefore, may have exerted adjuvant effects alongside trichlorfon by potentially reducing insect contact with the lesions and promoting tissue repair. However, because fly abundance and contact with the lesions were not evaluated during the study, the contribution of this potential insecticidal or repellent effect to the observed treatment response could not be determined. Another important consideration is that, since these components were administered as a combined formulation, it was not possible to determine the individual contribution of each substance to the observed lesion regression. Furthermore, the final trichlorfon concentration of 32.7% in the FPT was higher than the 6% concentration used in previous study, and it was formulated with an ointment vehicle rather than vaseline (Miyakawa et al. 2012).

There was no significant difference between treatment groups, and although lesion area decreased in both groups over the 60-day period, complete healing occurred in only two cows (one from each group), indicating that the treatment period was insufficient to achieve consistent lesion resolution. Similar findings were reported by Deola Filho et al. (2024), who observed that topical treatment alone with HoofCare® did not significantly reduce lesion area.

One possible explanation for the absence of statistical difference in lesion area is the inclusion of lesions varying sizes within the same evaluation groups, which may have influenced the statistical outcomes. A similar limitation applies to the regression index, as lesions with different initial sizes may exhibit distinct reduction patterns. This limitation has also been noted in previous studies (Silva et al. 2010; Miyakawa et al. 2010). Therefore, future studies should consider stratifying lesions according to their initial size to allow more accurate assessment of lesion regression and treatment effectiveness, for example by grouping lesions as mild, moderate, and severe (Rai et al. 1994). This type of classification was not feasible in the present study due to the limited number of lesions within each category, with lesions exceeding 25cm^2 being evaluated together with those measuring as little as 5cm^2.

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It should also be noted that the classification by lesion size there was not performed for the thermographic analysis, which may have contributed to the lack of significant difference observed. Previous studies have demonstrated that the thermographic examination enables early detection of inflammatory process through subtle temperature changes (Oliveira et al. 2022; Baccili et al. 2024), and a positive correlation has been reported between udder temperature values and somatic cell count, with up to 95% sensitivity for detecting subclinical mastitis (Polat et al. 2010; Sathiyabarathi et al. 2016). Therefore, further studies are needed to investigate temperature variation in the udder and its relationship with stephanofilariasis lesions, as well as its association with somatic cell count in affected cows.

It is important to highlight that thermographic analysis of ulcerative udder lesions proved to be a practical, rapid, and reliable tool for data collection, as previously described by Ribeiro et al. (2023). However, physical restraint of the animals and the assistance of at least two people were required to perform the procedure, as some lesions were difficult to locate, especially those positioned between udder quarters. This differs from other studies that did not require animal restraint for udder thermography in mastitis detection (Ribeiro et al. 2023; Velasco-Bolaños et al. 2021).

Although *Stephanofilaria* spp. is recognized as the primary agent associated with the development of these lesions, other factors may contribute to their progression and severity. Naseem et al. (2022) isolated *Staphylococcus agnetis* and *Staphylococcus hyicus* from skin lesions associated with *Haematobia irritans* infestation in cattle and identified virulence genes potentially associated in lesion development. Subsequently, Naseem et al. (2023) observed greater epidermal disruption and ulcerative lesions associated with bacteria, suggesting that *Staphylococcus* spp. may exacerbate cutaneous damage. However, the detection of *Staphylococcus* spp. in skin lesions must be interpreted with caution regarding their role in pathogenesis.

Species of this genus, such as *S. aureus* and *S. hyicus*, are known components of the normal skin microbiota in cattle and frequently act as opportunistic pathogens in presence of compromised skin. This colonization of the skin wounds is caused a mixed bacterial microbiota, including also *Streptococcus* spp., *Corynebacterium* spp., and *Trueperella* spp. This reinforces that the presence of bacteria in ulcerative lesions may reflect a polymicrobial process secondary to disruption of the skin barrier rather than the involvement of a single etiological agent. Furthermore, although *Staphylococcus* spp. may be involved in udder dermatitis, studies indicate that these bacteria are not considered predominant in the pathological process (Foster 2012; Mala et al. 2021).

The involvement of bacteria was not investigated in this study, however, secondary bacterial involvement may have present, especially because some cows exhibited areas of tissue necrosis accompanied by a foul odor. This finding is consistent with previous studies on udder dermatitis in dairy cows that identified several bacterial species, particularly *Trueperella pyogenes* and *Bacteroides pyogenes*, associated with necrotic tissue (van Engelen et al. 2021; Vermeersch et al. 2025). Thus, in the context of stephanofilariasis, the presence of these bacteria may represent colonization or secondary infection of a previously compromised skin barrier, although their contribution to lesion progression, severity, and persistence remains to be clarified.

To the best of our knowledge, no studies conducted in the Americas have longitudinally monitored the clinical progression of these lesions while simultaneously investigating the role of bacteria in their progression. This knowledge gap is particularly relevant considering the limited information available on the interactions between *Staphylococcus* spp. and other bacteria, as well as on the predisposing factors involved in the establishment and development of skin diseases in cattle (Foster 2012). Therefore, prospective studies including bacterial culture, molecular identification, and characterization of bacterial isolates at different stages of lesion progression are needed to clarify whether these bacteria act predominantly as secondary infections or as aggravating factors capable of interfering with lesion progression and the healing process.

Studies by Naseem et al. (2022, 2023) suggest that bacterial infections and hypersensitivity reactions to fly bites may contribute to the development of cutaneous lesions. However, both studies were conducted in beef cattle, in which the lesions were located near the medial canthus of the eyes and on the neck, shoulders, lateral body surfaces, and abdomen.

Nevertheless, neither study specifically evaluated udder lesions or reported the lactational status of the animals. In contrast, all animals included in our study were lactating dairy cows with udder lesions. Furthermore, the detection of nematode structures compatible with *Stephanofilaria* spp. by direct examination of lesion sediment supports the involvement of the parasite in this specific clinical presentation and anatomical location. Therefore, although other agents may act as contributing factors, the available evidence is insufficient to attribute the development of the udder lesions observed in the present study to these agents. Further studies are needed to investigate the involvement of other agents in the development and progression of udder lesions in lactating cows.

Regarding the use of HoofCare® for lesion treatment, the product contains zinc, copper, and tea tree oil, components associated with antimicrobial properties. Studies on bovine digital dermatitis have demonstrated favorable results with topical application and footbath protocols (Mendonça et al. 2024; 2025; 2026). The greater reduction in lesion area observed in the FPT group compared with the HC group may suggest that bacterial infection by *Staphylococcus* spp. was not the primary factor responsible for the lesions. This interpretation is based on the antimicrobial activity of HoofCare® (Mendonça et al. 2024; 2025). Thus, if *Staphylococcus* spp. played a

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primary etiological role, a more pronounced response to HoofCare® treatment might have been expected. However, a better response was observed with the trichlorfon-containing formulation, an antiparasitic compound with previously demonstrated efficacy (Miyakawa et al. 2012). Although this finding does not exclude the involvement of *Staphylococcus* spp. as a secondary agent, it makes bacterial infection less likely to have been the primary determinant of the lesions.

Nevertheless, the depth of penetration of the components of HoofCare® and the formulation containing ointment and trichlorfon following topical application has not yet been well established. Therefore, it remains unclear whether these compounds reach *Stephanofilaria* spp. or bacteria located in the deeper layers of the lesions at effective concentrations, or whether their effects remain predominantly restricted to the wound surface. Studies evaluating their tissue penetration and activity against *Stephanofilaria* spp. and *Staphylococcus* spp. at different lesion depths are needed to better elucidate their mechanisms of action.

At the end of the 60-day treatment, all 30 cows remained positive for *Stephanofilaria* spp. in the direct sediment examination, demonstrating that treatment protocol was ineffective in eliminating the parasite. Although complete lesion healing was observed in two cows, both remained positive for the parasite. The persistence of the parasite likely prevented sustained resolution of the lesions. In some cases, lesions may temporarily regress but reappear within 30 days, as observed in this study. Larvae can persist in the lesion for at least four weeks. Furthermore, the presence of the parasite, whether dead or alive, continues to stimulate the local inflammatory responses through release of histamine, causing a hypersensitivity reaction. In addition, secondary bacterial or fungal infections may occur, further contributing to lesion persistence and progression (Pugh et al. 2014; Santos et al. 2016).

Therefore, systemic anthelmintic therapy should be considered as an adjunct to topical treatment to improve therapeutic outcomes, especially since previous studies have demonstrated the effectiveness of associating parenteral anthelmintics with topical treatments for stephanofilariasis lesions. For instance, the administration of levamisole (9-12 mg/kg of body weight) in a single dose combined with daily topical zinc oxide until complete healing resulted in 100% resolution of the lesions (Rai et al. 1994).

CONCLUSION

The therapeutic protocols consisting of topical applications every 48 hours of HoofCare® and formulated paste with triclorfon were ineffective in promoting complete healing of stephanofilariasis udder lesions within a 60-day period. These findings suggest that the inclusion of parenteral anthelmintic therapy may be necessary to achieve parasite elimination and improve lesion healing.

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Conflict of interest statement.- The authors declare that even though this study received funding and technical support from the manufacturer of the product studied (Salmix), the company did not exert any influence on the data analysis or the interpretation of the results.

Credit author statement.- Raymis B.R. Moura, Ana P.A. Mendonça, Piero H.M. Teodoro, Júlio A.N. Lisbôa, and Celso A. Rodrigues developed the study conception and methodology. Raymis B.R. Moura and Ana P.A. Mendonça performed the preparation of the material and data collection. Selwyn A. Headley performed histopathological laboratory analyses of the fragments. Raymis B.R. Moura and Ana P.A. Mendonça performed the analyses and interpretation. Raymis B.R. Moura, Ana P.A. Mendonça and Celso A. Rodrigues were responsible for the writing and revision of the manuscript. All authors contributed to the final manuscript and have read and approved the submitted version.

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Figures

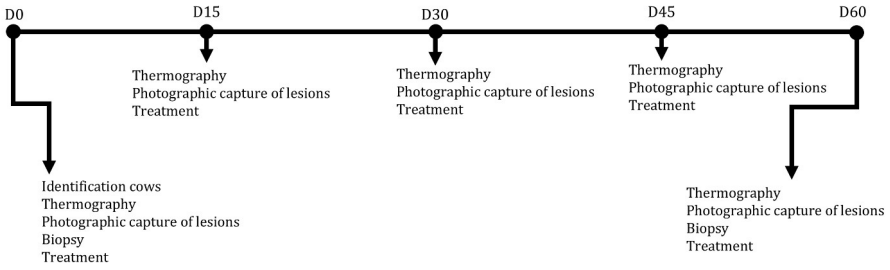


Fig.1. Schematic representation of the study design, showing the time points for biopsy, treatment, and lesion evaluation.

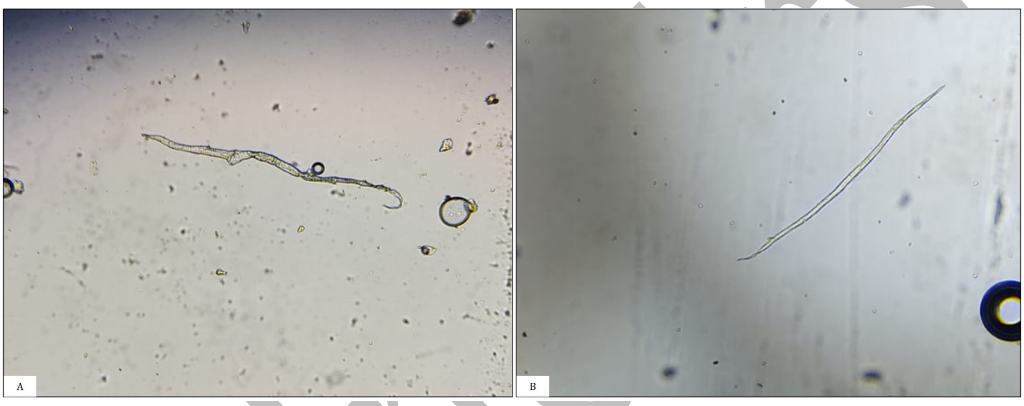


Fig.2. (A-B) Nematode structures compatible with *Stephanofilaria* spp. observed under light microscopy at 400× magnification during direct sediment examination of isotonic saline solution (0.9% NaCl) in which the biopsied tissue was immersed.

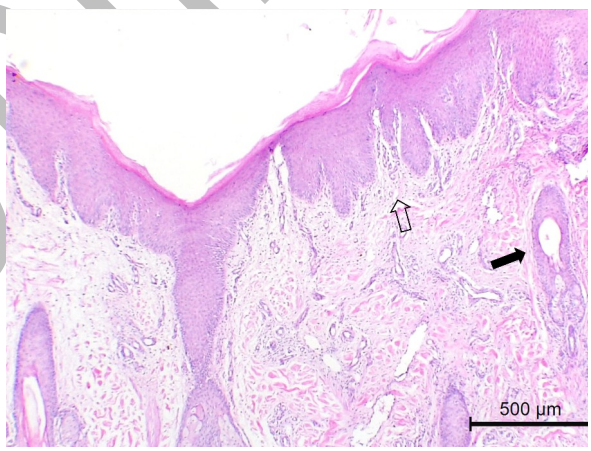


Fig.3. Histopathological examination of tissue biopsied from an udder lesion positive to stephanofilariasis of lactating cows, showing eosinophils, granulation tissue, and inflammatory cells adjacent to the epidermis (arrowhead), as well as vascular proliferation (arrow) (H&E, 10x).

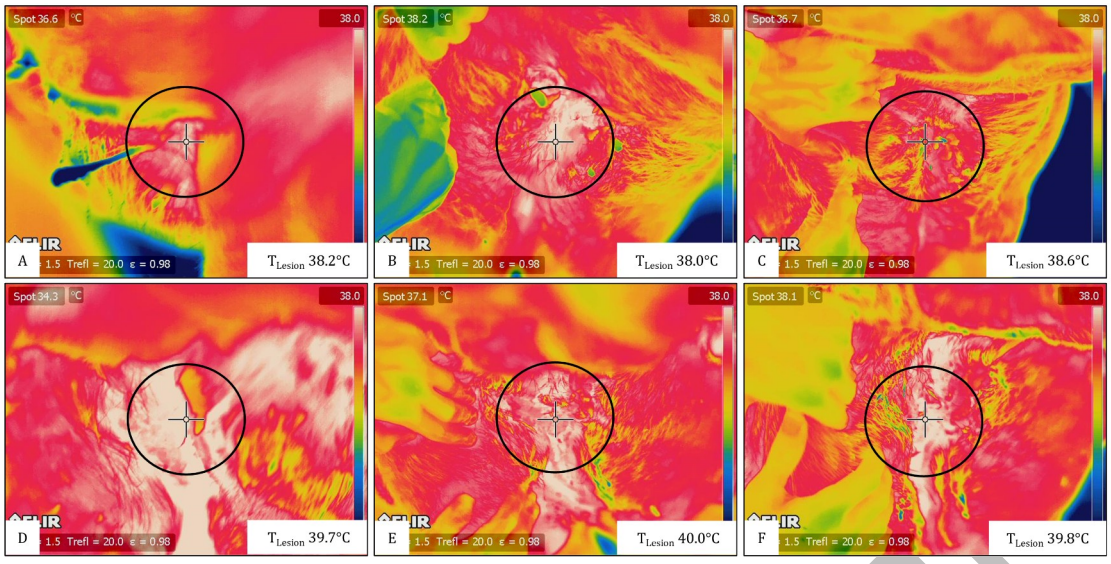


Fig. 4. Comparison of the corrected and standardized thermographic temperature of ulcerative udder positive to stephanofilariasis of lactating cows and subjected to topical treatment with HoofCare® (A-C) and positive control (D-F). A and D: D0 (before treatment); B and E: D30 (30th day of treatment); C and F: D60 (end of treatment).

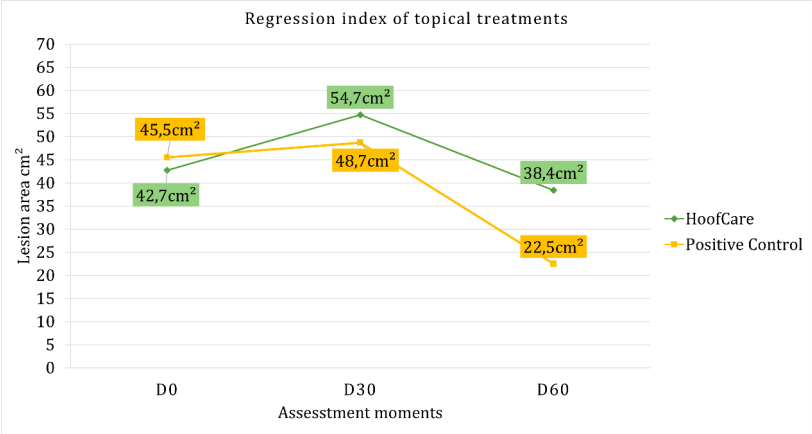


Fig.5. Evolution of the lesion regression index (area) of ulcerative udder lesions positive to stephanofilariasis of lactating cows and subjected to topical treatments with HoofCare® (HC) and positive control (FPT) at time points D0, D30 and D60.

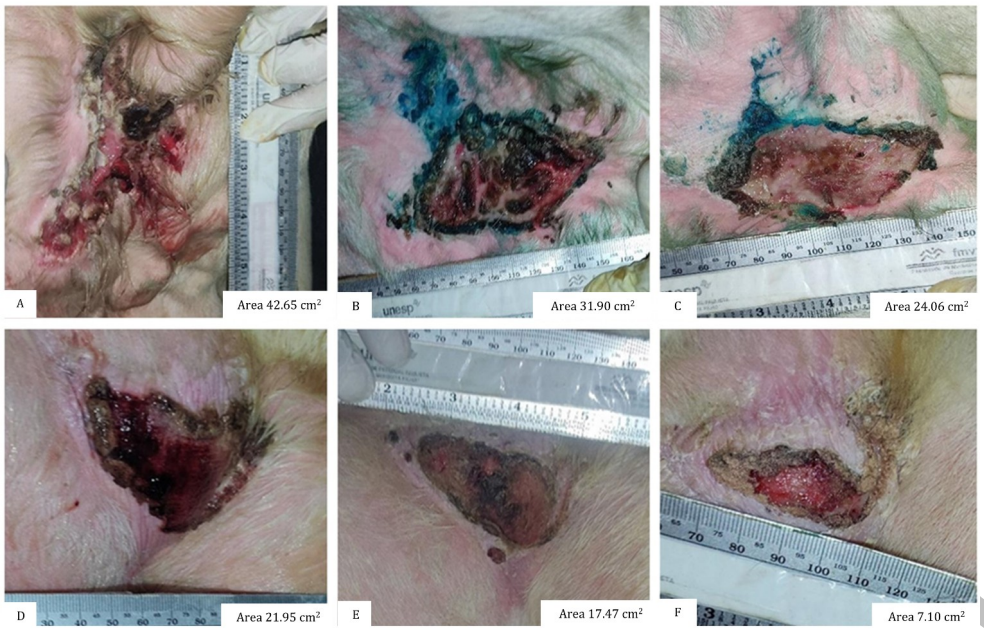


Fig.6. Comparison of morphometric measurements of ulcerative udder lesions positive to stephanofilariasis of lactating cows and subjected to topical treatments with HoofCare® (A-C) and positive control (D-F). A and D: D0 (before treatment); B and E: D30 (30th day of treatment); C and F: D60 (end of treatment).

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Table 1. Comparison of the medians [Q1 – Q3] of corrected and standardized thermographic temperatures (°C) of ulcerative udder lesions positive to stephanofilariasis of lactating cows and subjected to topical treatment with HoofCare® and positive control

Assessment time points	Corrected and standardized temperature (C°)	
	HoofCare®	Positive control
D0	38.2°C [37.5° - 38.4°] ^{Aa}	37.5°C [37.4° - 38.4°] ^{Aa}
D15	38.3°C [37.7° - 38.7°] ^{Aa}	37.9°C [37.5° - 38.4°] ^{Aa}
D30	38.1°C [37.7° – 38.7°] ^{Aa}	37.7°C [38.3° – 38.5°] ^{Aa}
D45	38.31°C [37.5° – 38.6°] ^{Aa}	37.9°C [37.5° - 38.5°] ^{Aa}
D60	38.6°C [37.7° – 38.7°] ^{Aa}	37.7°C [37.4 – 38.6] ^{Aa}
P	0.2579	0.2579

Different lowercase letters in the column indicate a statistical difference (P < 0.05); Different uppercase letters in the row indicate a statistical difference (P < 0.05).

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Table 2. Comparison of the medians [Q1 – Q3] of lesion area (cm^2) in ulcerative udder lesions positive to stephanofilariasis of lactating cows and subjected to topical treatment with HoofCare® and positive control

Assessment time points	Treatment groups	
	HoofCare®	Positive control
D0	42.7 [28.9 – 62.6] ^{Aa}	45.5 [31.9 – 77.5] ^{Aa}
D15	40.8 [16.5 – 63.9] ^{Aa}	50.6 [23.8 – 74.4] ^{Aa}
D30	54.7 [21.9 – 87.7] ^{Aa}	48.3 [25.1 – 76.3] ^{Aa}
D45	44.2 [16.1 – 101.6] ^{Aa}	34.6 [15.1 – 65.1] ^{Aa}
D60	38.4 [15.8 – 72.9] ^{Aa}	22.6 [13.7 – 35.6] ^{Ab}
P	0.9729	0.0570

Different lowercase letters in the column indicate a statistical difference ($P < 0.05$); Different uppercase letters in the row indicate a statistical difference ($P < 0.05$).

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Table 3. Comparison of the medians [Q1 – Q3] of the regression index of ulcerative udder lesions positive to stephanofilariasis of lactating cows and subjected to topical treatment with HoofCare® positive control

Assessment time points	Treatment groups	
	HoofCare®	Positive control
D30	24.7%^{Aa}	24.3% ^{Aa}
D60	19.2%^{Aa}	15.7% ^{Bb}

Different lowercase letters in the column indicate a statistical difference (P < 0.05); Different uppercase letters in the row indicate a statistical difference (P < 0.05).

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