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Digital dermatitis-associated *Treponema* species detection and quantification in migratory tundra caribou (*Rangifer tarandus*)

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ABSTRACT

Treponema spp. are associated with infectious lameness in livestock and wild ruminants. While extensive research has been conducted on cattle, investigations in wild ruminants are scarce. Hoof disease is common in caribou populations (*Rangifer tarandus*), but investigations are limited due to the remoteness of the Arctic. Our study aimed to assess the presence of *Treponema* spp. associated with bovine digital dermatitis in caribou. DNA was extracted from coronary band tissues from forty-eight caribou without visible hoof lesions and analyzed using two PCR methods (qPCR and nPCR). *Treponema* spp. were detected in low copy numbers/mg of tissue (3.6 to 6.6 $\times$ 10^1). *T. phagedenis* was the most prevalent and abundant species in 58% of samples by qPCR, followed by *T. medium* (44%), and *T. pedis* (10%). The qPCR and nPCR agreement ranged between 65% and 75% (Cohen's kappa 0.22–0.51). Sanger sequencing of thirteen nPCR products confirmed that treponemes in caribou are remarkably similar to those found in domestic ruminants and wild elk. Our study highlights the colonization of treponemes in healthy hooves of a wild ruminant in the Arctic, where there is no presence of livestock, and expands knowledge on the host range and distribution of treponemes. These findings also emphasize the need for further research into the multifactorial nature of treponema-associated hoof diseases and the putative role of treponemes in infectious lameness affecting caribou.

Treponemes are spirochetal bacteria that can colonize a wide range of hosts and tissues, causing different clinical symptoms, from tooth surfaces in gingival crevices during human periodontitis to human skin and organs in syphilis, as well as ruminant foot skin. Specifically in digital dermatitis (DD) lesions, three *Treponema* spp. are frequently detected, *Treponema phagedenis, Treponema medium*, and *Treponema pedis* (Beninger et al., 2018; Evans et al., 2009). These lesions are characterized by a granular skin ulcer on the heel bulb area, affecting dairy and beef cattle, sheep, and goats (Caddey et al., 2021; Cheli and Mortellaro, 1974; Collighan, 2000; Sullivan et al., 2015a; Sullivan et al., 2013). These *Treponema* spp. have been also detected in healthy foot skin (Beninger et al., 2018; Caddey et al., 2021; Frosth et al., 2023); however, studies including samples from healthy animals are limited. Yet, the etiology of DD is still under investigation, and its multifactorial origin remains unresolved.

Treponema spp. have also been associated with an emerging syndrome called *Treponema*-associated hoof disease (TAHD) in free-ranging elk (Clegg et al., 2015; Han et al., 2019), and with a novel hoof disease in captive European bison (Hoby et al., 2021). Although DD has not been diagnosed as such in caribou, hoof infections are not rare and outbreaks with significant morbidity and mortality have been reported (Tomaselli et al., 2018; Valkenburg et al., 2003). *Fusobacterium necrophorum*, another DD-associated bacterial species, is commonly identified in these hoof lesions in *Rangifer* (Handeland et al., 2010; Tryland and Kutz, 2018), but comprehensive health investigations in remote areas where caribou live are not always possible.

In this study, we aimed to detect three DD-associated *Treponema* spp. in caribou healthy foot tissue, employing two molecular techniques, to expand knowledge on treponemes distribution in wild ruminants.

We analyzed forty-eight coronary band skin tissue samples from tundra caribou belonging to the endangered Dolphin and Union caribou herd (*Rangifer tarandus groenlandicus x R. t. pearyi*). This herd is endemic to the central Arctic of Canada and has declined by more than 80% in the last two decades (Campbell, 2021). The causes of this decline are not well understood, although multiple health determinants, including infectious diseases, are suspected (Aguilar et al., 2023).

Left hind leg samples from caribou (from the metatarsus to the hoof) were collected from hunted caribou during its migration through the

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Wentzel river area (Kitikmeot region, Nunavut, Canada) in April and early May 2018 and 2019. Samples were collected by Inuit hunters from Kugluktuk (Nunavut, Canada) through a community-based wildlife health surveillance program involving Indigenous communities, the Government of Nunavut, and the Kutz lab at the University of Calgary (Aguilar et al., 2023). Environmental temperatures in the Arctic in April–May are still below 0 °C and samples were immediately frozen, individually placed in separate bags and stored and transported frozen to the University of Calgary. At the necropsy room, stringent measures were taken during processing of samples to avoid cross-contamination, including the use of fresh gloves for each sample, sterile scalpel blades, and new tweezers for every animal.

The sterile scalpel blades were used to shave and collect a piece of skin of approximately 1 × 0.3 cm comprising the coronary band. Samples were introduced directly into cryovials without contacting any other surface.

Bacterial genomic DNA (gDNA) was extracted from weighed tissue samples using the DNeasy Blood and Tissue kit (Qiagen, Canada) following the manufacturer's protocol. Briefly, approximately 25 mg of tissue was digested with proteinase K and ATL buffer, and the DNA was further purified using spin columns. Extracted gDNA was subjected to species-specific real-time quantitative (qPCR) targeting *T. phagedenis, T. pedis*, and *T. medium* (Beninger et al., 2018). This protocol was previously validated using spiking experiments in biopsy tissue samples. In this study, negative controls were added to control for contamination involving an extraction control (EC, extraction kit reagents without samples) and a negative template control (NTC, qPCR reaction reagents with ultra-pure water). Also, positive controls consisted of plasmids with cloned target DNA sequences constructed as described in Beninger et al. (2018). If amplification was detected in the NTC or EC, a new run or extraction from stocked tissues was performed, respectively. Bacterial quantities were calculated based on a standard curve generated from serially diluted plasmids with cloned target DNA in each run, and the results were normalized by the amount of extracted DNA and expressed per milligram of tissue.

To confirm the results, samples were also analyzed with a widely used nested PCR (nPCR) for *T. phagedenis*-like, *T. medium/T. vicentii*-like, and *T. pedis* groups (Evans et al., 2009). The nPCR products, encompassing 400–450 bp of the 16S rRNA gene, were observed by electrophoresis on 1% agarose gel. Positive (gDNA for nPCR and plasmid with cloned gene of interest for qPCR) and negative controls (non-template and extraction control) were used to validate both PCR results.

We compared bacterial species detection between qPCR and nPCR using Cohen's kappa coefficient. A set of thirteen nPCR products were submitted for Sanger sequencing in the Centre for Health Genomics and Informatics at the University of Calgary. Geneious prime version 2023.0.3 was used to align sequencing results with previously published sequences of isolates from cattle, sheep, elk, dogs, and humans using MAFFT method. A phylogenetic tree was built using the Maximum Likelihood tree method and the Jukes-Cantor model with bootstrap values from 10,000 interactions and edited using iTOL online tool (Letunic and Bork, 2021). 16 s rRNA gene sequences in this study are available in GenBank (accession numbers OR504432 to OR404444).

T. phagedenis was the most prevalent and abundant bacterial species in healthy foot tissue from caribou, followed by *T. medium*, and *T. pedis* (Table 1). The three targeted treponemes were detected together in 10% (5/48) of samples by qPCR and in 15% (7/48) of samples by nPCR. When we analyzed each bacterial species, we observed a higher level of agreement between the methods for *T. phagedenis*, followed by *T. pedis* and *T. medium*. Except for *T. pedis*, more positive samples were detected with the qPCR than the nPCR (Table 1).

We detected a median of 23.0, 12.0, and 6.2 copy numbers/mg of tissue in healthy foot tissue from caribou for *T. phagedenis, T. medium*, and *T. pedis*, respectively. In cattle, *Treponema* spp. were also detected in small amounts in healthy foot tissue (Beninger et al., 2018; Caddey et al., 2021; Frosth et al., 2023), from 1 to 100 copy numbers/mg of tissue. While *Treponema* spp. were absent in elk healthy foot tissue by nPCR, spirochetes-like organisms were observed by phase-contrast microscopy, suggesting that *Treponema*-like organisms may also occur in apparently healthy elk (Clegg et al., 2015). Given the limited number of samples and no active lesions analyzed in this study, no conclusions regarding the implication of *Treponema* in foot disease can be inferred. Recently, a digital necrobacillosis outbreak was reported in alpine reindeer in Norway associated with exceptional hot and wet weather (Mysterud et al., 2023). Crowding and wet and muddy grounds are also often associated with digital necrobacillosis outbreaks in domestic reindeer (Tryland and Kutz, 2018) and DD in cattle (Evans et al., 2016). Thus, the presence of *Treponema* spp. in caribou should be further investigated, especially considering hoof diseases during wetter summer conditions in the Arctic.

Treponema spp. presence is not the only factor necessary to induce DD as it has been observed in experimental infections (Gomez et al., 2012; Krull et al., 2016). The scientific community has recognized the multifactorial nature of this disease (Orsel et al., 2018). Although possible factors affecting the establishment of the infection have not been studied in detail and their specific role is still unknown, collective observations suggest that the infection might be related to the presence of determinants affecting the skin barrier, immune status of the animals, or presence of a specific microbiota type including the presence of several anaerobic groups or specific *Treponema* species composition (Arrazuria et al., 2020; Beninger et al., 2018; Caddey et al., 2021; Orsel et al., 2018; Watts et al., 2018).

All 13 amplified 16S rRNA nPCR products were similar to each other, with more than 97% identity, and matched those previously isolated from cattle, sheep (Sullivan et al., 2015b), and elk (Clegg et al., 2015) (Fig. 1). These results suggest that the bacterial species present in healthy foot tissue from caribou do not differ much from those found in DD lesions in other ruminants. These results are consistent with other studies on the low genetic diversity of DD-associated *Treponema* spp. strains in different hosts and countries (Clegg et al., 2016). Interestingly, we found two single nucleotide polymorphisms (SNPs) in *T. medium* in caribou samples, suggesting potential strain differences between hosts. Additional samples, particularly from foot lesions in caribou and whole genome analysis will reveal more about these genetic differences.

This study used molecular techniques to detect and quantify fastidious bacteria, providing relevant information to better understand the causal agents of infectious hoof diseases in ruminants. The species-specific qPCR test could be a useful tool for detecting and quantifying DD-associated *Treponema* spp. in wildlife, as validated by a widely used nPCR for livestock and wildlife. The qPCR was more sensitive in detecting *T. phagedenis* and *T. medium* compared to nPCR; however, more positive samples were observed for *T. pedis* by nPCR than qPCR.

Table 1
Frequency of positive caribou (*Rangifer tarandus)* coronary band tissue samples for *Treponema phagedenis* (Tphg), *Treponema medium* (Tmed), and *Treponema pedis* (Tped) detected by nested PCR (nPCR), quantitative PCR (qPCR), percentage of agreement and Cohen's kappa coefficient between the tests, median and interquartile range (IQR) of gene copy numbers/mg of tissue for each bacterial species by qPCR.

Bacterial species	Frequency of positive nPCR	Frequency of positive qPCR	% agreement	Cohen's Kappa	Median	IQR
Tphg	20/48 (42%)	28/48 (58%)	75	0.51	2.3×10^1	$9.7–6.6 \times 10^1$
Tmed	12/48 (25%)	21/48 (44%)	65	0.24	1.2×10^1	$6.2–1.9 \times 10^1$
Tped	15/48 (31%)	5/48 (10%)	69	0.22	6.2	$3.6–1.1 \times 10^1$

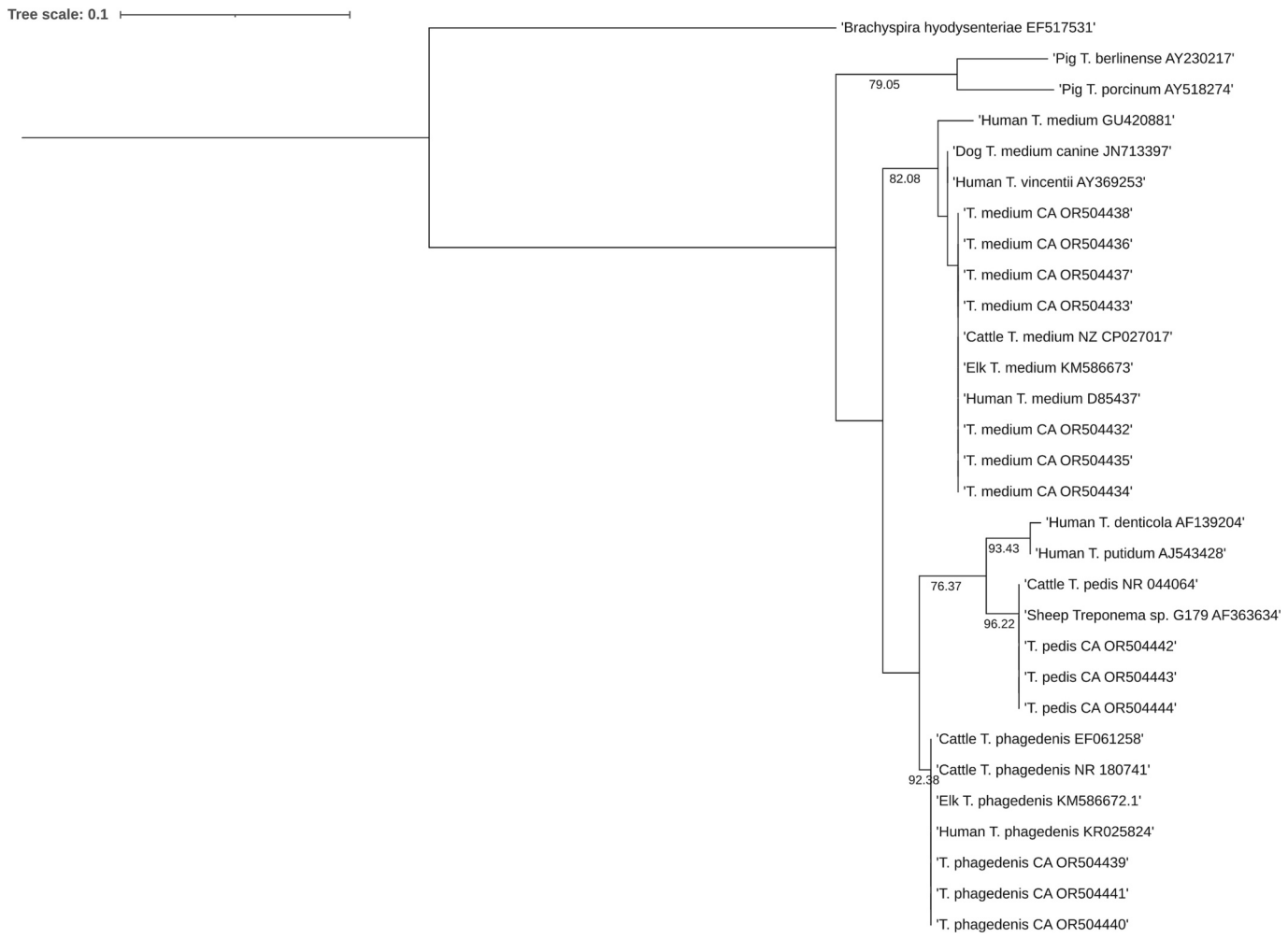

Fig. 1. A maximum likelihood tree that compares the *Treponema phagedenis, T. medium*, and *T. pedis* sequences obtained from healthy foot tissue (coronary band) from caribou with those species isolated from cattle, elk, sheep, dogs, humans, and pigs. The sequences from this study are denoted as "CA" with their accession numbers, while the sequences from GenBank are shown with their respective accession numbers. Bootstrap proportions values are shown on branches (10,000 interactions). *Brachyspira hyodysenteriae* was added as an outgroup.

Notably, qPCR exhibited 100% specificity in detecting targeted treponemas (Beninger et al., 2018), whereas nPCR might have lower specificity due to the high similarity of the targeted genes between human and bovine treponemes (Evans et al., 2009). Cross-reactivity with other treponemal species may be also occurring in the nPCR. Furthermore, some *T. pedis* strains may lack the target sequence (Frosth et al., 2023). Since no isolates were obtained by culture in this study, further conclusions cannot be drawn. However, it needs to be considered that the two PCRs utilized in this study target distinct genes. Due to the limited exploration of genetic diversity within *Treponema* associated with wild animals and/or caribou, it is not possible to evaluate the sensitivity and specificity of both employed PCRs in such samples.

To the best of our knowledge, this is the first report of DD-associated *Treponema* spp. detection in caribou tissue samples. These results expand our knowledge on the geographic distribution of *Treponema* spp. in Arctic regions without livestock, and a novel host description in a wild ungulate. Our study confirmed that DD-associated treponemes may frequently colonize caribou foot tissue. Hoof diseases in caribou populations require further investigation as lameness not only limits animals' access to food, causing weight loss due to impaired mobility but also makes them vulnerable to predation; thus, it is a life-threatening condition for these animals (Mysterud et al., 2023). Research is needed to further investigate the presence and role of *Treponema* in caribou infectious hoof diseases and their impact on climate and environmental change.

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CRediT authorship contribution statement

Angelica P. Dias: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing, Formal analysis, Visualization. **Xavier F. Aguilar:** Conceptualization, Investigation, Methodology, Project administration, Writing – review & editing, Supervision. **Jeroen De Buck:** Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Susan Kutz:** Funding acquisition, Investigation, Resources, Supervision, Validation, Writing – review & editing. **Rakel Arrazuria:** Conceptualization, Data curation, Formal

analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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