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Isolation of Digital Dermatitis Treponemes from Hoof Lesions in Wild North American Elk (*Cervus elaphus*) in Washington State, USA

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Since 2008, a large increase in the numbers of cases of lameness have been seen in wild North American elk (*Cervus elaphus*) from Washington State, USA. The most recent cases manifested as foot lesions similar both clinically and pathologically to those seen in digital dermatitis (DD) in cattle and sheep, a disease with a bacterial etiopathogenesis. To determine whether the same bacteria considered responsible for DD are associated with elk lameness, lesion samples were subjected to bacterial isolation studies and PCR assays for three phylogroups of relevant DD treponemes. The DD treponemes were isolated from lesional tissues but not from control feet or other areas of the diseased foot (including the coronary band or interdigital space), suggesting that the bacteria are strongly associated with DD lesions and may therefore be causal. In addition, PCR analysis revealed that all three unique DD treponeme phylotypes were found in elk hoof disease, and in 23% of samples, all 3 DD-associated treponemes were present in lesions. Sequence analysis of the 16S rRNA gene showed that the elk lesion treponemes were phylogenetically almost identical to those isolated from cattle and sheep DD lesions. The isolates were particularly similar to two of the three culturable DD treponeme phylotypes: specifically, the *Treponema medium*/*Treponema vincentii*-like and *Treponema phagedenis*-like DD spirochetes. The third treponeme culturable phylogroup (*Treponema pedis*), although detected by PCR, was not isolated. This is the first report describing isolation of DD treponemes from a wildlife host, suggesting that the disease may be evolving to include a wider spectrum of cloven-hoofed animals.

Diseases shared between wildlife and domesticated farm animals, such as brucellosis (1) and bovine tuberculosis in white-tailed deer (2), are notoriously difficult to manage. When wild animals are involved in the epidemiology of a disease which affects domestic animals, the effects on disease spread and control can be profound.

Treponemes can infect a wide range of hosts and tissues, causing a spectrum of diseases from syphilis in humans, periodontal disease in both companion animals and humans, and digital dermatitis (DD) in animals (3–5).

DD is an infectious foot disease causing severe lameness both in dairy and beef cattle worldwide (6, 7) and in sheep from the United Kingdom (8) and Ireland (9, 10). Although many bacteria can be isolated from a DD lesion, the most commonly observed bacteria belong to the genus *Treponema*. Cattle DD lesions generally contain spirochetes from several *Treponema* phylogroups, with previously isolated and characterized phylogroups identified as "*Treponema medium*/*Treponema vincentii*-like," "*Treponema phagedenis*-like," and "*Treponema denticola*/*Treponema putidum*-like" bovine (DD) spirochetes (11), with the latter now recognized as a new species, *Treponema pedis* (12). In addition, the same three unique, isolated phylogroups have been identified in bacterial cultures from DD foot lesions in sheep (9). The DD-associated treponemes are found in abundance in all DD lesions and are considered highly specific for DD lesions in both cattle and sheep, being undetectable in normal foot tissues. Current evidence suggests potential roles for the bovine gastrointestinal (GI) tract, manure and slurry, and hoof trimming equipment in the transmission of DD (13–15).

Currently, DD is very common in dairy cattle worldwide, particularly in those countries with intensive farming systems (16, 17). Furthermore, DD is present in beef cattle (18) and increasing in incidence in sheep (10) in the United Kingdom. Taken together, these data suggest that all cloven-hoofed animals are potential hosts for DD treponemes, a situation with similarities to that of the foot-and-mouth disease virus (7). Despite the identification of this widening host range, there have been no reports of treponemes being implicated in lameness in wild animals.

An outbreak of lameness in wild North American elk (*Cervus elaphus*) in Washington state, USA, has been reported since the mid-1990s, with an increased prevalence since 2008. Grossly, affected elk have deformed hooves that are asymmetrical, markedly elongated, and curved or broken or with sloughed horn. The disease pathology for elk showing such clinical signs has been described in detail (19).

Anecdotal information suggests that up to 80% of elk groups in the affected geographical area contain lame elk and that between 30 and 90% of individuals within a group are lame (20). This current study was designed to determine if this elk disease had the same infectious treponemal etiology as the DD lesions found in domesticated hooved species.

Received 8 August 2014 **Returned for modification** 13 September 2014
Accepted 14 October 2014

Accepted manuscript posted online 29 October 2014

Citation Clegg SR, Mansfield KG, Newbrook K, Sullivan LE, Blowey RW, Carter SD, Evans NJ. 2015. Isolation of digital dermatitis treponemes from hoof lesions in wild North American elk (*Cervus elaphus*) in Washington State, USA. J Clin Microbiol 53:88–94. doi:10.1128/JCM.02276-14.

Editor: B. W. Fenwick

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doi:10.1128/JCM.02276-14

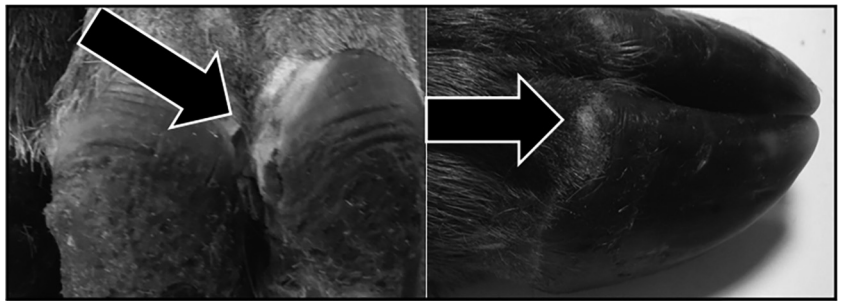

FIG 1 Photograph of an affected elk hoof with an early macroscopic lesion (indicated with an arrow) on the coronary band (right side) and a more typical foot lesion (left side) which shows more visual similarities to digital dermatitis.

MATERIALS AND METHODS

Animal distribution. Elk were sampled in 2013–2014 in southwest Washington. The study area included areas grazed by domestic cattle (*Bos taurus*) and sheep (*Ovis aries*); the DD status of the animals on this pasture was not determined. The terrain and study area have been discussed in more detail recently (19).

Sample collection. In the primary investigation, a variety of tissues were taken from seven young elk, representing four control animals (i.e., two unaffected animals from unaffected areas [elk 17 and 18] and two [elk 21 and 25] unaffected elk from an affected area) and three affected elk (elk 22 to 24). Biopsy samples were taken from the interdigital space, coronary band, and early gross macroscopic foot lesions (as judged by the attending veterinarian), where present (see Table 1). In addition, control samples were taken from the contralateral unaffected foot of affected animals (see Table 1). After the foot surface was cleaned by brushing and washing with sterile saline, a 3-mm punch biopsy specimen was taken from the center of the lesion and placed immediately in oral treponeme enrichment broth (OTEB) (Anaerobe Systems, Morgan Hill, CA, USA) containing rifampin (5 μg/ml) and enrofloxacin (5 μg/ml)). These samples were then transported with ice packs by courier from Washington to the University of Liverpool (~3 or 4 days) for microbiological analysis and were processed immediately for spirochete culture and DNA extraction for PCR. In addition, a second group of samples was collected from seven foot lesions and analogous foot tissues from 13 control tissues with no signs of lesions. These were processed blind, and results were collated after experimental work had been completed.

Isolation of spirochetes. Spirochete isolation attempts were made with all tissues taken from affected elk feet (coronary band, interdigital space, and lesions) and control elk samples. These bacterial isolations were done immediately upon arrival of samples, as described previously for cattle samples (11), using OTEB including rifampin (5 μg/ml) and enrofloxacin (5 μg/ml). Samples were inoculated into OTEB containing fetal calf serum (FCS) (Gibco, Paisley, United Kingdom) to maximize growth of *T. phagedenis*-like and *T. pedis* treponemes or containing rabbit serum (RS) (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) to maximize growth of *T. medium*/*T. vincentii*-like treponemes. All isolation attempts were carried out in an anaerobic cabinet (85% N_2, 10% H_2, and 5% CO_2; 36°C).

Passage of isolates was continued via fastidious anaerobe agar (FAA) plates, supplemented with 5% defibrinated sheep blood and antibiotics as described above, and single colonies from the plates were inoculated into further OTEB tubes to allow pure bacterial cultures to be obtained.

The second group of 20 elk samples, taken from 11 different animals, was inoculated into OTEB for culture as described above. The cultures were then examined by phase-contrast microscopy and analyzed by specific nested PCR assays to identify any specific treponeme phylogroups present, as described below.

DNA extraction. For isolation of bacterial genomic DNA from OTEB cultures, 2 ml of each culture was centrifuged (5,000 × *g*, 10 min, 4°C) in a bench-top centrifuge. DNA was then extracted from the cell pellet using Chelex-100, as previously described (21), and stored at −20°C.

PCR assays. Foot tissue and culture samples were subjected to nested PCR assays specific for the three DD-associated treponeme groups, "*T. medium*/*T. vincentii*-like," "*T. phagedenis*-like," and *T. pedis*, described previously (11, 12), with resulting PCR products encompassing 300 to 500 bp of the 16S rRNA gene. All hoof samples were also subjected to the *Treponema* genus PCR assay (22).

To validate the PCR assays, each experiment included positive controls (bovine DD treponeme genomic DNA from each of the three unique bovine DD treponeme phylogroups) and a negative control (water) as described previously (12), with all assays performed in triplicate. Characterization of isolates used PCR and gene sequencing of nearly the entire 16S rRNA gene, as described previously (11), with the sequencing outsourced to a commercial company (Beckman Coulter Genomics, Takeley, Essex, United Kingdom).

Sequencing and sequence analysis. Amplified PCR products were sequenced commercially, and the fragments of the 16S rRNA were assembled using the Chromas Pro sequence analysis package (Technelysium Pty. Ltd.) to produce a consensus gene sequence. Gene sequences were aligned using the software program CLUSTALW as implemented in the program MEGA 5.0 (23). The DNA alignment was subjected to analysis using the software program Modeltest, as implemented in the Topali interface (24), which revealed that the best-fit model was general time reversible (GTR). This was used to produce nucleotide maximum likelihood phylogenetic trees (bootstrap values based on 10,000 iterations).

Nucleotide sequence accession numbers. 16S rRNA gene sequences of isolates analyzed in this work are available in GenBank (accession numbers KM586666 to KM586673).

RESULTS

The pathology of lesions taken from elk feet has been described in detail recently (19), and an example is shown in Fig. 1. Briefly, a macroscopic description of the lesion pathology identified erosive lesions at the coronary band, underrun horn of the wall and sole, erosion of the pedal bone, and a red stippled appearance of exposed corium. It was this last appearance that initially suggested the similarity to DD lesions.

Spirochete isolations. Samples were taken from lesions, coronary bands, and interdigital spaces (IDS) from seven elk, three of which showed macroscopic coronary band lesions (Table 1). All six samples of lesional material taken from these three animals were positive for treponeme culture, subsequently confirmed by PCR. All control samples from unaffected elk feet (12 samples in total) were negative by DD treponeme-specific PCR assays and by culture. There was 100% correlation between PCR and isolation results, since every culture which was isolation positive was also

TABLE 1 Lesion and normal samples obtained from various foot sites from seven different elk[a]

Elk no.	Geographic location	Foot area	Isolation of treponemes using:		PCR result			
			FCS	RS	DD1	DD2	DD3	Treponeme whole genus
17	GH	Control	−	−	−	−	−	−
18	GH	Control	−	−	−	−	−	−
21	Lewis	IDS	−	−	−	−	−	−
22	Lewis	Control[b]	−	−	−	−	−	−
		Lesion 1	+ (elk 22af)	−	+	+	−	+
		Lesion 2	+ (elk 22f)	+ (elk 22R)	+	+	+	+
		IDS	−	−	−	−	−	−
23	Lewis	Lesion 1	+ (elk 23f)	+ (elk 23R)	+	+	−	+
		Lesion 2	−	+ (elk 23aR)	+	+	+	+
		Coronary band	−	−	−	−	−	−
		Control[b]	−	−	−	−	−	−
		Coronary band	−	−	−	−	−	−
24	Lewis	Control[b]	−	−	−	−	−	−
		Lesion 1	−	+ (elk 24R)	−	+	−	+
		Lesion 2	+ (elk 24f)	−	+	−	−	+
		Coronary band	−	−	−	−	−	−
25	Lewis	Coronary band	−	−	−	−	−	−
		IDS	−	−	−	−	−	−

[a] IDS, interdigital space. All samples were collected in summer 2013. Some elk had lesions on more than one foot, and each lesional sample was treated separately. Isolate names are shown in parentheses, and these are listed in the phylogenetic tree shown in Fig. 2 (where "f" indicates isolation using FCS, and "R" indicates isolation using RS). Control samples were taken from elk with no lesions found in an area considered to be unaffected, e.g., GH (Grays Harbor County), or from elk with no lesions found in areas known to be affected, e.g., Lewis County. All samples were cultured for treponeme isolation and analyzed by treponeme PCR, with only lesional material giving positive results. All other tissues, including control samples, were negative. DD1, DD2, and DD3 refer to the DD treponeme phylogroups, where DD1 is "*T. medium/T. vincentii-like*," DD2 is "*T. phagedenis*-like," and DD3 is "*T. pedis*."

[b] Each control sample was taken from the same anatomical area where the lesion was found but on an unaffected foot of the same elk. These were all found in Lewis County, WA.

PCR positive. Upon examination of the cultures by phase-contrast microscopy, the lesions were not highly contaminated with other bacteria, so it was possible to isolate a single discrete treponeme, which was analyzed further by 16S rRNA gene sequencing.

Spirochete isolations were also attempted from the second group of 20 biopsy samples taken from 11 elk. Thirteen of these samples were taken from elk not showing any signs of lameness or lesions (known as control elk), and seven samples were from foot tissues showing signs of potential DD-like disease (Table 2). Control samples were taken from the normal contralateral foot of animals with lesions, from normal feet of unaffected animals living within the area of endemicity (elk 4 and 5), and from normal feet of unaffected animals living in an unaffected area (elk 11 and 12).

As previously, all control elk samples were negative by isolation and by PCR (Table 2). However, three of the samples (33, 34, and 35) did have a bacterial organism which appeared to have a spirochetal morphology when viewed by phase-contrast microscopy but was subsequently shown by the diagnostic PCR assays not to be a treponeme. This organism requires further investigation. Of the seven elk showing signs of DD-like disease, spirochetes were isolated from five animals, with three of the samples containing two different phylogroups (*T. medium*/*T. vincentii*-like and *T. phagedenis*-like) of treponemes (Table 2). When cultured in OTEB, these samples proved to be highly contaminated with other unknown bacteria, so isolation of an individual treponeme for sequencing was not possible. The source of this bacterial contamination is unknown, but it may be due to delays in sample transport or to other bacteria present in lesion tissues. A negative-control OTEB tube remained free from bacterial growth, so contamination during culturing seems unlikely.

In total, for the second batch of 13 lesions investigated with the PCR assays, *T. medium*/*T. vincentii*-like, *T. phagedenis*-like, and *T. pedis* treponemes were detected in 54% ($n = 7$), 69% ($n = 9$), and 38% ($n = 5$), respectively. Three lesions contained three phylogroups, four contained two, and four contained just one phylotype.

16S rRNA gene analysis. Nine pure treponeme culture isolates were obtained from lesions taken from elk tested in the first group of samples and were subjected to 16S rRNA gene amplification with PCR prior to sequencing. One sequence produced an unreadable electropherogram and was excluded from future analysis. To determine the relationship of the eight elk treponeme isolates to those commonly found in domestic livestock (sheep, and cattle), the 16S rRNA gene sequences were compared to those from domestic livestock using phylogenetic analysis, with the results shown in Fig. 2. The sequences from these isolates are available in GenBank (see above).

Four treponemes with 16S rRNA gene sequences highly similar to those of *T. medium*/*T. vincentii*-like and four with high similarity to *T. phagedenis* were isolated from the elk foot tissues. The *T. phagedenis*-like elk spirochete 16S rRNA gene sequences were identical to each other and to isolate sequences from cases of clinical cattle DD, as well as sheep and similar human isolates.

Three of the four treponemes were closely related to *T. medium*, sharing 100% 16S rRNA gene nucleotide sequence identity. While the 16S rRNA gene sequence of one elk isolate was identical to dairy cattle *T. medium*/*T. vincentii*-like DD spirochete sequences from the United Kingdom (T19, T56m etc. [11]), the other three elk *T. medium*/*T. vincentii*-like DD spirochetes were more similar to human *T. medium* (25).

TABLE 2 Presence of spirochetes and PCR results from 20 elk samples taken from 11 different animals[a]

Elk no.	Sample no.	Sample type	Culture for spirochete growth				PCR result for treponeme group		
			FCS		RS				
			Spirochetes present	Treponeme whole genus	Spirochetes present	Treponeme whole genus	DD1	DD2	DD3
1	26	Lesion	+	+	+	+	+	+	+
1	37	Control	−	−	−	−	−	−	−
2	28	Lesion	−	−	−	−	−	−	−
2	50	Control	−	−	−	−	−	−	−
3	39	Lesion	−	−	−	−	−	−	−
3	40	Control	−	−	−	−	−	−	−
4	42	Control	−	−	−	−	−	−	−
5	47	Control	−	−	−	−	−	−	−
6	44	Control	−	−	−	−	−	−	−
6	38	Lesion	+	+	−	−	−	+	−
8	45	Lesion	+	+	−	−	−	+	+
8	41	Control	−	−	−	−	−	−	−
11	33[b]	Control	+	−	−	−	−	−	−
11	35[b]	Control	−	−	+	−	−	−	−
12	34[b]	Control	+	−	−	−	−	−	−
12	46	Control	−	−	−	−	−	−	−
13	29	Control	+	−	+	−	−	−	−
13	31	Lesion	+	+	−	−	−	+	−
16	36	Control	−	−	−	−	−	−	−
16	43	Lesion	+	+	+	+	+	−	+

[a] All samples were collected in January 2014. Where a lesion was present on one foot, a control sample was taken from the same animal, but from an unaffected foot ($n = 7$). In addition, four elk were tested which were unaffected by lameness and had no evidence of lesions. Culture using rabbit serum resulted in two treponemes from group 1, whereas culture using fetal calf serum resulted in four group 2 treponemes and three group three treponemes. Some of the lesions proved to be polytreponemal by PCR, whereas others were monotreponemal. DD1, DD2 and DD3 refer to the DD treponeme phylogroups, where DD1 is "*T. medium*/*T. vincentii*-like," DD2 is "*T. phagedenis*-like," and DD3 is "*T. denticola*/*T. putidum*-like." FCS is fetal calf serum, and RS is rabbit serum used for isolation of spirochetes.

[b] This control sample contained bacteria which appeared spirochetal when examined microscopically but later proved not to be treponemes when tested by PCR.

DISCUSSION

This is the first report of the isolation of DD-associated *Treponema* spp. from wild animals, with previous reports describing isolation from domesticated animals, including sheep, humans, and cattle (9, 26). The data presented here suggest that the range of hosts which treponemes are known to infect is expanding to now include elk.

The clearly detectable association of DD treponemes with elk foot lesions, based on detection and isolation of treponemes from only the lesion and no other part of the foot, or control feet, suggests that these bacteria are likely to be involved in the pathogenesis of the lesions. These lesions have many clinical and pathological (19) similarities with bovine DD and contagious ovine DD (CODD), as seen in cattle and sheep, respectively (9, 27). Recent studies have shown that isolated treponemes were capable of producing DD-like lesions in cattle feet, nearly fulfilling Koch's postulates for these spirochetal bacteria (26). In addition, there are a growing number of fluorescent *in situ* hybridization studies that substantially implicate the specific treponeme phylogroups as the considered etiological agents of DD (28–30).

Moreover, a range of metagenomic studies have identified the association of specific treponeme phylogroups with DD lesions in Europe, Japan, and the United States (31–34). In each of these studies, other bacterial genera were identified in DD lesions; however, only for the treponemes were there strong association data across all these studies.

In the elk, the high association of DD treponemes with the foot lesions and the lack of treponemes in unaffected tissues and control feet strongly suggest that DD treponemes may be implicated in this elk hoof disease, as they are in cattle and hoof diseases of other domestic livestock.

Nested PCR assays specific for three culturable DD treponeme phylogroups confirmed the isolation results for 9 of the 12 bacterial cultures grown in OTEB. The other three samples, although containing a spirochete-like microorganism when viewed microscopically, were in fact treponeme negative when tested by diagnostic PCR assays. This organism was not analyzed further. Due to the contaminated nature of these samples, 16S rRNA gene sequencing was not possible for these cultures.

In addition, and similarly to cattle and sheep lesions, the lesions from elk feet are generally polytreponemal, with bacteria belonging to two or three of the DD treponeme phylogroups according to the specific nested PCR assays. Previous studies have indicated that most DD lesions in cattle are polytreponemal (12, 22, 29, 30), and this is in agreement with lesions seen in elk described here. In this study, only 23% (3/13) of lesions were found to contain all three treponeme phylogroups when analyzed by PCR. This is significantly lower than the 74.5% of lesions reported for cattle. This may be due to wild animals having substantially less direct contact with animals (and their feet) infected with treponemes than is the case for housed dairy cattle, which usually show a much higher prevalence of DD than cattle on pasture (35).

Sequences of the 16S rRNA gene of the treponemes isolated from elk suggest that the bacteria found in the lesions are very similar and in some cases identical to those found in lesions in cattle and sheep (9, 35). This may suggest that elk are experiencing

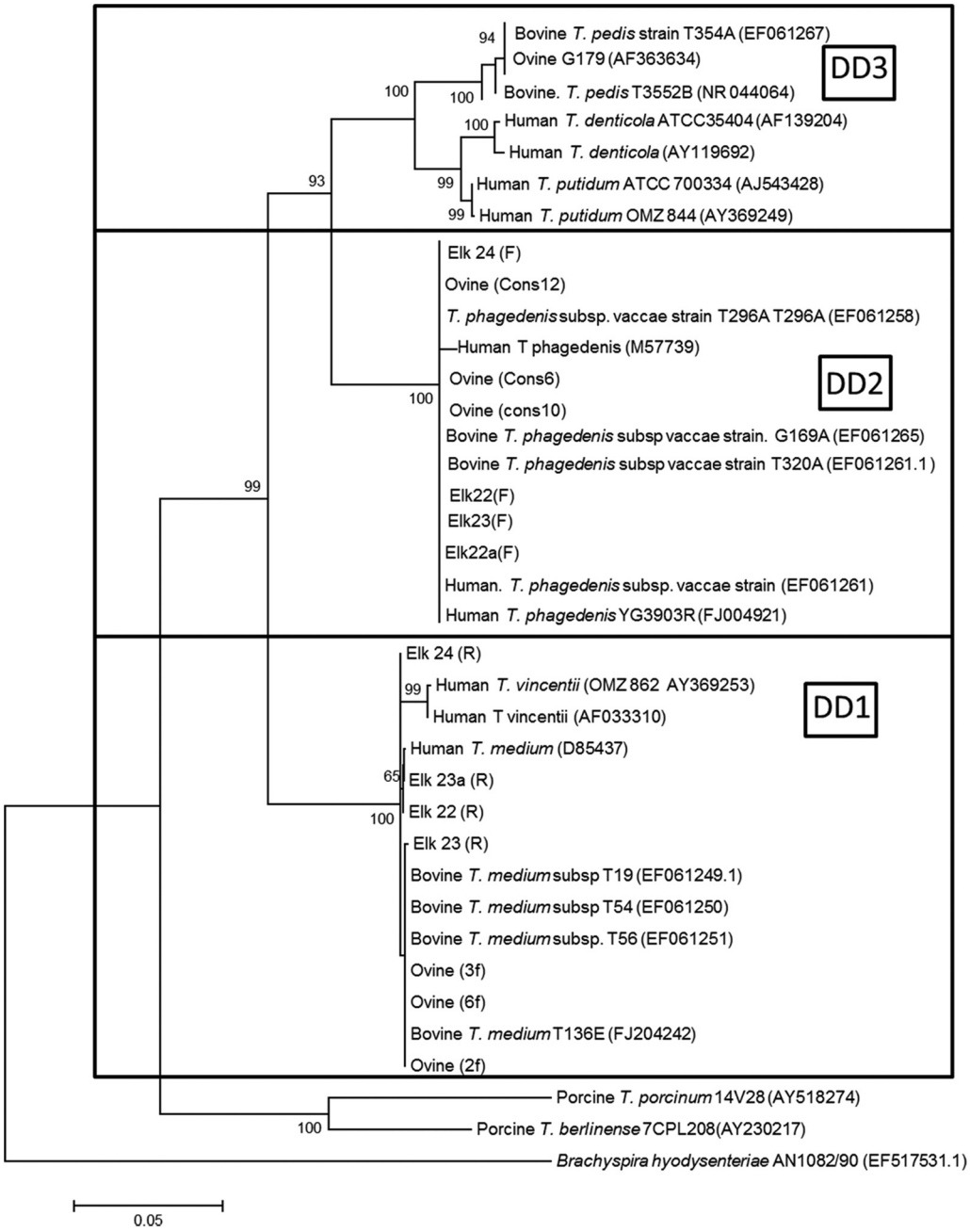

FIG 2 A maximum-likelihood tree (bootstrapped 10,000 times) for comparison of treponeme sequences isolated from elk to those isolated from cattle, humans, and sheep. (For clarity, bootstrap values below 65 were removed). Sequences from GenBank of human treponemes and other related treponemes are also shown, with the accession numbers in parentheses. The sequences from isolates in this study are labeled with the elk number and "F" or "R," indicating if they were isolated using fetal calf serum or rabbit serum. Key: DD1, DD2, and DD3 refer to the DD treponeme phylogroups, where DD1 is "*T. medium*/*T. vincentii*-like," DD2 is "*Treponema phagedenis*-like," and DD3 is "*T. denticola*/*T. putidum*-like."

a disease similar to that of farm ruminants, caused by the same bacteria, raising issues for potential transmission of disease between host species.

The clinical presentation of the lesions in elk is directly comparable with that of the lesions seen in DD in cattle and sheep. In sheep, the disease is frequently presented as severe lesions on the coronary band at the front of the hoof (36, 37). In dairy cattle, DD is mainly reported as a lesion at the rear of the foot between heel bulbs. However, there are many reports (in both Europe and the United States) showing that DD in cattle frequently manifests as a coronary band lesion at the front of the hoof in a manner similar to that of the initial lesion seen in sheep (36, 37). Whatever the presentation, the clear association of DD treponemes strongly suggests that we have identified another manifestation of the disease. Interestingly, DD treponemes have recently been associated with newly identified severe, nonhealing lesions in cattle feet, such as nonhealing white line disease and sole ulcers (38). This suggests that the DD treponemes are potent opportunistic secondary in-

vaders of other primary lesions, and this may be occurring in the elk feet. However, the extremely strong association of the DD treponemes with the elk lesions does suggest that they are primary invaders, as in cattle and sheep with DD, and lead to the ensuing severe pathogenesis.

Elk are wild animals, and their movement is currently uncontrolled. Thus, it is likely that they will travel much larger distances than domesticated cattle and sheep, which generally have much more controlled movements. While it might be considered that the elk may have originally contracted the bacteria while grazing on farmland previously used by sheep and cattle, they may now be considered to act as a potential reservoir of infection, spreading disease to other animals. The large territorial range of elk may mean that they have the potential to spread the bacteria over a larger range than domesticated animals, with implications for control, biosecurity, and disease management in both wild and domesticated animals (39).

This first-reported treponemal infection in wild animals may have far-reaching consequences for other animals, both wild and domesticated, and for disease management. Additionally, it suggests an expanding host range for the DD treponemes and that all cloven-hoofed animals could be susceptible to DD. Further studies will determine what preventative approaches and treatment measures can be considered to attempt to control the spread of this disease in elk and reduce the infection risk in other wildlife species.

ACKNOWLEDGMENTS

This work was funded by a Biotechnological and Biological Sciences Research Council (BBSRC) grant (BB/K009443/1). In addition, the Washington Department of Fish and Wildlife provided some funding for laboratory consumables.

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