Isolation of digital dermatitis treponemes from hoof lesions in wild North American elk (*Cervus elaphus)* in Washington State, USA.

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Abstract

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 manifest foot lesions similar clinically and pathologically with those seen in digital dermatitis (DD) in cattle and sheep, a disease with a bacterial aetiopathogenesis. To determine whether the same bacteria considered responsible for DD are present in elk lameness, lesion samples were subjected to 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 foot (including coronary band, or interdigital space), suggesting that the bacteria are associated with the lesions and may therefore be causal. In addition, PCR analysis specific for the three unique treponeme groups reveled that all three unique phylotypes were found in elk hoof disease, with some lesions being polytreponemal. Cattle and sheep lesions are commonly polytreponemal, with around 75% of cattle lesions commonly containing multiple phylotypes, compared to 23% of elk lesions. Sequence analysis of the 16S rRNA gene of treponeme isolates from elk lesions showed that the elk lesion treponemes are phylogenetically near identical to those isolated from cattle and sheep DD lesions. The isolates were highly 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.

Introduction

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 hoof disease causing severe lameness both in dairy and beef cattle worldwide (6, 18) and in sheep from the UK (7) and Ireland (8, 9). 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 characterised phylogroups identified as "*Treponema medium*/*Treponema vincentii*-like", "*Treponema phagedenis*-like" and "*Treponema denticola*/ T*reponema putidum*-like" bovine digital dermatitis (DD) spirochetes (10), with the latter now recognised as a new species, *Treponema pedis* (11). In addition, similar bacteria belonging to the same three unique, isolated phylogroups have been identified in DD spirochete cultures from hoof lesions in sheep (8). The DD-associated treponemes are found in abundance in DD lesions and are considered highly specific for DD lesions in cattle and sheep, being undetectable in normal foot tissues. Current evidence suggests a role for the bovine GI tract, manure and slurry and hoof trimming equipment in the transmission of DD (12-14).

Presently, DD is very common in dairy cattle worldwide, particularly in those countries with intensive farming systems (15, 16). Furthermore, DD is present in beef cattle (17), and sheep (9) in the UK. Taken together, these data suggest that all cloven hoofed animals are potential hosts for DD treponemes; a situation with similarities to the foot and mouth disease virus (18). 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-1990's, with an increased prevalence since 2008. Grossly, affected elk have deformed hooves that are asymmetrical, markedly elongated, and curved or broken, as well as hooves 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 30-90% of individuals within a group are lame (20). This current study was designed to determine if this elk disease had the same infectious treponemal aetiology as the DD lesions reported in domesticated hooved species.

Methods

Animal distribution.

Elk were collected between 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 has 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 22-24). Samples were taken from 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 (Table 1). After cleaning the foot surface by brushing and washing with sterile saline, a 3 mm punch biopsy was taken from the centre of the lesion and placed immediately into oral treponeme enrichment broth (OTEB: Anaerobe Systems, Morgan Hill, CA, USA) containing rifampicin (5 µg/ml) and enrofloxacin (5 µg/ml)). These samples were then then transported with ice packs by courier from Washington to the University of Liverpool (~3-4 days) for microbiological analysis and were processed immediately for spirochete culture and DNA extraction for PCR. In addition, a second group of samples were collected from seven foot lesions and analogous foot tissues from thirteen control tissues with no signs of lesions. These were processed blind, and results collated after experimental work had been carried out.

Isolation of spirochetes

Spirochete isolation attempts were carried out on all tissues taken from affected elk feet (coronary band, inter digital space and lesions) and control elk. These bacterial isolations were carried out immediately upon arrival of samples as described previously for cattle samples in oral treponeme enrichment broth (OTEB) (10) including rifampicin (5 µg/ml) and enrofloxacin (5 µg/ml). To maximise isolation attempts, samples were inoculated into OTEB containing foetal calf serum (FCS) (Gibco, Paisley, UK), to maximise growth of *Treponema phagedenis*-likeand "*Treponema pedis treponemes* and rabbit serum (RS) (GE Healthcare Life Sciences, Buckinghamshire, UK) to maximise growth of *Treponema medium*/*Treponema vincentii*-like treponemes. All isolation attempts were carried out in an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36ºC)

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

The second group of twenty elk samples taken from eleven different animals were inoculated into OTEB for culture as described above. The cultures were then examined by phase contrast microscopy and analysed 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 the culture was centrifuged (5000 X *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

Foot tissue and culture samples were subjected to nested PCR assays specific for the three DD-associated treponeme groups, "*Treponema medium*/*Treponema vincentii*-like", "*Treponema phagedenis*-like" *and Treponema pedis* described previously (10, 11) with resulting PCR products encompassing 300 to 500bp 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 (11) with all assays carried out in triplicate. Characterisation of isolates used PCR and gene sequencing of the near entire 16S rRNA gene as described previously

(10) with the sequencing carried out by a commercial company (Beckman Coulter Genomics, Essex, UK).

Sequencing and sequence analysis

Amplified PCR products were sequenced commercially (Beckman Coulter Genomics, Essex, UK) and the fragments of the 16S rRNA were assembled using Chromas Pro sequence analysis package (Technelysium Pty ltd) to produce a consensus gene sequence. Gene sequences were aligned using CLUSTALW as implemented in MEGA 5.0 (23). The DNA alignment was subjected to Modeltest, as implemented in Topali (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 10000 iterations).

Results

Pathology and location of lesions taken from elk feet is discussed in detail recently (19), and is shown in figure 1. Briefly, a macroscopic description of the lesion pathology consisted of small erosive lesions at the coronary band, under run horn of the wall and sole, erosion of the pedal bone and a red stippled appearance of exposed corium. It was this latter appearance that suggested the similarity to DD lesions.

Spirochete isolations

Samples were taken from lesions, coronary band and interdigital space (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 on culture and subsequent PCR. All control samples, IDS and coronary band (12 samples in total) were negative by DD treponeme specific PCR assays and by isolation. Only lesional tissues showed evidence of treponemes, with all IDS, coronary band and control samples from the healthy feet giving negative results (Table 1). There was 100% correlation between PCR and isolation results, as every culture which was isolation positive was also PCR positive. Upon examination of the culture by phase contrast microscopy, these lesions were not highly contaminated with other bacteria, so it was possible to isolate a single discrete treponeme which was analysed further by 16s rRNA gene sequencing.

Spirochete isolations were also attempted from the second group of 20 elk samples taken from eleven elk. Thirteen elk samples were taken from elk not showing any signs of lesions (known as control elk), and seven 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 endemic area (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 using 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 (*Treponema* *medium*/*Treponema vincentii*-like and *Treponema 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 may be due to delays in sample transport, or may be due to other bacteria present in lesion tissues. A negative control OTEB tube remained free from bacterial growth, so contamination during culturing seems unlikely. These samples will however be subject to future research into potential bacterial lameness causes.

In total, for the 13 lesions investigated with the PCR assays *Treponema medium*/*Treponema vincentii*-like, *Treponema phagedenis*-like and *Treponema pedis* treponemes were present in 54% (n=7), 69% (n=9) and 38% (n=5) respectively. Three lesions contained three phylogroups, four contained two, and four 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 Figure 2. The sequences from these isolates are available on Genbank (Accession numbers KM586666-KM586673)

Four treponemes with 16S rRNA gene sequences highly similar to *T. medium* and four with high similarity to *T. phagedenis* were isolated from the elk 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. Whilst 16S rRNA gene sequence of one elk isolate was identical to dairy cattle *T medium*-like DD spirochete sequences from the UK (T19, T56 etc: (10)), the other three elk *T. medium*-like DD spirochetes were more similar to the human *T. medium* isolate (25).

Discussion

This is the first report of isolation of DD-associated *Treponema* spp. from wild animals, with previous reports being from domesticated animals, including sheep, humans and cattle (8, 27). The data presented here suggests 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 a lesion 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 (8, 26). Recent studies have shown that isolated treponemes were capable of producing digital dermatitis-like lesions in cattle feet, near fulfilling Koch's postulates for these spirochetal bacteria (27). In addition there are a growing number of fluorescent in situ hybridisation studies that substantially implicate the specific treponeme phylogroups as the considered aetiological 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 USA (31-34) which all report the presence of other bacterial genera; however, only for the treponemes is there good 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 suggests 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 in nine of the 12 bacteria grown in OTEB. The other three samples, although containing spirochete-like micro-organisms when viewed microscopically, were in fact treponeme negative when tested by diagnostic PCR assays. This organism was not tested further. Due to the contaminated nature of the 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 phylogroups according to the specific nested PCR assays. Previous studies have indicated that most DD lesions in cattle are polytreponemal (11, 22, 29, 30) and this is in agreement with lesions seen in elk reported here. In this study, only 23% (3/13) of lesions were found to contain all three treponeme phylogroups when analysed 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 when compared to 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 on cattle and sheep (8, 35). This may suggest that elk are experiencing a similar disease to 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 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 showing that DD in cattle frequently manifests (reported in both Europe and USA) as a coronary band lesion at the front of the hoof in a similar manner to the initial lesion seen in sheep (36, 37). Whatever the presentation, the clear association of DD treponemes strongly suggest that we have identified another manifestation of the disease. Interestingly, DD treponemes have recently been associated with newly identified severe, non-healing lesions in cattle feet such as non-healing white line disease and sole ulcers (38). This suggests that the DD treponemes are potent opportunistic secondary invaders 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 as their movement is currently uncontrolled, and as such it is likely that they will travel much larger distances than domesticated cattle and sheep which generally have much more controlled movements. Whilst 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 report of 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.

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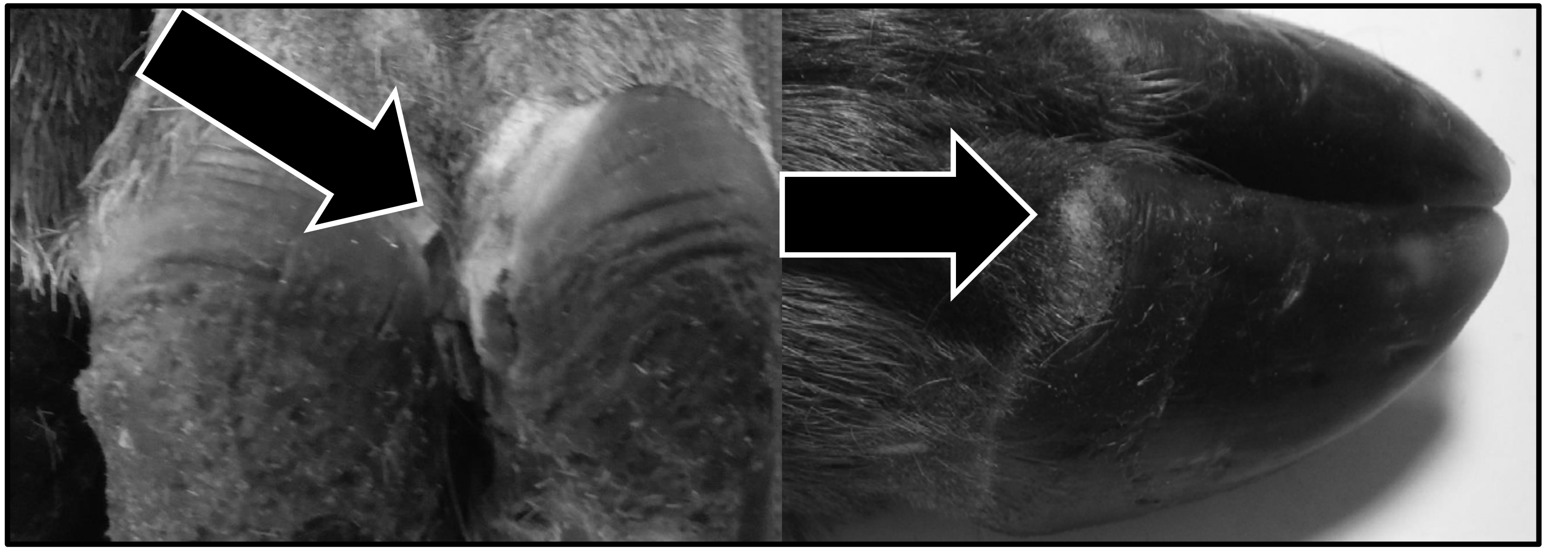


Figure 1. Photograph of an affected elk hoof with an early macroscopic lesion (indicated with an arrow) on the coronary band (right hand side) and a more typical foot lesion (left hand side) which shows more visual similarities to digital dermatitis.

					PCR			
Elk Number	**Geographic location**	**Foot area**	**Isolation of treponemes using FCS**	**Isolation of treponemes using RS**	**DD1**	**DD2**	**DD3**	**All Trep**
17	GH	Control	-	-	-	-	-	-
18	GH	Control	-	-	-	-	-	-
21	Lewis	IDS	-	-	-	-	-	-
22	Lewis	Control #	-	-	-	-	-	-
		Lesion 1	+ (Elk22af)	-	+	+	-	+
		Lesion 2	+ (Elk22f)	+ (Elk 22 p)	+	+	+	+
		IDS	-	-	-	-	-	-
23	Lewis	Lesion 1	+ (Elk 23 f)	+ (Elk 23 p)	+	+	-	+
		Lesion 2	-	+ (Elk 23a p)	+	+	+	+
		Coronary band	-	-	-	-	-	-
		Control #	-	-	-	-	-	-
		Coronary band	-	-	-	-	-	-
24	Lewis	Control #	-	-	-	-	-	-
		Lesion 1	-	+ (Elk 24 p)	-	+	-	+
		Lesion 2	+ (Elk 24 f)	-	+	-	-	+
		Coronary band	-	-	-	-	-	-
25	Lewis	Coronary band	-	-	-	-	-	-
		IDS	-	-	-	-	-	-

Table 1. Lesion and normal samples obtained from various foot sites from seven different elk (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. The names shown in parentheses show the isolate name, and these are shown in the phylogenetic tree shown in Figure 2 (where f indicates isolation using FCS and p 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

These samples were 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.

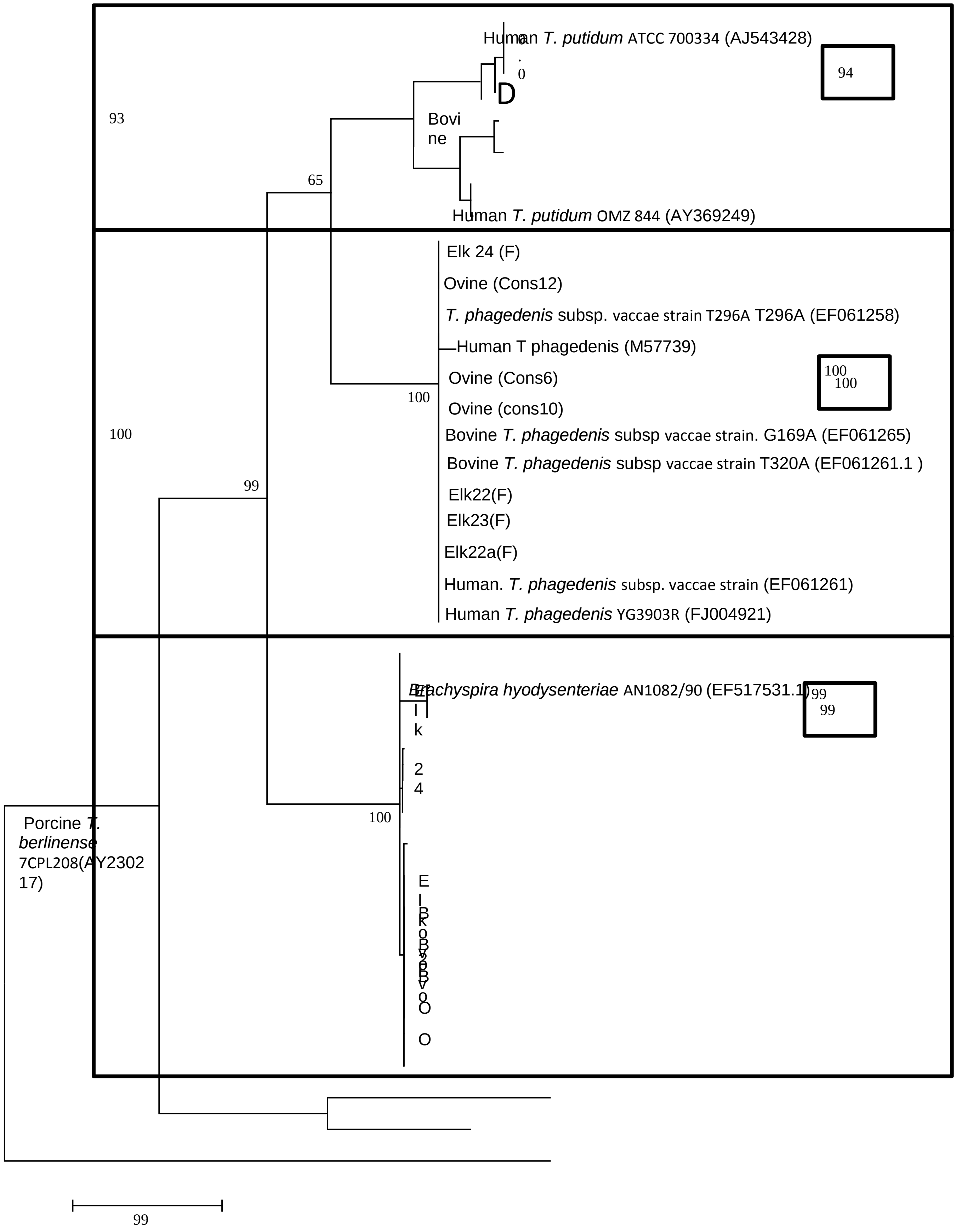
All samples were cultured for treponeme isolation and analysed by treponeme PCR, with only lesional material giving positive results. All other tissues, including control samples were negative. Key: DD1, DD2 and DD3 refer to the DD treponeme phylogroups, where DD1 is "*Treponema medium*/*Treponema vincentii*-like", DD2 is "*Treponema phagedenis*-like" and DD3 is "*Treponema pedis*". FCS is foetal calf serum, and RS is rabbit serum used for isolation of spirochetes

			Cultures for spirochete growth				PCR		
			Foetal calf serum (FCS)		Rabbit serum (RS)		Treponeme groups		
Elk Number	Sample Number	Lesion/ Control	Spirochetes present	All Trep PCR	Spirochetes present	All Trep PCR	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 #	Control	+	-	-	-	-	-	-
11	35 #	Control	-	-	+	-	-	-	-
12	34 #	Control	+	-	-	-	-	-	-
12	46	Control	-	-	-	-	-	-	-
13	29	Control	+	-	+	-	-	-	-
13	31	Lesion	+	+	-	-	-	+	-
16	36	Control	-	-	-	-	-	-	-
16	43	Lesion	+	+	+	+	+	-	+

Table 2. Presence of spirochates and PCR results from 20 elk samples taken from 11 different animals. 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 foetal 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.

In addition, three control samples (33, 34 and 35) contained bacteria which appeared spirochaetal when examined microscopically, but later proved not to be treponemes when tested by PCR. These are indicated with a # on the table.

Key: DD1, DD2 and DD3 refer to the DD treponeme phylogroups, where DD1 is "*Treponema medium*/*Treponema vincentii*-like", DD2 is "*Treponema phagedenis*-like" and DD3 is "*Treponema denticola*/ T*reponema putidum*-like". FCS is foetal calf serum, and RS is rabbit serum used for isolation of spirochetes



Human T. putidum ATCC 700334 (AJ543428)
94
93
Bovine
65
Human T. putidum OMZ 844 (AY369249)
Elk 24 (F)
Ovine (Cons12)
T. phagedenis subsp. vaccae strain T296A T296A (EF061258)
Human T phagedenis (M57739)
Ovine (Cons6)
100
100
Ovine (cons10)
100
Bovine T. phagedenis subsp vaccae strain. G169A (EF061265)
Bovine T. phagedenis subsp vaccae strain T320A (EF061261.1)
99
Elk22(F)
Elk23(F)
Elk22a(F)
Human. T. phagedenis subsp. vaccae strain (EF061261)
Human T. phagedenis YG3903R (FJ004921)
Brachyspira hyodysenteriae AN1082/90 (EF517531.1)
99
99
Elk 24
100
Porcine T. berlinense 7CPL208(AY230217)
99

Figure 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 number in parentheses. The sequences from isolates in this study are labelled with Elk number, and F or R, indicating if they were isolated using foetal calf serum or rabbit serum.

Key: DD1, DD2 and DD3 refer to the DD treponeme phylogroups, where DD1 is "*Treponema medium*/*Treponema vincentii*-like", DD2 is "*Treponema phagedenis*-like" and DD3 is "*Treponema denticola*/ T*reponema putidum*-like"