

Association of Unique, Isolated Treponemes with Bovine Digital Dermatitis Lesions[∇]

Nicholas J. Evans,^{1,2*} Jennifer M. Brown,¹ Ibrahim Demirkan,¹† Prem Singh,^{2,3} Brian Getty,⁴
Dorina Timofte,² W. Daan Vink,² Richard D. Murray,² Roger W. Blowey,⁵ Richard J. Birtles,¹
C. Anthony Hart,⁴ and Stuart D. Carter^{1,2}

Department of Veterinary Pathology, Faculty of Veterinary Science, University of Liverpool, Liverpool L69 3BX, United Kingdom¹;
Department of Veterinary Clinical Science, Faculty of Veterinary Science, University of Liverpool, Leahurst, Neston CH64 7TE,
United Kingdom²; Department of Veterinary Surgery and Radiology, CCS Haryana Agricultural University, Hisar 125004,
Haryana, India³; Department of Medical Microbiology, Duncan Building, University of Liverpool,
Liverpool L69 3GA, United Kingdom⁴; and Wood Veterinary Group,
St. Oswald's Road, Gloucester GL1 2SJ, United Kingdom⁵

Received 3 October 2008/Returned for modification 24 November 2008/Accepted 3 January 2009

This study used a PCR-based approach targeting 16S rRNA gene fragments to determine the occurrence and association of the three bovine digital dermatitis (BDD) treponeme phylogroups within lesions found in cattle from the United Kingdom. Examination of 51 BDD lesions collected from infected cattle across the United Kingdom revealed that BDD treponeme group 1 (*Treponema medium*/*Treponema vincentii*-like), group 2 (*Treponema phagedenis*-like), and group 3 (*Treponema putidum*/*Treponema denticola*-like) were present in 96.1%, 98%, and 76.5% of BDD lesions, respectively. The three phylogroups were present together in 74.5% of lesions. The PCR assays enabled the isolation of further treponeme strains from previously mixed primary BDD lesion cultures. Here a representative from each of the three distinct treponeme phylogroups was isolated from a single BDD lesion for the first time. These data highlight the extent to which this disease is polytreponemal. Immunohistochemistry and electron microscopy were used to investigate lesional hoof tissues, resulting in treponemes being identified copiously in hair follicles and sebaceous glands, suggesting a potential route of exit and/or entry for these pathogens. This study gives further evidence for the importance of the three treponeme groups in BDD pathogenesis and reiterates the value of molecular genetic approaches for isolating and identifying fastidious anaerobes.

Bovine digital dermatitis (BDD) is a worldwide disease responsible for lameness in cattle. The disease is exceptionally painful for the animal involved and has economic implications such as reduction in milk yield and reproductive performance (1, 15, 16, 34). It has now been more than 30 years since the disease was first recognized, and while regular foot bathing has been shown to have some efficacy, an effective preventative measure or treatment that completely eliminates the disease has yet to be identified (19). The result is that the disease is endemic in cattle across the world, and one recent report suggested that the economic impact resulting from milk production in the United States alone is a loss of as much as \$190 million per year (20).

The environmental conditions of housed dairy cows, whereby they stand in slurry continuously, are likely to result in the isolation of a plethora of different microorganisms from BDD lesions. Indeed, various types of bacteria have been identified within BDD manifestations including spirochetes, *Bacteroides* species, *Campylobacter* species, *Fusobacterium* species, and *Peptococcus* species (2, 3, 10, 18, 25, 28). The result of this

complex bacteriological environment is that the exact etiology of BDD remains unresolved. However, identification of the bacteria involved in the initial infection would allow specific targeting treatment and, hopefully, eradication of this disease. Treponemes are the only bacteria for which there is considerable evidence for a consistent presence and involvement within BDD lesions. Cloning of bacterial 16S rRNA genes from BDD lesions in Germany indicated five phylogroups of treponemes present within lesions (5). We recently isolated a large number of treponemes from BDD lesions in the United Kingdom and demonstrated that the isolates belong to three distinct phylogenetic/taxonomic groups (13). We designated the BDD treponemes group 1, group 2, and group 3, which correspond to *Treponema medium*/*Treponema vincentii*-like, *Treponema phagedenis*-like, and *Treponema putidum*/*Treponema denticola*-like spirochetes, respectively. These isolated BDD treponemes are nearly identical to three phylotypes described by a smaller study in the United States (31) and are nearly identical to three of the five phylotypes identified in BDD lesions in Germany (5).

We previously identified a strong association of *Treponema* species with BDD using a PCR-based method (6). However, that study focused solely on group 1, *T. medium*/*T. vincentii*-like spirochetes. In this study, we used PCR assays specific for each of the three BDD-associated treponeme groups in order to determine the presence of each group in BDD lesions drawn from infected animals across the United Kingdom. Furthermore, the localization of BDD-associated treponemes within

* Corresponding author. Mailing address: Department of Veterinary Pathology, Faculty of Veterinary Science, University of Liverpool, Liverpool L69 3BX, United Kingdom. Phone: 44 151 794 4209. Fax: 44 151 794 4219. E-mail: evansnj@liverpool.ac.uk.

† Present address: Department of Surgery, Faculty of Veterinary Medicine, Afyon Kocatepe University, 03200 Afyonkarahisar, Turkey.

[∇] Published ahead of print on 14 January 2009.

TABLE 1. Collection of strains used to test sensitivity and specificity of BDD-associated *Treponema*-specific PCR assays^a

Species and/or group and strain	Result			
	Specific PCR for group:			<i>Treponema</i> PCR
	1	2	3	
Group 1 (<i>T. medium</i> / <i>T. vincentii</i> -like) strains T184, T19, T54, T56, and T18A	+	-	-	+
Group 2 (<i>T. phagedenis</i> -like) strains T167, T52A, T136, T119A, T257, T296A, T354B, T2721A, T320A, T380A, T323C, W35, G169A, and G187	-	+	-	+
Group 3 (<i>T. denticola</i> / <i>T. putidum</i> -like) strains T354A, T3552B, G819CB, and T18B	-	-	+	+
Bovine spirochete strain G356	-	+	-	+
Ovine spirochete strain G179	-	-	+	+
<i>T. medium</i> ATCC 700293 ^T	+	-	-	+
<i>T. phagedenis</i> CIP 62.29	-	+	-	+
<i>T. phagedenis</i> biotype Reiter	-	+	-	+
<i>T. denticola</i> ATCC 35405 ^T	-	-	-	+
<i>T. denticola</i> GM-1	-	-	-	+

^a The assays include a specific PCR for groups 1, 2, and 3 and a general *Treponema* PCR.

lesion material was investigated using immunohistochemistry and electron microscopy (EM).

MATERIALS AND METHODS

Bacterial strains and clinical samples. Twenty-three strains isolated from BDD lesions and characterized previously were used to validate the PCR tests (13). A previously isolated group 2 strain, G356 (8); a group 3 strain, G179, isolated from a case of contagious ovine DD (CDD) (7); and several reference strains considered to be the closest relatives were also included (complete list in Table 1).

Twenty-nine BDD lesion biopsies were collected from Holstein-Friesian cows at farms in Merseyside, Cheshire, Lancashire, Gloucestershire, and Shropshire, United Kingdom, from 2003 to 2007. All biopsies were used for DNA extraction, and 20 of the 29 biopsies were used in an attempt to cultivate treponemes. Biopsies for bacterial cultivation and DNA extraction were collected and transported back to the laboratory as described previously (13), placed immediately in an anaerobic cabinet, and divided into two samples for said procedures. Samples for DNA extraction only were transported on ice without transport medium. All samples for DNA extraction were stored at -80°C. The genomic DNAs from a further 22 BDD lesion biopsies collected during 1996 to 1997 and previously

investigated by this laboratory using a different PCR method (6) were also included in this investigation. Single rear-hoof skin biopsies were collected post-mortem from nine young bullocks and seven dairy cows (sent to slaughter) that did not have any evidence of BDD. For immunohistochemical and electron microscopical studies, biopsies from BDD lesions (three acute cases and one chronic case) and from healthy feet (nine sampled post-mortem) were taken immediately into 10% formalin-saline and paraffin embedded.

Strain isolation. Strain isolations from BDD lesional tissues, isolate 16S rRNA gene sequencing, and phylogenetic analyses were carried out as previously described (13).

DNA extraction. DNA was extracted from treponeme cultures as previously described (13). Tissues from infected and uninfected hooves were thawed, and DNA was extracted using a DNeasy kit (Qiagen, United Kingdom) according to the manufacturer's instructions, and the resulting extracted genomic DNA was stored at -20°C.

PCR assays. Nested PCR assays that were specific for the three BDD treponeme groups recently characterized and described by our laboratory were developed (13). The initial PCR step used a universal bacterial primer pair encompassing the majority of the 16S rRNA gene. PCR mixtures used *Taq* polymerase (Qiagen, United Kingdom) according to the manufacturers' instructions, with 1 µl of the DNA template per 25-µl reaction mixture volume and incubation at 95°C for 5 min, 25 cycles of 94°C for 1 min, 55°C for 3 min, and 72°C for 3 min, with a final extension step at 72°C for 7 min. The second/nested PCR step used primers encompassing smaller (300-to 500-bp) regions within the 16S rRNA gene. Primers were identified using a 16S rRNA gene CLUSTALW alignment of the 23 strains isolated in a previous study (13) with all known treponeme sequences present in GenBank. Stringent PCR conditions were identified using a Mastercycler gradient thermocycler (Eppendorf, Germany). The BDD treponeme-specific PCRs were applied to culture and tissue-derived DNA samples using 25-µl reaction mixes as described above with 1 µl PCR product template from the initial reaction. Temperature cycling entailed 95°C for 5 min followed by 40 cycles of 95°C for 1 min; annealing for either 2 min at 68°C for group 1 primers, 1 min at 64°C for group 2 primers, or 30 s at 68°C for group 3 primers; an extension step at 72°C for 2 min; and then a final elongation step at 72°C for 10 min. To ensure validity in each assay, water was used as a negative control, and positive controls included genomic DNA from each of the three treponeme groups. All biopsy extractions were further subjected to a *Treponema* genus-specific PCR assay as originally described (21). All PCR primers used are listed in Table 2.

Production of antitreponemal antibodies. Antigens were prepared from each of the three groups of treponemes by sonication and repeated freeze-thawing (9). These antigens were then pooled and supplied to a commercial concern for the generation of rabbit antisera. During this procedure, rabbits were immunized in a multisite regimen using Freund's complete and incomplete adjuvants over a period of 3 months. At the terminal bleed, the antisera were tested for reactivity by enzyme-linked immunosorbent assay and Western blotting against the purified antigens and were shown to cross-react with all three treponeme groups (data not shown).

TABLE 2. Primers used to detect the three BDD treponeme phylogroups and all treponemes

Primer specificity	Primer (sequence)	Predicted band size (bp)	Region of 16S targeted (positions) ^a	Reference or source
Universal	16S F (5'-AGAGTTTGATCCTGG-3') 16S R (5'-TACCTGTTACGACTT-3')	1,526	7-26 1491-1506	27
Group 1 (<i>T. medium</i> / <i>T. vincentii</i> -like)	TmF (5'-GAATGCTCATCTGATGACGGTAATCGACG-3') TmR (5'-CCGGCCTTATCTAAGACCTTCTACTAG-3')	475	472-500 1001-1029	This study
Group 2 (<i>T. phagedenis</i> -like)	TbF (5'-GAAATACTCAAGCTTAAGTGAATGCAATG-3') TbR (5'-CTACGCTACCATATCTCTATAATATTGC-3')	400	612-640 1006-1029	This study
Group 3 (<i>T. denticola</i> / <i>T. putidum</i> -like)	TpF (5'-GGAGATGAGGGAATGCGTCTTCGATG-3') TpR (5'-CAAGAGTCGTATTGCTACGCTGATATATC-3')	475	459-484 1017-1045	This study
<i>Treponema</i> sp.	TPF (5'-AARCATGCAAGTCGARGCGCAAG-3') TPR ₁ (5'-TCCATTGCGGAATATTCTTA-3')	335	49-71 365-384	21

^a Locations relative to those for the *Escherichia coli* 16S rRNA gene sequence reported under GenBank accession number M25588 (11) are given.

TABLE 3. PCR detection of treponemes in BDD lesion biopsies, 2003 to 2007

Sample	Biopsy date (day/mo/yr)	Description	Type ^a	Treponeme isolated (no. of isolates) ^d	Result			Treponema PCR
					Specific PCR for group:			
					1	2	3	
1	1/12/03	Cheshire, farm 1, cow 167, left hoof	Chronic	T167 ^b (2)	+	+	+	+
2	1/12/03	Cheshire, farm 1, cow 167, right hoof	Acute	IF	+	+	-	+
3	1/12/03	Cheshire, farm 1, cow 13		IF	+	+	-	+
4	26/1/04	Shropshire, farm 1, cow 136		T136 ^b (2) T136E ^c (1) T136P2 ^c (3)	+	+	+	+
5	26/1/04	Shropshire, farm 1, cow 52		T52A ^b (2) T52B ^c (1)	+	+	+	+
6	26/1/04	Shropshire, farm 1, cow 200		T200 (2) ^c	+	+	+	+
7	26/1/04	Shropshire, farm 1, cow 119		T119A ^b (2)	+	+	+	+
8	13/2/04	Merseyside, farm 1, cow 320		T320A ^b (2)	+	+	+	+
9	13/2/04	Merseyside, farm 1, cow 380		T380A ^b (2)	+	+	+	+
10	13/2/04	Merseyside, farm 1, cow 272		T272A ^b (2)	+	+	+	+
11	13/2/04	Merseyside, farm 1, cow 355		T3552B ^b (3)	+	+	+	+
12	26/4/04	Gloucestershire, farm 1, cow 819		G819CB ^b (3)	+	+	+	+
13	26/4/04	Gloucestershire, farm 1, cow 317		IF	+	+	+	+
14	23/4/04	Gloucestershire, farm 2, cow 187	IDD	G187 ^b (2)	+	+	-	+
15	16/5/04	Gloucestershire, farm 3, cow 1		IF	+	+	+	+
16	16/5/04	Gloucestershire, farm 3, cow 169		G169A ^b (2)	+	+	+	+
17	9/7/04	Merseyside, farm 1, cow 323		T323C ^b (2)	+	+	+	+
18	9/7/04	Merseyside, farm 1, cow 645		T645C3 ^c (2)	+	+	+	+
19	28/4/05	Merseyside, farm 2, cow 116		T116B ^c (2)	+	+	+	+
20	2/9/05	Cheshire, farm 2, cow 100		T100A ^c (2)	+	+	+	+
21	2/9/05	Cheshire, farm 2, cow 122		T122A ^c (2)	+	+	-	+
22	1/12/05	Cheshire, farm 2, cow 5		NIA	-	+	-	+
23	15/06/06	Cheshire, farm 2, cow 67, left hoof	Chronic	NIA	+	+	-	+
24	15/06/06	Cheshire, farm 2, cow 67, right hoof	Chronic	NIA	+	+	+	+
25	7/11/06	Cheshire, farm 1, cow 577		NIA	+	+	-	+
26	20/11/06	Cheshire, farm 3, cow 87	Chronic	NIA	+	+	+	+
27	20/11/06	Cheshire, farm 3, cow 574	Chronic	NIA	+	+	+	+
28	20/11/06	Cheshire, farm 3, cow 265	Acute	NIA	+	+	-	+
29	11/01/07	Lancashire, farm 1, cow 1		NIA	+	+	+	+

^a All lesions were on rear hoofs with typical BDD-type manifestation unless otherwise stated.

^b Isolations reported previously.

^c Previously unreported isolations.

^d IF, isolation failed; NIA, no isolation attempted.

Immunohistochemistry. Paraffin-embedded tissues were sectioned by a microtome, deparaffinized with xylene, and blocked with 3% hydrogen peroxide and 2% bovine serum albumin. Each slide was incubated with antitreponeme antibodies (1:1,000 dilutions) overnight, washed with phosphate-buffered saline three times and then probed with a 1-in-500 solution of goat anti-rabbit antibody conjugated to peroxidase for 2 h. After a second washing step, bound peroxidase was localized with chromogen (diaminobenzidine) for 30 min and then counterstained with hematoxylin and eosin prior to light microscopy.

Electron microscopy. For EM, paraffin-embedded tissues were prepared according to a previously described method (14). The resin-embedded tissues were sectioned with an ultramicrotome, stained with 2% uranyl acetate (pH 7), and subjected to EM.

Nucleotide sequence accession numbers. The 16S rRNA gene GenBank accession numbers determined as part of this study for BDD *Treponema* strains T645C3, T116, T122, T100, T200, T52B, T136E, and T136P2 are FJ204236 to FJ204243, respectively.

RESULTS

PCR validation. Within the original 23 spirochete strains used to characterize the three novel BDD treponeme groups, each PCR test detected only the group it was designed to identify (Table 1). When including the nearest designated relatives for each phylogroup, which were human oral and genital treponemes, the group 1 and group 2 PCR tests detected no

other strains except the nearest designated relatives, *T. medium* and *T. phagedenis*, respectively, while the group 3 PCR test did not cross-react with its nearest designated relative, *T. denticola*. The detection limit for the group 1, 2, and 3 assays were stock culture DNA extraction dilutions of 1×10^{-3} , 1×10^{-4} , and 1×10^{-4} , corresponding to 88, 11, and 33 treponemes per PCR, respectively.

Group-specific PCR survey of biopsied BDD lesions. From the 29 BDD lesions biopsies collected during the current study (2002 to 2007), group 1, 2, and 3 treponemes were present in 96.6%, 100%, and 72.4% of lesions, respectively (Table 3). The only two acute lesions tested and a single case of interdigital dermatitis were positive for only two phylotypes: groups 1 and 2. All three groups were present in 72.4% of BDD lesions. From the 22 BDD lesions previously surveyed for group 1 isolates only (6), the group 1, 2, and 3 treponemes were present in 95.5%, 95.5%, and 81.8% of lesions (Table 4), with all three groups present in 77.3% of lesions.

Including all 51 lesions tested, the group 1, 2, and 3 treponemes were present in 96.1%, 98%, and 76.5% of lesions, with all three groups present in 74.5% of lesions. All BDD lesion samples were positive for the general treponeme PCR.

TABLE 4. PCR screen of BDD biopsies from a previous study from 1996 to 1997

Sample	Biopsy date (yr)	Description	Result			
			Specific PCR for group:			<i>Treponema</i> PCR
			1	2	3	
1	1996	Cheshire, farm 1, cow 30	-	+	+	+
2	1996	Cheshire, farm 1, cow 16	+	+	-	+
3	1996	Cheshire, farm 1, cow 197	+	+	+	+
4	1997	Cheshire, farm 1, cow 31	+	+	+	+
5	1997	Cheshire, farm 1, cow 39	+	+	+	+
6	1997	Cheshire, farm 1, cow 50	+	+	+	+
7	1997	Cheshire, farm 1, cow 66	+	+	+	+
8	1996	Cheshire, farm 2, cow 17	+	+	-	+
9	1997	Cheshire, farm 2, cow 25	+	+	+	+
10	1997	Cheshire, farm 2, cow 310	+	+	+	+
11	1997	Cheshire, farm 2, cow 502	+	+	+	+
12	1996	Cheshire, farm 3, cow 84	+	+	+	+
13	1997	Cheshire, farm 3, cow 328	+	-	-	+
14	1997	Cheshire, farm 3, cow 464	+	+	+	+
15	1996	Cheshire, farm 3, cow 579	+	+	+	+
16	1997	Cheshire, farm 3, cow 612	+	+	+	+
17	1997	Cheshire, farm 3, cow 623	+	+	+	+
18	1997	Cheshire, farm 3, cow 625	+	+	+	+
19	1997	Cheshire, farm 3, cow 593	+	+	+	+
20	1996	Cheshire, farm 4, cow 15	+	+	+	+
21	1997	Cheshire, farm 4, cow 54	+	+	+	+
22	1997	Cheshire, farm 4, cow 227	+	+	-	+

PCR survey of healthy foot tissues. Healthy (non-BDD) foot tissues were obtained from nine young bullocks, none of which had been reported to have suffered or had any symptoms of BDD. None of the biopsies from the young bullocks tested positive for any of the BDD-associated treponeme groups (Table 5). In order to test animals that had been present in the farmyard environment but did not have current BDD lesions, seven non-BDD cows that had been on farms that had endemic BDD were tested for the presence of the BDD treponemes in hoof tissues. For six of the seven samples tested, the PCRs did not reveal BDD treponemes in healthy hoof skin (Table 5). The single exception was one sample that tested positive for all three of the treponeme groups. Interestingly, all healthy hoof tissues, including that of the bullocks, tested positive by general treponeme PCR.

TABLE 5. PCR detection of treponemes in hoof tissues from cows without BDD

Sample (s)	Biopsy date (day/mo/yr)	Description	Result			
			Specific PCR for group:			<i>Treponema</i> PCR
			1	2	3	
1-9	15/12/05	Cheshire, farm 1, single rear foot from 8 young bullocks	-	-	-	+
10	17/1/07	Lancashire, farm 1, cow 182265	-	-	-	+
11	17/1/07	Lancashire, farm 1, cow 00645	+	+	+	+
12	17/1/07	Lancashire, farm 1, cow 600309	-	-	-	+
13	17/1/07	Lancashire, farm 1, cow 391	-	-	-	+
14	17/1/07	Lancashire, farm 1, cow 100233	-	-	-	+
15	17/1/07	Lancashire, farm 1, cow 20560	-	-	-	+
16	17/1/07	Lancashire, farm 1, cow 111B	-	-	-	+

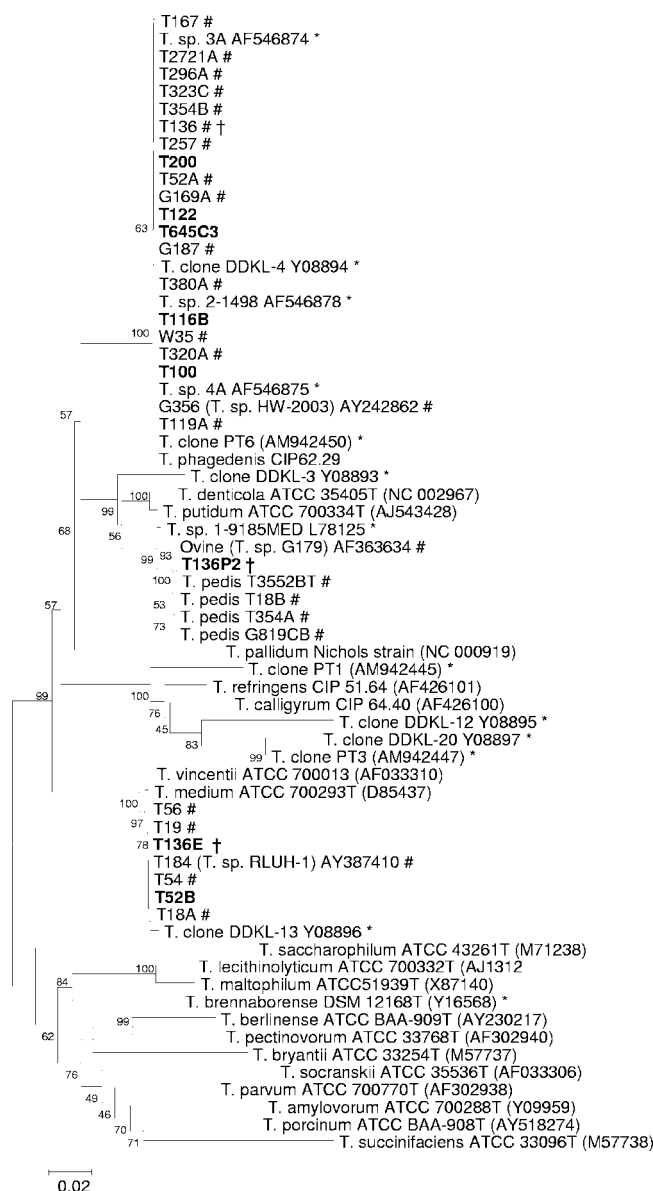


FIG. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison over ~1,330 aligned bases showing relationships between strains isolated here (boldface type) and related 16S rRNA gene sequences. Bootstrap confidence levels are shown as percentages of nodes, and only values above 40% are shown. GenBank accession numbers are shown in parentheses next to each strain or 16S rRNA gene fragment clone. *, previously reported 16S rRNA gene sequences from BDD lesions; #, previously reported isolated BDD treponemes from this laboratory; †, strains from three different groups isolated from the same lesion.

Isolation of further treponeme isolates. As part of this study, we isolated several more spirochetes, which are listed along with previous isolations from biopsies in Table 3. From the isolations listed, 12 of the 20 were reported previously (13). Strains T100, T122, and T116B were isolated as described previously and were confirmed as group 2 isolates, the most commonly isolated strains from BDD lesions (Fig. 1).

It was hypothesized that the successful development of group-specific PCRs would enable further isolations from pri-

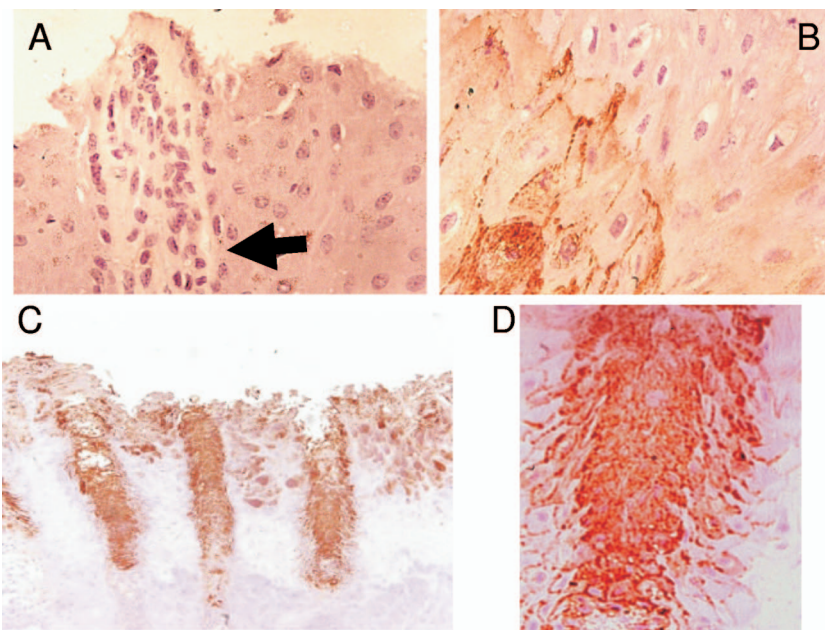


FIG. 2. Immunohistochemical staining of treponemes in digital dermatitis lesions. (A) Staining of healthy hoof skin (hair follicle is identified by the arrow). (B) Staining of deep dermal layers showing treponemes tracking between cells. (C) Staining of hoof skin showing strong staining of hair follicles. (D) Staining of hair follicle showing tracking of infection from hair follicles into surrounding tissues.

mary BDD biopsy cultures, which we had considered to contain mixed cultures of two or more treponeme groups. After reviewing the data suggesting that many lesions contained all three phylogroups, we used the PCR assays to test original initial primary cultures of strains for the three phylogroups. We then used group-specific serum and growth conditions described previously (13), with several rounds of plating and subculture to isolate further strains. In this approach, the group-specific PCRs were a powerful tool for the identification of isolate purity. A group 2 isolate, T200, was isolated from a culture that had previously failed. A group 1 isolate, T52B, was isolated from a lesional culture which had previously yielded only a group 2 treponeme (T52A). In one instance, we managed to isolate a further two treponemes, one group 1 strain (T136E) and one group 3 strain (T136p1), from a culture from which we had already isolated one group 2 isolate (T136). All the newly found isolates had their 16S rRNA genes sequenced and clustered with their respective groups upon phylogenetic analysis (Fig. 1). In Table 3, where isolation attempts failed, this was typically due to heavy contamination, which we could not remove, as opposed to no treponemal presence (as determined by phase-contrast microscopy).

Immunohistochemistry of healthy and infected bovine foot tissues. Healthy foot tissues showed no treponemal presence by immunohistochemistry using antisera raised against the BDD treponemes (Fig. 2A). In comparison, lesional tissues from BDD cases displayed very strong staining with the anti-treponemal antisera. This was apparent particularly in the deep layers of the lesion (Fig. 2B) and, unexpectedly, in the hair follicles and sebaceous glands (Fig. 2C and D). This staining pattern was seen in all the cases tested. Differences were seen in the sublocalization of the treponemes: in the hair follicles, the treponemes appeared to be both intra- and extra-

cellular, and in the surrounding tissues, they were almost entirely extracellular in location.

EM of infected tissues. Confirmation that the antitreponeme antisera were detecting treponemes in these tissues was provided by EM studies, which provided characteristic treponemal morphology both in deep lesional tissues and in hair follicles (Fig. 3). No treponemes were observed by EM in healthy foot tissues (data not shown).

DISCUSSION

One of the main aims of this study was to design and implement PCR tests that were capable of detecting treponemes, which we have previously identified as being associated with BDD (13). This would provide a powerful molecular epidemiology tool to help understand the involvement of individual organisms in the disease process. Immunohistochemistry and EM were also used to further delineate some relationships between the treponemes and foot infection in cows with BDD.

The development of the PCR tests was successful, and phylogroup-specific primers and PCR conditions were readily determined. The first use of these assays was to identify the distribution of the three phylogroups within lesions from cattle from different regions of the United Kingdom. This indicated the presence of two (or, most commonly, three) phylogroups in BDD lesions. Isolation data from previous studies suggested that the group 2 isolates (*T. phagedenis*-like) might be the only phylogroup specifically associated with BDD, as they have been the most commonly isolated ones (13, 32, 33). The current data clearly refute this premise, as the new PCR tests indicated the presence of group 2 treponemes in nearly all BDD lesions (98%) and detected group 1 treponemes (*T.*

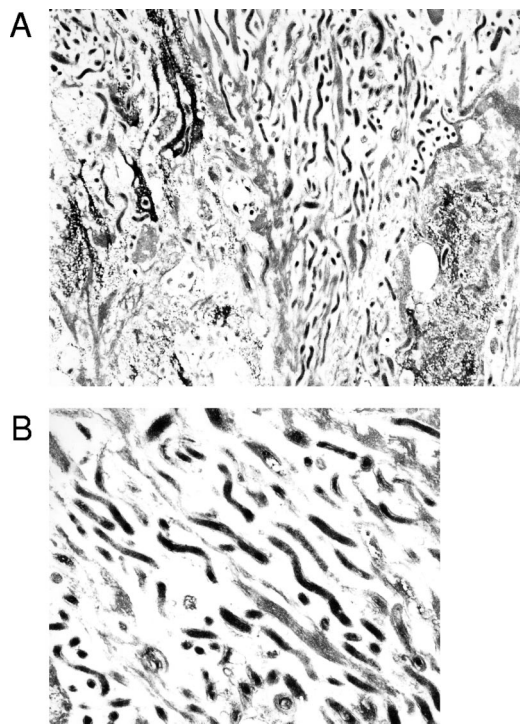


FIG. 3. EM of hair follicle from hoof skin of a cow with BDD. (A) Treponemes tracking in parallel inside follicle at a 1:9,000 magnification. (B) Characteristic treponeme morphology in follicle at a 1:22,500 magnification.

medium/T. vincentii-like) in the majority (96.1%) of BDD lesions. In comparison, the group 3 treponemes (*T. putidum/T. denticola*-like) were present in 74.5% of lesion biopsies. Previously, group 3 treponemes were identified only at the exterior of lesions, suggesting that they may prefer outer skin layers, while group 1 and group 2 isolates have been identified deeper within lesions (22). Therefore, we are unsure whether the comparatively lower association of group 3 isolates with BDD represents variations in sampling location and/or lesion age or whether the data reduce the importance of the group 3 isolates in the etiology of the disease. Interestingly, the healthy foot tissues were universally negative for any of the BDD-associated treponemes, although other treponemes were readily detected in these samples.

The high level of association of the group 1 and group 2 isolates with BDD is very interesting, especially as the association of oral spirochetes with periodontal infections is typically reported at a considerably lower percentage. For example, using a nested PCR method similar to that described here, *T. denticola* was found to be present in ~79% of human endodontic abscesses, while *Treponema socranskii* was the next most prevalent at 26%, and *T. medium* was present in only 5% of samples (30). Similar percent associations were also previously reported for human primary root canal infections (26). Hence, the results presented here argue that the infection etiology of this disease may not be as polymicrobial in origin as that encountered with oral infections and implicate group 1 and 2 treponemes as being highly associated with infection. It would be interesting to postulate that these two treponemes

are required together as a pathogenic complex to cause BDD, which might explain why the disease has been transmitted using lesion material (24) but why no single isolate has yet been reported to fulfill Koch's postulates.

Despite attempts to locate primers at unique sites on the 16S rRNA gene locus, the group 1 and group 2 treponeme primers still cross-reacted with their nearest relatives (*T. medium* and *T. phagedenis*, respectively). We expected cross-reactions with the nearest relatives of group 1 and 2 treponemes, as the respective 16S rRNA gene sequences are nearly identical. In fact, the taxonomic relationship with the closest relatives has still not been answered, although we have recently proposed the group 3 isolates as a novel species, "*Treponema pedis*" (12). The nearly identical 16S rRNA genes of these bovine and human treponemes could be considered a limitation, and future PCR methods might warrant the use of alternative genetic loci. It should be noted that the different *Treponema pallidum* subspecies that cause very different diseases in humans (e.g., syphilis and yaws) are distinguished thus far only by genes encoding putative surface-exposed proteins (4).

Retrospective isolations were carried out on initial cultures when the PCR assays identified more than one phylogroup present. Using the PCR protocol and further isolations, we have obtained several new strains from initial cultures, and when detected, we have been able to isolate these strains. To verify that the strains isolated belong to their expected PCR groups, these new isolates had their 16S rRNA genes sequenced, and it was confirmed that they belong to their respective groups. This is further validation of this PCR-based approach.

The PCR assays that we have developed can now be used to investigate environmental samples for potential transmission routes as well as whole-animal surveys for possible infection reservoir identifications. Furthermore, preliminary evidence presented here showed that acute lesions may be the result of only two phylotypes of treponeme, *T. medium*-like and *T. phagedenis*-like. Evidence of *T. medium*-like and *T. phagedenis*-like spirochetes being located deep within lesions has been identified before using fluorescent in situ hybridization (FISH) (22). Further studies of acute versus chronic lesions would give more evidence of whether this is a significant association and might allow the identification of the primary invader(s) so as to specifically target and eradicate BDD once and for all. Further PCR tests for other treponemes isolated from BDD lesions, such as *Treponema brennaborensis* (29), and a global survey of BDD lesions would also be very valuable. These PCR assays could also be used to further investigate the ovine manifestation of DD called CO DD. One of our isolates (G179) is from a CO DD lesion, and the current work confirms it as a group 3 treponeme, as previously suggested (13). This result adds strength to the suggestion that the sheep form of the disease has been transmitted from the cow or a shared intermediary host (9).

In terms of the etiopathogenesis and molecular epidemiology of BDD, the most striking finding is that 75% of BDD lesions contain representatives of all three BDD treponeme groups. This is not readily explainable, unless a symbiotic relationship is optimal for disease induction and maintenance. Symbiosis of treponemes in BDD lesions has been used to

explain recent results from FISH studies (17, 23), and further research into such a symbiosis might be appropriate. As a result of this study, we may be one step closer to dissecting such a symbiotic pathogenesis given that we have been able to isolate members of all three groups from the same lesion. However, it is important that a recent German BDD FISH study identified five groups as being the most important in BDD etiology (23), with three of the groups resembling the treponemes that we have isolated. Furthermore, a larger variety of treponemes have been identified in Danish BDD lesions, although only three groups were identified as being the most prevalent (17) and only one of the groups resembles the treponemes which we isolated, suggesting regional differences in BDD etiology.

Both PCR and immunohistochemistry suggest that healthy tissues on bovine feet do not (apart from one cow tested) harbor the BDD treponemes, although other treponemes are readily detected. Hence, the BDD treponemes do not appear to be commensals and/or opportunistic invaders. Indeed, it is likely that they are highly developed to exist in the environmental niche of the bovine foot, although they may also be present in large numbers in slurry.

If the treponemes are the primary infectious agent in BDD, they will need to be transmitted from a reservoir to foot tissues. While the reservoir for these organisms has not yet been identified, the immunohistochemistry shown here clearly indicates that the treponemes may be entering (and may be exiting) via hair follicles and/or sebaceous glands. This route of transmission would explain how they are able to breach such a hard physical barrier to establish infection in deep-lying tissues. This finding is also possibly relevant to the polytreponemal nature of BDD in that in most foot tissues, the organisms appear to be largely extracellular, and in hair follicles, they are extracellular and intracellular. The data presented in this paper justifies further investigation to attempt to identify routes of transmission of each of the three treponeme groups and how that may lead to the initiation of the BDD disease process.

ACKNOWLEDGMENTS

This work was funded by a Department for Environment, Food, and Rural Affairs animal welfare grant (grant AW1010) and a Biotechnology and Biological Sciences Research Council CEDFAS research grant (grant BBE0189201).

REFERENCES

- Arguez-Rodriguez, F. J., D. W. Hird, J. Hernandez de Anda, D. H. Read, and A. Rodriguez-Lainz. 1997. Papillomatous digital dermatitis on a commercial dairy farm in Mexicali, Mexico: incidence and effect on reproduction and milk production. *Prev. Vet. Med.* **32**:275–286.
- Blowey, R. W., S. H. Done, and W. Cooley. 1994. Observations on the pathogenesis of digital dermatitis in cattle. *Vet. Rec.* **135**:115–117.
- Blowey, R. W., and M. W. Sharp. 1988. Digital dermatitis in dairy cattle. *Vet. Rec.* **122**:505–508.
- Centurion-Lara, A., B. J. Molini, C. Godornes, E. Sun, K. Hevner, W. C. Van Voorhis, and S. A. Lukehart. 2006. Molecular differentiation of *Treponema pallidum* subspecies. *J. Clin. Microbiol.* **44**:3377–3380.
- Choi, B. K., H. Nattermann, S. Grund, W. Haider, and U. B. Gobel. 1997. Spirochetes from digital dermatitis lesions in cattle are closely related to treponemes associated with human periodontitis. *Int. J. Syst. Bacteriol.* **47**:175–181.
- Demirkan, I., S. D. Carter, R. D. Murray, R. W. Blowey, and M. J. Woodward. 1998. The frequent detection of a treponeme in bovine digital dermatitis by immunocytochemistry and polymerase chain reaction. *Vet. Microbiol.* **60**:285–292.
- Demirkan, I., S. D. Carter, C. Winstanley, K. D. Bruce, N. M. McNair, M. Woodside, and C. A. Hart. 2001. Isolation and characterisation of a novel spirochaete from severe virulent ovine foot rot. *J. Med. Microbiol.* **50**:1061–1068.
- Demirkan, I., H. F. Williams, A. Dhawi, S. D. Carter, C. Winstanley, K. D. Bruce, and C. A. Hart. 2006. Characterization of a spirochaete isolated from a case of bovine digital dermatitis. *J. Appl. Microbiol.* **101**:948–955.
- Dhawi, A., C. A. Hart, I. Demirkan, I. H. Davies, and S. D. Carter. 2005. Bovine digital dermatitis and severe virulent ovine foot rot: a common spirochaetal pathogenesis. *Vet. J.* **169**:232–241.
- Dopfer, D., A. Koopmans, F. A. Meijer, I. Szakall, Y. H. Schukken, W. Klee, R. B. Bosma, J. L. Cornelisse, A. J. van Asten, and A. A. ter Huurne. 1997. Histological and bacteriological evaluation of digital dermatitis in cattle, with special reference to spirochaetes and *Campylobacter faecalis*. *Vet. Rec.* **140**:620–623.
- Ehresmann, C., P. Stiegler, G. A. Mackie, R. A. Zimmermann, J. P. Ebel, and P. Fellner. 1975. Primary sequence of the 16S ribosomal RNA of *Escherichia coli*. *Nucleic Acids Res.* **2**:265–278.
- Evans, N. J., J. M. Brown, I. Demirkan, R. D. Murray, R. J. Birtles, C. A. Hart, and S. D. Carter. *Treponema pedis* sp. nov., a novel spirochete isolated from bovine digital dermatitis lesions. *Int. J. Syst. Evol. Microbiol.*, in press.
- Evans, N. J., J. M. Brown, I. Demirkan, R. D. Murray, W. D. Vink, R. W. Blowey, C. A. Hart, and S. D. Carter. 2008. Three unique groups of spirochetes isolated from digital dermatitis lesions in UK cattle. *Vet. Microbiol.* **130**:141–150.
- Glauert, A. M. 1975. Fixation, dehydration and embedding of biological specimens. Elsevier, North-Holland Biomedical Press, Amsterdam, The Netherlands.
- Hernandez, J., J. K. Shearer, and D. W. Webb. 2002. Effect of lameness on milk yield in dairy cows. *J. Am. Vet. Med. Assoc.* **220**:640–644.
- Hernandez, J., J. K. Shearer, and D. W. Webb. 2001. Effect of lameness on the calving-to-conception interval in dairy cows. *J. Am. Vet. Med. Assoc.* **218**:1611–1614.
- Klitgaard, K., M. Boye, N. Capion, and T. K. Jensen. 2008. Evidence of multiple *Treponema* phylotypes involved in bovine digital dermatitis as shown by 16S rRNA gene analysis and fluorescence in situ hybridization. *J. Clin. Microbiol.* **46**:3012–3020.
- Koniarova, I., A. Orsag, and V. Ledecy. 1993. The role anaerobes in dermatitis digitalis and interdigitalis in cattle. *Vet. Med. (Praha)* **38**:589–596. (In Slovak.)
- Laven, R. A., and D. N. Logue. 2006. Treatment strategies for digital dermatitis for the UK. *Vet. J.* **171**:79–88.
- Losinger, W. C. 2006. Economic impacts of reduced milk production associated with papillomatous digital dermatitis in dairy cows in the USA. *J. Dairy Res.* **73**:244–256.
- Moore, L. J., M. J. Woodward, and R. Grogono-Thomas. 2005. The occurrence of treponemes in contagious ovine digital dermatitis and the characterisation of associated *Dichelobacter nodosus*. *Vet. Microbiol.* **111**:199–209.
- Moter, A., G. Leist, R. Rudolph, K. Schrank, B. K. Choi, M. Wagner, and U. B. Gobel. 1998. Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. *Microbiology* **144**:2459–2467.
- Nordhoff, M., A. Moter, K. Schrank, and L. H. Wieler. 2008. High prevalence of treponemes in bovine digital dermatitis—a molecular epidemiology. *Vet. Microbiol.* **131**:293–300.
- Read, D. H., and R. L. Walker. 1996. Experimental transmission of papillomatous digital dermatitis (footwarts) in cattle. *Vet. Pathol.* **33**:607.
- Read, D. H., R. L. Walker, A. E. Castro, J. P. Sundberg, and M. C. Thurmond. 1992. An invasive spirochaete associated with interdigital papillomatosis of dairy cattle. *Vet. Rec.* **130**:59–60.
- Rocas, I. N., J. F. Siqueira, Jr., A. F. Andrade, and M. Uzeda. 2003. Oral treponemes in primary root canal infections as detected by nested PCR. *Int. Endod. J.* **36**:20–26.
- Rurangirwa, F. R., P. M. Dilbeck, T. B. Crawford, T. C. McGuire, and T. F. McElwain. 1999. Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order Chlamydiales: proposal of *Waddliaceae* fam. nov., *Waddlia chondrophila* gen. nov., sp. nov. *Int. J. Syst. Bacteriol.* **49**:577–581.
- Sabo, J., A. Hudac, and E. Fendtova. 1988. Ecology of anaerobic non-sporulating bacteria in relation to dermatitis digitalis in beef cattle. *Vet. Med. (Praha)* **33**:265–272. (In Slovak.)
- Schrank, K., B. K. Choi, S. Grund, A. Moter, K. Heuner, H. Nattermann, and U. B. Gobel. 1999. *Treponema brennaborense* sp. nov., a novel spirochaete isolated from a dairy cow suffering from digital dermatitis. *Int. J. Syst. Bacteriol.* **49**:43–50.
- Siqueira, J. F., Jr., and I. N. Rocas. 2004. *Treponema* species associated with abscesses of endodontic origin. *Oral Microbiol. Immunol.* **19**:336–339.
- Stamm, L. V., H. L. Bergen, and R. L. Walker. 2002. Molecular typing of papillomatous digital dermatitis-associated *Treponema* isolates based on

- analysis of 16S-23S ribosomal DNA intergenic spacer regions. J. Clin. Microbiol. **40**:3463–3469.
32. **Trott, D. J., M. R. Moeller, R. L. Zuerner, J. P. Goff, W. R. Waters, D. P. Alt, R. L. Walker, and M. J. Wannemuehler.** 2003. Characterization of *Treponema phagedenis*-like spirochetes isolated from papillomatous digital dermatitis lesions in dairy cattle. J. Clin. Microbiol. **41**:2522–2529.
33. **Walker, R. L., D. H. Read, K. J. Loretz, and R. W. Nordhausen.** 1995. Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. Vet. Microbiol. **47**:343–355.
34. **Whay, H. R., A. E. Waterman, and A. J. Webster.** 1997. Associations between locomotion, claw lesions and nociceptive threshold in dairy heifers during the peri-partum period. Vet. J. **154**:155–161.