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1 **Three unique groups of spirochetes isolated from digital dermatitis lesions in UK**
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Abstract

Bovine digital dermatitis (BDD) is a severe infectious cause of lameness which has spread through dairy cattle populations worldwide, causing serious welfare and agricultural problems. Spirochetes are the main organisms implicated and have previously proven difficult to isolate. This study aimed to isolate and characterise the range of spirochetes associated with BDD in the UK. Twenty three spirochete isolates were obtained from 30 BDD lesions, which by 16S rRNA gene and *flaB2* gene analysis clustered within the genus *Treponema* as three phylogroups; groups 1 (*Treponema medium*/*Treponema vincentii*-like), 2 (*Treponema phagedenis*-like) and 3 (*Treponema denticola*/*Treponema putidum*-like). The treponemes displayed large genotypic and phenotypic diversity between phylogroups and differed from named treponeme species. A previously isolated contagious ovine digital dermatitis spirochete was located within one of the three phylogroups, group 3, and could also be identified within this group on the basis of phenotype testing, suggesting BDD and contagious ovine digital dermatitis may share the same aetiological agent. A strain isolated from a bovine interdigital dermatitis lesion, could be identified as part of BDD isolate group 2, suggesting bovine interdigital dermatitis and BDD may have the same causative agent. Two common enzyme activities, C4 esterase and C8 esterase lipase, were identified in all BDD associated treponemes suggesting common metabolic pathways for sharing this novel niche or even common virulence traits. Further studies are required to determine whether the three groups of novel treponemes are representative of new treponeme taxa and to delineate how they interact with bovine tissues to cause disease.

Keywords: Bovine digital dermatitis, *Treponema*, 16S rRNA gene, phenotyping

1. Introduction

Bovine digital dermatitis (BDD) is an ulcerative foot disease found in dairy cattle, initially reported in Italy in 1974 (Cheli and Mortellaro, 1974) and which has subsequently been identified worldwide. The main clinical feature of BDD is lameness resulting from a lesion immediately above the coronet between the heel bulbs (Blowey and Sharp, 1988). BDD results in large welfare and economic problems; hence prevention and treatment of this disease is of great importance (Demirkan et al., 2000; Read et al., 1992). The etiology of BDD has not yet been completely determined; however, the majority of evidence suggests involvement of spirochetes. Spirochetes have frequently been found in large numbers, deep inside BDD lesions (Blowey et al., 1992; Demirkan et al., 1998; Read et al., 1992) and molecular methods have further implicated and identified them as belonging to the genus *Treponema* (Choi et al., 1997; Demirkan et al., 1998).

The *Treponema* species are very difficult to maintain in culture, although some progress has been made in isolation from BDD lesions. Eight spirochetes were isolated from BDD lesions in the USA, with seven of these isolates forming a distinct phenotypic group (Walker et al., 1995). A further four USA BDD spirochetes were identified as similar to *Treponema phagedenis* (Trott et al., 2003). A spirochete isolated from a BDD lesion in the UK was identified as similar to the USA isolates (Demirkan et al., 2006), whilst a German BDD spirochete (*Treponema brennaborensis*) has been identified as quite different (Schrack et al., 1999). We deduced that there was a need for further isolation of BDD treponemes given the small number of isolations and that a previous molecular survey (Choi et al., 1997) suggested a more diverse treponemal community

than the above isolation data suggests. In the present study, we attempted to determine the range of spirochetes present in BDD lesions from a number of farms in the UK. We isolated and characterised 23 spirochete strains and compared against other relevant treponemes.

2. Materials and Methods

2.1 Isolation and cultivation.

Single biopsies were taken from 30 Holstein-Friesian cows with BDD from Merseyside (n=12), Cheshire (n=9), Shropshire (n=4) and Gloucestershire (n=5), UK, with a total of 9 farms included in the study (Tables 1 and 2). The BDD biopsies were collected during the housing period when BDD cases are reported to peak (Blowey and Sharp, 1988; Somers et al., 2005); in this case from October 2003 through to July 2004. All farms sampled were medium-sized commercial dairy operations (~200-300 cows) using cubicle type housing and had suffered recent outbreaks of BDD. All biopsies were from typical bovine digital dermatitis (BDD) lesions, except for one sample taken from a case of bovine interdigital dermatitis (IDD) (see Table 1). After cleaning the foot surface by brushing and washing with sterile PBS, a 3 mm punch biopsy was taken from the centre of the lesion, washed in sterile phosphate buffered saline (pH 7.4), placed in oral treponeme enrichment broth (OTEB: Anaerobe Systems, Morgan Hill, CA, USA) containing rifampicin (5 µg/ml) and enrofloxacin (5 µg/ml) and transferred to the laboratory. The biopsy was transferred into an anaerobic cabinet (85% N₂, 10% H₂ and 5% CO₂, 36°C) and diced into small fragments (~1 mm³). The fragments were inoculated into fresh OTEB supplemented with 10% fetal calf serum (FCS), antibiotics as above and

incubated for 24 hours. Bacteria were then subcultured on fastidious anaerobe agar (FAA) plates (LabM, Bury, UK) supplemented with 5% defibrinated sheep blood, 10% FCS and antibiotics as above, for ~2 weeks. Single colonies were inoculated into growth media without antibiotics and subculture only repeated if cultures were not deemed pure by phase contrast microscopy and 16S rRNA gene sequencing. Isolates were stored at -80°C in growth medium containing 10% glycerol.

2.2 Reference strains.

Reference strains *Treponema vincentii* ATCC 35580, *T. vincentii* D2A2 and *Treponema medium* ATCC 700293^T were a kind donation from Dr. H. E. Allison, School of Biological Sciences, University of Liverpool, UK. *Treponema phagedenis* CIP62.29 was obtained from the Institut Pasteur, France.

2.3 Gene sequencing and phylogenetic analyses.

For isolation of genomic DNA, 7 ml of exponential phase spirochete 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 (Chua et al., 2005) and stored at -20°C.

Treponema 16S rRNA gene and *flaB2* gene PCR were carried out as described previously (Demirkan et al., 2001). Amplified PCR products were sequenced commercially and complete genes assembled using the Staden sequence analysis package (Staden, 1996). Gene sequences were aligned using CLUSTALW (Thompson et al., 1994) and phylogenetic trees were calculated with the neighbor joining method (bootstrap values based on 1000 iterations) using nucleotide substitution rates calculated according to the Kimura two-parameter model implemented in MEGA2 (Kumar et al., 2001).

2.4 Enzyme activities.

Enzyme profiles for each strain were determined using the APIZYM system (bioMérieux, Lyon, France) according to the manufacturer's instructions, with each test in triplicate. Validation of APIZYM testing used *Treponema vincentii* ATCC 35580 (LA-1) as a test microorganism with identical enzyme activities identified to those previously reported (Schrack et al., 1999).

2.5 Nucleotide sequence accession numbers.

The complete list of Genbank accession numbers for 16S rRNA and *flaB2* gene sequences determined as part of this study are shown in Table 1 except: *T. phagedenis* CIP62.29 16S rRNA gene accession EF645248, *T. medium* ATCC 700293, *T. vincentii* ATCC 35580 and *T. vincentii* D2A2 *flaB2* gene sequences accession numbers EF061285, EF061286 and EF061287 respectively.

3. Results

3.1 Spirochete isolation.

Twenty three spirochete isolates were obtained from BDD lesions (Table 1). Most cultures required 2-3 repeated passages onto supplemented FAA plates before non-contaminated, single spirochete strain cultures were obtained. To identify optimal serum for growth in liquid medium, isolated strains were inoculated into OTEB containing 10% FCS or rabbit serum (RS). From the 23 spirochetes isolated, several strains exhibited better growth (substantial increase in total cell number) with RS (than FCS) as an OTEB growth supplement (Table 1). We failed to isolate spirochetes from every biopsy (Table

2), as might be expected with fastidious anaerobe isolations. However, two different strains were isolated from single lesion biopsies from two cows.

3.2 Growth characteristics.

Isolated strains inoculated onto FAA plates only demonstrated growth with blood and/or serum present suggesting that growth of the spirochetes was serum dependent. Optimal growth (substantial increase in colony size and number) was achieved with both serum and sheep blood FAA supplements present. The spirochetes could be divided into three groups (groups 1, 2 and 3) on the basis of growth characteristics in OTEB (Table 1) and on sheep blood FAA plates. When group 1 isolates were inoculated onto FAA plates supplemented with 10 % RS; nine days incubation produced translucent, circular, convex, colonies between 0.1 and 3 mm in size. After a further week, colonies were larger (2-3 mm) with β -hemolysis underneath and a lawn had swarmed between colonies. A second group of isolates (group 2) demonstrated swarms of individual, circular colonies when inoculated onto FCS-supplemented FAA. After 14 days, colonies were typically a final size of 0.2-2 mm in size, convex, circular and translucent with no local hemolysis. When group 3 isolates were inoculated onto FCS-supplemented FAA, the agar surface was covered in translucent, circular, convex single colonies of ~0.2 mm after just 4 days, which after a further week grew to a final size of ~0.5-2 mm. After 3 weeks, distinct, β -hemolysis appeared underneath colonies which was more penetrative (visible from the under side of the plate) than that exhibited by group 1 isolates. Furthermore, irregular shaped projections appeared to have grown from many colonies. The projections had a characteristic metallic sheen and after a further week the protrusions had swarmed across the entire plate. An ovine spirochete strain G179 isolated in this laboratory previously

(Demirkan et al., 2001) demonstrated identical growth characteristics to the group 3 isolates.

3.3 16S rRNA gene analysis.

Approximately 1420 bp of the 16S rRNA gene were sequenced for each of the 23 isolates and sequences aligned against a large number of relevant 16S rRNA gene sequences (Fig. 1). On phylogenetic tree construction, the 23 isolates separated into three distinct phylogroups corresponding exactly to the three groups identified using growth characteristics. 16S rRNA gene sequence identity shared within each phylogroup was high with individual members of groups 1, 2 and 3 sharing a minimum sequence identity of 100%, 99.9% and 99.7% respectively. In contrast, phylogroups displayed a large amount of 16S rRNA gene sequence diversity between them, with group 1 and group 2 being least similar with 90.1% identity whilst group 2 and group 3 were most similar although with only 92.3% sequence identity. Group 1 strains were most closely related to DDKL-13 (an uncultivated German BDD spirochete sequence) with 99.84% sequence identity, then *T. medium* ATCC 700293^T (99.6%) and to a lesser extent *T. vincentii* ATCC 35580 (99.0%). All group 2 isolate 16S rRNA gene sequences were identical, except T167, and were identical to an uncultivated German BDD spirochete sequence DDKL-4, several previously reported American BDD isolates *Treponema* sp. 2-1498, *Treponema* sp. 3A, *Treponema* sp. 4A (Trott et al., 2003; Walker et al., 1995) and G356 (*Treponema* sp. HW-2003) isolated from a BDD lesion in the UK (Demirkan et al., 2006). T167 contained a single nucleotide substitution difference from the identical sequences. The group 2 isolate 16S rRNA sequences were also identical to the closest designated species *T. phagedenis* CIP62.29 except for a single nucleotide substitution and

were related to a lesser extent to *Treponema pallidum* spp.. Group 3 isolates were closely related to a spirochete isolated in this laboratory from a case of contagious ovine digital dermatitis (CODD) (Demirkan et al., 2001) with ~99.8% sequence identity, then to bovine isolate *Treponema* sp. 1-9185MED (~99.0%) from America (Walker et al., 1995), then to *Treponema putidum* ATCC 700334^T (~96.6%), *Treponema denticola* ATCC 35405^T (~95.7%) and then an uncultivated German BDD spirochete sequence DDKL-3 (~94.05%).

3.4 *flaB2* gene analysis.

Further phylogenetic analysis was undertaken by comparing *flaB2* across a number of isolates. Approximately 510 bp of the *flaB2* gene were sequenced in 14 BDD isolates and reference strains *T. medium* ATCC 700293^T, *T. vincentii* ATCC 35580 and *T. vincentii* D2A2. Initially, the *flaB2* gene sequences were aligned against a number of reported treponeme *flaB2* sequences and trimmed to the shortest length. After phylogenetic tree construction (Fig. 2), the 14 isolates could be divided into the same three distinct groups already identified by growth characteristics and 16S rRNA gene analysis. Amongst group 1 isolates there was 100% nucleotide sequence identity in the *flaB2* gene. The strains were placed closest to *T. medium* ATCC 700293^T and grouped to a lesser extent with *T. vincentii* strains (corresponding to 97.6% and 96.8% *flaB2* nucleotide sequence identity respectively). The group 2 isolates were located together in the *flaB2* phylogenetic tree as a group and two distinct subgroups could be identified within the phylogroup. Interestingly *T. phagedenis* Kazan 5 was situated in one subgroup with group 2 BDD isolates, as it had 100% nucleotide sequence identity with T119A and T320A. The second subgroup consisted of 4 isolates which shared between 97.0% and

99.4% nucleotide sequence identity. In agreement with 16S rRNA gene phylogenetic analysis, using *flaB2*, identified group 3 isolates as most closely related to a spirochete isolated from a CODD lesion. The CODD isolate actually clustered within the group 3 isolates with the ovine isolate *flaB2* and strain T3552B *flaB2* sharing 99.4% sequence identity whilst T354A and T3552B *flaB2* only shared 97.8% sequence identity. The group 3 isolates are then shown to be most closely related to a *T. denticola* isolate (ATCC 33521) sharing only ~80.9% sequence identity.

3.5 Enzyme activities.

The enzyme activities of the BDD associated spirochetes, compared with other relevant treponemes, showed (Table 3) that the three groups had specific enzyme patterns and were different to patterns for previously designated *Treponema* species. The enzyme profiles for each of the previously categorised three groups were identical within each group and different between groups, in good correlation with the genetic analyses and growth characteristics. Only C4 esterase and C8 esterase lipase enzyme activity was present in all three BDD treponeme groups. Interestingly, the previously reported ovine isolate which shared identical 16S rRNA gene sequence with the group 3 isolates also shared an identical enzyme profile with the group 3 isolates.

4. Discussion

This study has isolated and characterised a large panel of spirochetes associated with BDD in dairy cattle. This has been a significant advance as studies of this emerging and spreading disease have been previously severely hampered by the difficulties in isolating and maintaining these organisms *in vitro*. Although previous reports have implicated BDD as a polyspirochetal infection (Choi et al., 1997; Moter et al., 1998) only

on two (of 21) occasions were we able to isolate more than one spirochete phylotype from a single BDD lesion; although this might be expected given the fastidious nature of these anaerobes. The BDD spirochetes' 16S rRNA gene sequences identified the isolates as all belonging to the genus *Treponema*, in agreement with previously reported phylogenetic analyses (Choi et al., 1997; Demirkan et al., 1998). From five different spirochete 16S rRNA gene fragment sequences previously identified in BDD lesions (Choi et al., 1997), three of the gene fragments clustered with the 16S rRNA gene sequences of the strains isolated here. DDKL-3, DDKL-4 and DDKL-13, were reported by the authors to be most similar to *T. denticola*, *T. phagedenis* and *T. vincentii* respectively on the basis of 16S rRNA gene sequence similarity. In this study, the isolates showed similar relationships; with the group 1, 2 and 3 isolates sharing high 16S rRNA gene sequence similarities to *T. medium*/*T. vincentii*, *T. phagedenis* and *T. denticola*/*T. putidum* respectively, all isolated from human tissues (Chan et al., 1993; Smirbert, 1984; Umemoto et al., 1997; Wallace et al., 1967; Wyss et al., 2004). The reason only three of five potential phylogroups identified as populating BDD lesions are isolated here may be the result of culture bias given the fastidious nature of treponemes.

The results presented here clearly indicate that there is heterogeneity between these isolated BDD associated treponemes and that they generally fall into one of three well defined groups. It is intriguing that, by each of the means of characterisation used in this study, they divided into these groups and that each assay provided the same clustering, be it growth characteristics, gene sequences or enzyme activity patterns. Given the large number of similarities within groups and the differences to currently designated taxa, we believe further studies are required to delineate whether these BDD associated

treponemes represent new taxa. Whilst the group 3 isolates could be proposed as a new species as they are within the 97% 16S rRNA sequence identity limit to closest relative (Stackebrandt and Goebel, 1994), the group 1 and 2 isolates have nearly identical 16S rRNA gene sequences to closest relatives suggesting that DNA:DNA hybridisations and further phenotypic studies are required before it can be determined whether they represent novel taxa. Certainly the taxonomic status of the group 2, *T. phagedenis*-like treponemes have been discussed before without final taxonomic proposition (Trott et al., 2003) and such taxonomy is deterred as *T. phagedenis* itself is not currently in J. Euzéby's list of valid bacterial names (Euzéby, 1997).

The ovine spirochete isolate clustered with the group 3 isolates in 16S rRNA gene and *flaB2* phylogenetic trees, and also lay within this group of isolates on the basis of growth characteristics and enzyme activities. This is in agreement with previous work from this laboratory proposing that BDD and CODD may share a common etiological agent (Dhawi et al., 2005). Whilst the majority of strains were isolated from typical BDD lesions, strain G187 was isolated from an IDD lesion, a different clinical manifestation of the bovine foot. Strain G187 was identical to other group 2 BDD isolates on the basis of genotypic and phenotypic analysis suggesting that BDD and IDD share such treponemes as common etiological agents, as reported previously (Walker et al., 1995). All group 1 isolates were from a single farm (Table 1) suggesting this group may not be present in all BDD lesions or that culture bias has prevented subsequent isolation. All treponemes isolated from BDD lesions to date, including the three groups isolated here and the quite different *T. brennaborensis*, have C4 esterase and C8 esterase lipase enzyme activity. Further investigation is required to determine if these enzyme activities represent

common metabolic pathways required for sharing this novel niche or maybe even common virulence traits.

The three phylogroups are related to quite different treponeme species (Fig. 1), with group 1 and 3 isolates related to treponemes involved in human periodontal disease (Asai et al., 2002; Wyss et al., 2004) and group 2 isolates related to a human commensal treponeme from the human urogenital tract (Wallace et al., 1967). A fluorescence *in situ* hybridization (FISH) study has suggested that the *T. phagedenis*-like and *T. medium*-like spirochetes were found deep inside lesions whilst *T. denticola*-like spirochetes were only found in the superficial layers (Moter et al., 1998). As the group 2, *T. phagedenis*-like isolates have been identified in BDD lesions from UK, USA and Germany (Choi et al., 1997; Demirkan et al., 2006; Walker et al., 1995) and are most commonly isolated; the largest proportion of recent BDD research has been centred on them with some evidence of pathogenic potential obtained (Elliott et al., 2007; Zuerner et al., 2007). However, as the group 1 and 3 isolates are most closely related to human periodontal disease treponemes and in this study are reported to produce β -hemolysis, a virulence characteristic of pathogenic spirochetes (Hyatt et al., 1994; Lee et al., 2002); the importance of these 2 groups of treponemes may be currently underestimated. Hence, given the fastidious nature of treponemes, a large molecular investigation (e.g. using PCR) is needed to further delineate the relationship of the three phylogroups and further treponemes with BDD more effectively. Also, whilst disease has been experimentally transmitted from foot to foot using lesion material (Read and Walker, 1996), experiments using single isolates/mixes of the BDD treponemes should be attempted to try and fulfil

Koch's postulates. Hopefully, a combination of the aforementioned studies would finally identify the role that each of these treponeme phylotypes plays in BDD lesions.

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- 402

403 **Tables:**

404 TABLE 1. Spirochetes isolated from BDD lesions in this study.

Strain ^a	Isolation date	Location	Serum ^b	Group ^c	16S rRNA gene accession No.	<i>flaB2</i> gene accession No. ^d
T184	6/10/03	Merseyside, Farm 1, cow 1	RS (9)	1	AY387410	-
T19	7/10/03	Merseyside, Farm 1, cow 2	RS (9)	1	EF061249	EF061271
T54	7/10/03	Merseyside, Farm 1, cow 3	RS (9)	1	EF061250	EF061272
T56	7/10/03	Merseyside, Farm 1, cow 4	RS (9)	1	EF061251	EF061274
T18A	7/10/03	Merseyside, Farm 1, cow 5	RS (9)	1	EF061252	EF061273
T18B	7/10/03	Merseyside, Farm 1, cow 5	FCS (4)	3	EF061270	-
T167	8/12/03	Cheshire, Farm 1, cow 1	FCS (7)	2	EF061253	EF061279
T257	12/1/04	Cheshire, Farm 2, cow 1	FCS (7)	2	EF061257	EF061280
T296A	12/1/04	Cheshire, Farm 2, cow 2	FCS (7)	2	EF061258	-
T354A	12/1/04	Cheshire, Farm 2, cow 3	FCS (4)	3	EF061267	EF061283
T354B	12/1/04	Cheshire, Farm 2, cow 3	FCS (7)	2	EF061259	-
T136	4/2/04	Shropshire, Farm 1, cow 1	FCS (7)	2	EF061255	EF061282
T119A	4/2/04	Shropshire, Farm 1, cow 2	FCS (7)	2	EF061256	EF061275
T52A	4/2/04	Shropshire, Farm 1, cow 3	FCS (7)	2	EF061254	EF061281
T320A	24/2/04	Merseyside, Farm 2, cow 1	FCS (7)	2	EF061261	EF061276
T380A	24/2/04	Merseyside, Farm 2, cow 2	FCS (7)	2	EF061262	-
T2721A	24/2/04	Merseyside, Farm 2, cow 3	FCS (7)	2	EF061260	EF061277
T3552B	24/2/04	Merseyside, Farm 2, cow 4	FCS (4)	3	EF061268	EF061284
W35	17/3/04	Cheshire, Farm 3, cow 1	FCS (7)	2	EF061264	-
G819CB	7/5/04	Gloucestershire, Farm 1, cow 1	FCS (4)	3	EF061269	-
G187	12/5/04	Gloucestershire, Farm 2, cow 1	FCS (7)	2	EF061266	-
G169A	13/5/04	Gloucestershire, Farm 3, cow 1	FCS (7)	2	EF061265	EF061278
T323C	20/7/04	Merseyside, Farm 2, cow 5	FCS (7)	2	EF061263	-

405 ^a All strains were isolated from typical bovine digital dermatitis (BDD) lesions, except
 406 G187 which was isolated from a case of bovine interdigital dermatitis (IDD).

407 ^b Preferred OTEB serum supplement for optimal growth is described (as determined by
 408 total cell numbers). The number of days for optimal growth is shown in brackets.

^c Group designation according to growth characteristics.

^d A dash indicates that the sequence was not determined.

TABLE 2. Spirochete isolations compared with the number of biopsies sampled.

Location	Cows sampled ^a	Spirochetes isolated	Cows yielding more than one isolate ^b
Merseyside, Farm 1	6	6	cow 5 (2)
Merseyside, Farm 2	6	5	-
Cheshire, Farm 1	2	1	-
Cheshire, Farm 2	5	4	cow 3 (2)
Cheshire, Farm 3	2	1	-
Shropshire, Farm 1	4	3	-
Gloucestershire, Farm 1	2	1	-
Gloucestershire, Farm 2	1	1	-
Gloucestershire, Farm 3	2	1	-

^a A single biopsy was taken from each cow.

^b The total number of spirochetes obtained is indicated in brackets.

TABLE 3. Enzyme activities of the 23 bovine spirochetes compared with other relevant treponemes.

Species/Group ^a	Strain	Enzyme Activity ^b																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
<i>T. brennaborensis</i> ^c	DSM 12168 ^T	+	+	+	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-
<i>T. medium</i> ^d	ATCC 700293 ^T	+	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
<i>T. vincentii</i> ^c	ATCC 35580	-	-	-	-	+	-	-	-	-	+	+	-	+	-	-	-	+	-	-
<i>T. phagedenis</i> ^e	Reiter	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+	-	-
<i>T. putidum</i> ^f	ATCC 700334 ^T	+	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+	-	-	-
<i>T. denticola</i> ^f	ATCC 35405 ^T	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Group 1	5 isolates	+	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Group 2	14 isolates	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	+
Group 3	4 isolates	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Ovine spirochete ^d	G179	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Bovine isolates ^g	7 isolates	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-
Bovine isolate ^g	1-9185 MED	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-

^a Nearest species designated relatives (according to 16S rRNA gene sequence identity) are shown above the bovine treponemes and previously reported bovine and ovine isolates are shown below.

^bAs determined by the APIZYM system. Enzymes tested: 1, alkaline phosphatase; 2, C4 esterase; 3, C8 esterase lipase; 4, C14 lipase; 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, chymotrypsin; 10, acid phosphatase; 11, naphtholphosphohydrolase; 12, α -galactosidase; 13, β -galactosidase; 14, β -glucuronidase; 15, α -glucosidase; 16, β -glucosidase; 17, N-acetyl- β -glucosaminidase; 18, α -mannosidase; 19, α -fucosidase.

^c APIZYM results have been previously reported (Schrack et al., 1999).

^d determined in this study.

^e APIZYM results have been previously reported (Dettori et al., 1995).

436 ^f APIZYM results have been previously reported (Wyss et al., 2004).

437 ^g APIZYM results have been previously reported (Walker et al., 1995).

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Figure Captions:

FIG. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison over ~1420 aligned bases showing relationship between strains isolated here (bold type) and related 16S rRNA gene sequences. Bootstrap confidence levels are shown as percentages of nodes and only values above 40% are shown. Accession numbers are shown next to each strain/16S rRNA gene fragment clone in brackets.

* Previously reported 16S rRNA gene sequences from Bovine Digital Dermatitis lesions.

FIG. 2. Phylogenetic tree based on *flaB2* gene sequence comparison over 510 aligned bases showing relationship of bovine spirochetes isolated in this study (bold type) with other reported treponeme sequences. Accession numbers are shown next to each strain in brackets. Bootstrap confidence levels are shown as percentages of nodes and only values above 40% are shown.

*Oral treponeme *flaB2* genes sequenced as part of this study.



