BACTERIAL PATHOGENICITY

Isolation and characterisation of a novel spirochaete from severe virulent ovine foot rot

I. DEMIRKAN, S. D. CARTER, C. WINSTANLEY*, K. D. BRUCE†, N. M. McNAIR‡, M. WOODSIDES§ and C. A. HART*

*Departments of Veterinary Immunology and *Medical Microbiology and Genitourinary Medicine, University of Liverpool, Liverpool, †Division of Life Sciences, Franklin-Wilkins Building, Kings College, London, ‡Veterinary Research Laboratory, Belfast, Northern Ireland and §Clare Veterinary Group, Ballyclare, Northern Ireland*

A novel spirochaete was isolated from a case of severe virulent ovine foot rot (SVOFR) by immunomagnetic separation with beads coated with polyclonal anti-treponemal antisera and prolonged anaerobic broth culture. The as yet unnamed treponeme differs considerably from the only other spirochaete isolated from ovine foot rot as regards morphology, enzymic profile and 16S rDNA sequence. On the basis of 16S rDNA, it was most closely related to another unnamed spirochaete isolated from cases of bovine digital dermatitis in the USA, raising the possibility of cross-species transmission. Further information is required to establish this novel ovine spirochaete as the cause of SVOFR.

Introduction

Severe virulent ovine foot rot (SVOFR) is a recently identified and important disease of sheep, and an increasing cause of lameness in sheep in the UK [1]. SVOFR is a chronic, necrotising disease of the epidermis of the interdigital skin, in some cases involving the entire hoof matrix. As severely affected tissue is destroyed the hooves become detached from the underlying matrix of the foot, resulting in lameness [2].

Various aetiological agents of ovine foot rot have been described and it is thought that initial invasion of the epidermis by *Dichelobacter nodosus* and *Fusobacterium necrophorum* subsp. *necrophorum* paves the way for invasion by secondary opportunists including *Bacteroides fragilis*, *Prevotella* spp. and several other strict anaerobes [3–7]. Spirochaetes have been observed over many years in cases of foot rot in pigs, horses and sheep [3, 8]. In a recent microbiological investigation of unusually severe ovine foot rot [1], *D. nodosus* was not detected but *F. necrophorum*, *Bact. fragilis* and *Prevotella* spp. were isolated [9]. Motile spirochaetes were also observed and successfully cultured [9, 10]. Recently, spirochaetes have been cultured successfully from cases of a related disease

of cattle, bovine digital dermatitis, in the USA [11], Germany [12] and the UK [13]. The latter study used immunomagnetic separation to isolate a spirochaete from affected cattle [13]. The present study employed a similar approach in a case of SVOFR and described the morphological, biochemical and genotypic characteristics of the novel treponeme isolated.

Materials and methods

A flock of sheep from Northern Ireland with epidemic lameness due to SVOFR was selected for study. This flock had no known prior contact, directly or indirectly, with cattle. A biopsy (c.1 g) of a lesion was taken from the right rear foot and washed in sterile phosphate-buffered saline (PBS), pH 7.4. Care was taken to ensure that the biopsy was full skin thickness, including both epidermis and dermis. After washing, the biopsy was immediately placed in Oral Treponema Enrichment Broth (OTEB; Anaerobe Systems, Morgan Hill, CA, USA) supplemented with rifampicin 5 mg/L and nalidixic acid 500 mg/L. The biopsy in OTEB was shipped to Liverpool by surface mail (48-h journey time). On arrival, the biopsy was placed in an anaerobic cabinet maintained at 37°C and diced into small fragments with sterile instruments. To check viability, a smear of the material was examined by dark-field microscopy and was found to contain motile spirochaetes. The rest of the material was placed in

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Corresponding author: Professor C. A. Hart (e-mail: cahmm@liv.ac.uk).

fresh antibiotic-containing OTEB supplemented with fetal calf serum (FCS) 10% v/v and incubated in an anaerobic cabinet (Don Whitley Scientific, Shipley, West Yorkshire) at 37°C for 24 h.

Immunomagnetic separation

The method used was as described previously for the isolation of a spirochaete from a case of bovine digital dermatitis [13]. Briefly, immunomagnetic beads (2.8 µm diameter) covalently coated with polyclonal anti-rabbit IgG were obtained from Dynech (Billingshurst, Sussex). The beads (1.5×10^7 /ml) were incubated overnight at 4°C with a mixture of polyclonal rabbit antiserum to *Treponema denticola* and *T. vincentii* (200 µl of each). After washing in PBS, the coated beads (200 µl) were mixed with the 24-h biopsy culture (1 ml) by slow tilting and rotation for 60 min at 4°C. The beads were then collected by means of a magnetic particle concentrator and the supernate was discarded. The beads were washed in PBS incorporating FCS 10% v/v and re-collected magnetically. The beads were then placed into fresh OTEB supplemented with antibiotics as above and some were streaked on to Fastidious Anaerobe Agar incorporating defibrinated sheep blood 5% v/v (FABA) plates (LabM, Bury), supplemented with rifampicin 1 mg/L and enrofloxacin 1 mg/L. Broth and plates were incubated in an anaerobic cabinet at 37°C for 7 days. After initial isolation the culture was continued in OTEB without added antimicrobial agents. The isolated treponemes were stored on Protect Beads (Technical Service Consultants, Heywood) at -80°C until required for further analysis.

Electron microscopy

Carbon re-inforced, formvar-coated copper specimen support grids (400 square mesh) were coated with a drop of a 48-h OTEB ovine spirochaete culture suspension. The grid was air-dried then washed three times in a drop of distilled water on a microscope slide (to remove crystallised salts). It was then negatively stained with EM grade potassium phosphotungstate, pH 7.0 (Agar Scientific, Standstead) 1%. Grids were examined with a Philips 301 electron microscope.

Enzyme profile

A 48-h OTEB culture (10 ml) without antibiotics was centrifuged at 12 000 *g* for 20 min. The supernate was removed and the pellet was washed twice in PBS, pH 7.2. The pellet was suspended in PBS to a density equivalent to MacFarland turbidity standard 5. The suspension was inoculated into cupules of the API-ZYM strip system (bioMérieux, Basingstoke), which contains 19 different substrates. The strip was incubated and reactions were interpreted according to the manufacturer's instructions. Catalase activity was determined by mixing a portion of the pellet with

H₂O₂ 3% v/v and observing it for evolution of bubbles of oxygen.

DNA analysis

DNA was extracted from 7-day OTEB (antibiotic-free) cultures (20 ml). Bacteria were pelleted by centrifugation at 12 000 *g* for 20 min and washed twice in PBS. The pellet was suspended in 1 ml of lysis buffer (500 mM Tris, pH 9; 20 mM EDTA; 10 mM NaCl; sodium dodecyl sulphate 1% w/v and freshly prepared proteinase K 0.5 mg/ml) and incubated at 42°C for 4 h on a shaker. DNA was extracted with phenol, precipitated with 5 M NaCl and absolute ethanol, suspended in 100 µl distilled water and stored at -20°C.

16S rRNA gene sequencing and analysis

Primers pA (pA: 5'-AGA GTT TGA TCC TGG CTC AG-3') and pH' (pH': 5'-AAG GAG GTG ATC CAG CCG CA-3'), developed by Edwards *et al.* [14] and supplied by MWG-BIOTECH UK (Milton Keynes) were used to amplify 16S rDNA as follows. Genomic DNA (1 µl) was used directly in 50-µl volumes containing *Taq* DNA polymerase (Roche, Lewes, East Sussex) 1.25 U, 200 nM of each primer (pA and pH'), 1× PCR reaction buffer (supplied by Roche) and 100 µM nucleotides (dATP, dCTP, dGTP, dTTP). Amplifications were performed in a Perkin Elmer 2400 thermal cycler (Applied Biosystems, Warrington, Cheshire) for 30 cycles consisting of 95°C (1 min), 55°C (1 min) and 72°C (2 min) with an additional extension time at 72°C (10 min) after completion of 30 cycles. At the end of the amplification, 8-µl samples were subjected to electrophoresis on a standard TAE agarose 0.7% w/v gel and stained after electrophoresis with 2 µl of ethidium bromide 10 mg/ml in 200 ml of TAE buffer, to confirm the presence of an amplified product. This sample was purified with QIAquick PCR purification columns (Qiagen, Crawley, West Sussex) and subjected to sequencing at King's College Oral Microbiology Department with oligonucleotide primers pA and pH'. The accession code number for this sequence is AF363634.

The phylogenetic relationship of the ovine sequence was established by comparison with 28 treponeme and related sequences, taken from the Ribosomal Database Project (RDP) [15], selected to cover this group within the Bacterial domain. In addition to these sequences, the two sequences providing the closest currently available FASTA matches (Wisconsin package version 10.1, Genetics Computer Group (GCG), Madison, WI, USA) to the ovine sequence were also included, along with two sequences from treponemes identified previously as causing similar disease [10, 12]. Following alignment of these sequences, a dendrogram was generated from the 1326 aligned bases by dnaml (PHYLIP version 3.573c – updated from Felsenstein

[16]). The information from this dnaml analysis is displayed as a phylogram generated by Tree View (version 1.6.1) software [17], with *Escherichia coli* 16S rDNA sequence data providing information for the outgroup.

Flagellin gene sequencing

Degenerate oligonucleotide primers were designed after alignment of published flagellin gene sequences from *T. phagedenis*, *Serpulina hyodysenteriae* and *Borrelia burgdorferi*. Oligonucleotide primers (N-terminal region forward primer, SpiroF, 5'-ATTAATCGWG CYGGNGAYGATGC-3'; and C-terminal reverse primer, SpiroR, 5'-ATTWGCTTGNGCAANCATTGC-3') were obtained from Genosys Biotechnologies (Cambridgeshire). Genomic DNA (1 µl) was used directly in 25-µl volumes containing 2 units of Dynazyme (Flowgen Instruments, Sittingbourne, Kent), 200 nM of each primer (SpiroF and SpiroR), IX Dynazyme buffer and 100 µM nucleotides (dATP, dCTP, dGTP, dTTP). Amplifications were performed in an Eppendorf Mastercycler Gradient thermal cycler (Helena Biosciences, Sunderland, Tyne and Wear) for 30 cycles consisting of 95°C (1 min), annealing temperature (1 min) and 72°C (2 min) with an additional extension time at 72°C (10 min) after completion of 30 cycles. To determine optimum conditions, the annealing temperature was varied over a gradient of 48.6–64.7°C. At the end of the amplification, 5-µl samples were subjected to electrophoresis on a standard agarose 1.0% w/v gel to confirm the presence of an amplified product. Samples with single amplified products were pooled, purified in Microspin S-400 HR columns (Amersham Pharmacia Biotech, Little Chalfont, Bucks) and subjected to sequencing at the University of Liverpool DNA Sequencing Service with oligonucleotide primers SpiroF and SpiroR.

Nucleotide sequence alignments and trees were constructed with PILEUP, DISTANCES and GROWTREE from the GCG sequence analysis software package (Genetics Computer Group, University of Wisconsin). Trees were constructed by the UPGMA method.

A 687-bp sequence from the ovine spirochaete encompassing the central region of the flagellin gene was used in the analysis.

Results

Bacteriological and morphological characterisation

After immunomagnetic separation (IMS), growth was visible in OTEB after culture for 10 days as fine cotton-like strands. Colonies appeared on FABA 2–3 weeks after IMS, but on subculture it took 10 days for colonies to appear. Colonies were grey-white semi-translucent in appearance, domed and circular (1–

3 mm diameter) and with irregular filamentous edges after longer incubation. A zone of β -haemolysis was visible around the colonies. On dark-ground microscopy the bacteria were highly motile, exhibiting corkscrew-like and flexuous motility. The spirochaete has been designated G179.

Spiral bacteria were readily visible on negative-stain electron microscopy. The cells were 6–8 µm long and 0.23–0.28 µm in diameter (Fig. 1a). They had tapered ends with three-to-five helical turns of *c.* 2 µm amplitude. There were four axial periplasmic flagella subterminally attached at each pole (Fig. 1b). The enzymic profile of the ovine treponeme was different from all other organisms in the APIZYM database and from the profiles described for the spirochaetes isolated from German cases of bovine digital dermatitis [12]. The spirochaete produced trypsin, chymotrypsin, C₄ esterase, C₈ esterase, lipase, leucine arylamidase and acid phosphatase enzymic activity. None of the other enzymic activities (alkaline phosphatase, C₁₄ lipase, valine arylamidase, cystine arylamidase, naphtholphosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, n-acetyl glucosaminidase, α -mannosidase, α -fucosidase) nor catalase were detected.

Genotypic characterisation

rRNA gene. 1541 bp of the 16S rRNA from the ovine spirochaete was amplified with oligonucleotide primers pA and pH'. An aligned 1326-bp region was generated for the ovine spirochaete sequence together with the 16S rDNA sequences in Table 1. The dendrogram resulting from dnaml analysis is shown in Fig. 2.

The sequences displaying the highest level of overall similarity to the sequences in FASTA searches of all bacterial entries were the 16S rDNA sequences of GB_BA2:SRKDRRNA corresponding to Spirochaete isolate 1-9185MED (98.6% identity in 1492 nt) and GB_BA2:AF139203 corresponding to *T. denticola* strain ATCC35405 strain (95.1% identity in 1522 nt). The former sequence (GB_BA2:SRKDRRNA) corresponds to an unpublished sequence derived from an unidentified spirochaete from papillomatous digital dermatitis lesions in cattle in the USA [11].

When compared by using gap (from GCG), the ovine spirochaete sequence showed 85.5% identity to the 16S rDNA sequence of *T. brennaborensis* [12] and 90.5% identity to the *T. vincentii*-like 16S rDNA sequence reported by Collighan *et al.* [10].

Flagellin gene. The central region of a flagellin gene from the ovine spirochaete was amplified with oligonucleotide primers SpiroF and SpiroR. A 687-bp nucleotide sequence derived from the amplicon was aligned with equivalent regions of flagellin genes from *T. pallidum*, *T. phagedenis*, *T. maltophilum*, *S. hyody-*

Fig. 1. Negative-stain electron microscopy of the ovine spirochaete showing (a) a cluster of bacteria and (b) a close-up of the four axial flagella (arrow).

Table 1. Accession codes for rRNA genes used in this study

Accession codes	Bacterial species
AB015887	<i>Treponema socranskii</i> subsp. <i>socranskii</i>
AF001693	Unidentified rumen bacterium RC1
AF023028	<i>Treponema</i> sp. III:A:D24
AF023031	<i>Treponema</i> sp. II:C:T1
AF023035	<i>Treponema</i> sp. II:11:C57
AF023037	<i>Treponema</i> sp. II:I:C53
AF023053	<i>Treponema</i> sp. I:F:D13
AF023057	<i>Treponema</i> sp. I:E:U17A
AF023059	<i>Treponema</i> sp. I:J:T19
AF033303	<i>Treponema</i> sp. Smibert-5
AF033304	<i>Treponema</i> sp. Smibert-2 7:A:D96NR3
AF050550	Uncultured eubacterium WCHB1-91 16S
AF050551	Uncultured eubacterium WCHB1-30
AF056338	<i>Treponema</i> sp. I:O:AF16
AF056343	<i>Treponema</i> sp. VI:G:G47
AF061347	<i>Treponema</i> sp. I:Q:AT20
AF061349	<i>Treponema</i> sp. I:S:AT39
AF061350	<i>Treponema</i> sp. I:T:AT24
AF068334	Uncultured <i>Treponema</i> clone RFS11
AF068338	Uncultured <i>Treponema</i> clone RFS21
AF068342	Uncultured <i>Treponema</i> clone RFS6
AF068429	Uncultured <i>Treponema</i> clone RFS2
AF093251	<i>Treponema</i> sp. ZAS-1
AF104475	<i>Olavius loisae</i> endosymbiont 4
AF139204	<i>Treponema denticola</i> strain ATCC35404
AF182834	<i>Treponema</i> sp. 5:C:AT040
AF182836	<i>Treponema</i> sp. 3:3:AT013
AJ009476	Uncultured bacterium SJA-69
AF363634	Novel ovine spirochaete
SRKDRRNA	Spirochaete isolate 1-9185MED (closest FASTA match)
AF139203	<i>Treponema denticola</i> strain ATCC35405 (second closest FASTA match)
AJ010951	<i>Treponema vincentii</i> -like sequence
Y16568	<i>Treponema brennaborensense</i>
EC16SRRN	<i>E. coli</i>

senteriae and *B. burgdorferi* (Fig. 3). The alignment produced three separate groupings for *B. burgdorferi*, *S. hyodysenteriae* and *Treponema* spp. Within the *Treponema* spp. group there was no clear clustering of species. The flagellin gene sequence derived from the ovine spirochaete aligned most closely to *T. maltophilum* *flaB2*. However, the other *T. maltophilum* flagellin gene included in the analysis, *flaB3*, clustered separately. Although *T. pallidum* *flaB1* and *flaB3* clustered together, *T. pallidum* *flaB2* clustered separately, with *T. phagedenis* *flaB2*.

Discussion

Ovine foot rot is an important disease both in terms of animal welfare and economic damage. Classic foot rot follows interdigital dermatitis caused by *F. necrophorum* and results from invasion by *D. nodosus* [3–7]. Indeed, a vaccine containing whole killed *D. nodosus* has been used to control the disease. Recently, a severe virulent form of foot rot (SVOFR) has emerged in the UK [1, 18] which can affect up to 80% of the sheep in a flock [1]. Cases occurred despite administration of the foot rot vaccine [1, 18]. The clinical picture of SVOFR was much more aggressive and appeared to begin higher up the limb around the coronary band, producing angry red and swollen feet. *D. nodosus* has

not been isolated from SVOFR, but numerous motile spirochaetes were observed in lesions and a spirochaete has been isolated [9] which was biochemically and enzymically similar to isolates from bovine digital dermatitis [11]. The present study used immunomagnetic separation with polyclonal anti-treponemal antisera to enrich, followed by anaerobic culture to isolate a novel treponeme. It is noteworthy that culture was possible despite a 48-h delay between sample collection and processing.

After primary isolation, culture for 10 days on FABA at 37°C under strictly anaerobic conditions was required to produce visible colonies (1–3 mm). On electron microscopic examination the organism had a typical spirochaete appearance but differed from the only other ovine isolate [10] in having four rather than three axial flagella. This novel ovine isolate differed from other bovine [11, 12] or ovine [9] isolates as regards six or seven enzymic activities.

Differences and similarities were found on comparing the organism's 16S rDNA gene sequence with those of other treponemes (Fig. 2). The closest identity (98.6%) was to a spirochaete isolated from bovine digital dermatitis in the USA, followed by an isolate of *T. denticola* (95.1%). However, the novel ovine isolate was only distantly related (90.5%) to the other ovine

Fig. 2. A dendrogram showing the relatedness of 16S rDNA sequences from various spirochaetes in comparison to the ovine isolate.

spirochaete [10] and even less so (85.5%) to *T. brennaborensen*, a German isolate from bovine digital dermatitis [12].

Flagellin gene sequence comparisons have been used extensively for phylogenetic analysis of many bacterial species [19], including *Borrelia* spp. [20–22]. The flagella of *Borrelia* spp. differ from those of other spirochaetes in that they appear to comprise a single flagellin protein and lack the outer sheath layer (FlaB) [23]. In contrast, flagella of *Treponema* spp. comprise multiple flagellins of different types (FlaA and FlaB) [24]. In this study, available gene sequences encoding FlaB-type flagellins were aligned with the flagellin gene sequence derived from the ovine spirochaete. In the alignment analysis there was no clear clustering of

Treponema spp. flagellins from the same species. The ovine spirochaete flagellin gene was most closely related to *T. maltophilum* *flaB2*, but the two available *T. maltophilum* flagellin genes did not cluster together in the analysis. Although the FlaB2 and FlaB3 proteins of *T. pallidum* and *T. maltophilum* all fall within a similar size range (31–35 kDa), they differ in isoelectric point values (FlaB2: 7.9 and 6.98 [predicted]; FlaB3 4.9 and 4.75 [predicted] for *T. pallidum* and *T. maltophilum* respectively) [25]. This difference between FlaB-type proteins may account for the variation between *flaB* sequences being greater than variation between species. Therefore, it seems reasonable to assume that the ovine spirochaete flagellin sequence is from a *flaB2*-type gene and that of those species for which flagellin gene sequences are available, the ovine

Fig. 3. A dendrogram of relatedness of flagellin gene sequences. Bbfla, *B. burgdorferi* flagellin gene (accession no. X16833); SdflaB1, *S. hyodysenteriae* flaB1 (accession no. L34861); SdflaB2, *S. hyodysenteriae* flaB2 (accession no. X63513); Tphfla, *T. phagedenis* flaB2 (accession no. M94015); TpaflaB1, *T. pallidum* flaB1 (accession no. AE001257); TpaflaB2, *T. pallidum* flaB2 (accession no. AE001250); TpaflaB3, *T. pallidum* flaB3 (accession no. X63965); TmflaB2, *T. maltophilum* flaB2 (accession no. Y18889); TmflaB3, *T. maltophilum* flaB3 (accession no. Y18889).

spirochaete is most closely related to *T. maltophilum*. Interestingly, the 16S rDNA of *T. brennaborense* was also most closely related to that of *T. maltophilum* [12].

Clearly, it is not possible to determine that this novel spirochaete is the cause of SVOFR on the basis of one case. However, the evidence that a spirochaete is involved in the pathogenesis of SVOFR is growing [9, 10, 18]. If spirochaetes are the cause it would appear that at least two different *Treponema* spp. are involved, as this isolate differs considerably from that described by Collighan and colleagues [10] in morphology (four rather than three flagella), metabolic activity (by seven enzymic activities) and 16S rDNA sequence (90.5% similarity). Studies isolating spirochaetes from a larger number of sheep with SVOFR from different parts of the UK and from other countries are required to address some of the questions. The fact that this spirochaete, at least, is able to survive transport by surface carriage should make such studies

easier. Finally, on comparison of 16S rDNA sequences, this novel ovine *Treponema* sp. is most closely related to an unnamed spirochaete which was isolated from a case of bovine digital dermatitis [12]. This raises the possibility that bovine digital dermatitis and SVOFR are caused by the same pathogen and the possibility of cross-species transmission. However, the two spirochaetes were very dissimilar by API ZYM profiles. Longitudinal studies in cattle and sheep are under way to help resolve some of the above uncertainties.

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References

1. Harwood DH, Cattell JH, Lewis CJ, Naylor R. Virulent foot rot in sheep. *Vet Rec* 1997; **140**: 687.
2. Egerton JR, Roberts DS, Parsonson IM. The aetiology and pathogenesis of ovine foot-rot. A histological study of the bacterial invasion. *J Comp Pathol* 1969; **79**: 207–215.
3. Beveridge WIB. Diseases caused by non-sporing anaerobes: ovine foot-rot necrobacillosis. *Bull Off Int Epizoot* 1967; **67**: 1597–1601.
4. Roberts DS, Egerton NR. The aetiology and pathogenesis of ovine foot-rot. II The pathogenic association of *Fusiformin nodosus* and *F. necrophorus*. *J Comp Pathol* 1969; **79**: 217–227.
5. Thomas JH. The bacteriology and histopathology of foot-rot in sheep. *Aust J Agric Res* 1962; **13**: 725–731.
6. Piriz Duran S, Ceunca Valera R, Valle Manzano J, Vadillo Machota S. Isolation and identification of anaerobic bacteria from ovine foot rot in Spain. *Res Vet Sci* 1990; **49**: 245–247.
7. Hurtado MA, Piriz S, Valle J, Jimenez R, Vadillo S. Aetiology of ovine footrot in Spain. *Vet Rec* 1998; **142**: 60–63.
8. Osborne HG, Ensor CR. Some aspects of the pathology, aetiology and therapeutics of foot-rot in pigs. *N Z Vet J* 1955; **3**: 91–99.
9. Naylor RD, Martin PK, Jones JR, Burnell MC. Isolation of spirochaetes from an incident of severe virulent ovine footrot. *Vet Rec* 1998; **143**: 690–691.
10. Collighan RJ, Naylor RD, Martin PK, Cooley BA, Bulley N, Woodward MJ. A spirochete isolated from a case of severe virulent ovine foot disease is closely related to a *Treponema* isolated from human periodontitis and bovine digital dermatitis. *Vet Microbiol* 2000; **74**: 249–257.
11. Walker RL, Read DH, Loretz KJ, Nordhausen RW. Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. *Vet Microbiol* 1995; **47**: 343–355.
12. Schrank K, Choi B-Y, Grund S *et al.* *Treponema brennaborense* sp.nov. a novel spirochaete isolated from a dairy cow suffering from digital dermatitis. *Int J Syst Bacteriol* 1999; **49**: 43–50.
13. Demirkan I, Carter SD, Hart CA, Woodward MJ. Isolation and cultivation of a spirochaete from bovine digital dermatitis. *Vet Rec* 1999; **145**: 497–498.
14. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 1989; **17**: 7843–7853.
15. Maidak BL, Cold JR, Lilburn TG *et al.* The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* 2000; **28**: 173–174.
16. Felsenstein J. PHYLIP – Phylogeny Inference Package (Version 3.2). *Cladistics* 1989; **5**: 164–166.
17. Page RDM. Tree View: an application to display phylogenetic trees on personal computers. *CABIOS* 1996; **12**: 357–358.
18. Winter A. Virulent foot rot in sheep. *Vet Rec* 1997; **141**: 27.
19. Winstanley C, Morgan JAW. The bacterial flagellin gene as a biomarker for detection, population genetics and epidemiological analysis. *Microbiology* 1997; **143**: 3071–3084.
20. Fukunaga M, Koreki Y. A phylogenetic analysis of *Borrelia burgdorferi sensu lato* isolates associated with Lyme disease in

- Japan by flagellin gene sequence determination. *Int J Syst Bacteriol* 1996; **46**: 416–421.
21. Fukunaga M, Okada K, Nakao M, Konishi T, Sato Y. Phylogenetic analysis of *Borrelia* species based on flagellin gene sequences and its application for molecular typing of Lyme disease borreliae. *Int J Syst Bacteriol* 1996; **46**: 898–905.
22. Masuzawa T, Fukui T, Miyake M *et al.* Determination of members of a *Borrelia afzelii*-related group isolated from *Ixodes nipponensis* in Korea as *Borrelia valaisiana*. *Int J Syst Bacteriol* 1999; **49**: 1409–1415.
23. Charon NW, Greenberg EP, Koopman MB, Limberger RJ. Spirochete chemotaxis, motility, and the structure of the spirochetal periplasmic flagella. *Res Microbiol* 1992; **143**: 597–603.
24. Norris SJ and the *Treponema pallidum* Polypeptide Research Group. Polypeptides of *Treponema pallidum*: progress toward understanding their structural, functional, and immunologic roles. *Microbiol Rev* 1993; **57**: 750–779.
25. Heuner K, Grosse K, Schade R, Göbel UB. A flagellar gene cluster from the oral spirochaete *Treponema maltophilum*. *Microbiology* 2000; **146**: 497–507.