Successful *in vitro* cultivation of *Borrelia duttonii* and its comparison with *Borrelia recurrentis*

S. J. Cutler,¹ C. O. K. Akintunde,¹ J. Moss,¹ M. Fukunaga,² K. Kurtenbach,³ A. Talbert,⁴ H. Zhang,¹ D. J. M. Wright¹ and D. A. Warrell⁵

Author for correspondence: S. J. Cutler. Tel: +44 181 846 7570. Fax: +44 181 846 7261.
e-mail: s.cutler@ic.ac.uk

¹ Imperial College School of Medicine (Infectious Diseases & Medical Microbiology; Central Biomedical Services; Department of Histopathology; and Molecular Pathology), Charing Cross Campus, London, UK

² Department of Molecular Microbiology, Fukuyama University, Hiroshima, Japan

³ The Wellcome Trust Centre for the Epidemiology of Infectious Disease, University of Oxford, Oxford, UK

⁴ Mvumi Hospital, PO Box 32, Mvumi, Dodoma, Tanzania

⁵ Centre for Tropical Medicine, John Radcliffe Hospital, Oxford, UK

***Borrelia duttonii*, the cause of East African tick-borne relapsing fever, has until now been refractory to growth in laboratory media. This spirochaete has only been propagated in mice or by tissue culture, restricting both yield and purity of cells available for research. The successful isolation of five clinical isolates of *B. duttonii* from patients in Central Tanzania and their comparison with *Borrelia recurrentis* is reported. Electron microscopy revealed spirochaetal cells with pointed ends, a mean wavelength of 1.8 µm with an amplitude of 0.8 µm, similar to the findings for *B. recurrentis*. Cells contained 10 periplasmic flagella inserted at each end of the spirochaete, again comparable with the counts of 8–10 flagella found in *B. recurrentis*. PFGE revealed a chromosome of approximately 1 Mb, a large plasmid of approximately 200 kb, and a small plasmid of 11 kb in all strains of *B. duttonii* and in *B. recurrentis*. *B. duttonii* possessed a further 7–9 plasmids with sizes ranging from 20 to 90 kb. In two isolates of *B. duttonii*, the profiles were identical. In contrast, all 18 isolates of *B. recurrentis* fell into one of five plasmid patterns with 3–4 plasmids ranging from 25 to 61.5 kb in addition to those of 11 and 200 kb described above. Analysis of the SDS-PAGE profiles of *B. duttonii* strains revealed a high-molecular-mass band of 33.4–34.2 kDa in four strains (variable large protein, VLP) and a low-molecular-mass band of 22.3 kDa in the remaining strain (variable small protein, VSP). This resembles the protein profiles found in *B. recurrentis*. The G+C ratio of *B. duttonii* was 27.6 mol%. Nucleotide sequence of the *rrs* gene (16S rRNA) from four *B. duttonii* isolates revealed 100% identity among these strains and 99.7% homology with three strains deposited by others in GenBank. The *rrs* gene of eight representative clinical isolates of *B. recurrentis* confirmed their close similarity with *B. duttonii*.**

Keywords: *Borrelia duttonii*; *Borrelia recurrentis*

INTRODUCTION

Borrelia duttonii, the cause of East African relapsing fever, is transmitted by the bite of the soft-bodied tick *Ornithodoros moubata*. Tick-borne relapsing fever is endemic in central Tanzania, particularly the Singida,

Dodoma, Kigoma and Mwanza regions (Jongen *et al.*, 1997). In the Dodoma region, the Gogo form the predominant tribe. Most are small farmers and live in traditional dwellings (Tembe) constructed of mud, with flat, earth-covered roofs. The occupants sleep on the floor on animal skins, and frequently share their accommodation with small livestock. Approximately 87.2% of traditional mud dwellings are infested with *O. moubata* (Barclay & Coulter, 1990) living in close proximity with man. Nocturnal tick feeding usually passes unnoticed, with feeding times of up to 30 min (Walton, 1962). Infection with *B. duttonii* is the sixth highest cause of admissions and the seventh highest

Abbreviations: VLP, variable large protein; VSP, variable small protein.

The GenBank accession numbers for the *rrs* gene sequences of *B. duttonii* and *B. recurrentis* strain A1 are AF107363–AF107366 (for *B. duttonii* strains Ku, Ly, La and Ma, respectively), AF107367 (for the A1 strain of *B. recurrentis*) and AF107356–AF107362 (for *B. recurrentis* strains A4, A5, A8, A10, A17, A11 and A16, respectively).

cause of mortality among children at Mvumi Hospital in the Dodoma region of Tanzania (Talbert *et al.*, 1998). The annual incidence in this area has been estimated at 163/1000 children under 5 years, and 384/1000 children under 1 year (Barclay & Coulter, 1990). While in pregnancy tick-borne relapsing fever is associated with a considerable perinatal mortality (436/1000 births) and foetal miscarriage rate of 475/1000 pregnancies (Jongen *et al.*, 1997).

Relapsing fever may range from subclinical with mild spirochaetemia to severe form, with a mortality of 4–5% (Mekert, 1991). Higher spirochaete counts are usually associated with a poorer prognosis. As seen in the louse-borne disease, the level of spirochaetemia correlates with the disease severity. Adult acute complications include jaundice, hepatomegaly, pneumonia and mental confusion (Mekert, 1991).

Both *B. duttonii* tick-borne and louse-borne relapsing fever are considered to be diseases in which man is the only reservoir. Both spirochaetes are fastidious and until recently were considered non-cultivable. However, unlike *Borrelia recurrentis*, *B. duttonii* will infect laboratory mice. Prior to this study, research on *B. duttonii* has been restricted by the amount of material that can be collected from mice. An *in vitro* cell culture method was described using Sf1Ep cells, which could provide more cellular material, but has not been widely adopted (Konishi *et al.*, 1993).

We now describe the cultivation of *B. duttonii* from patients in Mvumi, Central Tanzania, despite earlier failed attempts to grow *B. duttonii* as recently as 1997 (Dupont *et al.*, 1997). We used the same technique by which we were able to culture *B. recurrentis* for the first time (Cutler *et al.*, 1994). The initial characterization of these clinical isolates of *B. duttonii* are presented and compared with the closely related louse-borne *B. recurrentis*.

METHODS

Tick-borne relapsing fever patients. Samples were collected over a 2 week period in May 1998, shortly after the Tanzanian rainy season. Patients were brought to Mvumi hospital from the surrounding catchment area. All patients were demonstrated to be spirochaetemic by blood film microscopy using Field's stain. Blood samples were collected from 12 patients either by venepuncture or collected in glass capillary tubes. Four patients were female and the remaining eight were male. Ages ranged from 4 months to 5 years (ages not recorded for two patients). Blood was inoculated into BSK II and incubated at room temperature (incubator not available). Cultures were blindly passaged every third day into fresh BSK II.

Bacterial strains and culture conditions. All spirochaetes were cultured in BSK II medium (Barbour, 1984), using BSA fraction V recommended by Sigma for inclusion in BSK II medium. It was necessary to test each batch of albumin for its ability to grow relapsing fever borrelia as over two-thirds failed to promote sufficient growth. Strains were maintained by either twice or thrice weekly subculture incubated at 33 °C. *Borrelia* were passaged 6–15 times *in vitro* before

analysis. This was the minimum level of passages to obtain sufficient material to undertake the study. The *B. recurrentis* strains used in this study have been described elsewhere (Cutler *et al.*, 1997).

Contaminated cultures were passaged through antibiotic-containing BSK II either containing 100 µg rifampicin ml⁻¹ and 100 µg 5-fluorouracil ml⁻¹ or additionally containing 50 µg colistin sulphate ml⁻¹. One culture contaminated with a filamentous fungus was purified using 100 µg amphotericin B ml⁻¹.

SDS-PAGE and immunoblotting. Borrelian proteins were stacked using a 4% (v/v) stacking gel, then separated by discontinuous SDS-PAGE using 14% (v/v) gels run at 150 V (mini-gels) or 200 V for large gels according to previously described methods (Cutler *et al.*, 1997).

Mini SDS-PAGE gels, as described above, were blotted onto nitrocellulose membranes as described elsewhere (Cutler *et al.*, 1993). The membranes were incubated at room temperature overnight with a genus-specific anti-borrelian flagellin mAb H9724 (Barbour *et al.*, 1986), used at a 1:1000 (v/v) dilution to locate this band in *B. duttonii* isolates. After washing the membrane was incubated with a 1:3000 (v/v) dilution of goat anti-mouse-alkaline phosphatase conjugate (Bio-Rad), washed and developed using NBT/BCIP (*p*-nitro blue tetrazolium chloride 0.3 mg ml⁻¹ and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt 0.15 mg ml⁻¹). A polyclonal rabbit anti-*Borrelia hermsii* VSP33 (kindly provided by Dr Alan Barbour; Barbour *et al.*, 1983), was used to test its reactivity with the isolates at a 1:400 (v/v) dilution. For these immunoblots, anti-rabbit-alkaline phosphatase conjugate was used at 1:3000 (v/v) dilution.

PFGE. Cells for PFGE were adjusted to optical density 2 at 600 nm and prepared as agarose blocks, and the DNA extracted as described elsewhere (Cutler *et al.*, 1997). These blocks were then loaded into 200 ml 1.2% (w/v) agarose gels and run in 0.5% TBE (0.045 M Tris-borate and 0.001 M EDTA) using the Pharmacia Gene Navigator. Lambda ladder, 8–48 kb and a 5 kb ladder pulsed-field markers (Bio-Rad) were included on every gel. The gels were run using either 150 V with pulse times from 0.5 to 45 s for a total of 30 h, or 200 V with pulse times from 0.5 to 15 s for 17 h. Bands were visualized following ethidium bromide staining (1 µg ml⁻¹) for 1 h.

G+C Content of DNA. The G+C content of *B. duttonii* strain Ly, was determined using HPLC as described by Cutler *et al.* (1997).

DNA sequencing. The *rrs* gene was sequenced from four of the five *B. duttonii* strains. The remaining *B. duttonii* strain was omitted as it was regarded as identical to one included in sequencing (see Results and Discussion). Representative strains of *B. recurrentis* protein groups 1–4 and plasmid types 1–5 (Cutler *et al.*, 1997) were also sequenced. Following overnight cell lysis at 56 °C in buffer containing 10 mM Tris (pH 8), 60 mM EDTA (pH 8), 5% (w/v) SDS and 50 µg proteinase K ml⁻¹, DNA was phenol/chloroform-extracted, ethanol-precipitated and washed with 70% (v/v) ethanol. Initial amplification of the *rrs* gene used a 50 µl reaction volume with primers UniB and fD3 (0.5 µM); dNTPs 200 µM; Promega Buffer A [50 mM KCl; 10 mM Tris/HCl, pH 9.0; 0.1% (v/v) Triton X-100]; 2.5 mM magnesium chloride; 1 ng template DNA; and 0.125 U *Taq* polymerase (Promega). Amplification was done using a Perkin Elmer thermocycler (model 9600) with 35 cycles of denaturation at 94 °C for 1 min; annealing at 53 °C for 45 s; and extension

at 72 °C for 1.5 min. Products were purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. DNA quantification was by comparison with *Hae*III digested ϕ X174 DNA markers in 1.2% (w/v) agarose gels.

DNA was sequenced in each direction using the overlapping primers fD3-500R, 400F-900R, 800F-1050R and 1050F-rD1 described by Marti Ras *et al.* (1996). Primers were used at 3.2 pmol μ l⁻¹ with 4 μ l Bigdye enzyme, 1–3 μ l DNA (approx. 100 ng) made up to a final reaction volume of 10 μ l. With the exception of primers 1050 forward and reverse, the amplification conditions were 96 °C for 10 s; 50 °C for 5 s; and 60 °C for 4 min repeated for 25 cycles. For primers 1050 forward and reverse these conditions were modified by decreasing the annealing temperature to 46 °C and increasing the cycle number to 35.

Following cycle sequencing reaction products were ethanol-precipitated on ice for 10 min, and washed in 70% (v/v) ethanol. The pellet was dried before loading on sequencing gels. The sequencing gel electrophoresis was performed using a 373 ABI automated DNA sequencer (Applied Biotechnology Centre).

Data were edited using SeqEd (version 1.0.3) and aligned using CLUSTAL V. Consensus sequences derived from both directions were compared with sequences held in GenBank.

Electron microscopy. *B. duttonii* strain Ly grown in BSK II medium was harvested at 3000 g for 20 min, washed once in PBS (3000 g for 20 min), the pellet resuspended and fixed in 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, recentrifuged and incubated at 4 °C for 1 h. Cells were postfixed in 2% (w/v) osmium tetroxide, dehydrated in ascending grades of ethanol, and embedded in Spurr's resin. Ultrathin sections were stained with aqueous uranyl acetate and Reynold's lead citrate prior to examination with an electron microscope. To prepare for negative staining, the pellet was resuspended (one part to nine) in 1% ammonium molybdate neutralized with concentrated ammonium hydroxide. Prior to examination, 1 drop of suspension was allowed to air-dry at room temperature on carbon-coated Formvar films supported on copper grids.

RESULTS

Isolation of strains

Viable cultures were obtained from five of the 12 patients. Strains were designated La, Ly, Lw, Ma and Ku. Initially four of these were contaminated (La, Ly, Ma and Ku), but were successfully purified by passage through selective inhibitory BSK II.

SDS-PAGE

Coomassie blue-stained gels showed highly conserved profiles between the *B. duttonii* and *B. recurrentis* isolates with the exception of a major band. Complete identity was seen between strains Ly and Lw with their major protein band being 33.4 kDa (Fig. 1). Strains La and Ma also showed major protein bands within this range with molecular masses of 34.2 kDa and 33.7 kDa, respectively (Fig. 1). These protein patterns are most like those of protein group 3 of *B. recurrentis* which gave a major band of 35.5 kDa (strain A9; Fig. 1) (Cutler *et al.*, 1997). The remaining

Fig. 1. Coomassie blue-stained SDS-PAGE protein profiles of *B. duttonii* strains together with representative strains of *B. recurrentis*.

B. duttonii strain, Ku, revealed a band of 22.3 kDa, comparable with protein group 4a of *B. recurrentis* (strain A10, Fig. 1). In addition to the major band, strains La, Ma and Ku also showed an extra band in the 19–21 kDa range.

Immunoblotting with borreliar antibodies

The flagellin protein band located at 41 kDa was detected using mAb H9724 in all isolates of *B. duttonii* (data not shown). Size was determined by comparison to molecular mass markers, the B31 strain of *Borrelia burgdorferi sensu stricto*, and to *B. recurrentis*.

Immunoblotting with rabbit polyclonal serum raised against VSP33 of *B. hermsii* showed reactivity with VSP of *B. duttonii* (strain Ku) but failed to react with VLP-expressing strains. The antisera however, reacted to both VSPs (strains A15 and A16) and VLPs (strains A1 and A9) of *B. recurrentis* (Table 1).

PFGE

All strains had a linear chromosome with a size of approximately 1 Mb. All isolates of *B. duttonii*, like *B. recurrentis*, carried a large plasmid of approximately 200 kb (range 196–202 kb; Fig. 2), however, the patterns seen for plasmids of 20–90 kb showed more heterogeneity (Fig. 2). None of the *B. duttonii* isolates resembled *B. recurrentis* by PFGE. Two isolates, Ly and Lw, showed identical PFGE profiles to each other with 11 bands, but were different from the remaining strains with 9–10 bands. As found in *B. recurrentis*, a plasmid of 11 kb was present in all strains. This band was not always detectable with ethidium bromide-stained gels, only being seen in freshly prepared blocks. The sizes of plasmids belonging to each strain of *B.*

Table 1. Immunoblot reactivity of antisera raised against VSP33 of *B. hermsii* with representative strains of *B. duttonii* and *B. recurrentis*

Lw, Ly, La, Ma and Ku represent different strains of *B. duttonii*, while A1, A5, A9, A10, A12, A15 and A17 are different strains of *B. recurrentis* representative of the different sized VLP and VSP groups for this species (protein groups 1–4).

Major expressed protein	VLP	VLP	VLP	VLP	VSP	VLP Gp1	VLP Gp2	VLP Gp3	VSP Gp4a	VSP Gp4b	VSP Gp4c	VSP Gp4d
Strain	Ly	Lw	La	Ma	Ku	A1	A5	A9	A10	A12	A15*	A17
Rabbit serum	–	–	–	–	+	+	–	+	–	–	+	–

* *B. recurrentis* strain A16, which belongs to VSP protein group 4c, was also reactive with the VSP33 antiserum.

Fig. 2. PFGE patterns of *B. duttonii* compared with those of representative plasmid types of *B. recurrentis*.

duttonii are given in Table 2. The chromosome and large plasmids resolved as sharp bands and thus, like other members of the genus, probably represent linear DNA (Casjens *et al.*, 1995; Ferdows *et al.*, 1996).

rrs gene sequencing

The four strains of *B. duttonii* gave 100% identity with each other and 97.9–99.7% identity with those previously published for this species (99.7% homology with three GenBank submissions, but only 97.9% with sequence U28503). The four base substitutions in the *rrs* gene previously described for differentiation of *B. recurrentis* from *B. duttonii* were upheld by the

Table 2. Molecular sizes (kb) of plasmids in *B. duttonii* strains

Lw	Ly	La	Ma	Ku
201	199	198	202	202
76	74	76	88	90
67	67	58	45	59
54	52	45	33	38
48	47	38	28	33
39	38	28	24	29
32	31	25	20	26
29	28	11	11	23
26	25			11
11	11			

sequencing results of a further seven strains of *B. recurrentis*. In addition, two of these isolates, A11 and A16, showed a further change. The results for *B. recurrentis* strain A1 in this study revealed an additional cytosine omitted from the previously published sequence (Marti Ras *et al.*, 1996).

G + C content of *B. duttonii* DNA

The G + C content of strain Ly was 27.6 mol %. This is within the range 27–32 mol % reported for members of the genus *Borrelia* (Fukunaga *et al.*, 1995).

Electron microscopy

Electron microscopy showed characteristic borrelial cells (Fig. 3a). The cells, like *B. recurrentis*, had tapering ends (Fig. 3a) and a mean wavelength of 1.8 µm with an amplitude of 0.9 µm. Length was variable, but was approximately 13–23 µm and cells had a diameter of 0.25–0.35 µm (Fig. 3b, c). Flagella counts on transverse sections showed 10 flagellae (Fig. 3b, c). Negatively stained preparations of the central portion of spirochaetal cells showed 18–22 flagellae, presumably with the flagellae released at their point of cross-over (Fig. 3e). Insertion points were distinguished in the negatively stained preparations (Fig.

Fig. 3. Electron micrographs of *B. duttonii* strain Ly. (a) Shows the spirochaetal morphology of cells and tapering ends (negative staining); scale bar, 1 μm ; (b, c) transverse section of cell revealing the periplasmic flagellae, short arrow shows outer membrane while the long arrow marks periplasmic flagellae; scale bar, 0.1 μm ; (d) negative-stained preparation showing terminally associated flagellae with insertion points marked by arrow; scale bar, 0.05 μm ; (e) negative-stained preparation showing flagellae associated with the central portion of a cell; scale bar, 0.2 μm .

3d), however, no more than three could be resolved in any one frame.

DISCUSSION

Morphologically, similarity was seen between the *B. duttonii* strains described in this study and *B. recurrentis* reported in earlier literature (Cutler *et al.*, 1997). Electron microscopic results revealed cells with tapering ends (Fig. 3a), typical of those described for the genus *Borrelia*, however, slightly less pointed than those described for *B. burgdorferi* (Hovind Hougen, 1984; Hulínská *et al.*, 1989). The length of cells, diameter, wavelength and amplitude of strain Ly were similar to that reported earlier for *B. recurrentis* (Cutler *et al.*, 1997). Previously, we reported the disparity between our flagella counts of 8–10 flagella for the A1 strain of *B. recurrentis* with those reported in the literature describing 15–20 and 25–30 unsheathed flagella, respectively (Hovind Hougen, 1974; Hulínská *et al.*, 1989). Similar flagella counts have appeared in the literature for *B. duttonii* (Barbour & Hayes, 1986) and recently a tick-borne species of relapsing fever *Borrelia* from Spain (Anda *et al.*, 1996). The finding of 10 flagella in *B. duttonii* substantiates our earlier findings with *B. recurrentis* and is in keeping with the Lyme-associated members of the genus (Barbour & Hayes, 1986).

Sequencing studies of the *rrs* gene for *B. duttonii* confirm the identity of this spirochaete at the nucleotide level. Results of three strains used in an earlier study showed only a single-base difference from our results reported in this paper (Marti Ras *et al.*, 1996) and underline the highly conserved nature of this gene. A fourth strain of *B. duttonii* gave more divergence in its *rrs* gene sequence with 25 nucleotide differences. The four-base difference that differentiates *B. duttonii* from *B. recurrentis* was upheld by the results of this study. Furthermore, two groups among the *B. recurrentis* strains were identified based on the sequences of their *rrs* genes.

The PFGE demonstrated the presence of a chromosome of approximately one megabase present in all of the strains tested. This is similar to that described in other borrelial strains analysed (Baril *et al.*, 1989; Bergström *et al.*, 1991; Ferdows *et al.*, 1996; Kitten & Barbour, 1992). High-molecular-mass plasmids of 180 and 170 kb have previously been described in *B. hermsii* and *Borrelia turicatae* respectively (Ferdows *et al.*, 1996) and of slightly larger size (183–194 bp) in *B. recurrentis* (Cutler *et al.*, 1997). These were also demonstrated in *B. duttonii* by our analysis. The remainder plasmid profile of the *B. duttonii* isolates was very heterogeneous. Two isolates, Ly and Lw, shared the same plasmid profile, and also had major protein bands of the same molecular mass, but the remaining strains failed to show a common pattern.

The serospecificity of *Borrelia* in each clinical relapse is determined by their variable major proteins (variable

large proteins, VLPs, or variable small proteins, VSPs). The VLPs and VSPs present in *B. hermsii*, and indeed other species of spirochaetes causing relapsing fever, are of similar size to those which we have found in both *B. duttonii* and *B. recurrentis*, VLPs of 33–45 kDa or VSPs of 21–24 kDa. Sequencing studies of a portion of the VLP from the A1 strain of *B. recurrentis* have shown greatest similarity with VLP12 of *B. hermsii* (Vidal *et al.*, 1998). This would suggest that the major protein bands detected at 33 to 45 kDa by Coomassie blue staining are homologous to the VLPs of these isolates. An additional low-molecular-mass band of between 19–21 kDa was seen in strains La, Ma and Ku of *B. duttonii* and in strains A10 of *B. recurrentis*. It is possible that this may also be VSP-like, reflecting either the uncloned nature of these clinical isolates, or antigenic variation occurring in culture in a sub-population of cells.

Adaptation of *B. hermsii* to a culture-adapted variant VSP33 has been reported previously (Carter *et al.*, 1994). This VSP showed greater homology with VSPs of similar molecular mass from *B. hermsii* and OspC of *B. burgdorferi* than it did with other 35–39 kDa VLPs derived from the same isolate (Carter *et al.*, 1994). The findings of this study showed reactivity of polyclonal antisera raised against VSP33 with the VLP of two of three strains of *B. recurrentis*, VSP of one *B. duttonii* and two strains of four *B. recurrentis* demonstrating significant homology among the VLP and VSP proteins of relapsing fever borreliae.

Areas of homology exist between the different VLP and VSP genes of *B. hermsii* and indeed, have been shown to have homology with other relapsing fever species including *Borrelia turicatae* and *Borrelia crocidurae* (Cadavid *et al.*, 1997; Burman & Bergström, 1994; Hinnebusch *et al.*, 1998). Primers designed for amplification of *B. hermsii* VLPs have also amplified VLP genes from *B. turicatae*, *Borrelia parkeri*, *B. crocidurae* and from the presumptive agent of epizootic bovine abortion, *Borrelia coriaceae* (Hinnebusch *et al.*, 1998). Sequences of the VLP/VSP genes of *B. hermsii* are similar to those of other *Borreliae*, namely the OspC protein of *B. burgdorferi sensu lato* (Marconi *et al.*, 1993; Margolis *et al.*, 1994) and a VLP/VSP-like gene in *Borrelia myamotoi* (Hamase *et al.*, 1996). This could account for the cross-reactivity seen with anti-serum raised against VSP33.

ACKNOWLEDGEMENTS

We thank the staff of Mvumi Hospital for their assistance and the Special Trustees of Charing Cross Hospital for supporting the visit to Tanzania. We also thank Ian Shore for technical assistance with the electron microscopy.

REFERENCES

- Anda, P., Sánchez-Yebra, W., del Mar Vitutia, M., Pastrana, E. P., Rodríguez, I., Miller, N. S., Backenson, P. B. & Benach, J. L. (1996). A new *Borrelia* species isolated from patients with relapsing fever in Spain. *Lancet* **348**, 162–165.

- Barbour, A. G. (1984). Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med* **57**, 521–523.
- Barbour, A. G. & Hayes, S. F. (1986). Biology of *Borrelia* species. *Microbiol Rev* **50**, 381–400.
- Barbour, A. G., Barrera, O. & Judd, R. C. (1983). Structural analysis of the variable major proteins of *Borrelia hermsii*. *J Exp Med* **158**, 2127–2140.
- Barbour, A. G., Hayes, S. F., Heiland, R. A., Schrupf, M. E. & Tessier, S. L. (1986). A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. *Infect Immun* **52**, 549–554.
- Barclay, A. J. & Coulter, J. B. (1990). Tick-borne relapsing fever in central Tanzania. *Trans R Soc Trop Med Hyg* **84**, 852–856.
- Baril, C., Richaud, C., Baranton, G. & Saint-Girons, I. S. (1989). Linear chromosome of *Borrelia burgdorferi*. *Res Microbiol* **140**, 507–516.
- Bergström, S., Barbour, A. G., Garon, C. F., Hindersson, P., Saint-Girons, I. & Schwan, T. G. (1991). Genetics of *Borrelia burgdorferi*. *Scand J Infect Dis* **77**, (suppl.) 102–107.
- Burman, N. & Bergström, S. (1994). A variable antigen in the relapsing fever agent *Borrelia crocidurae* is expressed from a novel expression site. In *Antigenic variation in relapsing fever Borrelia*, thesis by N. Burman (Umeå University Medical Dissertations, New Series, no. 407). Umeå: Umeå University.
- Cadavid, D., Pennington, P. M., Kerentseva, T. A., Bergström, S. & Barbour, A. G. (1997). Immunologic and genetic analysis of VmpA of a neurotropic strain of *Borrelia turicatae*. *Infect Immun* **65**, 3352–3360.
- Carter, C. J., Bergström, S., Norris, S. J. & Barbour, A. G. (1994). A family of surface-exposed proteins of 20 kilodaltons in the genus *Borrelia*. *Infect Immun* **62**, 2792–2799.
- Casjens, S., Delange, M., Ley, H. L., III, Rosa, P. & Huang, W. M. (1995). Linear chromosomes of Lyme disease agent spirochetes: genetic diversity and conservation of gene order. *J Bacteriol* **177**, 2769–2780.
- Cutler, S. J., Wright, D. J. M. & Luckhurst, V. H. (1993). Simplified method for the interpretation of immunoblots for Lyme borreliosis. *FEMS Immunol Med Microbiol* **6**, 281–286.
- Cutler, S. J., Fekade, D., Hussein, K., Knox, K. A., Melka, A., Cann, K., Emilianus, A. R., Warrell, D. A. & Wright, D. J. M. (1994). Successful *in-vitro* cultivation of *Borrelia recurrentis*. *Lancet* **343**, 242.
- Cutler, S. J., Moss, J., Fukunaga, M., Wright, D. J. M., Fekade, D. & Warrell, D. (1997). *Borrelia recurrentis* characterization and comparison with relapsing-fever, Lyme-associated, and other *Borrelia* spp. *Int J Syst Bacteriol* **47**, 958–968.
- Dupont, H. T., La Scola, B., Williams, R. & Raoult, D. (1997). A focus of tick-borne relapsing fever in southern Zaire. *Clin Infect Dis* **25**, 139–144.
- Ferdows, M. S., Serwer, P., Griess, G. A., Norris, S. J. & Barbour, A. G. (1996). Conversion of a linear to a circular plasmid in the relapsing fever agent *Borrelia hermsii*. *J Bacteriol* **178**, 793–800.
- Fukunaga, M., Takahashi, Y., Tsuruta, Y., Matsushita, O., Ralph, D., McClelland, M. & Nakao, M. (1995). Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the Ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. *Int J Syst Bacteriol* **45**, 804–810.
- Hamase, A., Takahashi, Y., Nohgi, K. & Fukunaga, M. (1996). Homolog of variable major protein genes between *Borrelia hermsii* and *Borrelia miyamotoi*. *FEMS Microbiol Lett* **140**, 131–137.
- Hinnebusch, J., Barbour, A. G., Restrepo, B. I. & Schwan, T. G. (1998). Population structure of the relapsing fever spirochete *Borrelia hermsii* as indicated by polymorphism of two multigene families that encode immunogenic outer surface lipoproteins. *Infect Immun* **66**, 432–440.
- Hovind Hougen, K. (1974). Electron microscopy of *Borrelia merionesi* and *Borrelia recurrentis*. *Acta Pathol Microbiol Scand Sect B* **82**, 799–809.
- Hovind Hougen, K. (1984). Ultrastructure of spirochetes isolated from *Ixodes ricinus* and *Ixodes dammini*. *Yale J Biol Med* **57**, 543–548.
- Hulínská, D., Jirous, J., Valesová, M. & Herzogová, J. (1989). Ultrastructure of *Borrelia burgdorferi* in tissues of patients with Lyme disease. *J Basic Microbiol* **29**, 73–83.
- Jongen, V. H. W. M., Van Roosmalen, J., Tiems, J., Van Holten, J. & Wetsteyn, J. C. F. M. (1997). Tick-borne relapsing fever and pregnancy outcome in rural Tanzania. *Acta Obstet Gynecol Scand* **76**, 834–838.
- Kitten, T. & Barbour, A. G. (1992). The relapsing fever agent *Borrelia hermsii* has multiple copies of its chromosome and linear plasmids. *Genetics* **132**, 311–324.
- Konishi, H., Morshed, M. G., Akitomi, H. & Nakazawa, T. (1993). *In-vitro* cultivation of *Borrelia duttonii* on cultures of Sf1Ep cells. *Microbiol Immunol* **37**, 229–232.
- Marconi, R. T., Samuels, D. S., Schwan, T. G. & Garon, C. F. (1993). Identification of a protein in several *Borrelia* species which is related to OspC of the Lyme disease spirochetes. *J Clin Microbiol* **31**, 2577–2583.
- Margolis, N., Hogan, D., Cieplak, W., Schwan, T. G. & Rosa, P. A. (1994). Homology between *Borrelia burgdorferi* OspC and members of the family of *Borrelia hermsii* variable major proteins. *Gene* **143**, 105–110.
- Marti Ras, N., Lascola, B., Postic, D., Cutler, S. J., Rodhain, F., Baranton, G. & Raoult, D. (1996). Phylogenesis of relapsing fever *Borrelia*. *Int J Syst Bacteriol* **46**, 859–865.
- Melkert, P. W. (1991). Mortality in high risk patients with tick-borne relapsing fever analysed by the borrelia-index. *East Afr Med J* **68**, 875–879.
- Talbert, A., Nyange, A. & Molteni, F. (1998). Spraying tick infested houses with lambda-cyhalothrin reduces the incidence of tick-borne relapsing fever in children under five years old. *Trans R Soc Trop Med Hyg* **92**, 251–253.
- Vidal, V., Scragg, I. G., Cutler, S. J., Rockett, K. A., Fekade, D., Warrell, D. A., Wright, D. J. M. & Kwiatkowski, D. (1998). Variable major lipoprotein is a principal TNF-inducing factor of louse-borne relapsing fever. *Nat Med* **4**, 1416–1420.
- Walton, G. A. (1962). The *Ornithodoros moubata* superspecies problem in relation to human relapsing fever epidemiology. In *Aspects of Disease Transmission by Ticks* (Royal Zoological Society of London Symposium Series), pp. 83–153. London: Royal Zoological Society.