ZOONoses

Isolation of *Borrelia burgdorferi* from ticks in the Highlands of Scotland

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Borrelia burgdorferi, the causative agent of Lyme disease, was first isolated in 1982 and since then has been regularly isolated from ticks and clinical material in both Continental Europe and the USA. However, only three isolations have been reported in Britain. During the summer of 1997, 128 ticks were collected from two sites in the Highlands of Scotland and examined by the polymerase chain reaction (PCR) and culture. Eleven fresh isolates were obtained from culture and passed up to 22 times. Seven of the tick emulsions were also positive by flagellin gene PCR, and a further one was positive by PCR but negative on culture. All 11 isolate cultures were positive by the flagellin gene PCR. Further studies on four of these isolates confirmed their identity by immunofluorescence, but also detected possible differences between them and *B. burgdorferi* ACA-1 by enzyme profiles and by PCR with *OspA* gene primers. Culture of these new strains provides antigens that should improve diagnostic serological tests in Britain.

Introduction

Borrelia burgdorferi, the causative agent of Lyme disease, was first isolated from ticks in 1982 [1] and from human cases in 1983 [2, 3]. Since then, isolations from both ticks and clinical samples have been made regularly in both Europe and the USA [4–7]. However, great difficulty has been found in attempts to isolate local strains in Britain [8]. Lyme disease is endemic in several rural areas of Britain [9, 10] (New Forest, Hampshire; Thetford, Norfolk; the Scottish Highlands), and infected ticks have been found in two London parks [11]. Despite numerous attempts at isolation, only three successful isolations have been reported [12]. In one study, ticks were collected from sites of known Lyme disease in England, Scotland, Wales, Switzerland and Slovakia [8]. Only one of the 108 tick pools collected from British sites showed signs of spirochaetal growth. This strain was identified by immunofluorescence (IF) and polymerase chain reaction (PCR) as *B. burgdorferi*, but could not be maintained for more than six passages. When isolation of *B. burgdorferi* was attempted with 12 ticks collected in one area known to have 50–70% of ticks positive by PCR, all samples were positive by PCR after 2 weeks, but by 4 weeks only one sample was still PCR-positive

[8]. Motile spirochaetes were not seen by dark-ground microscopy. Isolates were obtained and maintained from Swiss and Slovak ticks with the same medium and cultural conditions [8]. This indicates that the strain of *B. burgdorferi* present in Britain may differ from those in Europe and that culture conditions may need to be adapted to achieve isolation.

Lyme disease is endemic in the Scottish Highlands [10] and ticks infected with *B. burgdorferi*, on the basis of PCR and IF positivity, have been detected in several areas [13, 14], with infection rates of between 20% (IF) and 35% (PCR) [14]. Experience at this laboratory has demonstrated an increasing awareness of Lyme disease among the public and a rise in the number of requests for laboratory confirmation of infection [15]. Serological evidence of infection in both rural and urban populations has been documented [15], but with a significantly higher rate in urban areas. Culturing *B. burgdorferi* ACA-1 in this laboratory has led to attempts to culture local strains, because such material might allow the development of more appropriate serological tests for Lyme disease.

Materials and methods

Collection and preparation of ticks

Ticks were collected by sheet dragging on three occasions during 1997. In May, a collection was made

from two sites east of Inverness at Urchany, Cawdor, a wooded area, and at Clunas Reservoir, an open grassy area. In July and September, ticks were collected from the same areas at Urchany and also from a wooded area of Culloden. Ticks were transported in plastic 28-ml screw-capped containers and prepared for examination on the same day the collections were made. Ticks were treated individually unless they were very small, when two were treated together. After being washed in methanol for 2 min and rinsed in normal saline (NS), the tick abdomen was shredded with sterile needles in NS. The larger particles were allowed to settle and half the emulsion (*c.* 200 μ l) was used for culture. The remainder (*c.* 200 μ l) was stored at -20°C until tested by PCR.

Culture of ticks and isolates

Tick homogenates were inoculated into 7 ml of Barbour-Stoenner-Kelly (BSK)-H medium (Sigma) with inactivated rabbit serum (Sigma) 6.6%, gelatin (Sigma) 1.4%, septrin (DBL; trimethoprim 16 mg/L, sulphamethoxazole 80 mg/L) and neomycin (Wellcome Diagnostics, Dartford, Kent) 100 mg/L in glass 7 ml screw-capped bottles. These were sealed with parafilm (Sigma) and incubated at 33°C . These culture conditions, *i.e.*, filling the bottles to the top and sealing them with parafilm, had been found by chance to give optimal growth conditions for the reference strain, ACA-1. The reduced oxygen concentration thus produced resembled micro-aerophilic conditions most closely. Cultures were examined at weekly intervals and any bottle showing signs of growth by changes of medium colour or turbidity was examined by dark-ground microscopy. Negative cultures were maintained for at least 8 weeks before being discarded. Positive cultures were maintained in the same medium and passaged at regular intervals. At several passages, portions of the cultures were stored both in liquid nitrogen and at -20°C after being centrifuged at 3000 *g* for 30 min, washed twice in phosphate-buffered saline (PBS) and finally resuspended in distilled water.

Investigation of isolates

Three drops of cultures of each of the four isolates that were investigated further were inoculated into 7 ml of BSK-H medium in glass bottles and incubated under micro-aerophilic (sealed with parafilm), aerobic and anaerobic conditions and in an atmosphere of air with CO_2 5% at 31°C and 36°C . Cultures were examined after 5 days. Also, 5 ml of a 4–5-day culture of each isolate were inoculated into an aerobic and anaerobic blood culture bottle and incubated in the VITAL blood culture system (bioMérieux, UK) for 5 days. Each of these four isolates was also cultured at 33°C for 5–6 days in 7-ml bottles containing 7, 6, 5, 4, 3 and 2 ml of BSK-H medium, sealed with parafilm.

Gram's and Giemsa's staining

After culture for 4–5 days, the four isolates were centrifuged (3000 *g* for 30 min), washed twice in NS, resuspended in 1 ml of NS and smears were made and air dried. For Gram's stain, the slides were heat-fixed and stained in a Midas staining machine. For Giemsa's stain, the slides were fixed in methanol for 5 min, stained in a 1 in 10 dilution of Giemsa's stain (Sigma) for 30 min, washed in distilled water and air dried. Slides were examined with a Leitz Ortholux microscope (Leitz UK) at a magnification of 100.

Flagellin gene PCR

A nested PCR assay was developed to amplify a sequence of the highly conserved flagellin-encoding gene of *B. burgdorferi* with four primers, as described previously [16]. The outer primer set (F1-5'-ATT AAC GCT GCT AAT CTT AGT-3' and F3-5'-GTA CTA TTC TTT ATA GAT TC-3') amplifies a product of 791 bp and the inner primer set (F6 -5'-TTC AGG GTC TCA AGC GTC TTG GAC T-3' and F8-5'-GCA TTT TCA ATT TTA GCA AGT GAT G-3') amplifies a 275-bp nested product. The first stage PCR was performed with 20 μ l of sample in a reaction volume of 50 μ l containing final concentrations of 10 mM Tris-HCl, (pH 8.5), 50 mM KCl_2 , 3.5 mM MgCl_2 (Applied Biotechnologies, Cambridge), 0.2 mM dNTP (Pharmacia Biosystems, Milton Keynes), 0.5 μM of each primer (Severn Biotech, Kidderminster) and 0.5 U taq polymerase (Applied Biotechnologies, Cambridge). The thermal cycling conditions were 35 cycles of 1 min at 94°C , 2 min at 41°C and 3 min at 66°C followed by a further extension period of 5 min at 72°C . For the nested PCR, 20 μ l of a 1 in 5 dilution of the first stage product was amplified in a 50- μ l reaction mix with a reduced concentration of MgCl_2 (2.5 mM). The thermal cycling conditions were 35 cycles of 1 min at 94°C , 1 min at 50°C and 1 min at 72°C with a final extension of 5 min at 72°C . The PCR products were electrophoresed in agarose 2.5% gels containing ethidium bromide 1 mg/L at 133 V for 20 min [17].

OspA gene PCR

A nested PCR was used to amplify part of a variable gene encoding an outer surface lipoprotein (OspA) of *B. burgdorferi*, with primers described previously [18]. The outer set (-5'-AAA AAA TAT TTA TTG GGA ATA GG-3' and -5'-GTT TTT TTG CTG TTT ACA CTA ATT GTT AA- 3'-) amplifies a 702-bp product and the inner set (-5'-GGA GTA CTT GAA GGC G-3'- and -5'-GCT TAA AGT AAC AGT TCC-3'-) amplifies a 345-bp nested product. The reaction mix for both stages was similar to that used for the flagellin gene nested PCR (with 2.5 mM MgCl_2). The thermal cycling conditions for the first stage were initial denaturation at 96°C for 2 min followed by 20 cycles of 30 s at 94°C , 30 s at 37°C , 2 min at 72°C with a final extension period of 5 min at 72°C . For the nested

PCR, a touchdown PCR was performed on the first stage product (20 µl; 1 in 100): initial denaturation for 2 min at 96°C; 10 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C; 10 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C; 10 cycles of 30 s at 94°C, 30 s at 50°C, 1 min at 72°C; five cycles of 30 s at 94°C, 30 s at 45°C and 1 min at 72°C with a final extension of 5 min at 72°C. Detection of the nested products was as described for the flagellin gene nested PCR.

Biochemical reactivity

Surface pre-formed enzyme activity of four isolates (each grown in four 7-ml bottles containing 7 ml of BSK-H medium for 6 days) and *B. burgdorferi* ACA-1 (grown in two 7-ml bottles containing 7 ml of BSK-H medium for 14 days), was measured with AP1-ZYM strips (bioMérieux), inoculated, incubated and read according to the manufacturer's instructions. Strains were centrifuged (3000 g for 30 min) and washed twice in hypotonic saline (bioMérieux) before testing.

Immunofluorescence

The four isolates grown for 4–5 days were centrifuged (2500 g for 20 min), washed twice in PBS, resuspended in 1 ml of PBS and air dried on multispot slides (Hendley UK; 40 µl/well). Slides were fixed in acetone for 5 min. Doubling dilutions (1 in 10–1 in 80) of high positive and negative anti-*B. burgdorferi* antisera (40 µl) were added and incubated at 37°C for 20 min. After the slides were washed in PBS and air dried, anti-human IgG antiserum conjugated to fluorescein isothiocyanate (SAPU Scotland; 40 µl; 1 in 50) with Evan's Blue was added and incubated at 37°C for 30 min. After being washed again in PBS, slides were mounted and examined with an Olympus microscope at a magnification of 40.

Results

Collection of ticks and isolation of *B. burgdorferi*

The number of ticks collected on each occasion is shown in Table 1. No ticks were found on the open grassy land at Clunas Reservoir. On each occasion the

weather was sunny and warm. Collection of ticks was found to be easiest in May and more were obtained from long grass in the shelter of trees; fewer were found on heather or on more open grassy areas. All the collections were done in areas close to footpaths regularly used by walkers. In the laboratory, the ticks were all prepared and inoculated into medium in areas where the routine strain of *B. burgdorferi* (ACA-1) was not handled. Only one culture from May and one from July were discarded because of contamination. Overall, 11 (8.5%) of 128 ticks yielded an isolate (Table 1). All isolates showed highly motile borrelia-like organisms under dark-ground microscopy. The July isolate (C7) was first detected after incubation for 4 weeks. From the September collection one isolate (E8) was detected after incubation for 1 week, seven (E9, E10, F2, G4, G5, H1, J1) after 2 weeks, one (F8) after 3 weeks and one (E5) after 5 weeks. Isolate J1 was the only one from the Culloden site and was the only one from a tick showing signs of a recent blood meal. The other 10 isolates, all from the Urchany site, were from ticks collected within a half mile area. Each letter (A–J) represents a number of ticks collected by one drag of the sheet (Table 1). All except one isolate (E5) have now completed at least nine passages with the earliest isolate (C7) having undergone 22 passages.

Four isolates were investigated at early passage (<10) representing different collection sites (J1, Culloden; C7, E8, G5, Urchany) and different times of collection (C7, July; E8, G5, J1, September). All four stained well with Giemsa's and Gram's stain (gram-negative), giving the typical appearance of borreliae (Fig. 1). The culture characteristics were examined by incubation in several different atmospheres and temperatures. Growth was obtained in BSK-H in micro-aerophilic, aerobic, anaerobic and CO₂ atmospheres at both 31°C and 36°C. The best growth was found with micro-aerophilic incubation at 33°C; the four isolates required passage at 4-day intervals after the initial 2–3 passages. However, no growth was detected in aerobic or anaerobic blood culture bottles in the VITAL blood culture system. No growth was found on solid media (blood or chocolate blood agar) at either temperature in any atmosphere. When the amount of BSK-H in the glass 7-ml bottles was reduced, growth was found at all volumes tested, but was better in volumes >6 ml.

Table 1. Collection of ticks and isolation of *B. burgdorferi* strains in 1997

Date	Location	Number of ticks collected	Number of <i>B. burgdorferi</i> isolates (%)
May	Urchany (A)	39	0 (0)
	Clunas (B)	0	0 (0)
July	Urchany (C)	44	1 (2.3)
	Culloden (D)	10	0 (0)
September	Urchany (E–H)	34	9 (26.5)
	Culloden (J)	1	1 (100)
Total		128	11 (8.5)

Flagellin gene PCR

All the tick emulsions from the three collections were examined by flagellin gene PCR [16] (Fig. 2). This PCR showed a sensitivity of one organism when a culture of *B. burgdorferi* ACA-1 was used (Fig. 1) and at least 10 organisms when a tick emulsion seeded with *B. burgdorferi* ACA-1 was used. All emulsions from May were negative, and C7 was the only positive one from the July collection. The results of the PCR and

Fig. 1. Tick isolate G5, Giemsa's stain. Bar = 2 μ m.

isolation from the September emulsions did not show full agreement. One emulsion (E4) was PCR-positive but did not grow; four emulsions (E5, E9, F8, G4) were PCR-negative but yielded growth of isolates and, with six emulsions, both PCR and culture were positive. However, PCR was positive with all isolates at early passage, i.e., the first or second passage.

OspA gene PCR

All four isolates (C7, E8, G5, J1) and the *B. burgdorferi* ACA-1 strain gave positive results in the primary PCR run (Fig. 2). However, two of the isolates (C7 and J1) failed to produce bands on the nested run (Fig. 2) on repeated testing. *B. burgdorferi* ACA-1 was detected to a level of 10 organisms in the nested run. The remaining isolates were not tested.

Biochemical reactions

On AP1-ZYM strips, reactions were found for eight enzymes with some variation in results between the four strains tested and *B. burgdorferi* ACA-1 and among the four strains themselves (Table 2). The remaining 12 enzyme wells on the AP1-ZYM strips were uniformly negative on three attempts. The four isolates were tested at the same culture density, but it was not possible to achieve as strong a density for the ACA-1 strain.

Immunofluorescence

All four isolates tested (C7, E8, G5, J1) gave positive results on immunofluorescence with a commercial human positive control serum. No fluorescence was seen with the commercial human negative control serum.

Discussion

Isolation of *B. burgdorferi* from 8.5% of local ticks during 1997 was somewhat surprising in view of the previous difficulties in isolation of this organism in Britain [8, 13, 14]. There is no obvious reason for this discrepancy. Previous attempts have used BSK II medium [8, 14], whereas BSK-H medium was used in the present study and great care was taken to create micro-aerophilic conditions by filling the bottles to the top, leaving only a small air space and sealing the cap with parafilm. Although low passage isolates grew in all atmospheres, the best growth was obtained in micro-aerophilic conditions and stricter control may be necessary for initial isolation. Some isolates appeared more quickly than others (range 1–6 weeks), indicating that there may be a difference in the level of infection in different ticks and confirming previous studies [8, 13] where it was suggested that low levels of infection may contribute to the difficulties in isolation.

Fig. 2. Demonstration of PCR products from isolates of *B. burgdorferi* with flagellin and OspA gene primers. Top half: flagellin gene PCR nested products. Lanes 1 and 14, 123-bp ladder (Sigma); 2, *B. burgdorferi* ACA-1 (one organism); 3, distilled water as negative control; 4–9, tick emulsions (E10, F2, H1, G4, E4, E9); 10–13, tick isolates (C7, E8, G5, J1); A, 246 bp. Bottom half: OspA gene primary (lanes 2–7) and nested (lanes 8–13) PCR products. Lanes 1 and 14, 123-bp ladder; 2 and 8, *B. burgdorferi* ACA-1 (10 organisms); 3 and 9, distilled water as negative control; 4–7, 10–13, tick isolates (C7, E8, G5, J1); B, 738 bp; C, 369 bp.

Table 2. Results of pre-formed enzyme reactions with four fresh isolates and *B. burgdorferi* ACA-1

Enzyme reaction	C7	E8	G5	J1	<i>B. burgdorferi</i> ACA-1
Alkaline phosphatase	+++	+	+	++	–
Esterase	+	+	+	+	+
Esterase lipase	+	+	++	+	+
Leucine arylamidase	+++	+++	+++	++++	++
Valine arylamidase	+	–	–	+	–
Phosphatase acid	++	++	+	+++	+
Naphthol-AS-B1-phosphohydrolase	+	+	+	+	+
α -Glucosidase	+	+	++	++	+

On this occasion, although initial isolation took several weeks in some ticks, after two or three initial passages, passage time for all isolates settled to 4–5 days. Continuous passage has been established for 9–22 passages. This is different from the passage time of the reference strain (ACA-1), which is 10–14 days, making it very unlikely that the ticks were contaminated with the ACA-1 strain. Great care had been taken

to avoid the latter during initial isolation stages from ticks.

An interesting finding was the large variation in isolation rate at different times of the year. The 26.5% rate in September agrees with previous reports of 25–35% tick infection rate as determined by PCR [14], but contrasts with the rates of 0% in May and

2.3% in July for the same area. This suggests that there may be a seasonal variation in the level of tick infection, which could have implications for human disease. The results of the present study also show that levels of infection in ticks vary between different sites in relatively close geographical areas. Further studies are required to define the characteristics of heavily infected tick areas as the number of ticks collected from some locations was very small.

All the isolates were identified as *B. burgdorferi* by flagellin gene PCR [16]. One culture-negative tick emulsion was PCR-positive, either because spirochaetes were non-viable or because of a low level of infection. Inhibition of the PCR reaction was the probable cause of two of the culture-positive, PCR-negative samples, because the tick emulsions spiked with *B. burgdorferi* ACA-1 gave only weak reactions. Another PCR-negative, culture-positive emulsion (E9) may have been due to cross-contamination with two other positive emulsions (E8, E10) which were prepared by the same person at the same time. There was no obvious explanation for the remaining PCR-negative emulsion. Further evidence for the identity of the isolates as *B. burgdorferi* was demonstrated by immunofluorescence, a method used in the initial identification of *B. burgdorferi* [2]. All four isolates tested reacted with positive control serum, but not with negative control serum. Staining by Gram's and Giemsa's techniques ruled out the possibility that the isolates were either treponemes or leptospira [19]. None of the cultures was contaminated by other bacteria, as incubation on solid media in all atmospheres was negative, as were the blood cultures. A previous British isolate was contaminated with *Xanthomonas maltophilia* [12].

Comparison of the profiles of pre-formed enzymes indicated a possible difference between *B. burgdorferi* ACA-1 and the four isolates tested. The ACA-1 strain did not possess alkaline phosphatase which was present in all four isolates or valine arylamidase which was present in some isolates. These results need to be interpreted cautiously, as it was not possible to obtain the same culture density of ACA-1 as with the four test isolates. All possessed high levels of leucine arylamidase, which agrees with one previous study [20]. Differences among isolates were also detected by PCR with OspA primers. Although specific bands were found at the primary run stage, only two isolates (E8, G5) gave bands at the nested stage. Differences in the OspA gene have been used to differentiate between the three European genospecies, *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii* [21]. *B. burgdorferi* ACA-1 is known to be *B. afzelii*. The fact that two of the isolates did not produce nested products may indicate that these are different genospecies. These two were from ticks collected either at a different time (C7, July 1997) or a different site (J1

Culloden) from the other two (E8, G5; Urchany September 1997).

The ability to grow so many local isolates of *B. burgdorferi* this summer has produced considerable quantities of antigen for further studies. It is possible that our strains may differ from those of the rest of Britain and that is the reason for their ease of isolation. It has been suggested that the immune response to infection with *B. burgdorferi* in the Highlands is different from that seen in Continental Europe or the USA [16]. Use of these locally isolated strains in diagnostic tests such as enzyme-linked immunosorbent assay and Western blotting may greatly improve serological diagnosis. Further characterisation of these strains will help to improve the understanding of *B. burgdorferi* infection in this country.

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