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Lyme disease: a search for a causative agent in ticks in south-eastern Australia

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SUMMARY

Attempts were made to identify the causative organism of Lyme disease in Australia from possible tick vectors.

Ticks were collected in coastal areas of New South Wales, Australia, from localities associated with putative human infections. The ticks were dissected; a portion of the gut contents was examined for spirochaetes by microscopy, the remaining portion inoculated into culture media. The detection of spirochaetes in culture was performed using microscopy, and immunochemical and molecular (PCR) techniques. Additionally, whole ticks were tested with PCR for spirochaetes.

From 1990 to 1992, approximately 12000 ticks were processed for spirochaetes. No evidence of *Borrelia burgdorferi* or any other spirochaete was recovered from or detected in likely tick vectors. Some spirochaete-like objects detected in the cultures were shown to be artifacts, probably aggregates of bacterial flagellae.

There is no definitive evidence for the existence in Australia of *B. burgdorferi* the causative agent of true Lyme disease, or for any other tick-borne spirochaete that may be responsible for a local syndrome being reported as Lyme disease.

INTRODUCTION

Lyme disease is a tick-borne zoonosis caused by the spirochaete bacterium *Borrelia burgdorferi*. Symptoms in humans may include a characteristic rash

known as erythema migrans, neurological, cardiac and skin sequelae, and an arthropathy which may develop months to years after infection [1].

In the northern hemisphere, small placental mammals act as reservoirs for the spirochaete and maintenance is achieved via their specific *Ixodes* ticks. Transmission to humans will only occur from ticks that feed first on infected reservoir hosts and then on humans. The only species of ticks shown to be competent vectors of *B. burgdorferi* to humans belong to the *Ixodes persulcatus/ricinus* complex. This includes *I. dammini* (recently relegated to synonymy with *I. scapularis* [2]), *I. scapularis* and *I. pacificus* in the United States, *I. ricinus* in western Europe, and *I. persulcatus* in eastern Europe and Asia [3].

Since first recognized as a distinct clinical entity in 1975, Lyme disease has become the most frequently reported tick-borne infection transmitted to humans, and accounted for 81% of all reported cases of arthropod-transmitted diseases in the USA during 1986–90. Since 1982 there have been over 30000 cases reported from the United States, with 9465 Lyme disease cases in 1991 [4]. Lyme disease has been diagnosed from Canada, Europe, Russia, China and Japan; it has also been reported from South-East Asia, South America, Africa [5] and Australia, although there remains doubt as to the existence of true Lyme disease in the southern hemisphere in general, and in Australia in particular.

Lyme disease in Australia

The first Australian cases of a syndrome consistent with Lyme disease were reported from the Hunter Valley region of New South Wales (NSW) in 1982 [6]. Further clinical cases were reported in 1986 from the south [7] and central coast of NSW [8]. In Queensland, in 1986–9, the State Health Laboratories tested 1247 patients for antibody response to *B. burgdorferi*, using an indirect fluorescent antibody test (IFAT), and reported 186 with positive ($\geqslant 64$) titres [9].

In 1988 a serological diagnostic service for Lyme disease was started at Westmead Hospital. Enzyme linked immunosorbent assay (ELISA) for IgG, and IFAT for IgG and IgM, were used with antigens derived from a North American strain (B31) of *B. burgdorferi*. From 1988 to October 1992, specimens were tested from 2446 patients referred with suspected clinical Lyme disease; only 66 (2·7%) showed positive results by both methods indicating possible Lyme disease [10]. These figures included seven patients infected outside Australia. More recent data from one of us (DD) indicate that, to August 1993, 75 (2·2%) of 3458 local patients tested were positive for IgG by both methods. Less than 1% of the patients referred with suspected Lyme disease conformed with the United States national surveillance case definition for Lyme disease [11]. It is well recognized that there are problems of specificity and sensitivity associated with serological testing for Lyme disease [12], and this must be particularly true for Australia where no local causative spirochaete has been isolated for use as a reference antigen.

With respect to possible vectors for local Lyme disease transmission, no ticks of the *I. persulcatus/ricinus* complex are known to occur in Australia. However, indigenous tick species such as *I. holocyclus* parasitise both native vertebrate hosts and humans, and are thus potential vectors for transmission of *B. burgdorferi* or a 'local' spirochaete causing a similar syndrome. Between January 1990 and December 1992, a field and laboratory programme was undertaken to identify vectors of the putative disease through detection and isolation of the causative

agent, *B. burgdorferi* or similar spirochaete. This paper reports on the results of that investigation.

MATERIALS AND METHODS

Collection of ticks

The study area comprised the coastal strip of NSW, from the Queensland border in the north to the Victorian border in the south. Tick collections were made in all seasons in each of the 3 years, particularly in areas associated with putative cases of Lyme disease, but also in other regions with habitats populated by ticks. Ticks were collected in natural habitats by dragging a one metre square of flannel cloth over vegetation, and were also collected by removal from native and domestic animals.

Detection of spirochaetes

This was undertaken by processing ticks for microscopy, culture of spirochaetes into media, and by direct testing of ticks for spirochaete DNA using the polymerase chain reaction (PCR).

For spirochaete culture, the ticks were stored live until processed; they were identified, surface sterilised by dipping in 70% ethanol, washed twice in sterile distilled water, and the midgut tissues dissected out aseptically. A portion of the midgut contents was examined by dark field microscopy for the presence of spirochaetes and the rest inoculated into *B. burgdorferi* culture media (BSKII), incubated at 33 °C for up to 3 months and examined weekly using dark field microscopy. Various factors (nutritional media components, and chemical and physical culture conditions) known to influence growth of spirochaete and other bacteria were manipulated in an effort to produce enhanced growth of spirochaete-like organisms. A variety of solid, semi-solid and liquid media were used under different cultural conditions (e.g. anaerobic, aerobic, microaerobic conditions; variable pH) with the addition of growth enhancers (e.g. vitamin K, haemin, cysteine, Skirrow's supplement, Isovitalex®). Purified isolates of spirochaete-like objects (SLOs) were sought by selective filtration from the cultures.

For direct testing of ticks for borrelia by PCR, a subsample of the total collections was selected to represent the different tick species and geographic areas within the study region, and gut contents and haemolymph were extracted for testing.

Molecular identification of culture isolates

Molecular characterization of SLOs was attempted with a variety of immunochemical techniques, including reactivity with monoclonal and polyclonal antibodies against antigens of *B. burgdorferi*, and PCR. Separate isolations of purified SLOs were tested against four polyclonal and three monoclonal antibodies by IFAT. The four polyclonal sera were rabbit anti-*B. burgdorferi*-FITC serum, rabbit polyclonal anti-P39 serum, rabbit polyclonal anti-P22-A serum, and rabbit anti-*B. burgdorferi* whole serum. The monoclonal antibodies were directed against the outer surface proteins OspA and OspB, and flagellin proteins. PCR was used to detect borrelia-specific DNA in purified and non-purified isolations of the SLOs. The primary targets for PCR detection were the OspA gene, the flagellin gene (Fla) and the 16S ribosomal RNA gene [13]. Strain 297 (a North American isolate) of

B. burgdorferi was used as a positive control, and DNA from *Leptospira interrogans* serovar *copenhageni* as a negative control. No-DNA controls were also included in each experiment in order to detect non-specific amplification products. OspA-specific primers (ospA2 and ospA4), fla-gene primers (fla1 and fla3) and primers specific for borrelia 16S rDNA (DD02 and DD03) [13] were used to amplify target sequences and the PCR products were resolved by high resolution agarose gel electrophoresis with *Hpa* II digests of ΦX174 as markers. In order to confirm the specificity of the PCR products obtained, the amplified DNA fragments were Southern blotted and hybridized to gene specific oligonucleotide probes as described [13, 14]. The ospA3, fla2 and DD04 probes were used to detect the OspA, Fla and 16S rDNA gene PCR products respectively [13]. Eubacterial specific primers (pA and pE), that are able to amplify rRNA genes from a wide variety of bacteria [15], were also used to amplify a 950 base pair fragment from 16S rDNA by PCR. As a control, genomic DNA from *Bacillus subtilis* was included. Restriction enzyme digestion was used as a confirmatory test in order to identify the PCR products produced. The PCR products were digested with *Eco*R I and the digestion products were separated by high resolution agarose gel electrophoresis.

Molecular analysis of ticks for spirochaetes

The target DNA for PCR amplification with the direct analysis on ticks was the borrelia-specific 41 kDa flagellin gene [13].

Electron microscopy of culture isolates

The SLOs were prepared for transmission electron microscopy by fixing in a 5% solution of formaldehyde, then a 1 ml sample was centrifuged for 30 min at 1500 g. No pellet was formed and higher centrifuge speeds were found to disrupt the SLOs, consequently the top 0·9 ml of the centrifuged sample was removed and discarded. One drop of the remaining 0·1 ml was placed on a palladium-carbon coated grid and allowed to dry slowly at 30 °C in a humidified atmosphere. The grid was coated with a 4% aqueous solution of uranyl acetate for 15 min, washed once with distilled water and observed for SLOs using a Phillips 201 transmission electron microscope.

RESULTS

Tick collections

Between January 1990 and December 1992, > 20000 ticks were collected. Approximately 11000, including all stages of four species, *Ixodes holocyclus*, *I. tasmani*, *Haemaphysalis bancrofti* and *H. longicornis* (Table 1) were dissected for spirochaete isolation. Each year the collections were dominated by *I. holocyclus*, with the larval stages being most abundant during the late-summer and autumn months, the nymphal stages most abundant from late-autumn to mid-spring, and the adults most common from mid-spring to early-summer. The other species showed similar stadial activity.

Spirochaete detection and isolation

No spirochaetes were detected by dark field microscopy of the gut contents of the unfed ticks collected from natural habitats for dissection. With the additional 1038 ticks (Table 2) tested using PCR, no amplification products which would

Table 1. *Total number of ticks dissected for spirochaete isolation, Jan. 1990–Dec. 1992*

Species	Larvae	Nymphs	Males	Females	Total
Amblyomma moreliae	0	4	7	3	14
A. triguttatum	0	0	0	2	2
Aponomma concolor	0	6	2	1	9
Haemaphysalis bancrofti	554	265	67	71	957
H. longicornis	232	914	18	748	1912
Ixodes cornuatus	0	0	1	1	2
I. fecialis	0	0	0	3	3
I. holocyclus	4862	1115	411	964	7352
I. tasmani	41	29	4	31	105
I. trichosuri	13	46	0	1	60
Ixodes species	529	0	0	0	529
Rhipicephalus sanguineus	0	0	18	7	25
Total	6231	2379	528	1832	10970

Table 2. *Total number of ticks examined by PCR for the presence of* borrelia, *Jan. 1990–Dec. 1992*

Species	Larvae	Nymphs	Males	Females	Total
Amblyomma moreliae	0	0	3	1	4
Aponomma concolor	0	0	0	1	1
Haemaphysalis bancrofti	2	119	6	8	135
H. longicornis	0	159	0	137	296
Ixodes holocyclus	2	279	53	236	570
I. tasmani	0	6	0	13	19
I. trichosuri	0	13	0	0	13
Total	4	576	62	396	1038

suggest the presence of *borrelia* were detected. In contrast, controls consisting of naturally infected *I. scapularis* (previously *I. dammini*) from the known Lyme disease endemic region of Westchester Co., New York, USA, yielded reproducible positive results under the test conditions.

Spirochaete-like objects (SLOs), at concentrations of 10^3–10^4/ml, were revealed by dark field microscopy in 92 cultures of gut contents of bloodfed ticks collected from various localities from southern Queensland through New South Wales to northern Victoria. The tick species yielding these SLOs were *I. holocyclus*, *H. bancrofti*, *H. longicornis* and *Amblyomma moreliae*. All SLOs were found in association with bacterial contaminants which came presumably from the tick's bloodmeal. Purified SLOs were obtained with 0·45 μm filters, but it was not possible to subculture them in the absence of bacterial contaminants nor improve their growth by manipulating culture conditions.

Molecular identification of culture products

All the monoclonal antibodies yielded negative results when tested against 18 SLOs, but were positive with the North American reference strain (B31) of *B. burgdorferi*. While a few positive results were obtained by IFAT using polyclonal antibodies, the results were both variable and inconsistent for the 18 SLOs tested. All the polyclonals tested positive with the control.

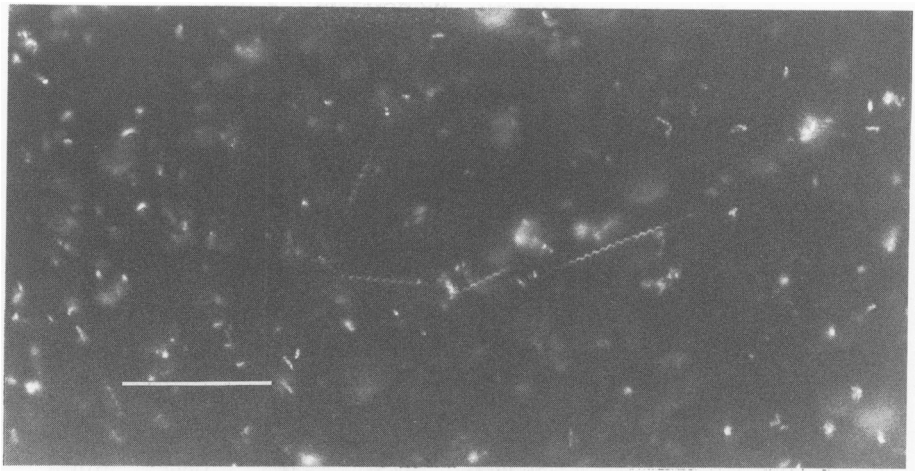

Fig. 1. Dark field micrograph of spirochaete-like objects in culture media with bacillus contaminants. Bar = 50 μm.

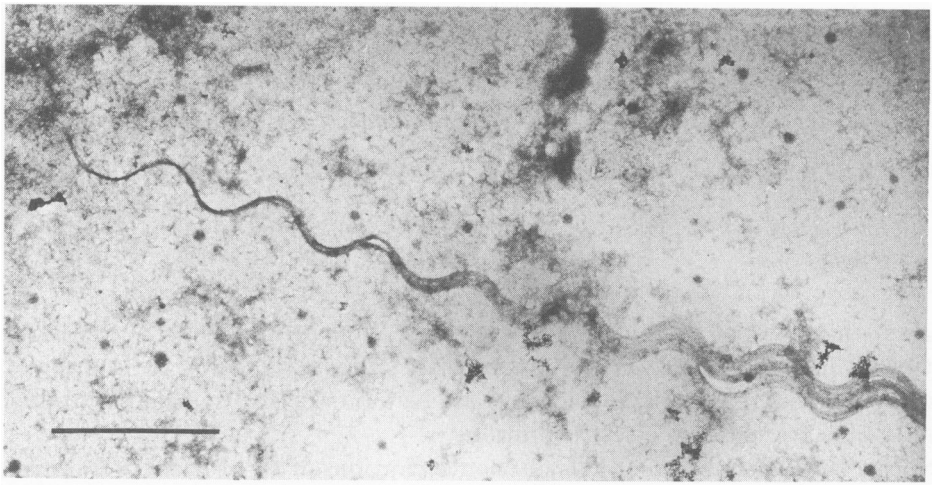

Fig. 2. Transmission electron micrograph of detail of spirochaete-like object showing fibre-like composition. Bar = 5 μm.

DNA from the SLOs, when amplified by PCR using the primer pairs osp2/osp4, fla1/fla3 and DD02/DD03 yielded no products that could be reproducibly attributed to *B. burgdorferi*. PCR using the eubacterial specific primers pA and pE successfully amplified a 950 bp fragment in 92 of 92 SLO cultures, however the fragments amplified produced characteristic enzyme digestion products of a *Bacillus* sp. and not a *Borrelia* sp.

Description of culture products

The SLOs were examined by light and electron microscopy. Under dark field microscopy the SLOs appeared straight, rigid and uniformly coiled (Fig. 1), varied in length (10–300 μm), and had 2–40 complete coils; all appeared to be non-motile.

Electron micrographs showed that these SLOs had no distinct cellular structure but were composed of fibre-like subunits (Fig. 2), and were not spirochaetes.

DISCUSSION

Earlier reports from this investigation [16, 17] and the final results reported herein fail to provide definite evidence for a spirochaete causing a Lyme disease-like syndrome in Australia.

It is possible that the monoclonal antibodies and PCR primers used in this study may not have been appropriate to identify indigenous Australian spirochaetes, however the dissected tick guts and the cultures from the gut contents were also negative for spirochaetes by microscopy. Although SLOs were detected in cultures (from bloodfed ticks only), dark field microscopy showed they were not typical of borrelia in shape or form. The electron micrographs of the SLOs were comparable with photographs of similar SLOs recovered from cultures of ticks from Missouri, USA, and comprised aggregations of bacterial flagella thought to originate from contaminating bacteria in the cultures [18]. Similar objects found in cultures from dissected bloodfed ticks taken from animals on the mid-north coast of NSW are claimed to be related to *B. burgdorferi* and to be the probable cause of Lyme disease in Australia, based on serological characterization and SDS–PAGE analysis [19, 20]; however, their descriptions suggest that they are identical with our SLOs and therefore may be similar aggregations of bacterial flagella.

There are some major differences between the northern hemisphere endemic areas and that of Australia, with respect to the natural history of Lyme disease. In the northern hemisphere all principal tick vectors to humans are members of the *Ixodes persulcatus/ricinus* complex; no ticks of this species complex occur in Australia. Of the ticks in Australia that might be associated with putative human infections, *I. holocyclus* is the logical candidate vector; this species has a wide host range and is the most common tick species biting humans along the east coast of Australia. *I. holocyclus* has been shown to be unable to maintain or transmit a North American strain of *B. burgdorferi* [21], but there is no information on the ability of *I. holocyclus* to transmit European strains and its association with any Australian spirochaetes remains unresolved.

Any consideration of reservoir hosts for *B. burgdorferi*, or similar spirochaete, in Australia should take into account that the native vertebrate fauna (primarily marsupial mammals) is dissimilar to that (placental mammals) of the Lyme disease endemic regions in the northern hemisphere. None of the mammal species identified as reservoir hosts for *B. burgdorferi* transmission in Europe, northern Asia and North America is present in Australia. There are reports of spirochaetes in Australian native animals [22, 23], and it is possible that some marsupials could act as a reservoir host for an indigenous spirochaete that might occasionally infect humans through a tick vector and product a clinical syndrome similar to Lyme disease, although none was detected in the approximately 12000 ticks processed during the present investigation.

With regard to human contact with possible tick vectors of a spirochaete, the field collections produced some interesting results. The developmental cycle for *I.*

holocyclus is generally completed within one year, with one or more stadia being active during virtually all months. The major period of activity for nymphs in south-eastern Australia occurred in the cooler months, and directly followed that of the larvae which were most active in summer and autumn. Nymphs, thought to be the most important stage for transmission of *B. burgdorferi* to humans in the northern hemisphere because they are more likely to be infected than larvae, are active predominantly in summer periods when humans are more likely to visit sylvan areas. In addition, they are much smaller than adult ticks and are thus less likely to be noticed and removed soon after attachment. In Australia, the period of greatest likelihood for contact between human or vertebrate host and nymphs (and therefore higher risk of possible infection) would appear to be during the cool seasons when human activity in sylvan areas is less. Also, there is little evidence that tick populations are increasing in, or near, residential areas of Australia. Rather, the opposite appears the case as native vertebrate hosts are driven from residential areas by urbanization and the introduction of non-indigenous feral animals and domestic pets, particularly cats and dogs.

The diagnosis of Lyme disease outside known endemic areas cannot be based solely on serology since false positives may be obtained due to cross reactions with other bacteria, notably various spirochaetes, and also in unrelated syndromes such as autoimmune diseases [24]. In addition, a lack of agreement as to what constitutes a positive serological result [25] has led to over-diagnosis of Lyme disease. Laboratory confirmation by culture of the causative organism from a local patient without travel history to an endemic area in the northern hemisphere has not been achieved in Australia.

Munro and Dickeson [26] compared the seroprevalance rates for *B. burgdorferi* infection in healthy blood donors from high tick exposure groups (rural residents) and low tick exposure groups (urban residents) in NSW; they found an overall seropositivity rate of 2·2% with no significant difference between rural and urban groups. This contrasts with similar studies in known Lyme disease endemic areas where rural human populations have typically considerably higher seropositivity rates than those in urban areas [27–30].

Barbour and Fish [31] report that in areas without Lyme disease, residents are between 1 and 2% seropositive, and they infer that this represents cross-reacting antibodies yielding false positive results. A review of serological data for patients from southeastern Australia with suspected Lyme disease referred to our laboratories has revealed only 2·2% (75 of 3458 in 1988–93) to be seropositive, and a serological suvey of dogs from both urban and rural areas in the study by one of us (D D, unpublished) has shown a similar seropositivity rate of 2·5% (6 of 239). These data do not suggest that the region is a Lyme disease endemic area.

Confirmation of clinically suspected cases of Lyme disease in Australia, based on positive serology from IFAT and ELISA, should thus continue to be questioned. Unfortunately, a 'more sophisticated' technique such as Western immunoblot testing may be no more helpful because of the lack of specificity of protein 'markers' such as the 41 kDa flagella protein and the outer surface proteins (such as OspA) which are known to be heterogeneous in North American and European borrelia [32–35]. Until more specific markers are defined for a causative agent for the Australian syndrome, preferably following isolation and characterization of an

organism from a local patient, the exact nature of 'Lyme disease' in Australia will remain an enigma.

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