SCOPING STUDY TO DEVELOP A RESEARCH PROJECT(S) TO INVESTIGATE THE PRESENCE OR ABSENCE OF LYME DISEASE IN AUSTRALIA

FINAL REPORT

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INDEX

TERMS OF REFERENCE

To produce a scoping paper that identifies the research needs for an investigation into whether a causative tick-borne microorganism (*Borrelia*) for Lyme disease exists in Australia. This would include a consultation with relevant stakeholders including:

- The Chief Medical Officer's Clinical Advisory Committee on Lyme disease to determine their views on the possible direction for any future research program;
- Lyme disease and *Borrelia* experts, including those overseas, to seek advice on research directions and current practices; and
- Identify researchers that are currently conducting or considering research projects that examine tick-borne disease in Australia and how their research may complement or inform any research program.

The major outcome from the scoping paper will be the development of an outline for a research project to seek whether a causative agent(s) of Lyme disease exists in Australia. This will also address:

- Optimal methods for identification and bacterial characterisation from appropriate haematophagous arthropod vectors; and
- Provide guidance on a diagnostic pathway.

INTRODUCTION

It is just over 30 years since the discovery of *Borrelia burgdorferi* as the aetiological agent of Lyme disease in North America (Burgdorfer et al 1982; Steere et al 1983; Benach et al 1983). Since that time, Lyme borreliosis has been recognised as an emerging disease with increasing numbers of cases across much of the temperate zones of the Northern Hemisphere, stretching from the Mexican border to southern Canadian provinces in North America, the whole of Europe and northern Asia (Hubalek 2009; Franke et al 2013). Occasional cases have also been recognised in Central and South America (Gordillo-Perez et al 2007; Carranza-Tamayo et al 2012), and in northern Africa (Hubalek 2009; Franke et al 2013). It is now recognised to be the most frequent cause of tick-borne disease with an estimated 65,000+ cases in Europe and a further 20,000+ cases in the United States, but this may be a significant underestimate with many cases unreported, and compounded by the small number of countries in Europe to make Lyme disease notifiable, and the actual total may be closer to 255,000 cases annually (Rudenko et al 2011; Radolf et al 2012). The clinical presentation varies depending on the stage of the illness: early disease includes erythema migrans and an influenza-like illness; early disseminated disease includes multiple erythema migrans, meningitis, cranial nerve palsies and carditis; and late disease present primarily as arthritis. In most patients, signs and symptoms resolve after appropriate treatment with antimicrobials in 2-4 weeks (Murray and Shapiro 2010), but in some patients a prolonged or 'late' disease may occur lasting over several months, and there have also been claims that 'chronic Lyme disease' may occur in some individuals with a wide range of unspecific symptoms (Cameron et al 2004; Franke et al 2013). In Australia, the presence of Lyme disease remains uncertain, equivocal, and evidence for the presence of *B. burgdorferi* or any other related aetiological agent remains confused or unsubstantiated. This uncertainty and confusion has spilt over into the public arena, fuelled in part by emotive and unsubstantiated reporting by the media, and has resulted in substantial public concern. It is becoming increasingly important to resolve this issue in order to provide public assurance, particularly for those whose life-styles or homes are associated with risks for exposure to tick bites, and to provide some degree of certainty to those suffering from symptoms which have been diagnosed as being due to Lyme disease. To ensure public confidence, it is essential that any investigation to determine the presence or absence of Lyme disease should be open and uncommitted, and that all possible scenarios should be canvassed. In approaching this, the current accepted knowledge of Lyme as it occurs in the United States, Europe and Asia must provide the basis of Australian studies, but with the acknowledgement that an Australian agent responsible for 'Lyme-like' disease might be significantly different from those described elsewhere in the world, including the possibility that it might be due to an infectious agent other than a *Borrelia* species, and that this might extend to differences in modes of transmission and to possible treatment protocols.

Scoping a research programme to prove or disprove the presence of Lyme borreliosis in Australia requires an understanding of the incidence, cause, transmission, pathogenesis, diagnosis, and epidemiology of Lyme disease. Thus this scoping study begins with a brief review of Lyme disease in the Northern Hemisphere, especially the United States and Europe, and then defines some of the questions needed to address the rationale of the study. Finally these questions are expanded to form a research programme. Sections in the Background specifically directed at the Australian scene or particularly relevant to determining the presence or absence of Lyme borreliosis in Australia, are shown in bold type.

BACKGROUND: BRIEF REVIEW OF LYME BORRELIOSIS.

(a) *Borrelia* species in Lyme disease and their vectors, reservoirs and genomes.

Lyme disease is a zoonotic tick-borne disease caused by a certain members of a group of related spirochaetes – *Borrelia burgdorferi* sensu lato (s.l.) – that are transmitted by specific *Ixodes* spp. ticks. The *B. burgdorferi* s.l. complex is a diverse group of more than 18 spirochaete species. Four species comprising *B. americana*, *B. andersonii*, *B. californiensis*, and *B. kurtenbachii* are found only in North America; eleven species occur in and are restricted to Eurasia comprising *B. afzelii*, *B. bavariensis*, *B. garinii*, *B. japonica*, *B. lusitaniae*, *B. sinica*, *B. spielmanii*, *B. tanukii*, *B. turdi*, *B. valaisiana*, and *B. yangtse*; and three species occur in both North America and Europe, *B. burgdorferi* sensu stricto (s.s.), *B. bissettii*, and *B. carolinensis* (Rudenko et al 2011). In North America, the primary and by far the most frequent cause of Lyme borreliosis has been *Borrelia burgdorferi* s.s. (Radolf et al 2012; Stanek and Reiter 2011; Rudenko et al 2011), although very occasional cases may be due to *B. andersonii* and *B. americana* (eg. Clark et al 2013), whereas in Europe five species, *B. afzelii*, *B. garinii*, *B. burgdorferi*, *B. spielmanii*, and *B. bavariensis*, have been shown to cause Lyme disease, with occasional cases associated with three other species, *B. bissettii*, *B. lusitaniae*, and *B. valaisiana* (Rudenko et al 2011; Stanek et al 2012). In ticks, *B. afzelii* and *B. garinii* are the most common genospecies circulating in Europe, followed by *B. burgdorferi* s.s. and *B. valaisiana* (Rauter and Hartung 2005). North American strains of *B. burgdorferi* s.s. are significantly more heterogeneous than those from Europe, with genetic diversity demonstrated by PCR-restriction fragment length polymorphism (Liversis et al 1999) and by sequence typing (Bunikis et al 2004), and different genotypes have been associated with disease severity (Travinsky et al 2010). Sequence typing has also shown genetic diversity for *B.afzelii* in Europe (Bunikis et al 2004). Of three main genospecies, *B. garinii* and *B. afzelii* are antigenically distinct from *B. burgdorferi* s.s., which may account for some of the variation in clinical presentation in different geographic regions. The *Borrelia* spp. in the Lyme borreliosis group, together with their vectors and reservoirs, are tabulated and discussed by Franke et al (2013).

New genospecies in the Lyme *Borrelia* complex are being recognised almost every year (Stanek and Reiter 2011) and more would be undoubtedly found if a concerted effort was made in collecting and processing ticks, especially in new areas. Examples of this have been demonstrated in Canada (Scott et al 2010; Ogden et al 2011), and in Uruguay (Barbieri et al 2013). The latter report is the first isolation of indigenous *B. burgdorferi* s.l. in the Southern Hemisphere, and also demonstrates that novel *Borrelia* genospecies in the *B. burgdorferi* s.l. complex may occur in new geographic areas.

Transmission of Lyme borreliosis is through injection of tick saliva during feeding. The disease is transmitted largely by four species of hard ticks in the *Ixodes ricinus* complex: the major vector in Europe is *I. ricinus* and in Asia is *I. persulcatus*, whereas in the United States the major vector in the north-eastern and mid-western states is *I. scapularis*, and in western US is *I. pacificus* (Stanek et al 2012; Radolf et al 2012). Other hard ticks do not appear to play any significant role in Lyme borreliosis; they are either inefficient in the acquisition of *Borrelia* spirochaetes from blood meals, or they are unable to maintain the spirochaete. The risk of infection in humans increases with length of time of exposure to the tick, approaching 100% on the third day (Biesiada et al 2012). It usually requires a feeding period of more than 36 hr for transmission of *B. burgdorferi* by *I. scapularis* or *I. pacificus* ticks in North America, but can be significantly shorter, often less than 24hr, for

transmission of *B. afzelii* by *I. ricinus* ticks in Europe (Hubalek 2009). Although *Borrelia* spirochaetes can be transmitted to humans by all three stages of tick development, the nymphal stage is responsible for the vast majority of infections, partly because it is often overlooked by those being bitten due to its small size, followed by the female adult tick. An exception to this is found with *I. persulcatus* in which adult female ticks are most frequently responsible for transmission (Stanek et al 2012). The mean prevalence of *Borrelia* infection in ticks is difficult quantify with any accuracy as it depends on locality, suitable sources of blood meals to ensure tick maintenance, prevalence of suitable reservoir species, climate, herbage and other environmental factors. Recent meta-analysis of surveillance data from Europe indicated that the overall mean prevalence was 13.7%, with a range from 0 to 49% (Rauter and Hartung 2005). Similar results have been observed in other European studies (eg: Reye et al 2010; Wilhelmsson et al 2010; Mysterud et al 2013) and in endemic areas of North America (Morshead et al 2006), although some studies found a higher incidence (32-39%) in questing ticks (Walker et al 1994; Hanincova et al 2006). The number of spirochaetes per *Borrelia*-infected tick ranged from 2 x 10^2 to 4.9 x 10^5 with a median of 7.8 x 10^3 (Wilhelmsson et al 2010). Similar levels of infection per tick were reported from the north-east United States (Wang et al 2003).

Although other tick species are believed to play no significant role in Lyme borreliosis, some species of *Amblyomma* can yield spirochaetes in the *B. burgdorferi* s.l. complex. In the United States, *B. burgdorferi* s.s. has been reported in *A. americanum,* the Lone Star tick (eg Schulze et al 2006; Clark et al 2013), but this tick species has been shown to be unable to transmit Lyme *Borrelia* (Piesman and Sinsky 1988; Ryder et al 1992).

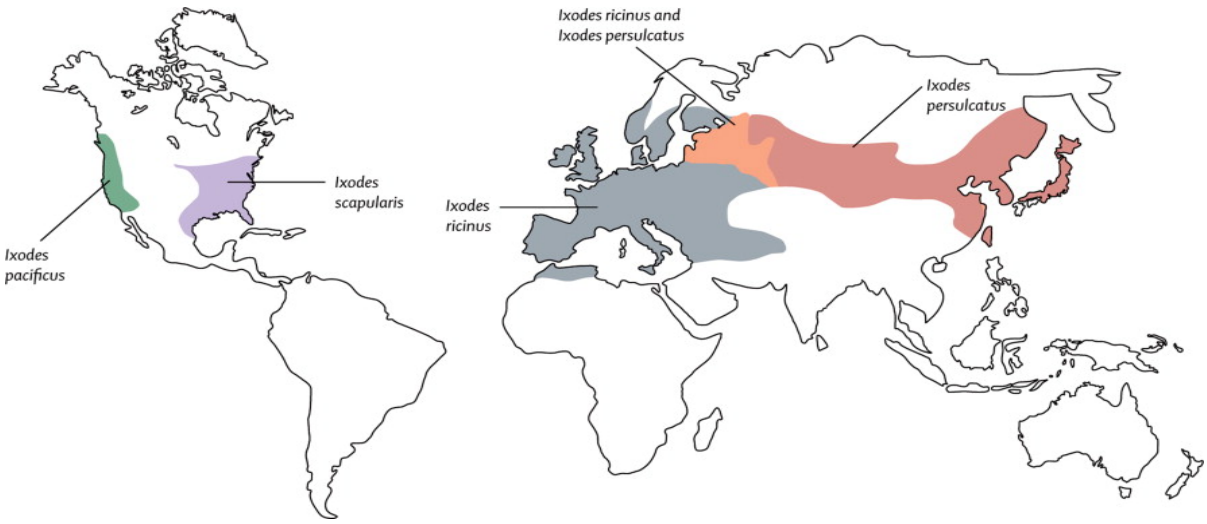

Figure 1. Global distribution of the vectors (*Ixodes ricinus* species complex) of Lyme *Borrelia*. From Stanek et al (2012), Lancet 379: 461-473.

Mites have also occasionally been implicated in the transmission of *B. burgdorferi* s.l., but their role in transmitting the spirochaetes to humans remains to be determined (Lopatina et al 1999; Kampen

et al 2004; Netusil et al 2005; Literak et al 2008). Mites (*Dermanyssus gallinae*) have been suspected of transmitting *B. anserina* to avian hosts in eastern Australia (J.Curnow, personal communication).

No member of the *I. ricinus* complex occurs in Australia, but the most plausible indigenous vector is *I. holocyclus* which is known to parasitise native vertebrate hosts, domestic animals and humans, and is the most common tick biting humans. Known as the paralysis tick, it is the most important medically, causing local irritation and allergic reactions, but paralysis in humans is now uncommon, and occurs most often in domestic animals. It is found in a 20-30 kilometre strip along the eastern coast of Australia, as well as in pockets up to 100km inland. To this date, there has only been one report of *Borrelia* species being found in *I. holocyclus* ticks, but the cultures were not confirmed and were unsustainable (Wills and Barry 1991). Experimental vector competence studies have demonstrated that this species of tick is unable to be infected by a North American isolate of *B. burgdorferi* (Piesman and Stone 1991), but further studies with other *Borrelia* species is warranted. The high spirochaete loads reported in infected ticks in both Europe and North America would have suggested that a similar finding might be expected in Australian ticks, so it is all the more surprising that they haven't been regularly detected in *I. holocyclus* or any other Australian tick species. In Western Australia where cases of Lyme-like disease have also been reported, the most common ticks biting humans are *A. albolimbatum*, *A. triguttatum* and *I. australiensis* (Mark Harvey, personal comunication), but none of these have been associated with borreliosis. Most of the 75+ species of Australian ticks are hard ticks, and other widespread examples are the brown dog tick (*Rhipicephalus sanguineus*) and the bush tick (*Haemaphysalis longicornis*), although there has been a report that Lyme spirochaetes have been detected in this latter species in China (Meng et al 2008). There are fewer soft ticks in Australia, and the most important belong to the *Ornithodoros* genus, examples being the *O. gurneyi*, the Kangaroo soft tick; *O. capensis*, a tick of birds, and *O. moubata* complex. Some members of this latter complex feed on animals, with one species infecting pigs and occasionally biting humans. The avian *O. capensis* has been shown to harbour the flavivirus, Saumarez Reef (see below). This genus of soft ticks is more important for the transmission of the relapsing fever *Borrelia* species, but *B. burgdorferi* s.l. have occasionally been found in *Ornithodoros* species (eg. Lane et al 2010; Adham et al 2010).

(b) The natural reservoirs of Lyme Borrelia species.

The reservoirs of Lyme *Borrelia* spp. are small mammals and some birds (reviewed in Piesman and Gern 2004; Rizzoli et al 2011; Franke et al 2013). Deer are not competent reservoirs but are essential in many areas for the maintenance of tick populations because they are one of a few wildlife hosts able to feed sufficient numbers of adult ticks (Stanek et al 2012). Other large domestic animals such as cattle and sheep are also not competent reservoirs. Lyme *Borrelia* do not cause disease in reservoir hosts, and other than humans, the only mammals known at this time to show disease symptoms are dogs and possibly horses. Indeed the use of ticks taken from dogs provides a good indication of the presence of Lyme disease in a given location, and dogs are an excellent sentinel species for estimating Lyme disease risk (Hamer et al 2009; Smith et al 2012). Although a broad spectrum of clinical signs have been attributed to infection with *Borrelia* in horses, actual cases of equine Lyme borreliosis are rare if they exist at all (Butler et al 2005).

Birds can act both as biological carriers of *Borrelia* and transporters of infected ticks, and aid in the dispersal and spread of *B. garinii* to new foci (Comstedt et al 2011). Certain Passerine songbirds are capable of being reservoir hosts of *Borrelia* species in the United States and Europe, especially members of the Family *Turdidae*, which includes the thrushes, Blackbirds, and the American robin, many of which are migratory so thus dispersing infected ticks and expanding the geographic range of the spirochaete (eg: Olsen et al 1995; Comstedt et al 2006; Kipp et al 2006; Poupon et al 2006; Dubska et al 2009;Brinkerhoff et al 2010; Scott et al 2010; Brinkerhoff et al 2011; Scott et al 2012). The most common *Borrelia* species found in *Ixodes ricinus* ticks taken from Passerine birds in Europe were *B. garinii*, *B. lusitaniae*, and *B. valaisiana*, whereas those in United States were *B. burgdorferi* in *I. scapularis* ticks, and occasionally in *I. pacificus*. A second bird-associated tick, *I. auritulus*, was also found to be infected with *Borrelia* species including *B. burgdorferi* s.l. and some novel *Borrelia* species in ticks collected from a number of songbird species from various sites across Canada (Morshed et al 2005; Scott et al 2010; Scott et al 2012). *I. auritulus* ticks have been found on birds in many parts of the world (eg: Gonzalez-Acuna et al; 2005; Eisen et al 2006; Kolonin 2009), but while they may not bite humans, they appear to play a significant role in Canada in the dispersal and maintenance of *Borrelia* species. With respect to dispersal by migratory passerines, it has been found that birds are able to carry the Lyme disease as a latent infection for several months which can be reactivated by migratory stress and passed on to ticks making the birds long-distance carriers of the spirochaete (Gylfe et al 2000).

The seabird tick, *I. uriae*, also maintains *B. garinii* in silent enzootic cycles in seabirds at their nesting sites demonstrating that these non-passerine seabirds also play a role in long-distance dispersal of *Borrelia* species over a wide area in both the Northern and Southern Hemispheres (Olsen et al 1993), and suggested a transhemispheric exchange of Lyme disease spirochaetes (Olsen et al 1995). However, *I. uriae* ticks (and *I. holocyclus*) might possibly have an Australian origin, and with a parsimony analysis indicating that southern hemisphere *I. uriae* ticks are paraphyletic in respect to northern *I. uriae* ticks, and the northern ticks are monophyletic, it would suggest that this tick is no longer carried across the equator, but further work is needed to clarify this (Gylfe et al 2001). More recently, phylogenetic studies of *Borrelia* species circulating in seabirds in the Northern Hemisphere have been undertaken by Duneau et al (2008) demonstrating the presence of two main clades, one associated with *B. garinii* and the other with *B. lusitaniae*, but there was no clear association between different *Borrelia* species and a given host seabird species. An additional sequence also clustered most closely with *B. burgdorferi* s.s. Other studies have described various tick-borne viruses in *I. uriae* ticks collected on Macquarie Island, but no attempt was made to examine the ticks for *Borrelia* infections (St George et al 1985; Major et al 2009). *I. uriae* do not bite humans, but it is probable that seabirds carrying the ticks could interact with shore birds at many sites which could introduce Lyme *Borrelia* to native avifauna, or the ticks could be introduced by gulls to shorebirds, but as avian ticks are not known to bite humans in Australia, it is likely that only occasional, sporadic human cases could result.

It is therefore plausible that certain *B. burgdorferi* s.l. strains could be brought to the Southern Hemisphere and enter local Australian ecosystems through intermingling between seabirds and land-based avian species, but most bird ticks do not bite humans, and if they did, would rapidly drop off before the opportunity to transmit the spirochaete. If cases of human infection were to result, they would be very occasional and localised.

(c) Some comments of the *Borrelia* genome.

Borrelia species have the most complex genomes of all known bacteria (Radolf et al 2012), comprising a relatively small chromosome and 20 or more linear and circular plasmids. The complete genome sequences of three major species of *Borrelia* have been described; strains of *B. burgdorferi* s.s. (Fraser et al 1997; Schutzer et al 2011), *B. garinii* (Glockner et al 2004; Casjens et al 2011), and *B. afzelii* (Glockner et al 2006; Casjens et al 2011), together with a number of their plasmids (Fraser et al 1997; Casjens et al 2000; Glockner et al 2006). In addition, whole genome sequences have also recently been reported of *B. bissettii*, *B. valaisiana*, and *B. spielmanii* (Schutzer et al 2012). Most of the housekeeping genes are on the chromosome which is fairly constant in organization and content across the genus (Casjens et al 2010). In contrast, the plasmids exhibit much greater variability in gene content and are not equally represented between all strains and are not all essential for the maintenance of the enzootic cycle. The core of all *Borrelia* species consists of the chromosome and two plamids (1p54 and cp26). The key genes thought to contribute to maintenance of the *B. burgdorferi* enzootic cycle, including the position of each with respect to the genetic element, and the function of their encoded protein, have been reviewed by Radolf et al (2012). As an obligate parasite, *B. burgdorferi* lacks the conventionally recognizable machinery for synthesizing nucleotides, amino acids, fatty acids, and enzyme cofactors, apparently scavenging these necessities from the host (Fraser et al 1997).

(d) Lyme *Borrelia* and human disease.

When an infected tick takes a blood meal, the ingested bacteria multiply and undergo a number of phenotypic changes , including the expression of the outer surface protein C (OspC) which allows them to invade the host tick's salivary glands – a process which takes several days and explains why transmission only occurs after a significant delay (Stanek et al 2012). The initial clinical manifestations of Lyme disease in both North America and Europe share some common features such as an erythema migrans (EM) rash and an influenza-like illness, with fatigue, headache, myalgia, arthralgia, and malaise, although some early infections may be completely asymptomatic. In about 90% of cases, the development of the characteristic skin lesion, EM, occurs at the site of the tick bite. The incubation period between tick bite and appearance of EM is typically 7 to 14 days, but may be as short as a day and as long as 30 days (Marques 2010). Atypical EM can occur in some patients, and be uncharacteristic (Marques 2010; Schutzer et al 2013). Erythematous lesions occurring within a few hours of a tick bite represent hypersensitivity reactions rather than EM. Subsequent manifestations may develop and correlate approximately with the infecting species: *B. burgdorferi* s.s. infection frequently leads to arthritis, whereas *B. garinii* leads more often to neurological manifestations, and *B. afzelii* to skin disorders, although these clinical associations are not absolute (van Dam et al 1993). A summary of clinical case definitions and a discussion of the various manifestations of Lyme borreliosis has been provided by Stanek et al (2011), and the primary and supporting diagnostic testing and supporting clinical findings for each Lyme manifestation were tabulated in summary form in Stanek et al (2012). As Lyme disease can take various forms, differential diagnosis is essential, and the most common of these are described by Stanek et al (2012).

To exemplify the presentation and disease course, one particular series of cases is described here that occurred over a 12 month period in an area of central Germany where Lyme disease is very common (Huppertz et al 1999). A total of 313 cases were diagnosed as Lyme disease, based on strict case definition criteria which required the presence of either EM lesions or lymphocytoma, or a positive serological test with the presence of another specific manifestation of Lyme borreliosis, giving an incidence of 111 cases/100,000 population. The highest rates of infection were in children (<18) and elderly adults (60-65 years old). EM was the most common clinical manifestation, occurring in 288 (92%) patients. It was the only manifestation in 279 (89%) patients of whom 26 had multiple EM lesions. Other specific manifestations of Lyme borreliosis with or without EM occurred in 34 (11%) patients. Fever was noted in only 26 patients. Of the 34 patients with manifestations other than EM alone, 15 had arthritis, 9 had neuroborreliosis, 6 had lymphocytoma, 4 had acrodermatitis chronica atrophicans (ACA), and one patient had carditis. In the 15 patients with Lyme arthritis, the large joints were most frequently involved, most commonly the knee, and most patients had had symptoms in more than one joint, usually the shoulders or elbows. Of the 9 patients with neuroborreliosis, 5 had symptoms of meningitis, one of whom also had facial nerve palsy; one patient had uveitis and oedema of the optic disk; and the remaining three had symptoms of radiculoneuritis, such as paresthesia or palsy of the lower extremities. Children were more likely to have manifestations other than EM alone, whereas adults were more likely to have EM as their only manifestation. A total of 185 (60%) patients reported a recent tick bite; those in children were more likely to be on the head and neck compared to adults where the bite was most frequently on the legs. All patients with symptoms other than EM alone experienced improvement or resolution after antibiotic therapy, except one patient with ACA who refused therapy.

The results in the above case study were similar to those in other studies in Europe, but differ slightly from enzootic areas in North-Eastern United States. The incidence of EM is similar in some studies (Shapiro and Gerber 2000) but is lower in others (Bacon et al 2008; Ertel et al 2012) in the United States. The most common late manifestation in the United States is arthritis; but neurological manifestations are less common, and ACA and lymphocytomas are extremely rare. The latter may be a reflection of the aetiological agent as they are usually caused by the European *Borrelia* species, *B. afzelii* or *B. garinii* (Huppertz et al 1999; Shapiro and Gerber 2000).

As with ***all*** infectious diseases, infection with *B. burgdorferi* s.l. leads initially to an IgM antibody response, followed 2-4 weeks later by an IgG antibody response. The IgM response tends to be relatively short-lived in most patients, but the IgG response remains for decades following infection (Glatz et al 2008; Kalish et al 2001).

A Lyme-like disease in Brazil, the Baggio-Yoshinari syndrome, has been associated with *B. burgdorferi* s.l. infection serologically and by PCR. The Brazilian cases present with EM and with other Lyme-like manifestations, such as arthritis, but with an increasing frequency of relapsing episodes in untreated patients. The main vector is *Amblyomma cajennense*, although ticks in the genus *Rhipicephalus* may also be involved (Gouveia et al 2010; Carranza-Tamayo et al 2012; Mantovani et al 2012). These Brazilian cases demonstrate that other disease manifestations may be important in Lyme borreliosis in novel geographic habitats, including problems in isolating and identifying infectious agents and in the role of tick species other than members of the *I. ricinus* complex.

Late Lyme disease occurs in a few patients with specific symptoms that can take several months to fully resolve. Thus Lyme neuroborreliosis may take weeks or months to resolve in a small number of patients with residual paresthesias or facial paresis, and in cases when Lyme neuroborreliosis diagnosis was made late in the course of disease, recovery from severe neural disease may be incomplete. Patients with acrodermatitis chronica atrophcans who sustained severe tissue damage prior to treatment may have atrophic lesions, peripheral neuropathy, and joint deformities. In a small number of Lyme arthritis patients, the arthritis may not respond to further antibiotic treatment, and it is likely that the arthritis in these patients is driven by immune-pathological mechanisms (Stanek et al 2011). Similar slow resolution of symptoms and signs can occur in patients with many other systemic infections (Hickie et al 2006), even if some symptoms persist. It should also be recognised that Lyme is not a fatal disease, although a few case reports have suggested that Lyme carditis might have contributed to a patient's death (Halparin et al 2013).

Chronic Lyme disease is a widely used but poorly defined term. It is frequently used as a diagnosis for patients with persistent pain, fatigue, or neurocognitive complaints without clinical evidence of previous acute Lyme borreliosis and in some instances even without serological identification of borrelial infection. However, some consider Lyme borreliosis to be a disease that may lead to an irreversible chronic stage, potentially leading to fibromyalgia or chronic fatigue syndrome, or worse. It is important for all concerned that every attempt is made to verify or not the diagnosis of chronic Lyme disease, and also that the 'Jury is out' over this contentious issue. There needs to be confirmatory testing in accredited laboratories to provide scientific evidence-based support to these cases; it is well-accepted that specific IgG remains at detectable levels for many years (Glatz et al 2008; Kalish et al 2001). In addition, there will need to be a long and extensive discussion to establish the correct balance between the demands of evidence-based medicine and other healing concepts (Stanek and Strle 2009), as was undertaken recently by the Robert Koch Institute, Germany's national Public Health Institute.

Lyme borreliosis has been reported in Australia (eg Mayne 2011; Hudson et al 1998; D Dickeson, personal communication; B Hudson, personal communication), but the vast majority of cases were patients who had travelled to Lyme endemic areas overseas. Confirmatory testing is needed for patients with no travel history, and where additional testing of putative positive specimens has been done in NATA-accredited Australian laboratories, the results could not be confirmed to international standards for Lyme diagnoses (D Dickeson, personal communication). A possible case of Lyme disease was recently reported with a neuropsychiatric presentation but no detail was provided on standard Lyme borreliosis diagnostic test procedures (Maud and Berk, 2013).

(e) **Other *Borrelia* species associated with disease.**

Other species of *Borrelia* have been associated with louse-borne and tick-borne relapsing fever (Larsson et al 2009; Cutler 2010). Louse-borne relapsing fever is caused by *B. recurrentis* and transmitted by the human body louse (*Pediculus humanus*), and is found in limited areas of Asia, Africa and South America. It is usually associated with crowding and poor hygiene, and with periods of famine, social disruption, and war, as well as among refugees from these events (Brouqui 2011; Badiaga and Brouqui 2012). However, homeless populations in developed countries may also be at risk. There is a sudden onset of fever lasting 3-6 days, and this is usually followed by a single, milder

episode. The fever often ends in “crisis”, consisting of chills, followed by intense sweating, falling body temperature and low blood pressure. Louse-borne relapsing fever is mainly a disease of the developing world, but without antibiotic treatment can result in a 10-70% fatality rate.

Tick-borne relapsing fever is caused by various *Borrelia* species depending on geographic area, and is found in Africa, parts of Asia and southern Europe, and in North and South America. It is characterised by multiple episodes of fever. The first episode of fever usually occurs after an incubation period of about 7 days, and lasts from 4 to 7 days. Subsequent episodes occur with up to 2 weeks between episodes. Frequent complaints include nausea, malaise, headaches and body aches, and sometimes with skin rashes and hepatomegaly, sometimes with jaundice. Neurological complications may occur as well as lymphocytic meningitis. Without antibiotic treatment, the disease may result in a 4-10% fatality rate. Soft ticks of the family Argasidae, usually *Ornithodoros* species, are the vectors of the tick-borne relapsing fever group, which includes *B. duttonii* in Africa and *B. hermsii* and *B. turicatae* in the United States. Unlike Ixodid ticks which usually attach to their host for days, *Ornithodoros* ticks feed quickly, completing their blood meal in less than an hour, and as their saliva contain painkillers, patients may be unaware that they have been bitten.

A second clade of *Borrelia* spirochaetes closely related to the relapsing fever spirochaetes phylogenetically are associated with hard tick vectors, including *B. theileri*, *B. lonestari*, and *B. miyamotoi*. *B. theileri* is a pathogen of cattle, the aetiological agent of bovine borreliosis, and transmitted by *Rhipicephalus microplus*, the cattle tick. It is found widely in Australia but is not believed to infect humans. *B. lonestari* is transmitted by *Amblyomma americanum* and has been associated with a Lyme-like disease in south-eastern and south-central United States which resembles Lyme disease clinically, but the patients have no evidence of infection with *B. burgdorferi* and do not develop the sequelae associated with Lyme disease (Armstrong et al 2001; James et al 2001). This syndrome is called southern tick-associated rash illness (STARI) (James et al 2001; Masters et al 1998). However, recent detailed studies of 10 patients with STARI have suggested that some cases of Lyme borreliosis in Florida and Georgia might be due to *Borrelia burgdorferi* s.l., and indeed DNA sequencing indicated *B. burgdorferi*, *B. andersonii*, and *B. americana* as infecting agents (Clark et al 2013). *B. miyamotoi* can be transmitted by a variety of *Ixodes* species including *I. persulcatus* in Japan, *I.ricinus* in Europe, *I. scapularis* and *I. pacificus* in North America. In the 8 years since *B. miyamotoi* was discovered in Japan, it has been found to have a wide geographic range in Eurasia and North America, but its role in human disease has only recently been demonstrated in Russia (Platonov et al 2011); of the 46 patients, all presented with an influenza-like illness with fever as high as 39.5^{o}C, and relapsing fever occurred in 5 (11%) patients, and erythema migrans in 4 (9%). A second study in Russia found that over 50% of cases of Lyme disease without erythema migrans were caused by *B. miyamotoi*, whereas *B. burgdorderi* s.l. predominated as a causative agent of the erythemic form of borreliosis (Kara et al 2010). Another recent case attributed to *B. miyamotoi* has been reported in a case of progressive mental deterioration in an older, immunocomprimised patient when the spirochaee was detected by microcopy and PCR in cerebrospinal fluid (CSF) (Gugliotta et al 2013). To date, *B. miyamotoi* has not been able be cultured *in vitro*. It has recently been shown to occur in ticks collected from passerine birds, particularly Northern Cardinals (*Cardinalis cardinalis*) in the United States, which might indicate that birds have a role in the geographic dispersal of this species (Hamer et al 2012).

Other new *Borrelia* species are being reported which are different to the *B. burgdorferi* s.l. or to the relapsing fever *Borrelia* species, although not yet associated with disease (eg Takano et al 2011; Mediannikov et al 2013), in a number of different tick species including *Amblyomma* spp. and *Hyalomma* spp..

The Louse-borne and tick-borne relapsing fevers have not been reported in Australia or New Zealand. *B. miyamotoi* appears to be widely dispersed now in the Northern Hemisphere, but there is no evidence of it being in the Southern Hemisphere. It would seem that an examination of soft ticks, particularly *Ornithodoros* species, in Australia for *Borrelia* and other pathogens is well overdue.

(f) *Borrelia* species in Australia.

Considerable amount of work was carried out from the late 1930s through to the early 1970s on *B. anserina*, the agent responsible for fowl spirochaetosis, transmitted by an *Argas* spp. tick in eastern and south-eastern Australia. The disease was largely controlled by controlling the tick vector, but it remains in some species in which it is transmitted by mites (J Curnow, personal communication). It had recently been thought to be a disease only of domestic species and wild birds were believed to be resistant (Mackenzie 1994; Ladds 2009), but this may be untrue, and the spirochaete may be found in various species of doves (J Curnow, personal communication). There have been two early reports of the detection of *Borrelia* species in rodents, native mammals and cattle (Mackerras 1959; Carley and Pope 1962; Pope and Carley 1956). Carley and Pope were able to culture a *Borrelia* species, *B.queenslandica*, from *Rattus villosissimus* collected near Richmond in north-western Queensland. However, they were not able to maintain it in culture. Spirochaetes morphologically similar and antigenically related *to Borrelia burgdorferi* were cultured from the gut contents of *I. holocyclus* and *Haemophysalis* spp. ticks by Wills and Barry (1991), but the cultures weren't sustainable and these results have not been able to be repeated from ticks collected more recently. However, there is little recent information, with the exception of a limited study by Russell (1995) in native rats, bandicoots and a marsupial mouse trapped on the south coast of NSW, but no evidence of any spirochaete was found. A major study of 12,000 ticks collected along the coastal strip of NSW was undertaken by Russell and Colleagues to investigate the presence of *Borrelia* species. About 11,000 ticks comprising more than 12 species, especially *Haemaphysalis bancrofti*, *H. longicornis*, *I. holocyclus*, and various other *Ixodes* species, were dissected and the gut contents examined by dark field microscopy and, in some cases, culture but no spirochaete of any kind was detected, although spirochaete-like objects were visualised from by dark-field microscopy. A further 1000 ticks were tested by PCR for the presence of *Borrelia* species, but once again, all were negative (Russell et al 1994; Russell 1995). More recently Mayne (2012) reported the detection of *B. burgdorferi* from four patients by PCR of EM biopsy specimens; surprisingly the PCR results indicated considerable diversity with sequence data suggesting three distinct strains of spirochaete.

Despite the recent indication of possible *B. burgdorferi* strains in Australia, further work is needed to verify these claims, and confrmatory evidence should be obtained in a second NATA-accredited laboratory.

(g) Laboratory diagnosis.

Laboratory support is an essential component of clinical diagnosis of Lyme borreliosis because of the non-specific nature of many clinical manifestations. A wide range of methods have been developed for the direct detection of *B. burgdorferi* s.l in clinical tissue specimens. These include microscopic examination, detection of specific proteins or nucleic acids, and cultivation. Culture of spirochaetes from patient specimens remains the gold standard for specificity, but it is a slow process with long incubation times, and because of the low numbers of viable spirochaetes in most biopsies and the fastidious nature of the organism, the results can be very variable, ranging from 1% in Lyme arthritis to 70% in EM skin lesions, and importantly, negative results may not exclude active infections (Stanek et al 2010; Brouqui et al 2004). However culture is seldom done or available because it is unnecessary for patients with EM and too insensitive for patients with extracutaneous manifestations (Stanek et al 2012). Direct nucleic acid tests utilise PCR-based molecular techniques that can rapidly and specifically confirm clinical diagnosis of Lyme disease, and identify the genospecies in clinical specimens and cultures (eg: Cerar et al 2008a; Cerar et al 2008b; Liveris et al 2002; Liveris et al 2012; Nocton et al 1996; O'Rourke et al 2013). The sensitivity varies depending on methodology and specimen source (Marques 2010), and will probably depend on genospecies responsible for the infection, particularly in non-endemic areas. In a study comparing the sensitivities of two PCR assays and culture for detection of *Borrelia* spp. in skin biopsies from patients with typical EM, nested PCR was found to be the most sensitive method for detecting *Borrelia* in skin lesions, followed by culture and a PCR targeting the flagellin gene. Standardisation is required as there are significant differences in methodologies and gene targets, and more clinical validations are needed, but the direct detection of *B. burgdorferi* s.l. by PCR is much more desirable than serology if the method can be developed to be reliable, easy-to-perform, economical, and sensitive (Stanek and Strle 2009). It is also important to recognise that a negative PCR result does not necessarily indicate the absence of *Borrelia* (Aguero-Rosenfeld et al 2005). Nevertheless, next generation PCR methodologies promise to make diagnosis of Lyme borreliosis more accurate and reliable in the future.

Indirect tests through serological assays for antibodies to *B. burgdorferi* s.l. are the mainstay of laboratory diagnosis, and the most common diagnostic methodologies employed; not only are the prerequisite laboratory facilities widely available, but specimens are easy to obtain. However, the complexity of the antigenic composition of *B. burgdorderi* s.l., and the temporal appearance of antibodies to different antigens, have made the sensitivity and specificity of serological tests questionable, although the use of newer recombinant antigens rather than whole cell lysates have substantially improved their reliability. Nevertheless, the limitations of serological tests must be recognised (Murray and Shapiro 2010; Evans et al 2010); the antibody response may be weak or absent, especially in EM and early in infection (Steere et al 2008) which due to a delayed IgM response or seroconversion may be ablated by early antibiotic treatment (Stanek and Strle 2003; Glatz et al 2006), and they do not distinguish between active and inactive infections (Kalish et al 2001). Most serological diagnostic protocols in the United States and Europe use a two tier system with the first stage most commonly an enzyme-linked immunosorbent assay or sometimes an indirect immunofluorescent-antibody assay, although the former is preferable as it is quantifiable and is significantly more sensitive. This is followed by a Western blot (CDC 1995; Aguero-Rosenfeld et al 2005; Brouqui et al 2004; Wilske et al 2007; Stanek et al 2011). If the serological test is positive or equivocal, then separate IgM and IgG immunoblots are done on the same serum sample. If

symptoms have persisted for 4 weeks, then the IgG Western blot should be positive – untreated patients who remain seronegative despite persisting symptoms are unlikely to have Lyme disease and other potential diagnoses should be considered (Stanek et al 2012). A positive specific antibody response persists for many years (Gratz et al 2008; Kalish et al 2001). Western blots are interpreted using standardised criteria, requiring at least two of three bands for a positive IgM Western blot, and five of ten bands for a positive IgG Western blot (Marques 2010), but the criteria for the United States (CDC 1995) are not applicable for European patients (Robertson et al 2000) as their immune response is restricted to a narrower spectrum of *Borrelia* proteins compared with that shown by American patients (Dressler et al 1994). There are problems in interpreting Western blots, particularly in Europe because of the number of different genospecies of *B. burgdorferi* s.l. with variant antigens being expressed with slightly different antigen sizes between different genospecies, and even between strains of a single species, which make standardisation of blotting procedures difficult (Robertson et al 2000; Evans et al 2011; Mavin et al 2011).

It is important that the 2-tier protocol is undertaken; if the first tier ELISA is omitted or interpretation of the Western blot is carried out using criteria that are not evidence-based, this will potentially decrease the specificity of the testing and lead to misdiagnosis. Interpreting the IgM Western blot can lead to false positive results if insufficient care is taken as non-specific weak bands can often occur.

The use of recombinant antigens, principally VlsE lipoprotein of *B. burgdorferi*, and the C6 peptide, which reproduces the invariable region 6 of VlsE, has been a major advance in Lyme disease serology. The C6 peptide ELISA has excellent sensitivity for acute-, convalescent-, and late-phase specimens as well as excellent specificity (Liang et al 1999; Bacon et al 2003; Marangoni et al 2008; Steere et al 2008; Wormser et al 2008). It has been suggested that the new recombinant antigen tests, particularly with C6, may make the 2-tier protocol redundant, but most evidence would indicate that this decision is too early and the 2-tier testing should continue for the foreseeable future, especially in Europe with several pathogenic genospecies with variability of immunodominant antigens (Stanek et al 2011). Other new methodologies show promise to provide new diagnosticsin the future (Kraiczy et al 2008; O'Rourke et al 2013; Colman et al 2011). Immunoblots should use recombinant antigens p100, p58, p41i, VlsE, OspC, and DbpA, including those expressed primarily in vivo (VlsE and DbpA) instead of whole cell lysates.

The accuracy and reproducibility of commercially produced Lyme disease kits has been generally poor (eg: Bakken et al 1997; Bakken et al 1992; Luger and Krauss 1990; Ang et al 2011; Busson et al 2012), and it is important that commercial laboratories utilise validated kits (CDC 2005; Klempner et al 2001). However, there has been very limited interassay standardisation, especially in the European market, and not unsurprisingly, different test methodologies can result in differences with respect to test quality. Indeed in Germany alone, at least 55 different companies provide a variety of diagnostic tests (Müller et al 2012) which can lead to a high number of both false negative and false positive results. There is an urgent need for improved interassay standardisation of commercially available test kits, and independent clinical evaluation of assays should be a legal requirement before they are marketed.

False-positive results of serological tests for Lyme disease can sometimes occur in the ELISA from cross-reactive antibodies from patients exposed to other spirochaetal infections, e.g., syphilis,

leptospirosis or relapsing fever (Shapiro and Gerber 2000). It is also possible that antibodies directed at sprochaetes that are part of the normal oral flora may cross-react with *B. burgdorferi* (Shapiro and Gerber 2000).There have also been false positive reports in cases of recent primary infection with varicella-zoster virus (Feder et al 1991), Epstein-Barr virus (Beradi et al 1988; Goossens et al , 1999), cytomegalovirus (Goossens et al 1999), Herpes simplex type 2 virus (Strasfeld et al 2005), and *Rickettsia rickettsia* (Beradi et al 1988). In addition to these examples associated with other infectious diseases, a case of subacute granulomatous thyroiditis (de Quervain's throiditis) was positive for Lyme disease in a screening ELISA and the reflexed Western blot was IgG negative but IgM positive, but once the fever and thyroid function test had returned to normal several weeks later, the tests for Lyme disease were all negative (Garment and Demopoulos 2010). False positive *Borrelia* serology and facial paralysis due to anaplastic lymphoma mimicking Lyme disease was recently reported showing an aggressive lymphoma may present with both clinical and serological Lyme characteristics (Deeren and Deleu 2012).

(h) Co-transmission of tick-borne organisms.

Ticks are hosts and vectors of a number of parasites, bacteria and viruses, and able to transmit more than one organism per blood meal. The main organisms which are transmitted by *Ixodes* spp. ticks, other than *Borrelia* species, are species of *Anaplasma*, *Babesia*, *Bartonella*, "Canditatus *Neoehrlichia mikurensis*", *Ehrlichia*, *Francisella*, *Rickettsia*, *Theileria*, and various viruses (Lotric-Furlan et al 2001; Swanson et al 2006; Coipan et al 2013), as well as multiple *Borrelia* spp. (Floris et al 2007; Ružić-Sabljic et al 2005; Herrmann et al 2013). Recently*, Leptospira* spp. have also been found in *I. ricinus* ticks, but the incidence and relevance is yet to be determined (Wojcik-Fatla et al 2012). The following organisms are those most commonly associated with ticks and believed to be co-transmitted. Only information on Australian examples of these organisms is shown, unless the organism has yet to be reported in Australia.

Anaplasma.**Two *Anaplasma* species occur in Australia, *A. platys* which causes canine anaplasmosis (Brown et al 2006; Jefferies 2006) and *A. marginale* which is one of the causes of bovine anaplasmosis or bovine tick fever in northern and eastern Australia and is transmitted by the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Rogers and Shiel 1979; Jonnson et al 2008), but neither are known to infect humans.**

Babesia*. Bovine babesiosis is a significant disease of cattle in Australia, having been introduced as early as 1829 by cattle imported from Indonesia, It currently costs the industry as much as $29 million each year in lost production**. **Two species of *Babesia* cause bovine tick fever, *B. bovis* and *B. bigemina*, and they are transmitted by *R.microplus*. The former is by far the most important causing 80% of outbreaks. Considerable work on *B. bovis* was undertaken by CSIRO scientists in the 1940s through to the 1960s, especially in livestock (J. Curnow, personal communication). The first report of locally-acquired case of human babesiosis, caused by *Babesia microti*, was in a 56 year old man who had never travelled and had no history of blood transfusions (Senanayake et al 2012). The origin of the aetiological agent is uncertain; it is most closely related to North American strains, and the patient was either bitten by an imported tick or a local tick might have transmitted an autochthonous infection, presumably originating from one or more species of introduced rodent. If it was a local tick, the most likely candidate would be *I. holocyclus* as *Ixodes

species are the usual vectors overseas. Canine babesiosis is a cause of anemia, thrombocytopenia, and a wide range of clinical signs ranging from mild to fatal infections in dogs, and three species of canine *Babesia* spp. occur in Australia, *B. canis, B. vogeli* and *B. gibsoni* (Brown et al 2006; Jefferies et al 2007; Irwin 2010; Mitrovic et al 2011). A *Babesia* species has been identified in the blood of wild captured woylies (*Bettongia penicillata ogilbyi*) in Western Australia (Paparini et al 2012), and a similar species has been found in ticks in eastern Australia.

Bartonella. ***Bartonella* species occur both in domestic and wild animals in Australia. *Bartonella henselae* and *B. clarridgeiae*, causative agents of cat scratch disease, have been reported in Australia, but are most frequently transmitted to humans by cat fleas rather than ticks (Flexman et al 1995; Barrs et al 2010). Several *Bartonella* species have been reported in ticks and fleas collected from marsupial hosts, including brush-tailed bettong or woylie (*Bettongia penicillata*), western barred bandicoots (*Perameles bougainville*), yellow-footed antechinus (*Antichinus flavipes*), and Eastern grey kangaroos (*Macropus giganteus*), as well as from various rodents (Fournier et al 2007; Saisongkorh et al 2009; Kaewmongkol et al 2011), and the most frequent tick vectors were *Ixodes* spp., including *I. australiensis*, *I. tasmani*, and *I. myrmecobii*, but it is uncertain whether these species of *Bartonella* can cause human disease, or whether their tick vectors bite humans. There has been no record of co-infection of *Bartonella* species with *B. borgdorferi* s.l. overseas.**

Candidatus *Neoehrichia mikurensis*. Ca*. Neoehrlichia mikurensis* is a newly recognised human pathogen. Small Gram-negative obligate intracelluar cocci, they belong to the *Anaplasmataceae* and lack cross-reactivity with other genera in the family, such as *Anaplasma* and *Ehrlichia* (Kawahara et al 2004). First recognised as a human pathogen by Wellinder-Olsson et al (2010), they have been shown to cause human infection in China and found there in ticks and rodents (Li et al 2012), and co-infection of *I. ricinus* ticks in Sweden (Andersson et al 2013), Denmark (Fertner et al 2012), Switzerland (Maurer et al 2013), and the cause of human disease in Germany (von Loewenich et al 2010). Interestingly it has been shown to exist widely in China where it exhibits significant genetic diversity.

This organism has not been found in Australia, but it almost certainly hasn't been looked for at this stage.

***Ehrlichia*. *Ehrlichia* species have not been recognised in Australia, although *E. canis* is an infection found in dogs worldwide except Australia due to effective quarantine regulations (Irwin 2007), but it is not known whether any species occur in native wildlife.**

Francisella. **The first evidence of a *Francisella tularensis* subsp. *novicida* in Australia was its identification from an environmentally-acquired foot infection sustained in the Northern Territory (Whipp et al 2003). This low pathogenicity subspecies of *Francisella tualarensis* is relatively rare, and this represents the first time it has been found in the Southern Hemisphere. A second infection, a case of ulceroglandular tularemia due to *Francisella tularensis* subsp. *holarctica*, occurred in a woman bitten by a ringtail possum (*Pseudocheirus peregrinus*) in Tasmania, suggesting an ecological niche for this organism in the native forests of western Tasmania (Jackson et al 2012). The first evidence of *Francisella* species in ticks in Australia was obtained in the Northern Territory using DNA isolated from pools of *Amblyomma fimbriatum* hard ticks (Vilcins et al 2009). The 16S rRNA gene sequences obtained from the ticks indicated that the *Francisella***

species grouped phylogenetically with *Francisella*-like endosymbionts in a cluster separate to pathogenic and free-living *Francisella* species. There is no evidence to suggest that these organisms are pathogenic for humans.

Rickettsia. **Several rickettsial diseases occur in humans in Australia (Graves et al 2006), but not all are tick-borne. The tick-borne human pathogens are Queensland tick typhus (*Rickettsia australis*) transmitted by *I. holocyclus* and *I. tasmani*; Flinders Island spotted fever (*R. honei*) transmitted by the reptile tick *Aponema hydrosauri* and humans are probably accidental hosts (Stewart 1991); and a variant of the latter caused by *R. honei* strain *marmionii* or *R. marmionii* (Unsworth et al 2007) but the tick vectors of which remain to be determined, although one case in north Queensland was transmitted by *Haemophysalis novaeguineae*; and Q fever (*Coxiella burnetii*) which is carried by several tick species, but most human cases are acquired by aerosol. However, an early investigation demonstrated the presence of *C. burnetii* in *I. holocyclus* ticks (Smith 1942) collected from bandicoots (*Isoodon macrourus*) in southeastern Queensland. This tick represents a potential vector for the transmission of *C. burnetii* from natural hosts to domestic animals, livestock, and humans. Another tick species of importance as a reservoir for *C. burnetii*, *Amblyomma triguttatum*, is primarily found on macropodids, but is also promiscuous in host species and has a wide distribution across Australia (McDiarmid et al. 2000). More recently, Cooper et al (2013) found *C. burnetii* DNA in *I. holocyclus* ticks collected from the common northern bandicoot (*Isoodon macrourus*) and in *A. triguttatum* collected from the eastern grey kangaroo (*Macropus giganteus*). Thus although most human infections with Q fever are acquired by aerosol, the potential also exists for transmission from wildlife through a tick bite. Perhaps the most interesting of these tick-borne pathogens is *R. marmionii* which has an apparently wide distribution but may also be associated with occasional chronic diseases, including a chronic fatigue-like illness. Wildlife species harbour various Rickettsia, including *R. gravesii* sp. nov. BWI-1 transmitted in Western Australia by *Amblyomma triguttatam trigattatum* (Owen et al 2006; Li et al 2010), and rickettsial DNA most closely associated with *R. tamurae* from *Amblyomma fimbriatum* reptile ticks collected in the Northern Territory (Vilcins et al 2009).**

Viruses. A number of viruses belonging to different families and genera are transmitted by ticks and are important human pathogens. Perhaps the most relevant for this discussion are the tick-borne flaviruses including Tick-borne encephalitis virus, which is commonly found in *Ixodes ricinus* and other *Ixodes* spp. ticks in Eurasia from France to Japan (reviewed by Hubalek and Rudolf 2012), and frequently co-transmitted with *Borrelia* spp.; Powassan virus an occasional pathogen in the United States (Romero and Simonsen 2008), and Kyasanur forest disease virus in Karnataka State in India (Holbrook 2012).

Various viruses have been isolated from ticks in Australia and Australian territories, especially from seabird ticks, and from neighbouring countries of south-east Asia (Mackenzie and Williams 2009). Two flavivuses, Gadgets Gully and Samaurez Reef, have been described in Australia and Australian territories. Gadgets Gully has been isolated from *Ixodes uriae* ticks collected on Macquarie Island (St George et al., 1985; Major et al 2009). Ticks were collected from areas inhabited by Royal penguins (Eudyptes chrysolophus schlegeli), but no disease association with seabirds has been established (St George et al 1985). Antibodies to Gadgets Gully virus have been reported in human sera from residents of the Great Barrier Reef (Humphery-Smith et al 1991).

Samaurez Reef virus was isolated from *Ornithodoros capensis* seabird ticks collected from nests of various seabird species on coral cays off the east coast of Queensland, and from *Ixodes eudyptidis* ticks taken from two dead Silver gulls (*Larus novaehollandiae*) in northern Tasmania (St George et al 1977). This latter investigation was initiated following reports of febrile illness in meteorological workers operating on Saumerez Reef who had been bitten by ticks, but no association could be found. Experimental infection of Little blue penguins (*Eudyptula minor*) with Saumarez Reef virus resulted in a fatal infection (Morgan et al 1985). Thus neither of these two flaviviruses has been associated with human disease. St George et al (1985) also isolated a novel Bunyavirus in the Phlebivrus genus, Precarious Point virus. More recently, three other novel viruses have been reported from *I. uriae* ticks on Macquarie Island, an Orbivirus and two Bunyaviruses from the Phlebovirus and Nairovirus genera. The novel Orbivirus was isolated from ticks collected from the King penguin colony and given the name of Sandy Bay virus; the novel Nairovirus was also obtained from ticks associated the King penguin colony, and named Finch Creek virus; and the novel Phlobovirus isolate was isolated from ticks associated with the Rockhopper penguins and named Catch-me-cave virus. This latter virus was found to be related to but distinct from Precarious Point virus (Major et al 2009). None of these viruses have been associated with illness in the penguins nor is there any evidence that they are infectious to humans.

Thus the role, if any, that these seabird-associated tick-borne viruses play in human disease is unknown, except for the antibodies to Gadgets Gully virus in some residents of Great Barrier Reef islands.

Co-infection concerns. Co-infection between *B. burgdorferi* s.l. complex species and other tick-borne organisms may lead to different and varied clinical manifestations and different levels of disease severity (Belongia 2002;Swanson et al 2006; Moro et al 2006), and abnormal laboratory test results may be frequently observed (Swanson et al 2006). Indeed co-infections are very often under diagnosed, although they occur frequently. Concurrent infection should be considered in a patient with unusually severe or atypical features of Lyme disease (Marques 2010). Humans infected with Lyme disease and babesiosis appear to have more intense and prolonged symptoms than those with Lyme borreliosis alone (Swanson et al 2006). There are many examples of ticks carrying Lyme *Borrelia* together with one or more additional organisms, including *Anaplasma phagocytophilum* (Hildebrandt et al 2003; Nieto and Foley 2009; Soleng and Kjelland 2013), *Babesia microti* (Schulze et al 2013), *Bartonella henselae* (Mietz et al 2011), *Ehrichia* (Levin and Fish 2000; Stanczak et al 2002), *Babesia microti*, *Borrelia miyamoto*, and Powassan virus (Tokarz et al 2010). There are also examples of double infections with Lyme *Borrelia* and tick-borne encephalitis virus and other agents in patients (Arnez et al 2003; Broker 2012).

MAJOR GAPS IN OUR KNOWLEDGE OF LYME-LIKE DISEASE IN AUSTRALIA.

There are a number of major gaps in our knowledge of Lyme disease in Australia which need to be investigated as a consequence of this scoping study. The essential questions can be enumerated as follows:

1. Does *Borrelia burgdorferi* s.l. occur in Australian ticks, and especially in *I. holocyclus*?

2. Do other Australian tick species transmit Lyme borreliosis?

3. Can Australian ticks be infected with, maintain, and transmit *B. burgdorderi* s.l.?

4. Can we find better diagnostic tools to search for Lyme borreliosis?

5. Is there an indigenous species of *Borrelia* in Australia able to infect humans and to cause Lyme-like disease?

6. Do other possible pathogens occurring in Australian ticks cause Lyme-like disease?

7. Are there any relapsing fever group *Borrelia* species in Australia?

8. Can *B. burgdorferi* s.l. be detected with any certainty in EM rashes following a tick bite, as demonstrated by PCR and/or culture of biopsy specimens?

9. Is there an immune response to *B. burgdorferi* s.l. or to any other possible agent in the sera of patients presenting with Lyme-like disease?

10. Are there any *B. burgdorferi*-specific IgG antibodies in the sera of patients with chronic Lyme borreliosis?

11. If there is evidence found to indicate the presence of Lyme borreliosis in Australia, what is the geographic spread of cases?

The above topics are just some of the broad issues, and there are many additional queries that need to be addressed, but most will emerge naturally as the information on the major issues becomes clearer.

RESEARCH PROGRAMMES TO DETECT/CONFIRM/DISPROVE THE PRESENCE OF LYME BORRELIOSIS IN AUSTRALIA.

A number of areas need to be addressed to fill in the uncertainties and lack of evidence-based, scientific information about Lyme-like disease in Australia. In respect of this scoping study, it should be considered that 'the Jury is out' and it is in everyone's best interests to come to an evidence-based answer which fulfils the criteria of Lyme disease or otherwise. Two initial actions need to be stressed: all research carried out in the search for evidence of Lyme borreliosis, or with any other organism that may be associated with Lyme-like symptoms, must agree to (a) sharing of specimens

that are believed to be positive for Lyme disease, whether the specimens are clinical material such as serum or whether they are ticks; and (b) with the permission of the patient and the attending physician, to undertake confirmatory testing of any positive clinical specimens using a NATA-accredited laboratory.

The European experience may be the most useful in assessing the Australian situation. More pathogenic strains of *Borrelia* are found in Europe than in North America, and they have therefore extensive expertise in uncovering new *Borrelia* species. Greater involvement with European experts would be a valuable resource, and assistance should be sought through the European Centre for Disease Control (ECDC) in Stockholm. It would be helpful if a panel of reference sera and reference organisms could be obtained from an accredited European laboratory and kept by the major Australian NATA-accredited laboratories. It would also be preferable if an accredited European laboratory could undertake some confirmatory testing of putative positive specimens, at least in the short term.

In addition, it would seem to be eminently sensible to ensure that specified laboratories are selected to be reference laboratories for Lyme borreliosis, and the obvious two initially would be the Institue for Clinical Pathology and Medical Research at Westmead and Royal North Shore Hospital. In addition, there is a strong case for a reputable, independent private laboratory and the most relevant would be the Australian Rickettsial Reference Laboratory, Geelong. If the panel of reference sera and organisms are obtained from overseas, they should be distributed to the three reference laboratories. It might also be that a further reference laboratory be established in Western Australia at PathWest.

The most obvious question about Lyme disease in Australia is whether or not *B. burgdorferi* exists in Australia, either endemically or epidemically, and if the latter, whether it needs to be re-introduced to cause sporadic infections, or is there a novel indigenous species of *Borrelia* which causes Lyme-like disease with occasional instances of relapsing disease-like symptoms. All other questions relevant to the occurrence of Lyme-like disease or the development of chronic Lyme disease are dependent on this initial question. Thus it follows that most, but not all, research efforts should be directed towards providing an answer to this. It should be noted, however, that it is always much harder to prove a negative!

The major research programmes required to accomplish the terms of reference of this scoping study are enumerated below.

1. **Experimental programme to determine whether there is a *Borrelia* species in ticks in Australia causing Lyme-like disease, or whether another tick-borne pathogen is involved in human Lyme-like disease.**

There is no confirmed agent of Lyme borreliosis in Australia at this time, and although there have been positive and negative reports of *B. burgdorferi* s.l. strains in Australia, confirmed and sustainable isolates remain elusive. A broad and detailed investigation of ticks for *Borrelia* spp. and other pathogens needs to be the major initial focus area for research, and should be conducted in more than one laboratory. The closest potential vector in Australia is *I. holocyclus*, the paralysis tick, which is the most common tick found biting humans in the coastal fringe of eastern Australia, but in a single report was found not to be able to support and transmit a North American strain of *B.*

burgdorferi s.s., although this does not preclude this species being a transmitter of other *Borrelia* species. In Western Australia where cases of Lyme-like disease have also been reported*, I. holocyclus* does not occur, but several other ticks commonly bite humans and need to be investigated. Thus the single most important issue to be addressed is whether *Borrelia* strains exist in Australia which can cause Lyme disease, or whether other pathogenic organisms are responsible, including *B. miyamotoi* which can cause EM in some patients and relapsing episodes in others.

In North America and Europe, ticks infected with *B. burgdorferi* s.l. are full of spirochaetes which can readily be detected and/or visualised. This does not appear to be the case with Australian ticks, and it will be important to address the question of spirochaete carriage using more sensitive detection techniques, such as nested PCR or next generation sequencing, for example, 454 high throughput sequencing. Ticks should be collected from NSW in coastal regions and from the south-west of Western Australia where Lyme-like disease has been reported, and obtained by various means from different sources including veterinary clinics (for ticks taken from dogs); general practice clinics where ticks have been removed from patients; blanket sweeps for collecting ticks in suitable habitats; from small animals/wildlife, especially rodents and bandicoots (the probable natural host species), with assistance from ecologists and zoologists (using on-going small animal collection studies where possible), archival sources (various museums, Commonwealth Scientific and Industrial Research Organisation (CSIRO), and entomology groups at Australian universities). Although the primary tick focus should remain *I. holocyclus* in eastern Australia, other tick species should be considered including *Ambylomma* species and *Ornithodoros* species, whereas in Western Australia the focus should be on *I. austaliensis*, and *A. triguttam* ticks. It is envisaged that several groups would explore ticks for possible spirochaetes, but as mentioned above, it's essential that potentially positive material should be shared between the groups as *Borrelia* species are often difficult to isolate and maintain in culture.

If an indigenous *Borrelia* species exists in Australia and is responsible for the Lyme-like disease, it is quite possible that current methods, primers, and antigens will not pick up the novel genospecies if it is significantly different from other members of *B. burgdorferi* s.l., and it is essential that new, techniques be developed to detect *Borrelia* species using a variety of genomic methodologies. These may include a relatively simple approach using broader and less stringent primers designed to bind to highly conserved sequences, or primers for the *flaB* and *gyrB* (Takano et al 2010), PCR-restriction fragment length polymorphism based on the *flaB* gene (Wodecka 2011), or it might include more sophisticated high throughput sequencing (454 and/or MySeq) of pooled tick DNA following quantitative PCR for *Borrelia* 16S rRNA. This latter approach is currently being developed at Murdoch University (P Irwin, personal communication). Other new *Borrelia* species have recently been described (Takano et al 2010), and any new techniques should incorporate this new species.

While the initial search is for *Borrelia* species, it is essential that other pathogens are not neglected and *Anaplasma*, *Babesia*, *Bartonella*, *Ehrlichia*, *Francisella*, *Neoehrlichia*, *Rickettsia*, and viruses should be considered and included in the detection process, both as individual pathogens and as examples of increased pathogensis in co-transmission. Some of these may be less likely as pathogens as they are not normally found in Australia (eg. *Ehrlichia*), some have not been looked for previously (eg.*Neoehrlichia*), and some have not been found as in co-transmission with *Borrelia*, but are pathogens in ther own right (eg. *Bartonella*). The viruses are in a different category. No tick-borne viral pathogens have been reported previously, and the only viruses from ticks collected in Australia

or Australian territories are the two flaviviruses Samaurez Reef and Gadgets Gully from *I. uriae* on seabirds. Gadgets Gully is able to infect humans although no disease symtoms have been recognised (Humphery-Smith et al 1991). Only one other flavivirus found occasionally in ticks of relevance to Australia is West Nile virus, although the Kunjin clade of West Nile has not been reported in ticks. Of other virus groups, some Orbiviruses are found in ticks from Macquarie Island including Nugget virus, a member of the Kemorovo group (Gorman et al 1984), a Bunyavirus from the Nairovirus genus, Taggert virus, a member of the Sakelin virus group (Doherty et al 1975), as well as two recent isolates, Sandy Bay and Finch Creek viruses which are related to Nugget virus and Taggert virus respectively (Major et al 2009). Other Orbivuses have been isolated from mosquitoes and *Culicoides* in Australia, such as Wallal, Warrego and Wongurr viruses. Tick-borne virus isolates belonging to the Bunyavirus family, *Phlebovirus* genus, have also been found in ticks from Macquarie Island. Thus the potential of finding a virus in the ticks is relatively high.

2. **Are Australian ticks competent to maintain and transmit *B.burgdorferi* s.l. genospecies, or other Borrelia species associated with relapsing fever?**

It would be important to determine whether common Australian tick species known to bite humans are able to be infected with, maintain, and transmit *Borrelia* genospecies. Early work had demonstrated that *I. holocyclus* ticks were unable to transmit a specific North American strain of *B. burgdorferi* s.s. (Piesman and Stone 1991), but there is no information of the competence of this species of tick to transmit European *Borrelia* genospecies, particularly B. garinii which has been found in the Southern Hemsphere, nor of the competence of other important Australian tick species to transmit *Borrelia* species. Thus vector competence studies should be carried out with some urgency to investigate whether *I. holocyclus* is able to transmit a wide spectrum of *Borrelia burgdorferi* s.l. genospecies, starting with *B. garinii*, and whether other Australian ticks of the *Ixodes*, *Haemaphysalis*, *Ornithodoros* and *Amblyomma* genera are competent to transmit examples of the major *B. burgdorferi* s.l. genospecies, and the relapsing fever species, including the species transmitted by soft ticks, *B. duttoni*, *B. crocidurae*, *B. hermsii*, and *B. hispanica*, and those transmitted by Ixodes species, particularly *B. miyamotoi*.

3. **Do we have the best reagents for detecting novel *Borrelia* species, including *B. miyamotoi*, especially in clinical specimens?**

It is possible that the PCR primers and other commonly used reagents cannot detect an indigenous strain or genospecies of *Borrelia* either in the tick or in clinical material. The former were briefly discussed above for detection in ticks, but the alternative route to investigate the presence of novel *Borrelia* species would be in biopsy material. If current PCR primers are ineffective with novel species, new methods will have to be developed. This might include a variety of methods, including a nested PCR using a broadly based, low stringency initial primer followed by more specific second round primer pairs based on common genetic sequences from known genospecies, perhaps in the rRNA gene or the flagellin gene, or some other conserved genetic element. Primer sets are also needed to detect and identify relapsing fever *Borrelia* species and the hard tick-transmitted

relapsing fever-like species such as *B. miyamotoi*. Biopsy material might also be examined by immunofluorescent antibodies to expressed flagellin protein *flaB*, and to *ospA*, or C6 peptide.

In addition to new PCR primers, it is also important to develop and verify novel serological techniques to ensure highly specific, sensitive yet broadly based IgG and IgM antibody detection systems using expressed antigens for ELISA and other assay systems for detecting specific antibody, and immunological methods for detecting *Borrelia* species in biopsy material as an alternatve to genomic methods, such as immunofluorescent antibodies to expressed flagellin protein, ospA, or C6 peptide. Archival biopsy specimens are available at Royal North Shore Hospital, and sera and other specimens are at Royal North Shore and Westmead.

4. Clinical studies of patients presenting with symptoms suggestive of Lyme or Lyme-like disease.

The second strand of the research should be a prospective study directed at detecting *Borrelia* spp. or other pathogens in human cases presenting with Lyme-like symptoms. This would need to undertaken with the consent, support and assistance of General Practitioners who see many of the relevant patients, as well as the patients themselves, and undertaken as a collaborative study with infectious disease/clinical microbiologists who have a specific interest in this area. There should be two major thrusts in this strand of the research programme – one is the collection and testing of biopsy material from EM, and the other is collection of paired sera from patients for assay of borrelial antigens using the two-tier protocol. It would be preferable if EM biopsy specimens could be taken from both the central bite region (Mayne 2012) and from the periphery or leading edge (Berger et al 1992). Tissue would then be tested by real-time PCR and culture (Aguero-Rosenfeld et al 2005; Ivacic et al 2007; O'Rourke et al 2013), and possibly other tests such as specific immunofluorescence using reference antisera as determined by the clinician.

There seems little doubt that some tick bites result in skin eruptions at the site of the bite which look like a form of EM and that this may progress in some instances to disease symptoms that may be reminiscent of Lyme borreliosis. Bites from *I. holocyclus* ticks can result in an allergic response (Gauci et al 1989), and the site of the bite can be erythemic and sometimes mimic EM. If the EM is indeed caused by *Borrelia* species, it will develop about 48hr after the bite of the tick, however if it is an allergic reaction to the tick bite, it should fully resolve within 24-48hr.

Patients with later symtoms suspected of being possibly due to disseminated Lyme borreliosis such as arthritis or neuroborreliosis, some of whom may not have had EM or instead had an atypical rash, should be tested using standard techniques, including culture, immunodiagnosis, and/or PCR of synovial fluids (eg.Nocton et al 1994; Priem et al 1998; Ivacic et al 2007; Li et al 2011) and for CSF (Skogman 2008; Cerar et al 2010), within the accepted guidelines (Mygland et al 2010). If patients present with repetitive episodes of sudden fever, myalgia, headache and nausea, relapsing fever should be considered, and although there is no evidence of relapsing fever group *Borrelia* species in Australia, the possibility of their actual presence should not be ignored both with respect to the normal relapsing species of *Borrelia*, but also *B. miyamotoi*.

5. Retrospective investigation of chronic cases of Lyme borreliosis.

As described in the Background review, this scoping study suggests that 'the jury is out' when considering the contentious issue of chronic Lyme borreliosis. However, it is in everyone's interest to attempt to verify the diagnosis of Lyme borreliosis in these cases, not least for the patients themselves, and thus retrospective studies are recommended. It is suggested that this be done in two distinct series of studies; the first seeking evidence of past infection with *B. burgdorferi* s.l., and the other reviewing the clinical case histories of selected cases to gain greater insight into the diagnoses. In both instances, it is essential that patients are willing to be included and fully aware of rationale of the studies, that General Practitioners caring for the patients are comfortable with the study protocols and agree to be part of the study team, and that the studies meet all human ethical requirements.

The study seeking evidence of past infections with *B. burgdorferi* s.l. should be undertaken by serological tests for IgG to *Borrelia* antigens. To provide a broad, strong result, this should be done with a 2-tier approach.

For the study seeking a better understanding of the background diagnoses, it is recommended that clinical case history notes be assembled anonymously and reviewed by a panel of infectious disease experts from within Australia and overseas.

An invitation to bring an acknowledged international expert to Australia would be an extremely useful avenue to assist in assessing projects in topics recommended above, but more importantly could be part of an international Lyme and Lyme-like diseases symposium under the auspices of a local partner organisation, such as the annual Communicable Diseases Conference, or with the Australian Society for Infectious Diseases (ASID), the Australian Society for Microbiology (ASM), or the Royal College of Pathologists of Australia annual meeting. An acknowledged expert in Lyme diseases and *Borrelia* ecology could also be asked to give a series of public lectures.

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