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Is there a Lyme-like disease in Australia? Summary of the findings to date

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ABSTRACT

Lyme Borreliosis is a common tick-borne disease of the northern hemisphere caused by the spirochaetes of the *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s. l.) complex. It results in multi-organ disease with arthritic, cardiac, neurological and dermatological manifestations. In the last twenty-five years there have been over 500 reports of an Australian Lyme-like syndrome in the scientific literature. However, the diagnoses of Lyme Borreliosis made in these cases have been primarily by clinical presentation and laboratory results of tentative reliability and the true cause of these illnesses remains unknown. A number of animals have been introduced to Australia that may act as *B. burgdorferi* s. l. reservoirs in Lyme-endemic countries, and there are some Australian *Ixodes* spp. and *Haemaphysalis* spp. ticks whose geographical distribution matches that of the Australian Lyme-like cases. Four published studies have searched for *Borrelia* in Australian ticks, with contradicting results. The cause of the potential Lyme-like disease in Australia remains to be defined. The evidence to date as to whether these illnesses are caused by a *Borrelia* species, another tick borne pathogen or are due to a novel or unrelated aetiology is summarised in this review.

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1. Introduction

Lyme Borreliosis is a common tick-borne disease of the northern hemisphere. It is caused by spirochaetes of the *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s. l.) complex. Typically, the disease first presents with an erythema migrans rash at the site of the tick bite, followed by flu-like symptoms and later by debilitating arthritic, dermatological and neurological manifestations. The bacteria are transmitted by *Ixodes* species ticks, although other Ixodidae ticks [1–4] and haematophagous arthropods [2,4–14] have been implicated in carrying the bacteria. Bacterial reservoirs of the disease are usually small mammals, birds and occasionally reptiles [15,16]. The presence or absence of Lyme disease (or a Lyme-like disease) in Australia remains a contentious issue with varying opinions being held by medical practitioners, scientists and lay stake holders while the aetiological agent remains undetermined.

In response to the continued controversy and media attention regarding the possibility of there being Lyme Borreliosis in Australia, the Australian Government Chief Medical Officer, Professor Chris Baggoley, established the Clinical Advisory Committee on Lyme Disease (CACLD) in 2013 [17]. The purpose of this committee was to advise the Chief Medical Officer on the following points:

1. The extent to which there is evidence of *Borrelia* species causing illness in humans in Australia
2. The most appropriate laboratory diagnostic testing algorithms (best world practice) for persons who have suspected Borreliosis in Australia
3. The most appropriate treatments for Borreliosis in Australia
4. The most appropriate ways to disseminate information to health professionals and the general public on Borreliosis/Lyme disease
5. The requirements for further research into Borreliosis in Australia, and the generation of appropriate new questions relevant to the terms of reference.

Furthermore, the Australian Government Department of Health commissioned a scoping study [18] to identify the gaps in scientific evidence surrounding the causative agent of the Australian Lyme-like disease. Subsequently, upon advice from the CACLD, the Australian public was called upon to review and contribute to the scoping study, and 36 submissions were obtained in total. All points raised were considered individually and then collated, culminating in the following twelve considerations [18]:

1. Does *B. burgdorferi* s. l. occur in Australian ticks, and especially in *Ixodes 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 able to cause a Lyme disease-like syndrome?
6. Do other possible pathogens occurring in Australian ticks cause a Lyme disease-like syndrome?
7. Are there any relapsing fever group *Borrelia* species in Australia?
8. Can *B. burgdorferi* s. l. be detected with any certainty in erythema migrans 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 a Lyme disease-like syndrome?
10. Are there any *B. burgdorferi*-specific IgG antibodies in the sera of patients with Lyme disease-like syndrome?
11. If there is evidence found to indicate the presence of Lyme Borreliosis or a Lyme disease-like syndrome in Australia, what is the geographic spread of cases?
12. Are there other potential vectors that could transmit *Borrelia* in Australia?

Further to the above identified knowledge gaps, during the course of this literature review, the authors will consider two further points of investigation:

1. Could native Australian animals act as reservoirs of *B. burgdorferi* s. l.?
2. Could introduced animals such as foxes, hares, placental mice and rats act as reservoirs of *B. burgdorferi* s. l. in Australia?

The purpose of this review is to assess the current situation of the controversial Lyme or Lyme-like illness reported by some to be present in Australia. The existing evidence is explored and areas require further investigation are identified. Alternative infectious and non-infectious diagnoses are also considered.

2. Potential reservoirs of Lyme Borreliosis-causing *Borrelia* species in Australia

If a *Borrelia* causing a Lyme-like disease is present in Australia, importation or native evolution are both possible origins of the causative agent. Such an agent might be a known *Borrelia* species or a novel, as yet undescribed microbial pathogen.

2.1. Borrelia in introduced animals

In the 1900s, two species of *Borrelia* were introduced to Australia via the agricultural industry. These were *Borrelia theileri*, the worldwide cause of bovine Borreliosis [19], and *Borrelia anserina*, the worldwide agent of avian spirochaetosis [20]. *B. theileri* has been reported in cattle of Queensland and New South Wales [21–23] and *B. anserina* has infected poultry of Victoria and the Northern Territory [23–25]. *B. theileri* is transmitted in Australia by the cattle tick *Rhipicephalus* (*Boophilus*) *australis* [21,26] while the vector of *B. anserina* is *Argas persicus* s. l. [27]. *Argas persicus* ticks have been observed in all states of Australia except for Tasmania, and *R. australis* is distributed along the northern and eastern coasts of Australia [26]. *R. australis* may occasionally bite humans [26]. Neither *B. anserina* nor *B. theileri* belong to the *B. burgdorferi* s. l. complex, nor have they ever been described as causing a Lyme-like illness in humans.

If Lyme Borreliosis was present in Australia, it is reasonable to expect that its presence would be prominent in livestock, domestic animals and particularly feral deer, as is the case with Lyme Borreliosis in the northern hemisphere. However, very few cases of a Lyme-like illness in Australian animals are present in the veterinary literature. Lyme Borreliosis was reported in two cows at Camden, New South Wales in 1989 [28]. These cows were previously infested with *Haemaphysalis longicornis* (see Section 2.5) and presented with fever, anaemia, poor condition and polyarthritis. The diagnosis of Lyme Borreliosis was made in the first cow on the presence of spirochaetes in the synovial stroma and the second by positive IFA Lyme serology. However, from the images of spirochaetes from the first case described in the paper it is unclear if these represent true spirochaetes or artefact. Ephemeral fever, chlamydiosis, *Mycoplasma bovis* and "other septicaemic bacteria" were ruled out in the cows, but it is unspecified if *B. theileri* was one of

the septicaemic bacteria considered. *B. theileri* antibodies may cross-react with *B. burgdorferi* s. l. [29]. This agent can cause fever and anaemia, although is not associated with polyarthritis [30–33]. Conversely, polyarthritis has been associated with *B. burgdorferi* s. l. in animals in the northern hemisphere [30]. However, true Lyme Borreliosis was not confirmed by the diagnostic techniques performed in these cases, and it is possible that these cases were *B. theileri* infection. Overall, the relative absence of reports of veterinary cases of Lyme or a Lyme-like disease in Australia suggests the absence of traditional Lyme Borreliosis causing agents in the country.

2.2. Borrelia in native animals

Reports of "*Borrelia* species" in Australian native animals appear to be localised to Queensland. In Brisbane, spirochaetes observed in blood films of bandicoots, and in western Queensland spirochaetes were seen in blood films of kangaroos were both identified as a novel *Borrelia* species [34]. The identification of these *Borrelia* to the species level was not determined, and their place in the phylogeny of the *Borrelia* genus remains unknown. Molecular characterisation methods were not available at the time and morphological appearance alone was used to classify these into the *Borreli*a genus. Due to their presence in blood films, it is hypothesized that these spirochaetes were likely to be relapsing fever *Borrelia* rather than Lyme-causing *Borrelia*, since the latter generally have a much lower spirochaetal load in the bloodstream than the former. Once again, modern phylogenetic analysis techniques that would have definitively placed these spirochaetes into Lyme-causing or relapsing fever *Borrelia*, or another genera of spirochaete altogether were not available to confirm these diagnoses. In 1956, Pope and Carley isolated a spirochaetes from one native rat (*Rattus villosissmus*) out of twenty-seven dead and dying rats tested in Richmond, north-western Queensland [35] and named it *Borrelia queenslandica* [36]. Attempts to infect a human volunteer with this spirochaetes were unsuccessful [37] and attempts to transmit the spirochaete from mouse to mouse via the Argasid tick *Ornithodorus gurneyi* were also unsuccessful [38]. Due to loss of all isolates, whether *B. queenslandica* is a part of the *B. burgdorferi* s. l., a relapsing fever group or another genus of spirochaete cannot now be determined. However, the lack of pathogenicity in the human volunteer are counterindicative of this organism being the causative agent of the Australian Lyme-like disease considered in this paper.

2.3. Spread of Borrelia by migratory birds

Birds play an important role in the perpetuation of ticks and *B. burgdorferi* s. l. in North America [39,40], Europe and Asia [41–43]. More significantly there is evidence that bird migration results in a wider dispersion of Lyme-causing *Borrelia*. Examples of this include the detection of *B. burgdorferi* s. l. in migratory songbirds across Canada [40] and the transport of *Borrelia garinii* via birds migrating from mainland Asia to Japan [42]. Most relevant to the Australian situation is the worldwide dispersal of seabird species and the seabird tick *Ixodes uriae* [44–46]. *B. garinii*, a species known to cause Lyme Borreliosis, has been detected in *I. uriae* not just in the northern hemisphere [46,47], but also in southern hemisphere locations including Campbell Island off New Zealand, the Crozet Islands in the southern Indian Ocean and the Falkland Islands off South America [46]. This transhemispheric dispersal of *B. garinii* may be not just due to the spread of infected ticks, but also by seabirds acting as *B. garinii* reservoirs. However, the theoretical spread of *B. garinii* from seabirds to humans and even other birds and mammals, is unlikely, as generally the seabirds and their ticks are restricted to the open sea, remote islands, and peninsulas where contact with other animals is rare [46]. The ticks of seabirds along the Australian coast have not to date been investigated for *Borrelia*.

2.4. Introduced animals identified as Lyme reservoirs overseas

A number of non-native mammals have been introduced to mainland Australia since its settlement [48], some of which are known reservoirs of *B. burgdorferi* s. l. in the northern hemisphere.

Several introduced animals found in Australia, including the black rat (*Rattus rattus*), the house mouse (*Mus musculus*), the brown hare (*Lepus europaeus*), several species of deer and to lesser extents the red fox (*Vulpes vulpes*) and the Norwegian rat (*Rattus norvegicus*) are known to be reservoirs of *B. burgdorferi* s. l. in the northern hemisphere. Most of these animals have established widespread populations in Australia since their introduction, excepting the Norwegian rat, which has established a localised population only [48]. In Australia, *R. rattus and R. norvegicus* in Australia are parasitised by *I. holocyclus* and *Ixodes tasmani* ticks [26,49], *M. musculus* is parasitized by *I. tasmani*, and *L. europaeus* is parasitized by *H. longicornis* [26]. No studies on the ticks commonly parasitising *L. europaeus* in Australia have been performed, but these hares tend to occur in open grassland, which is not a preferred habitat of *Ixodes* ticks. To date, no investigations have been conducted into the presence or absence of *B. burgdorferi* s. l. in introduced undomesticated animals of Australia.

2.5. Likely tick vectors of B. burgdorferi s. l. in Australia

Overall very little evidence exists of the transmission of a potential Lyme-like disease by Australian ticks. It is hypothesised that if ticks are transmitting *B. burgdorferi* s. l. in Australia, the tick species would parasitize a number of hosts including humans, and would likely (but not necessarily) be of the *Ixodes* genus, as this is the genus that transmits Lyme Borreliosis in the northern hemisphere. The following information is intended only to identify the need for further research in testing for the presence of *B. burgdorferi* s. l. in wild populations of these ticks, and if *B. burgdorferi* s. l. is present, their transmission competency.

In the northern hemisphere, the Lyme-causing *Borreliae* are transmitted mainly by *Ixodes* species ticks. Nineteen species of *Ixodes* have been described in Australia [49], many of which have only a small geographical distribution (e.g. *Ixodes vestitus* and *Ixodes myrmecobii* are localised to Western Australia) or a limited host range (e.g. *Ixodes vespertillionis* is confined to bats and *Ixodes ornithorhynchi* to the platypus) [49]. It should be noted that the main ticks that transmit Lyme Borreliosis in the northern hemisphere are the black-legged ticks (the *ricinus* complex [50]) and that there are none of this group found in Australia. *I. holocyclus* and *I. tasmani* appear to have the widest geographical spread of the Australian *Ixodes* species while also having a large range of potential hosts. Furthermore, *I. myrmecobii* occurs in WA and belongs to the same subgenus (*Sternalixodes*) as *I. holocyclus* [51].

I. tasmani is the most abundant species of tick in Tasmania but is also found throughout Victoria, along the coastal and sub coastal areas in New South Wales and Queensland and in parts of southeast South Australia and southwest Western Australia [26]. *I. tasmani* has a broad range of hosts, but rarely bite humans, making it a candidate as a tick-borne disease reservoir and bridge vector of any putative tick borne Lyme-like agent in Australia, but an unlikely candidate vector to humans. Examples of hosts it parasitises include possums, bandicoots, wallabies, native rats, introduced rats, dogs, cats, horses and humans. No work has been published regarding the potential vector competence of *I. tasmani* for *B. burgdorferi* s. l.

I. holocyclus, colloquially known as the "paralysis tick", has an extensive host range including, but not limited to, domestic animals such as cats, dogs, chickens and other fowl, ducks and man [26]. Native animal hosts include wallabies, kangaroos, bandicoots, possums and dingoes [26]. *I. holocyclus* is distributed along coastal areas of northern and eastern coasts of Queensland and New South Wales, Victoria and Tasmania. In southern Queensland and northern New South Wales its range also extends somewhat further inland [49]. This geographic distribution coincides with that of the Lyme-like disease cases reported in the scientific

literature (Fig. 1). Although there are anecdotal reports of a Lyme-like illness being present in Western Australia, outside of the range of *I. holocyclus*, no cases have been published in the scientific literature. However, in a vector competence experiment, *I. holocyclus* was able to ingest but not transmit the JD1 strain of *B. burgdorferi* s. s. [52]. Whilst this finding does not preclude the capacity of *I. holocyclus* to transmit other *B. burgdorferi* s. l. species or strains, it does infer a likelihood of poor vector competence for this species.

In the northern hemisphere, *B. burgdorferi* s. l. has also been detected in Ixodidae (hard tick) of genera other than *Ixodes* [1–4] and even in haematophagous arthropods including lice [2], fleas [4,5], keds [2,6], mites [7–9], flies [10–12] and mosquitoes [9,13,14]. While the transmission capability of these arthropods remains undetermined, it does raise the possibility of Lyme transmission by arthropods other than *Ixodes*. In Australia, other genera of hard ticks include *Amblyomma*, *Haemophysalis*, *Bothriocroton* and *Rhipicephalus*. These vary in their distribution and host range depending on the species. The "bush tick" *H. longicornis* is a native of the south-east coast of Russia, North and South Korea, Japan and China [26] and is believed to have been introduced to Australia in the nineteenth century from Japan [63]. In Australia, *H. longicornis* occupies a large coastline area spanning from central Queensland to south-eastern Victoria but is found especially in Kempsey, New South Wales [26]. A very small area in the south-western corner of Western Australia also contains *H. longicornis* [64]. Similar to *I. holocyclus*, *H. longicornis* parasitises a large number of hosts in Australia including but not limited to cattle, sheep, horses, dogs, cats, hares, domestic fowls, Australian magpies and marsupials [26], but only rarely bites humans [26]. The detection of *B. burgdorferi* s. l. in *H. longicornis* ticks of Japan [65] and China [3] supports the possibility that *H. longicornis* could carry Lyme Borreliosis in Australia.

2.6. Studies investigating Borrelia in Australian ticks

Four studies have been published that investigated the potential for *B. burgdorferi* s. l. in ticks, both employing culture with or without PCR and in the most recent studies, next generation sequencing.

2.6.1. Wills and Barry 1991

Wills and Barry [66] published preliminary results of their investigations into the presence of *Borrelia* in Australian ticks in a letter to the editor of *The Medical Journal of Australia* in 1991. One-hundred and sixty-seven ticks consisting of *I. holocyclus* and *H. longicornis* were collected from the Hunter Valley and Manning River districts of coastal New South Wales and their midguts were cultured in BSK-II media. Within 8 weeks incubation, motile, rigid spirochaete-like objects (SLOs) were observed in 44% of their *I. holocyclus* cultures and 35% of their *H. longicornis* cultures; a total of 70 Australian tick midgut positive cultures out of 167 cultured. The individual number of *I. holocyclus* midguts and *H. longicornis* midguts cultured was not specified. The presumptive spirochaetes were described as "large, coiled motile bacteria with an irregular rotational movement" and were "morphologically indistinguishable" to the reference strain *B. burgdorferi* (B31). At least four of the spirochaetes isolated shared antigenic epitopes with *B. burgdorferi* as demonstrated by ELISA, immunofluorescence and western blotting, suggestive of *Borrelia* species. However, details of the laboratory methods are not published and the organisms recovered were not made available for confirmation by another laboratory, rendering the experiment unable to be replicated. False positives in the ELISA, immunofluorescence and western blotting cannot be ruled out. No PCR or sequencing has been conducted to confirm the identity of the isolates, and positive *Borrelia* cultures from Australian tick samples have not been reproduced to date. Although this investigation was conducted as a part of the author's (Wills') PhD, no follow-up report to these preliminary findings was ever published in the scientific literature.

Fig. 1. Locations of Australian Lyme-like cases published in the scientific literature. Specific location based on town, suburb or GPS coordinates. Approximate location based on broad location description, e.g. "rural Victoria" or "Hunter Valley".

Alleged L-form variant "rigid" SLOs of *Borrelia* have been described in other papers, from cultured biopsy and synovial fluid samples from Lyme Borreliosis patients [67,68], in animal skin sample cultures [69] and in control *Borrelia* cultures subjected to antispirochaetal agents [70,71]. However, the SLOs in contaminated cultures observed under electron microscopy have been identified by some researchers [69,72,73] as large flagella aggregates from the contaminating bacteria, and therefore not indicative of the presence of *Borrelia* spirochaetes. Furthermore, cultures of *Bacillus* have been identified as capable of producing these structures [74]. It is possible that this is what was observed in the cultures conducted by Wills and Barry [66]. However, this does not explain the return of such rigid SLOs to normal, motile spirochaetes after multiple subcultures [66,67]. SLOs in uncontaminated cultures have been observed by others and can be explained by the flagella passing though filters that block whole bacteria and the flagella then coalesce to form the long SLOs (Doggett, S. pers. comm. 2016). The use of molecular techniques, especially sequencing, would be ideal for confirmation or dismissal of any cultured SLOs as *Borrelia*.

2.6.2. Russell et al. 1994

A comprehensive search for *Borrelia* in Australia conducted by Russell et al. [73] contradicted the findings of Wills and Barry [66]. Approximately 12,000 ticks were collected over three years along the New South Wales coast. Ticks were collected from natural habitats and removed from native and domestic animals, although the animal species are not disclosed. The majority of tick species collected were *I. holocyclus* (7922) followed by *H. longicornis* (2208) and *Haemaphysalis bancrofti* (1092). The remaining 786 ticks consisted of nine other species. Tick midguts were cultured in BSK-II media and screened by dark-field microscopy, although factors including nutritional media components, chemical and physical culture conditions were adjusted in an unspecified number of cultures. Ninety-two cultures of bloodfed ticks revealed SLOs. These SLOs were straight, rigid and uniformly coiled and non-motile and later determined to be bacterial flagella aggregates by electron microscopy. The authors describe "a few" of the 18 SLOs as having tested positive using polyclonal *B. burgdorferi* s. s. antibodies, though none reacted with monoclonal *B. burgdorferi* s. s. antibodies. The study found "no definitive evidence for the existence in Australia of *B. burgdorferi* the causative agent of true Lyme Borreliosis, or for any other tick-borne spirochaete that may be responsible for a local syndrome being reported as Lyme disease". The authors observed Wills' and Barry's [66] cultured *Borrelia* and found them to be identical

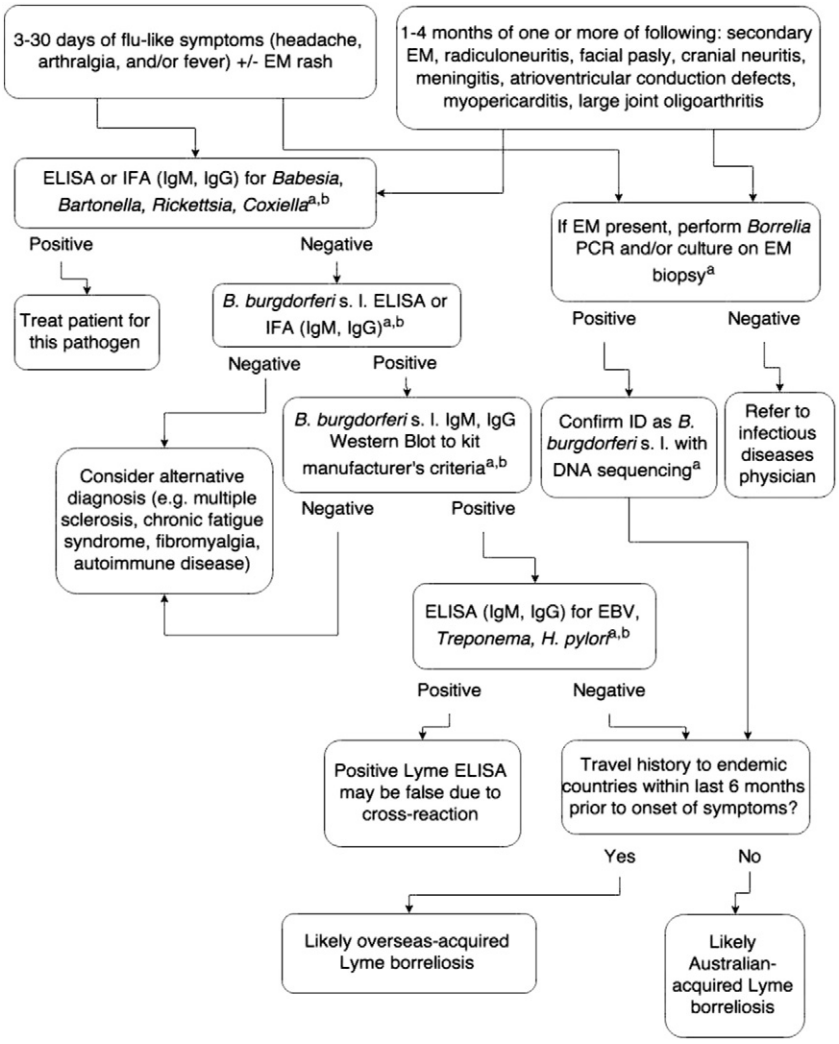

Fig. 2. Assessing the cause of a patient's Lyme-like disease. [a] Perform only in NATA-accredited laboratory. [b] Paired serum testing must be performed. Only consider positive if there is a 4-fold rise in titre, or seroconversion is observed. Positive results without 4-fold rise or seroconversion only indicate past exposure and not current infection. A third serum sample may be required if equivocal. EM, erythema migrans; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; and EBV, Epstein-Barr virus.

to their own SLOs, concluding that Wills' and Barry's cultured SLOs were also contaminant flagella aggregates. Russell et al. also had the advantages of *Borrelia* genus-specific PCR and a much larger sample size over Wills' and Barry's study. The conclusion of Russell et al.'s study – that no spirochaetes were able to be identified through culture or molecular methods in Australian ticks – therefore seems more plausible than the conclusions of Wills and Barry.

2.6.3. Gofton et al. 2015a

A recent study by Gofton et al. found no *B. burgdorferi* s. l. in Australian *I. holocyclus* ticks, but did detect a novel relapsing fever group *Borrelia* [75]. This study tested 109 *I. holocyclus* from around New South Wales, collected over a ten year period. DNA extracted from these ticks was subjected to next generation sequencing to determine the bacteriome of the ticks. Thirty *Ixodes ricinus* ticks collected in Germany were included for comparative purposes. Whilst *B. burgdorferi* s. l. sequences were not recovered from any Australian *I. holocyclus* ticks, nine (30%) of the German *I. ricinus* samples yielded 16SrRNA sequences homologous to either *B. burgdorferi* s. s. or *Borrelia afzelii* [75]. A single Australian *I. holocyclus* taken from an echidna yielded 16SrRNA sequences of an unknown *Borrelia* species, clustering within the relapsing fever group and not the *B. burgdorferi* s. l. group of Borreliae [75].

This work provides further evidence that the cause of the Lyme-like illness in Australia may not be a member of the *B. burgdorferi* s. l. complex. The finding of a novel relapsing fever *Borrelia* in an Australian monotreme does provide evidence for the presence of Borreliae in Australia, but it is not known if this organism can infect humans, and should it do so, it is likely that it would present as a relapsing fever illness rather than with Lyme-like symptoms. These factors limit the likelihood that this novel *Borrelia* species is the cause of the Lyme-like illnesses seen in Australia. The study was limited by the relatively low number of ticks sampled and the limited geographic range from which they were collected. No data was presented regarding the distribution of collection sites (urban, rural or wilderness) within that state.

2.6.4. Gofton et al. 2015b

In the above study, only one species of tick, *I. holocyclus*, was õsampled in this study [76]. Although it is assumed that this is the most likely vector candidate in Australia by many researchers, as noted in Section 2.5 of this review, this species has been shown not to be able to transmit *B. burgdorferi* s. s. in vector competence studies. *H. longicornis*, with its wider geographic range and known competence as a vector of Lyme-causing *Borrelia* in Japan, would be a superior candidate for potential *B. burgdorferi* s. l. transmission in Australia, except that it very rarely bites humans. Further work using the same protocol on a

larger cohort of ticks, from an Australia-wide catchment and including other tick species (particularly *H. longicornis*) is warranted. Gofton et al. addressed this requirement in a recently published study of 460 ticks collected from below the line of the tropic of Capricorn in Western Australia and the seaboard Eastern Australia (though one from inland Queensland was included). The ticks were identified as being 279 *I. holocylcus*, 167 *Amblyomma triguttatum*, seven *H. bancrofti* and a further seven *H. longicornis*. Midguts of all ticks were subjected to 16s ribosomal RNA PCR and next generation sequencing. A *Borrelia* genus specific *flaB* nested PCR was also performed on all ticks recovered. None of the ticks concerned yielded any *Borrelia* sequences or PCR products [76].

3. Relevance of diagnostic techniques to Australia

3.1. Diagnosis in the endemic setting

In the Lyme Borreliosis endemic United States of America (USA), serology for Lyme Borreliosis is the diagnostic technique recommended by the Centers for Disease Control and Prevention (CDC) [54]. Serology is conducted by a two tiered approach: firstly, an enzyme-linked immunosorbent assay (ELISA) or immunofluorescence antibody (IFA) test is performed, and if positive, this is followed secondly by an immunoblot. The ELISA or IFA tests may give false-positive reactions in the presence of other infectious, autoimmune or inflammatory conditions [53,54]. Similarly, not performing the ELISA or IFA step will increase the likelihood of false positives in the immunoblot [57].

The interpretation of the immunoblot depends on the number of bands present. In the USA, where *B. burgdorferi* sensu stricto (*B. burgdorferi* s. s.) is the only causative agent of Lyme Borreliosis, the following criteria are required for diagnosis: An IgM immunoblot is positive if two of the three bands are present: 24 kDa (OspC), 39 kDa (BmpA), and 41 kDa (Fla) [56]. An IgG immunoblot is considered positive if five of the following 10 bands are present: 18 kDa, 21 kDa (OspC), 28 kDa, 30 kDa, 39 kDa (BmpA), 41 kDa (Fla), 45 kDa, 58 kDa (not GroEL), 66 kDa, and 93 kDa [55]. In patients with acute Lyme Borreliosis (less than 30 days) within the USA, the IgM blot has a sensitivity of 58.5% and specificity of 92% to 94% [56]. In patients greater than 30 days after initial infection, the IgG blot has a sensitivity of 83% and specificity of 95% [55].

In Europe and Asia there are a greater number of *B. burgdorferi* s. l. species and strains that cause Lyme Borreliosis than there are in the United States [60,61]. Different strains of *B. burgdorferi* s. l. may express only some of the antigens detected in immunoblot, may constitutionally lack certain genes for certain proteins, or comprise immunodominant antigens of molecular weights that differ from those typically used in the immunoblot. For these reasons, the immunoblot interpretation using a method developed at one geographic area may not be applicable to other geographic areas. Consequently, standardisation of immunoblotting methods for Lyme Borreliosis diagnosis in Europe and Asia is unfeasible [60,61]. A number of commercial immunoblot kits and interpretative criteria are available with varying specificity and sensitivity [62].

3.2. Confounding factors in serological diagnosis in the non-endemic setting

The CDC diagnostic serological method used for *B. burgdorferi* s. s. is inappropriate for use in the Australian context except for patients with a travel history to endemic countries [59]. It is possible that any theoretical Australian *B. burgdorferi* s. l. species would cause a different serological response in a Lyme Borreliosis patient than the American, Asian or European species. Such antigenic differences could result in false negative serology results. It has been shown that chronic Lyme Borreliosis patients may test seronegative even if they are PCR confirmed or culture confirmed to be infected by *B. burgdorferi* s. l. [58,77]. This does not necessarily mean that these patients lack an antibody response, but rather the banding pattern in an immunoblot is merely different to that of the standard diagnostic criteria [78]. This must be considered in regard to almost all of the purported Lyme-like illness cases seen in Australia, which almost exclusively [79–82] present with clinical symptoms correlating to the late (greater than 30 days duration) stage of Lyme Borreliosis.

It is important to consider that in areas not endemic for Lyme Borreliosis, the positive predictive value of the serology test will be low [59]. In endemic areas, patients with other illness and even healthy donors may display at least 5 of the 10 bands required for a positive anti-*B. burgdorferi* IgG western blot result [56]. Furthermore, in the non-endemic setting of Papua New Guinea, 50% of 84 individuals screened for Lyme Borreliosis fitted the CDC serological criteria for Lyme Borreliosis [83]. Further testing of these samples for antibodies to *Treponema pallidum* by microhaemagglutination assay, rapid plasma reagin test, fluorescent treponemal antibody-absorption test, and Western blot all yielded negative results. The pattern of IgG bands seen differed from controls with confirmed Lyme Borreliosis and none of the patient sera inhibited the growth of *B. burgdorferi in vitro*, whilst 69% of Lyme patient sera will do so [83]. It was thought that the false positive Lyme serology results were the consequence of high levels of immunoglobulin or cross-reactive antibodies residents of tropical regions [83]. It is possible this same phenomenon may occur in Australia. While the causative agent of the putative Lyme-like disease remains unknown, any positive or negative Lyme serology results are unreliable.

3.3. The RCPA protocol for the diagnosis of Lyme Borreliosis in Australian patients

The many confounding factors influencing Lyme Borreliosis diagnosis in Australia led to the release in 2014 by the Royal College of Pathologists of Australasia (RCPA) of a position statement on the diagnostic laboratory testing for Lyme Borreliosis [59]. This position statement sought to address misinformation regarding the Lyme Borreliosis in Australia and to provide guidance to clinicians in regard to ordering tests for the diagnosis of potential Lyme Borreliosis cases. This very balanced statement noted that Australia was amongst several countries in which the presence of local Lyme Borreliosis had not been confirmed. It outlined the expected clinical symptoms of a patient with Lyme Borreliosis, summarised the diagnostic difficulties in inherent in laboratory diagnosis, particularly the potential for false positive results in low or zero prevalence such as Australia. The position statement also made several recommendations for laboratory investigation of suspected Lyme Borreliosis cases in Australian patients [59]. The Lyme Disease Association of Australia put out its own position statement which was critical of the RCPA's, however it is interesting to note that they too are now labelling this disease as Lyme-like [156].

It was recommended in the RCPA's position statement that serological diagnosis of Lyme Borreliosis in Australia should consist of an EIA followed by a confirmatory western blot. It is noted that Australian reference laboratories can effectively diagnose Lyme Borreliosis in affected patients who have returned from a known Lyme endemic area who contracted the infection over four weeks previously. Laboratory tests with unconfirmed efficacy of diagnosis, such as measurement of CD57 lymphocyte counts and PCR on urine for the detection of *B. burgdorferi* s. l. DNA, were not indicated as relevant to the diagnosis of Lyme Borreliosis. Importantly, the report recommended that testing should only be performed in NATA/RCPA accredited laboratories, and patients and their doctors were advised to exercise caution in the interpretation of result from non-accredited laboratories in Australia and overseas that have not been validated to diagnose Lyme Borreliosis based upon international consensus documents. The position statement did allow for the culture and PCR of erythema migrans-type rash biopsies collected by interested doctors from patients with no travel history outside Australia for research purposes [59]. The authors of this review encourage such testing (see Fig. 2), as it will allow the collection of data,

specimens and (potentially) cultures that may assist in the elucidation of the cause of the Lyme-like illness reported in Australia. If no infectious agents were recovered, over time and with sufficient specimen numbers, a large body of negative evidence by molecular and phenotypic methods from such testing would almost definitively exclude *B. burgdorferi* s. l. as the cause of this illness in Australia.

4. Lyme-like case reported in Australia

A literature search for Australian Lyme-like cases was performed using the Google scholar search bar at https://scholar.google.com.au/ and the PubMed Advanced Search Builder at http://www.ncbi.nlm.nih.gov/pubmed/advanced. A boolean search involving "Lyme", "Disease" and "Australia" was used. The search was limited to Academic Journals only and no time frame was set for the search. A further boolean search using the same limiters was then performed using the terms "*Borrelia*" and "Australia". Finally, any further Australian-relevant articles referenced within these articles that did not come up in the initial search were obtained. Papers that had Australian authors but were otherwise irrelevant to Australia were removed. At least 525 human cases [79–82,84–89] and two bovine cases [28] of Lyme-like illness have been mentioned in the scientific literature. Only the Lyme-like cases with specified locations are portrayed in Fig. 1, and only those with detailed case presentation, diagnosis and location are presented in Table 1. It should be noted that the majority of these are Lyme-*like* cases that are suspected, but *not confirmed* to represent cases of Lyme Borreliosis. Unreliability of the published case reports in their diagnostic methods means the evidence for Australian Lyme-like cases remains quite unsubstantial and unconvincing.

4.1. Serology from patients

Several patients have been diagnosed as having likely Lyme Borreliosis in Australia solely upon the basis of positive results by one of several methods. The limitations of Lyme serology in Australian patients are discussed in Section 3.2. Over 200 Australian patients (and one Australian cow) presenting with a Lyme-like disease have tested "positive" for Lyme Borreliosis serology [28,79,80,88,89]. However, 32 of these were diagnosed by IFA or EIA only [28,79,89]. None of these one-tiered tested patients (EIA or IFA only) can be definitively considered to have Lyme Borreliosis without further confirmatory testing. Of the 28 positives described by Mayne [80], 15 were immunoblotted without a supporting IFA result being published, severely hindering the validity of these results. A further nine "positive" IgM results are ruled out because of the lack of diagnostic value of the IgM results when the clinical syndrome has been present for greater than 30 days. The remaining four positives had only four or less of the 10 bands required for positive IgG. A further 19 IFAs, 100 IgM immunoblots and 75 IgG immunoblots have also been reported as positive in Australian patients presenting with a Lyme-like condition but also showing concurrent positive antibody titres in several other infectious disease serology tests [88]. It must be reiterated that in a non-endemic or low-endemicity setting, cross reaction of non-specific antibodies due to the presence of other diseases will often lead to the visualisation of false-positive immunoblot bands. In summary, none of the published Lyme-like illness cases from Australian patients diagnosed by serology alone have met the minimum criteria for serological diagnosis of Lyme Borreliosis as described in Section 3.1.

4.2. Culture from patients

Although biopsies of erythema migrans have been taken from numerous Australian patients for histology or PCR [81,82,85,87], there has only been one published report of *Borrelia* culture been successful [84]. The case involved a patient that had sustained a tick bite while walking in bushland of Pittwater Shire, Sydney. This was followed by erythema migrans formation, headache and fever, and later generalised arthalgias and myalgias, insomnia and recurrent skin lesions. Over 18 months after the initial tick bite a biopsy of one of the patient's secondary erythema migrans lesions was cultured in BSK-II media. Spirochaetes were present after three weeks incubation and were identified by direct immunofluorescent staining as *B. garinii*. Although the disease appeared to follow the tick bite contracted in New South Wales, this patient had also travelled to three Lyme-endemic countries in Europe 17 months before the onset of his symptoms [84]. Whilst this published case demonstrates a culture confirmed Lyme Borreliosis-causing *Borrelia* isolate in an Australian patient, Australian acquisition could not be confirmed.

4.3. Molecular detection of B. burgdorferi s. l. from patients

Borrelia burgdorferi s. l. DNA has been detected and sequenced in five Australian patients presenting with Lyme-like disease. Three patient erythema migrans biopsies were tested for *B. burgdorferi* s. l. using primers coding for Borrelial *rpoC* [87]. The publication stated that sequencing of the products revealed a 99% homology with *B. burgdorferi* s. s. One of these patients had never left Australia. However, the primer sequences were not published and the three sequences differed significantly in size, being 206 bp, 336 bp and 165 bp long [87], suggesting non-specific cross-priming. Another erythema migrans rash biopsy was tested using a duplex PCR targeting borrelial *ospC* and *16SrRNA*. The paper states that the *ospC* PCR yielded an amplicon 83 bp long [87]. However, analysis of the *ospC* primers utilized in the study using the NCBI Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) shows an expected amplicon of 104–113 bp in length. Thus, non-specific amplification may have led to the positive PCR reaction. The same author reported elsewhere an Australian erythema migrans biopsy yielding a product with a *Borrelia 16SrRNA* PCR once again having a 99% homology with *B. burgdorferi* s. s. [86]. The sequence of the amplicon was not provided, and the primer sequences were also withheld [86]. The laboratory concerned has, to date, yet to share their primer sequences, nor any DNA or isolates with other researchers for independent verification. A further 126 positive *Borrelia* PCRs on blood samples and 46 on urine samples have been reported, but no sequencing was performed to confirm the amplicon identities, and the primers were once again not disclosed [88]. Many of the abovementioned patients also had overseas travel histories [88]. Given the controversy surrounding the possibility of Lyme Borreliosis transmission in Australia, unequivocal demonstration of the local acquisition of *B. burgdorferi* s. l. within this country would be best supported by both a cultured isolate (stored for analysis by other laboratories, including a recognised reference laboratory skilled in the identification of such isolates) and positive direct molecular identification from clinical material (confirmed by sequencing) from a patient with absolutely no history of overseas travel.

4.4. Seroprevalence in the population

It would be expected that if the putative Lyme-like disease in Australia is caused by *B. burgdorferi* s. l., there would be a high seropositive rate in the Australian population and an even higher seroprevalence in reservoir hosts. However, the seroprevalence rate of *B. burgdorferi* s. l. using IgG ELISA in residents of coastal New South Wales was found to be 2.2% (9/400) and in dogs of this area the prevalence was 2.5% (6/239) [90]. Conversely, in Westchester County, New York (endemic for Lyme), 49.2% of dogs were seropositive, ranging from 6.5% to 85.2% depending on the municipality [91] and in New Jersey by IFA, 34.7% of asymptomatic dogs were seropositive [92]. In the Aland Islands of Finland (also Lyme endemic), 19.7% of residents were positive for *B. burgdorferi* s. l. IgG with ELISA [93].

Table 1
Geographic distribution of Australian Lyme-like cases from peer-reviewed scientific literature.

Location	Travel history	Symptoms	Laboratory findings	Diagnosis by culture/PCR[a]	Reference
Lower Hunter Valley, NSW	ND	Insect bite followed by EM with secondary lesions, relapsing arthritis with swelling and pain in the knee and left hip, behavioural change, headaches, memory loss, urinary retention, tachycardia.	Biopsy showed perivascular lymphocytic infiltrate. Synovial fluid from left knee 50×10 [6]/L, 70% lymphocytes. CSF raised protein (1.24 g/L) normal cell count. EEG and CAT scan unremarkable. Diagnosed with mild meningoencephalitis. ECG-documented supraventricular tachycardia without evidence of atrioventricular block. Negative arbovirus serology (RRV, Sindbis virus, Australian encephalitis virus)	NP/NP	[82]
Guerilla Bay near Moruya, NSW	ND	Insect bite followed by EM. Weeks after treatment, EM recurred.	Biopsy showed superficial and deep perivascular infiltrate of lymphocytes.	NP/NP	[81]
North Bendalong (between Nowra and Ulladulla), NSW	ND	One month EM, lassitude, polyarthralgia, headaches.	Biopsy showed dense perivascular infiltrate of lymphocytes in full thickness of the dermis, some with eosinophils.	NP/NP	[81]
Gorokan, NSW	ND	3 weeks of increasing lethargy, malaise, intermittent fevers, multiple EM, severe occipital headache, sore throat.	Biopsy showed mixed acute and chronic infiltration in superficial dermis. No spirochaetes on silver staining. ESR and C1q binding assay elevated. Negative culture. Syphilis serology, antinuclear factor, antistreptolysin O titre and immunoglobin all normal levels Paired sera CDC ELISA showed optical density ratio of 0.02 (acute serum) and 0.05 (convalescent serum) when compared with strongly positive reference serum. This is below the 0.2 ratio expected in patients with late or complicated Lyme disease (but this patient only early Lyme disease).	Negative/NP	[85]
Pittwater Shire, Sydney	17 months prior to tick bite, visited 3 countries in Europe known to be endemic for Lyme. Did not recall any tick bites or exposure to ticks. EM appeared at the Australian tick bite site.	EM at tick bite site. Mild headache, malaise and low grade fever, non-pruritic rash, insomnia, generalised arthralgias, myalgias, insomnia, difficulty with memory and "thinking clearly", secondary EM lesions. Duration >18 months	ELISA *B. burgdorferi* s. s. antibody negative. Western blot 2+ antibodies (one level below strongest staining intensity) to outer surface protein A (OspA) of *B. garinii* only. Biopsy of secondary lesion showed mild, mainly perivascular lymphohistiocytic inflammatory cell infiltrate in superficial dermis, minimal exocytosis, a little pigment incontinence, no organisms demonstrated with PAS, Gram or Warthin–Starry	Positive/positive (NS)	[84]

(continued on next page)

Table 1 (*continued*)

Location	Travel history	Symptoms	Laboratory findings	Diagnosis by culture/PCR[a]	Reference
			stains. Culture of biopsy grew spirochaetes Direct immunofluorescence antibody staining to flagellin protein of *B. burgdorferi* s. l. and PCR of the flagellin and 16S rRNA identified *B. garinii* more closely related to European rather than Asiatic *B. garinii* strains.		
152.8E, 31.66S	Yes	EM, no systemic illness	*rpoC* PCR positive	NP/positive-sequencing showed 99% identity match to *B. burgdorferi* strain N40	[87]
152.7E 31.73S	Never left Australia	EM, systemic illness	*rpoC* PCR positive	NP/positive-sequencing showed 99% identity match to *B. burgdorferi* strain N40	[87]
151.3E, 33.74S	Yes	EM, fever, meningism, severe headache worse with coughing and shaking of head, photophobia and retro-orbital pain.	*rpoC* PCR positive Follow-up testing post-treatment revealed: *B. burgdorferi* IgA, G, M negative. *B. burgdorferi* multiplex PCR negative (primer targets not specified). *Babesia* and *Bartonella* serology negative.	NP/positive-sequencing showed 98% identity match to *B. burgdorferi* strain N40	[87]
152.8E, 31.32S	Never left Australia	EM, no systemic illness	Multiplex primer set *16S rRNA* and *OspC* used — but only one product sequence is provided. Unclear if both or only one set was positive.	NP/positive-sequencing result inconclusive.	[87]
Rural Victoria	ND	Fever, regular presumed viral illness, chronic fatigue syndrome. Severe arthritis in hands, auditory hypercusis, poor concentration, irritability and emotional lability, episodic sleep disturbances, two episodes of severe generalized body pain without cause, one episode of auditory hallucinations and paranoid ideas. Duration: 8 years	Diagnosed with fibromyalgia at 17 yrs ld Lyme serology IgG titre 80 and IgM titre 10.	NP/NP	[79]
Mid-north coast of NSW	Travelled from Byron Bay NSW to Eastlakes Victoria. No overseas travel.	Lyme-like presentation	Lyme IgM western blot bands 23–25, 39 and 41 kDa. *B. henselae* IgM serology positive (titre 1:40).	NP/NP	[80]
QLD	Travelled to northern NSW and Sydney, NSW; Melbourne, Victoria; Hobart, Tasmania. No overseas travel.	Lyme-like presentation	Lyme IFA 1:40. Lyme IgM western blot bands 31 and 41 kDa. Positive *Borrelia* plasmid PCR. *Babesia duncani* IgG serology positive 1:40, *Babesia microti* IgG serology positive 1:40, *Bartonella henselae* IgM serology positive 1:40.	NP/positive (NS)	[80]
Armstrong beach, QLD	Karratha, WA. No overseas travel.	Lyme-like presentation	Lyme IFA serology 1:80, Lyme IgM western blot bands 34 and 41 kDa, *Bartonella* IgG serology positive 1:40.	NP/negative	[80]
NSW	Victoria, Queensland, South Australia. No overseas travel.	Lyme-like presentation	Lyme IgM western blot bands 31 and 41 kDa. *Babesia duncani* IgG positive 1:40.	NP/negative	[80]

ND, no data; NP, not performed; NS, not sequenced; EM, erythema migrans; ESR, erythrocyte sedimentation rate; PCR, polymerase chain reaction; EEG, electroencephalogram; CAT, computerized axial tomography; ECG, electrocardiogram; CSF, cerebrospinal fluid; RRV, Ross river virus; NT, northern territory; WA, western Australia; QLD, Queensland; NSW, New South Wales; VIC, Victoria; and TAS, Tasmania.

[a] Serological confirmation of Lyme Borreliosis in the context of non-endemicity is questionable; diagnosis by culture and molecular identification methods are preferable for confirmation in the Australian setting.

5. Differential diagnoses

5.1. Infectious diseases

It is assumed by many that the causative agent of Lyme-like illness in Australia must be tick-borne. As noted in a previous section, almost all Australian Lyme-like illness predominantly present with a condition analagous to chronic Lyme Borreliosis. Indeed, it is unusual that not more acute Lyme Borreliosis cases are identified in humans and animals within Australia if the organism causing this illness was indeed B. burgdorferi s. l. Any putative agent of the Australian Lyme-like disease would be capable of producing a syndrome similar to Lyme Borreliosis, with a clinical presentation including flu-like symptoms followed by arthralgic, neurological, dermatological and/or cardiac complications. Some Australian bacteria, parasites and viruses individually, or in co-infection with other pathogens, might produce such a syndrome. A summary of known Australian endemic infectious agents that might be considered in the differential diagnosis of an Australian patient with a Lyme-like presentation is presented below.

The clinical presentations of the Australian Rickettsioses are quite similar to each other and atypical presentations may mimic an acute Lyme Borreliosis. Symptoms include headache, chills, malaise, fever, lymphadenopathy, maculopapular rash and an eschar found at the tick bite site [94,95]. Sometimes arthralgias and myalgias may also be present [94,96]. In some cases, the eschars may be absent [96,97] and the rash may appear as varicelliform [94] or petechial [97]. In rare cases, the rash will not develop at all [97]. Rickettsial infections presenting without a maculopapular rash could be mistaken for a Lyme-like illness.

In Australia, *Babesia canis vogeli* is found throughout northern and central Australia and is spread by *Rhipicephalus sanguineus* ticks [98]. *Babesia gibsoni* has been described in dogs in Victoria [98]. *Babesia bovis* and *Babesia bigemina* in cattle are spread by the Australian cattle ticks *R. australis* [99,100]. *Babesia equi* (later known as *Theileria equi* [101]) was briefly introduced to Australia in 1976 [102,103] but this did not spread and become established due to the absence of suitable vectors [106]. *B. bovis* has been reported as a rare cause of infection in humans [104]. The first definitive case of human Babesiosis acquired in Australia was reported in 2012 and was caused by *Babesia microti* [105]. To date, *B. microti* has not been identified in any Australian ticks. *Babesia* infection can be atypically associated with rheumatoid muscular pains, and nervous complications including incoordination of legs and hysteria, restlessness and nervousness [106]. It therefore appears that *Babesia* is capable of mimicking a Lyme-like syndrome. Like *B. burgdorferi* s. l., *Babesia* is also capable of establishing long-term, persistent infection [107].

Coxiella burnetii may also be considered in patients with tick bite history and reporting Lyme-like symptoms. The majority of cases of *C. burnetii* infection are asymptomatic, but in symptomatic infections the most prevalent acute symptoms include fever (95%), headaches (53%) and myalgia (38%) [108]. Other manifestations may include hepatitis, pneumonia, meningitis, meningoencephalitis, pericarditis and myocarditis [108–110]. Chronic infection may manifest as endocarditis, vascular infections, osteoarticular infections, chronic hepatitis, pericarditis and very rarely as adenopathies, lung or splenic pseudotumours, or chronic neuropathy [108,111–114]. Therefore Q fever may sometimes present as an infection similar to Lyme carditis or Lyme neuroBorreliosis.

Many tick species have been shown as capable of carrying *Bartonella* spp. including: *I. ricinus*, *Dermacentor occidentalis*, *Dermacentor variabilis*, *Dermacentorreticulatus*, *H. longicornis*, *Harperocallis flava*, *Ixodes nipponensis*, *Ixodes pacificus*, *Ixodes persulcatus*, *I. ricinus*, *Ixodes scapularis*, *Ixodes turdus*, *Ixodes antechini*, *Ixodes australiensis*, *I. tasmani*, *Ixodes trichosuri* and *Rhipicephalus sanguineus* [115–119]. Presently, only *Bartonella henselae* [120–124] and *Bartonella quintana* [125] have been reported to cause disease in Australian residents. However, a number of other *Bartonella* species of unknown clinical significance have been identified in Australian animals and their parasites [116,117,119,125,126].

B. henselae infection (cat scratch disease) is typically associated with isolated lymphadenopathy with fever without any other symptoms [128]. However it is now recognised that *Bartonella* may cause a wide spectrum of atypical manifestations even in immunocompetent patients [127–130]. Atypical manifestations may mimic a Lyme-like illness [131] including rheumatic manifestations [131–133], fibromyalgia and chronic fatigue syndrome [131,134], neurological disease [135–137] and endocarditis [138,139]. *B. henselae* been associated with erythema marginatum rashes [130] that may be mistaken for an erythema migrans rash. Like *B. burgdorferi* s. l., *B. henselae* is capable of sustaining chronic infection [134,140,141].

DNA sequences of a newly discovered organism, *Candidatus Neoehrlichia*, were recovered from fifteen New South Wales *I. holocyclus* ticks tested by Gofton, et al. [75]. These sequences did not conform to the emerging tick-borne pathogen *Ca.s Neoehrlichia mikurensis*, but did cluster within two clusters belonging to the *Ca. Neoehrlichia* group [75] and later designated "*Ca. Neoehrlichia* species A and B" [76]. The two species were detected in 248 *I. holocyclus* ticks from both eastern and Western Australia by 16s rRNA next generation sequencing, though when a *Ca. Neoehrlichia* species A and B specific nested PCR was applied to the same samples, only 36 were positive [76]. *Candidatus Neoehrlichia mikurensis* has previously been detected in rodents, humans and ticks from Europe and Asia [75]. A review of eleven human cases in Europe showed that all but one patient were actively immunosuppressed, and most were asplenic [142]. Symptoms included fever, myalgia, arthralgia, neutrophilia and anaemia combined with vascular events such as transient ischaemic attacks and deep vein thrombosis [142]. Only five of the patients recalled being bitten by a tick [142]. While some of these described symptoms may be confused with a Lyme-like illness, further work must be performed to determine the host range, infectivity and clinical presentation of the two novel *Ca. Neoehrlichia* species detected in Australian *I. holocyclus* ticks before these may be confirmed as potential Lyme-like disease candidates. Furthermore, other novel candidate infectious agents such as the three new species each of *Anaplasma* and *Ehrlichia* that have been identified by next generation sequencing of Australian ticks, though at much lower prevalence than the novel species of *Ca. Neoehrlichia* species, also require investigation [76].

5.2. Non-infectious diseases

It is important that potential non-infectious causes are considered in the investigation of Australian patients presenting with a Lyme-like illness. Fibromyalgia, chronic fatigue syndrome, delusional parasitosis and multiple sclerosis are examples of conditions that may be misdiagnosed as a Lyme-like disease, especially in Australia where the infectious aetiology for this condition has not been elucidated. This list is by no means exhaustive.

It should be noted that antigens in *I. holocyclus* saliva alone may cause an erythematous rash to develop in bitten patients [143]. Of forty-two volunteers inoculated by pin-prick with an extract of *I. holocyclus* salivary glands, 36% developed a local erythematous lesion at that site within minutes or hours [143]. In most cases, the rash was >50 mm in diameter and persisted for up to 7 days or more [143]. Such a hypersensitivity rash might easily be mistaken for an erythema migrans lesion in patients recently bitten by *I. holocyclus* ticks [143]. These findings do raise a question as whether the Australian presentations of a Lyme-like illness may in some cases be an allergic response by some individual patients to antigens found within local tick saliva.

Symptoms of fibromyalgia include widespread musculoskeletal pain, hyperalgesia, fatigue, insomnia, memory loss and poor concentration, depression, headache and irritable bowel syndrome [144–146]. Since diffuse arthralgia, cognitive difficulties and fatigue are common

in chronic Lyme Borreliosis, it is possible for fibromyalgia to be mistaken for Lyme borrelioisis and *vice versa* [147,148].

Chronic fatigue syndrome is very similar to fibromyalgia in that it is a syndrome of unknown aetiology characterised by persistent fatigue, musculoskeletal pain, insomnia and cognitive impairment and headaches [149–151]. Both syndromes are more common in women than men, and the two syndromes commonly co-occur. It has even been suggested that the two syndromes are merely symptom amplification of the same somatic syndrome [149]. Fibromyalgia is diagnosed based on widespread musculoskeletal pain, sensitivity in a number of "tender spots", and the presence of other associated symptoms such as headaches, sleep disturbances and memory loss [152]. Chronic fatigue syndrome diagnosis is based on onset of unexplained persistent or relapsing chronic fatigue that is not substantially alleviated by rest, accompanied by symptoms such as short term memory or poor concentration, sore throat or lymph nodes, muscle or joint pain and headaches [150]. Chronic fatigue and fibromyalgia may present as sequelae of infections with *C. burnetii*, *Chlamydophila pneumoniae*, Epstein-Barr virus and Parvovirus B19 [150].

Delusional parasitosis is a psychiatric disorder where a patient has the false but fixed belief that they are being infested by parasites [153,154]. It may present as a primary somatic disorder or secondary to other conditions such as drug use, schizophrenia or dementia. Primary delusional parasitosis occurs most commonly in middle-aged women, and except for their delusion the patient may otherwise be rational and mentally healthy [153]. Patients may describe sensations of parasitic activity on or under their skin such as crawling, biting or burrowing (collectively known as formication), and may bring in objects such as hair, lint or skin as evidence of their infestation despite unremarkable findings on examination [153,154].

There has been one published Australian case of delusional parasitosis in which the patient was convinced she had Lyme Borreliosis [155]. The patient brought evidence of "ticks" to her doctor and presented with rashes as a result of scratching and disinfecting. The patient had shaved off all her hair and fumigated her house in an attempt to be rid of the arthropods. However after several months of cognitive behavioural therapy and 150 mg of venlafaxine, her paranoia and symptoms were successfully alleviated [155].

6. Conclusion

Suggestions that a Lyme-like disease may exist in Australia [17] remain controversial and no study to date has definitively identified the presence of a *Borrelia* species infecting humans that have a locally acquired Lyme-like syndrome. It is unclear whether the causative agent of this purported condition is a *B. burgdorferi* s. l. related organism, another pathogen altogether or of non-infectious aetiology. Over 500 Lyme-like cases from Australian patients have been published in the scientific literature [79–82,84–89] and two bovine cases [28] but upon investigation, these diagnoses were highly questionable due to significant flaws in the diagnostic process or presentation of results. Only in one instance has a Lyme Borreliosis-causing *Borrelia* species been cultured from an Australian patient or animal [84]. This patient had a history of travel to a Lyme endemic area of the northern hemisphere [84] so overseas acquisition cannot be ruled out. Serology has a low positive predictive value in non-endemic areas and cannot be relied upon for diagnosis. The reported culture of possible *Borrelia* spirochaetes from 109 Australian ticks [66] was not reproduced in over 10,000 ticks [73]. *B. burgdorferi* s. l. has never been cultured from an Australian patient that could not have acquired the infection overseas and therefore there is currently no proof that *B. burgdorferi* s. l. or any other kinds of *Borrelia* species are infecting humans in Australia. If there is a Lyme-like disease that exists in Australia it may well be of a different aetiology. It is recommended by the authors that in the non-endemic context such as Australia, in addition to following the RCPA protocol for the diagnostic laboratory testing of Borreliosis [59], a minimum of live *Borrelia* culture combined with a positive, sequenced *B. burgdorferi* s. l. specific PCR and independent verification of the identity of that organism by an experienced reference laboratory is required to confirm any future diagnosis of Australian acquired Lyme Borreliosis.

Disclaimer

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Inhibition of the endosymbiont "*Candidatus* Midichloria mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in *Ixodes* ticks from Australia

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Abstract

Background: The Australian paralysis tick (*Ixodes holocyclus*) is of significant medical and veterinary importance as a cause of dermatological and neurological disease, yet there is currently limited information about the bacterial communities harboured by these ticks and the risk of infectious disease transmission to humans and domestic animals. Ongoing controversy about the presence of *Borrelia burgdorferi* sensu lato (the aetiological agent of Lyme disease) in Australia increases the need to accurately identify and characterise bacteria harboured by *I. holocyclus* ticks.

Methods: Universal PCR primers were used to amplify the V1-2 hyper-variable region of bacterial 16S rRNA genes present in DNA samples from *I. holocyclus* and *I. ricinus* ticks, collected in Australia and Germany respectively. The 16S amplicons were purified, sequenced on the Ion Torrent platform, and analysed in USEARCH, QIIME, and BLAST to assign genus and species-level taxonomy. Initial analysis of *I. holocyclus* and *I. ricinus* identified that > 95 % of the 16S sequences recovered belonged to the tick intracellular endosymbiont "*Candidatus* Midichloria mitochondrii" (CMM). A CMM-specific blocking primer was designed that decreased CMM sequences by approximately 96 % in both tick species and significantly increased the total detectable bacterial diversity, allowing identification of medically important bacterial pathogens that were previously masked by CMM.

Results: *Borrelia burgdorferi* sensu lato was identified in German *I. ricinus*, but not in Australian *I. holocyclus* ticks. However, bacteria of medical significance were detected in *I. holocyclus* ticks, including a *Borrelia* relapsing fever group sp., *Bartonella henselae,* novel "*Candidatus* Neoehrlichia" spp., *Clostridium histolyticum*, *Rickettsia* spp., and *Leptospira inadai*.

Conclusions: Abundant bacterial endosymbionts, such as CMM, limit the effectiveness of next-generation 16S bacterial community profiling in arthropods by masking less abundant bacteria, including pathogens. Specific blocking primers that inhibit endosymbiont 16S amplification during PCR are an effective way of reducing this limitation. Here, this strategy provided the first evidence of a relapsing fever *Borrelia* sp. and of novel "*Candidatus* Neoehrlichia" spp. in Australia. Our results raise new questions about tick-borne pathogens in *I. holocyclus* ticks.

Keywords: Tick, Vector-borne disease, Zoonoses, Metagenomics, 16S community profiling, *Ixodes holocyclus*, *Ixodes ricinus*, *Candidatus* Midichloria, *Borrelia*, *Candidatus* Neoehrlichia

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Background

Ticks are the second most important vector of pathogens to humans after mosquitoes and the chief cause of vector-borne diseases in domestic animals and wildlife [1–3]. Ticks also vector the greatest diversity of pathogenic microorganisms of any haematophagous arthropod, including members of the bacterial genera *Anaplasma* [4], *Bartonella* [5], *Borrelia* [6], *Ehrlichia* [7], *Francisella* [8], *Rickettsia* [9], and "*Candidatus* Neoehrlichia" [10]. Furthermore, bacterial co-infections in ticks are common and provide diagnostic and therapeutic challenges for medical and veterinary practitioners [11–13]. In Europe, North America, and Australia the incidence of tick-borne diseases is rising due to a combination of factors including perturbation in climate, increasing populations and movement of humans and domestic animals, and increased human encroachment into tick habitats [14].

In Australia there is a long-standing controversy concerning the presence of Lyme disease and its aetiological agents, *Borrelia burgdorferi* sensu lato. First reported in the 1980s [15, 16], intensive efforts to determine the aetiological agent of Australian "Lyme-like" illness found no evidence for *B. burgdorferi* sensu lato in ticks or wildlife [17, 18], yet numerous victims of tick bites continue to present with Lyme-like symptoms in Australia [19]. Thus there is a pressing need to apply contemporary next-generation sequencing (NGS) techniques to better understand bacterial pathogens harboured in Australian ticks.

In Australia *I. holocyclus* is the most important tick species from both a medical and veterinary perspective [20, 21]. Its enzootic range is limited to a narrow strip along Australia's eastern seaboard that extends several thousand kilometres from Cape York to eastern Victoria, and includes most of Australia's most densely populated regions [22]. *Ixodes holocyclus* is commonly found on domestic animals in which it causes life-threatening paralysis. *Ixodes holocyclus* is also the most common tick found on people in its range and impacts human health by causing weakness, paralysis, allergic reactions, and is a vector for the spotted fever pathogens *Rickettsia australis* and *R. honei* [23].

Together with known vector-borne pathogens, ticks also harbour closely related endosymbiotic bacteria such as *Coxiella* spp. [24–26], *Francisella* spp. [27–29], *Wolbachia* spp. [30, 31], *Rickettsia* spp. [32–35], and the recently discovered "*Candidatus* Midichloria mitochondrii" (CMM) [36–39]. These bacterial endosymbionts often dominate the microbial population within their arthropod hosts and can affect the transmission dynamics of pathogenic species [40–42].

CMM is an intracellular endosymbiont that was first discovered in the European sheep tick *Ixodes ricinus* [36] but has since been detected in other ticks including *I. holocyclus* [37, 43–47], as well as tabanid flies [48], bed bugs [49], and mites [50]. In ticks, CMM resides in high numbers in female reproductive tissues and is transmitted to all offspring where it infects 100 % of larvae, nymphs, and females [36, 51]. Male *I. holocyclus* ticks also appear to inherit and harbour CMM, however, *I. ricinus* males fail to establish stable CMM populations [36, 38, 51]. In addition to this, CMM is found in *I. ricinus* salivary glands from where it is introduced during feeding to vertebrate hosts, including humans [52, 53]. However, the consequences of CMM infection in vertebrate hosts, if any, are unknown [52].

Next-generation sequencing and bioinformatics advances have greatly increased our ability to accurately identify trace amounts of DNA in highly heterogeneous samples, making them excellent tools for molecular epidemiological studies of pathogens that may be present in low abundance. In particular, the application of 16S rRNA gene (hereafter referred to as 16S) community profiling has been particularly successful for characterising bacterial assemblages from a wide variety of sources, including ticks [30, 54–62]. With this methodology, a short region (100-500 bp) of the 16S gene is amplified using PCR primers that bind to orthologous regions either end of a hyper-variable region of the gene. Because the primers bind to orthologous regions of the 16S gene numerous bacterial taxa within a heterogeneous sample can be targeted simultaneously, and the hyper-variable region proximal to the primers permits taxonomic discrimination of those taxa [63, 64].

A limitation of 16S community profiling in ticks is that a high proportion of sequences generated during PCR will belong to bacterial endosymbionts [40]. These over-abundant endosymbiont 16S sequences can mask the presence of less abundant bacterial 16S sequences including pathogens, resulting in biased results and a decreased detected bacterial diversity. This limitation can be overcome to some extent by deeper sequencing to increase detection of low abundant sequences. However, this approach fails to address the source of the problem and is costly, making it difficult to study a large number of samples. In addition, various factors such as biases in PCR amplification efficiency and inter-specific variation of the 16S copy number are known to skew the measured proportion of NGS reads, and limits the use of sequence abundance to infer actual bacterial abundance in the original sample [65, 66].

As part of an ongoing study into tick-borne diseases in Australia, we developed a primer that inhibits amplification of CMM 16S sequences, enabling us to identify other less abundant bacteria in *I. holocyclus* and *I. ricinus*. This approach has provided insights into the bacterial microbiome of *I. holocyclus* and is readily applicable

to other arthropod vectors of plant and animal diseases where overabundant species prove problematic to the identification of important taxa.

Methods

Sample collection

A total of 196 individual specimens of *I. holocyclus*, were collected from mammalian ($n = 85$) and avian ($n = 2$) hosts, and from the environment ($n = 109$) in various locations in New South Wales, Australia, between 2004 and 2014 (Table 1). All host-seeking *I. holocyclus* ticks were collected by flagging, using standard techniques [67], and either preserved frozen, stored in 70 % ethanol, or used immediately. In addition, 20 nymph and ten female *I. ricinus* ticks were collected by flagging in suburban parks in the cities of Freising and Leipzig, Germany, in 2013, and were immediately placed in 70 % ethanol and shipped to Murdoch University. All ticks were identified morphologically using standard keys [68, 69].

Ethics statement

This research complies with the *Australian Code for the Responsible Conduct of Research, 2007* and the *Australian Code for the Care and Use of Animals for Scientific Purposes, 2013*. Removal of ticks from animal hosts was approved by the Murdoch University Animal Ethics Committee; collection from domestic animals ($n = 35$) and wildlife species ($n = 26$) was opportunistic, from individuals that were presented to veterinarians, or were dead as a result of unrelated accident or injury. Ticks ($n = 26$) were removed from humans by the person themselves or by medical professionals during outpatient treatment.

DNA extraction

Total genomic DNA from individual ticks was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's recommendations (Qiagen Supplementary Protocol: Purification of total DNA from insects). Before extraction, individual ticks were surface sterilised in 10 % sodium hypochlorite, washed in 70 % ethanol and DNA-free PBS, frozen in liquid nitrogen for 1 min, and homogenised with 5 mm steel beads in a Tissue Lyser LT (Qiagen, Germany) for 1 min at 40 Hz. DNA-free equipment and tubes were used for each step and equipment was decontaminated between samples with DNAaway (Life Technologies, USA). Extraction reagent blanks were performed in parallel with all DNA extractions in order to determine background bacterial populations (one extraction reagent blank for every 23 samples). To prevent potential cross-contamination by known *I. ricinus* pathogens, DNA extractions from these ticks were performed in a separate laboratory to *I. holocyclus* DNA extractions.

Table 1 *Ixodes holocyclus* and *I. ricinus* ticks collected from different hosts and the environment

Tick Instar or Sex	Number of ticks	Hosts or Questing (number of ticks)
Ixodes holocyclus	196	
Nymph	15	Questing (15)
Male	41	Questing (41)
Female	140	*Bos taurus* (4), *Canis familiaris* (26), *Corvus coronoides* (1), *Cracticus tibicen* (1), Echidna (Family: *Tachyglossidae*) (1), *Felis catus* (5), *Homo sapiens* (26), *Macropus spp.* (9), *Trichosurus vulpecula* (14), Questing (53).
Ixodes ricinus	30	
Nymph	20	Questing (20)
Female	10	Questing (10)

Blocking primer design

In pilot 16S community profiling experiments, over 95 % of the sequences generated from each sample, from both *I. holocyclus* and *I. ricinus* ticks, belonged to CMM regardless of the sequencing depth, PCR primers, or sequencing platform used (data not shown). To inhibit amplification of these overabundant sequences during PCR, we developed a CMM-specific blocking primer (*Mid*Blocker) [70] to be used in conjunction with the 16S universal primers 27F-Y (Fig. 1) and 338R (5'-TGC TGCCTCCCGTAGGAGT-3') that amplify the V1-V2 16S region [71]. The *Mid*Blocker primer was designed from an alignment of 107 partial 16S sequences including known tick-borne pathogens and endosymbionts, ubiquitous environmental bacteria, and CMM (Fig. 1). The 5' end of the *Mid*Blocker primer has a 7 bp overlap with the 3' end of the 27F-Y primer, extends 15 bp downstream of the 27F-Y primer-binding site, and terminates polymerase elongation due to a C3 spacer at the 3' end of the primer (Fig. 1). *In silico* analysis (not shown) suggests that the *Mid*Blocker primer is specific to CMM and will not modulate the binding of the 27F-Y primer to other closely related Rickettsiaceae and Anaplasmataceae.

Validation of the *Mid*Blocker primer

Total DNA from host-seeking female *I. holocyclus* ($n = 10$) and *I. ricinus* ($n = 10$) were amplified by qPCR using the 27F-Y and 338R primers with and without 10 μM of *Mid*Blocker primer. Different concentrations (2-14 μM) of the *Mid*Blocker primer were trialled in pilot experiments on a subset of samples (data not shown). PCR conditions, fusion-primer architecture, semiconductor sequencing, and sequence analysis were the same as described below. Nonparametric Mann-Whitney U-tests were performed in Quantitative Insights Into Microbial

Name (NCBI accession)	Sequence (5'-3')
27F-Y Primer (This study)	AGAGTTTGATCCTGGCTYAG
*Mid*Blocker Primer (This study)	GGCTYAGAGTGAACGCTGGCGG/C3/
Candidatus Midichloria mitochondrii (CP002130)	AGAGTTTGATCCTGGCTCAGAGTGAACGCTGGCGG
Borrelia burgdorferi (B31_30245)	AGAGTTTGATCCTGGCTTAGAACTAACGCTGGCAG
Borrelia afzelii (CP009212)	AGAGTTTGATCCTGGCTTAGAACTAACGCTGGCAG
Borrelia duttonii (AF107364)	AGAGTTTGATCCTGGCTTAGAACTAACGCTGGCAG
Rickettsia rickettsia (CP000766)	AGAGTTTGATCCTGGCTCAGAACGAACGCTATCGG
Bartonella henselae (AJ223780)	AGAGTTTGATCATGGCTCAGAACGAACGCTGGCGG
Ehrlichia chaffeensis (CP007480)	AGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGG
Anaplasma phagocytophilum (CP006618)	AGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGG

Fig. 1 Alignment of partial 16S rDNA sequences and the 27F-Y and *Mid*Blocker primers. Alignment includes partial 16S sequences of seven tick-borne bacterial pathogens and "*Candidatus* M. mitochondrii" with the 27F-Y and *Mid*Blocker primers showing mismatches that allow specific blocking of "*Candidatus* M. mitochondrii"

Ecology (QIIME) [72] to determine the significance of differences in bacterial diversity between samples amplified with and without *Mid*Blocker; significance was set at $p < 0.05$ (Mann-Whitney U-Test).

16S community profiling qPCR

The primers 27F-Y and 338R amplified the 16S V1-2 hyper-variable regions (250-320 bp) [73] in *I. holocyclus* and *I. ricinus* DNA samples. 27F-Y and 388R primers also incorporated a six to eight base pair multiplex identifier (MID) sequence together with Ion Torrent sequencing adapters A and P1 (Life Technologies, USA). Each sample was amplified with primers containing a unique combination of forward and reverse MID sequences to allow multiplex sequencing and discrimination of sequences to samples in downstream analysis. All community profiling qPCRs were carried out in duplicate in 25 μl reactions containing 1 × PCR buffer (5 prime, Germany), 2 mM $MgCl_2$ (5 Prime, Germany), 0.25 mM dNTPs (Fisher Biotech, Australia), 0.01 mg BSA (Fisher Biotech, Australia), 0.4 μM of each 27F-Y and 338R primer, 10 μM of *Mid*Blocker, 0.12 × SYBR Green (Life Technologies, USA), 1 U of Perfect Taq Polymerase (5 Prime, Germany), 1 × ROX dye (Life Technologies, USA), and 2 μl of DNA (1-100 ng/μl). No-template control reactions and extraction reagent blank controls were included in every qPCR run and were incorporated in the sequencing libraries. All PCR amplifications were performed on a Step-One real-time qPCR machine (Applied Biosystems, USA) with the following thermal conditions: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C (30s), annealing at 62 °C (30s), and extension at 72 °C (45 s). Thermocycling was followed by a melt curve and final extension at 72 °C for 10 min.

Library preparation and NGS

16S amplicons from all samples and controls were pooled into one of four sequencing libraries in equimolar amounts. Amplicon libraries were then purified twice using 1.2 volumes of Agencourt Ampure XP beads (Agilent Technologies, USA) and quantified by qPCR using a known concentration of a serially diluted 152 bp synthetic oligonucleotide as a standard. qPCR reactions contained 1X Power Syber Green mastermix (Life Technologies, USA), 0.4 μM Ion Torrent primers A and P1, and 2 μl DNA template, and were run with the following thermal conditions: initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C (30 s), annealing and extension at 60 °C (45 s). Templating emulsion PCR and enrichment were performed according to the manufacturer's recommendations on the One-Touch 2 and One-Touch ES instruments (Life Technologies, USA). Sequencing was performed on an Ion Torrent PGM (Life Technologies, USA) using 400 bp chemistry and 316-V2 semiconductor chips, following the manufacturer's recommendations.

Sequence processing and analysis

Sequences were first processed in Geneious 8.0.4 [74] by retaining only reads with perfect 27F-Y and 338R primers and MID sequences (no mismatches allowed). Sequences were then de-multiplexed into individual samples based on their unique combination MID sequences. Primer sequences and distal bases were trimmed from each read, and reads shorter than the minimum reported length of the amplicon (<250 bp) were discarded. Remaining reads were quality filtered using USEARCH [75], allowing only reads with a < 1 % error rate to remain and singletons were removed on a per-sample basis. In order to identify bacterial genera present in samples operational taxonomic units (OTUs) were selected by clustering sequences at 97 % similarity with the UPARSE algorithm [76]. OTUs were checked against the ChimeraSlayer Gold reference database with the UCHIME algorithm [77] to ensure OTUs were not the result of chimeric reads. Genus level taxonomy was assigned to OTUs against the GreenGenes 16S database (August 2013 release) [78] in QIIME 1.8.0 [72] using the UCLUST algorithm [75] with default parameters. Only OTUs assigned to the genus level were used for further analysis. Bacterial genera that were identified in extraction reagent blanks and no-template controls were

removed from the dataset to eliminate background bacterial sequences.

16S sequences from genera that contained known tick-borne pathogens, known tick endosymbionts, or medically important bacteria that have not previously been associated with ticks, were compared against the NCBI GenBank Nucleotide database using BLAST [79] in an attempt to resolve species level taxonomy. Sequences were only assigned to a species if the query sequence matched only one species-specific reference sequence with a pairwise identity match ≥ 99 % with ≥ 99 % query coverage.

Sequences from the genera *Borrelia*, and "*Candidatus* Neoehrlichia" in this study were aligned with 16S sequences from known members retrieved from GenBank using the Geneious alignment tool [74] and refined with MUSCLE [80]. Alignments were trimmed to match the length sequences obtained in this study. *Borrelia* alignment contained 27 members with 313 bp sequences including gaps and the "*Candidatus* Neoehrlichia" alignment contained 43 members with 309 bp sequences including gaps. Neighbour-joining phylogenetic trees were constructed from these alignments in Geneious [74] using the Tamura-Nei genetic distance model and resampling 1000 bootstrap replicates. "*Borrelia*" and "*Candidatus* Neoehrlichia" sequences from "*I. holocyclus*" ticks used for phylogenetic reconstructions were deposited in GenBank (accessions KT203914-6).

Results

Validation of blocking primer

Comparison of unique sequences recovered from PCR amplification with or without the *Mid*Blocker primer revealed 46,698 vs. 14,154 sequences for *I. holocyclus* and 30,689 vs. 12,723 sequences for *I. ricinus*, respectively. *Ixodes holocyclus* and *I. ricinus* samples amplified without the *Mid*Blocker primer contained a total of 98.2 % and 99.6 % CMM sequences respectively, while amplification with the *Mid*Blocker primer decreased the number of CMM sequences to a total of only 2.3 % and 3.6 % of the reads respectively. Six of ten *I. holocyclus* samples and four of ten *I. ricinus* samples still contained CMM >sequences after amplification with the *Mid*-Blocker primer, however, these sequences comprised < 4 % of sequences in each of these samples.

Consequent to the blocking step, all samples had a significantly higher taxonomic diversity when amplified with the *Mid*Blocker primer than when amplified without the *Mid*Blocker primer ($p < 0.05$; Mann-Whitney U-Test). Amplification without the *Mid*Blocker primer resulted in the detection of 32 and 14 bacterial genera in *I. holocyclus* and *I. ricinus* samples respectively, while inhibition of CMM 16S sequences resulted in the detection of 103 and 89 additional bacterial genera in *I. holocyclus* and *I. ricinus* samples respectively (Fig. 2). Furthermore, the *Mid*Blocker primer did not appear to inhibit the amplification of other Rickettsiales closely related to CMM, as confirmed by the identification of members of the closely related *Rickettsia* and "*Candidatus* Neoehrlichia" genus in *I. holocyclus* and *I. ricinus* samples amplified with the *Mid*Blocker primer.

Bacterial pathogens in *I. holocyclus* and *I. ricinus* ticks

After sequence processing, a total of 2,441,958 and 412,130 sequences were generated for *I. holocyclus* and *I. ricinus* ticks, respectively. Sixty-five bacterial genera were detected in extraction reagent and no-template controls, of which 28 were also present in at least one tick sample (Additional file 1). These genera were all associated with ubiquitous environmental and commensal bacteria and were subtracted from samples in order to eliminate potential environmental contaminants from the dataset. After removing background taxa a total of 199 and 95 bacterial genera were identified in *I. holocyclus* and *I. ricinus* samples, respectively (Additional files 2 and 3). Most bacteria identified were environmental and free-living bacteria often associated with soil and leaf-litter environments, characteristic of tick habitats. CMM was still the most common bacterium identified in *I. holocyclus* ticks (75.5 %) and the second most common in *I. ricinus* ticks (70 %) after *Rickettsiella* spp. However, CMM sequences comprised an average of only 6.8 % and 4.3 % of sequences per sample for *I. holocyclus* and *I. ricinus*, respectively. Six genera of medical importance were found in tick samples including tick-borne pathogens in the genera *Anaplasma*, *Bartonella, Borrelia, "Candidatus* Neoehrlichia", and *Rickettsia*, and the free-living pathogens *Leptospira* and *Clostridium.*

Bartonella henselae was identified with 100 % sequence similarity to multiple known reference sequences [GenBank: AJ223779, HG726042, HG969191, JN646651] in one female *I. holocyclus* removed from a domestic cat. Additionally, a second *Bartonella* sp. was identified from a female *I. holocyclus* removed from a human. *Bartonella* sequences in this sample had multiple > 99 % matches to three *Bartonella* species *B. coopersplainsensis, B. australis,* and *B. rattaustraliani* [GenBank: EU111759, DQ538394, EU111751]; species reported to date only in native Australian wildlife. *Bartonella* species were not identified in any *I. ricinus* ticks.

Borrelia 16S sequences were obtained from ten questing *I. ricinus* ticks and a single *I. holocyclus* tick removed from a wild Echidna (Tachyglossidae sp.). *Borrelia* sequences derived from the *I. holocyclus* tick had 100 % sequence similarity, and clustered with high bootstrap confidence (91.1 %) into a group of pathogenic relapsing fever *Borrelia* species including *B. duttonii, B. recurrentis, B. parkeri*, and *B. crocidurae* (Fig. 3). *Borrelia* 16S sequences derived from one *I. ricinus* tick clustered with high bootstrap confidence (90.2 %) with

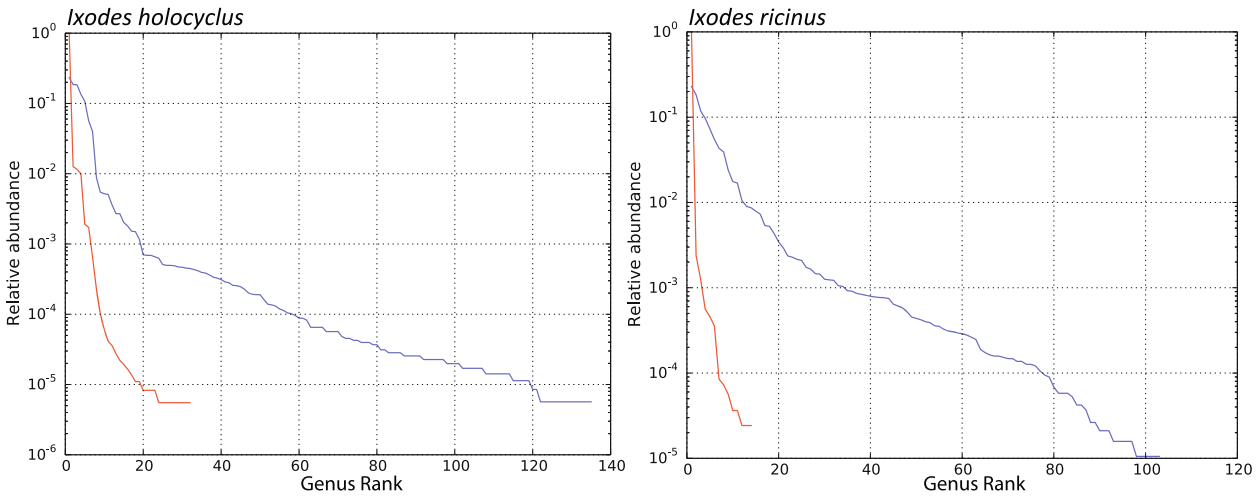

Fig. 2 Rank abundance plots of bacterial genera identified with and without blocking. The ranked relative abundance of bacterial genera identified in 10 *I. holocyclus* and 10 *I. ricinus* ticks when amplified with (blue lines) and without (red lines) the *Mid*Blocker primer. X-axis represents the number of bacterial genera identified

the pathogenic relapsing fever *Borrelia* spp. *B. miyamotoi* and *B. lonestari*, with 99.3 % and 97.7 % sequence similarity respectively. Sequences derived from nine other *I. ricinus* ticks had 100 % sequence identity and clustered with the Lyme borreliosis-causing *B. burgdorferi* and *B. afzelii* with bootstrap values of 93.4 % and 86.8 % respectively (Fig. 3).

Three *I. ricinus* ticks and 15 *I. holocyclus* ticks contained sequences from the genus "*Candidatus* Neoehrlichia" and all *I. ricinus*-derived sequences had > 98 % sequence similarity, and clustered with "*Candidatus* Neoehrlichia mikurensis" reference sequences (Fig. 4). *Ixodes holocyclus*-derived "*Candidatus* Neoehrlichia" sequences formed two distinct novel clades with high bootstrap confidence (94.2 % and 97.2 %) that did not group with any "*Candidatus* Neoehrlichia" sequences in GenBank (Fig. 4). Sequences within each of these novel "*Candidatus* Neoehrlichia" clades were less than 1 % dissimilar to each other but more than 6 % dissimilar to any known "*Candidatus* Neoehrlichia mikurensis" or "*Candidatus*

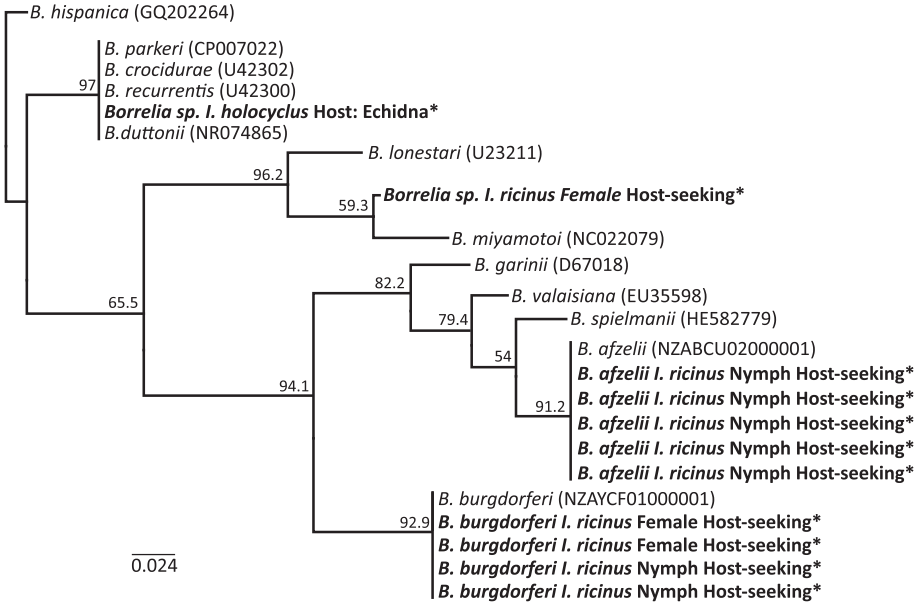

Fig. 3 Neighbour-joining tree of 16S V1-2 *Borrelia* sequences from *I. holocyclus* and *I. ricinus* ticks. Branch labels are bootstrap values inferred from 1000 replicated. Parenthesises after node labels refers to the GenBank accession number. * Indicates sequences from this study

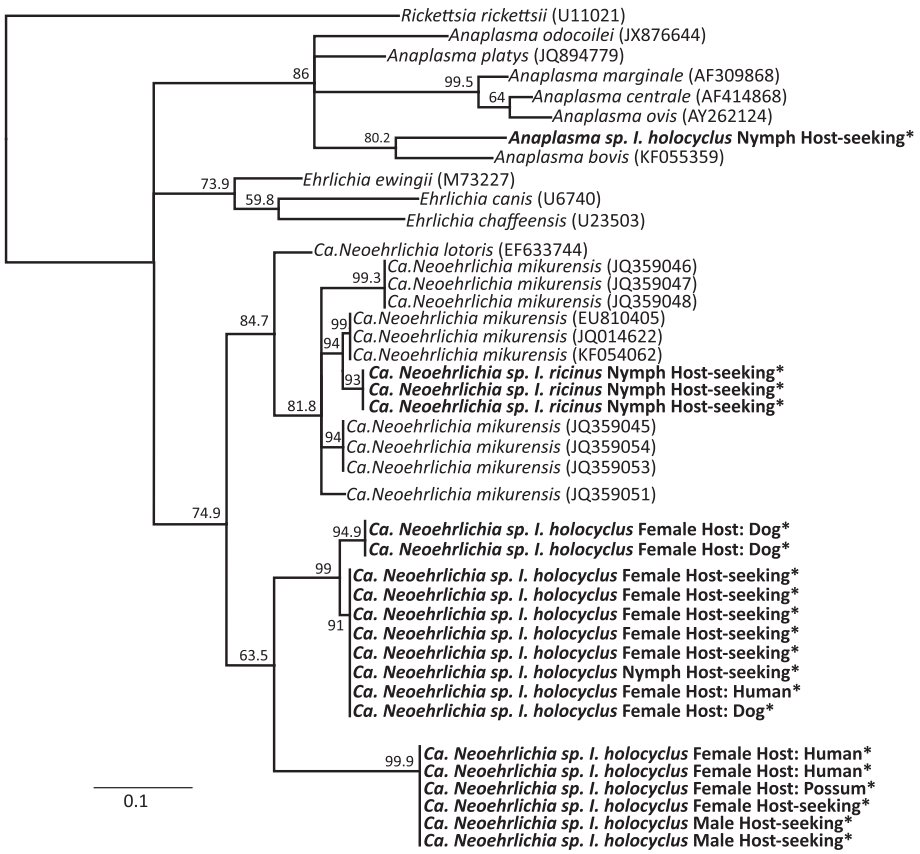

Fig. 4 Neighbour-joining tree of 16S V1-2 "*Candidatus* Neoehrlichia" sequences from *I. holocyclus* and *I. ricinus* ticks. Branch labels are bootstrap values inferred from 1000 replicated. Parenthesises after node labels refers to the GenBank accession number. * Indicates sequences from this study

Neoehrlichia lotoris" 16S sequences. One *I. holocyclus* tick also contained sequences that grouped with relatively high bootstrap confidence (75.1 %) with *Anaplasma bovis* within a clade that also includes the pathogens *A. platys, A. Phagocytophilum* and *A. odocoilei* (Fig. 4).

The genus *Rickettsia* was identified in five *I. ricinus* ticks and six *I. holocyclus* ticks. In two *I. ricinus* ticks, *R. helvetica* was identified with 100 % matches to reference sequences [GenBank: L36212, KJ740388, GQ413963] and no other matches > 97 %. Four *I. ricinus* ticks were infected with *Rickettsia* spp. that could not be identified to the species level due to high sequence homology (> 99 %) between many sequences including pathogenic and benign species: one of these ticks was also co-infected with *R. helvetica. Rickettsia* sequences in six *I. holocyclus* ticks were unable to be resolved to the species level due to high sequence homology (> 99 %) at the loci sequenced between many *Rickettsia* spp., including pathogenic and benign species.

The genera *Leptospira* and *Clostridium* were identified in 18 and 30 *I. holocyclus* ticks respectively. *Leptospira* sequences derived from all ticks had 100 % sequence similarity with *Leptospira inadai* [GenBank: NR115296, AY631891, AY631887] and did not match any other species-specific sequence > 98 %. *Clostridium* sequences from 15 *I. holocyclus* ticks matched with the pathogenic *Clostridium histolyticum* [GenBank: NR113187, NR104889] with sequence similarity (99.4 %), however species designation of sequences from the 10 other ticks were unable to be resolved due to high sequence homology (> 99 %) with between many *Clostridium* spp.

Bacterial endosymbionts in *I. holocyclus* and *I. ricinus* ticks

In addition to CMM mentioned previously, the genus *Francisella* was identified in three questing *I. holocyclus* nymphs. *Francisella* sequences from these ticks matched > 98 % with *Francisella*-like endosymbionts from *Amblyomma*, *Dermacentor*, and *Ornithodoros* ticks, and all sequences were > 6 % dissimilar from the zoonotic pathogen *Francisella tularensis.* The arthropod endosymbiotic genus *Rickettsiella* was also identified in eight *I. holocyclus* ticks and 15 *I. ricinus* ticks, however species-specific discrimination was not possible due to high sequence homology (> 99 %) between many *Rickettsiella*

species at the loci sequenced. The common arthropod endosymbiont *Wolbachia* was also detected in a single *I. holocyclus* tick, which matched > 94 % to *W. pipientis* and other *Wolbachia* endosymbionts of arthropods.

Discussion

Blocking primers are a useful tool in molecular microbiology studies, reducing amplification of overabundant sequences that would otherwise dominate sequencing results [70, 81–83]. The application here of a CMM-specific blocking primer significantly reduced the number of CMM sequences in *I. ricinus* and *I. holocyclus* samples, allowing identification of previously occult bacteria including other endosymbionts and potential pathogens.

Not unexpectedly, *Borrelia burgdorferi* and *B. afzelii* were detected in *I. ricinus* ticks. The prevalence of these bacteria is high in European tick populations [84] but these Lyme disease-causing agents were not detected in Australian *I. holocyclus* ticks. However, DNA of a relapsing fever *Borrelia* sp. was detected in a single *I. holocyclus* tick from a wild echidna that had 100 % identity to the known relapsing fever pathogens *B. duttonii, B. recurrentis, B. parkeri,* and *B. crocidurae.* The significance of this finding is uncertain; *Borrelia*-like organisms have been suggested in Australia previously [18, 85, 86] but this is the first report of a relapsing fever *Borrelia* species in Australia, a finding that may have public health implications. Symptoms of *Borrelia* relapsing fever can be severe, inducing fevers, myalgia, arthralgia, lethargy, petechial rash, photophobia, and facial palsy.

The organism "*Candidatus* Neoehrlichia mikurensis" is an emerging tick-borne pathogen that has been detected in rodents, humans, and ticks throughout Europe and Asia [87–90]. A second member of the genus designated "*Candidatus* Neoehrlichia lotoris" has also been described as a pathogen in the American racoon, *Procyon lotor* [91]. "*Candidatus* N. mikurensis" causes significant illness in immunocompromised humans including, but not limited to, anaemia, deep vein thrombosis, fever, diarrhoea, joint and muscle pain, pulmonary embolism, and arterial aneurysm [87–89]. Based on the partial 16S sequences reported here, the "*Candidatus* Neoehrlichia" spp. from *I. holocyclus* ticks are closely related to, but distinct from, "*Candidatus* N. mikurensis" and "*Candidatus* N. lotoris", and may therefore be a novel species. In fact, this is the first description of the "*Candidatus* Neoehrlichia" genus in Australia; the medical significance of this finding warrants further research to refine its phylogenetic position and investigate its pathogenicity, if any, in humans. Furthermore, the detection of an *Anaplasma* sp. in one *I. holocyclus* tick is also of significance, as only two species of *Anaplasma* have previously been detected in Australia; *Anaplasma marginale* in *Rhipicephalus microplus* ticks [92], and *Anaplasma platys* in *R. sanguineus* ticks in central and northern Australia [93].

Detection of *Leptospira inadai* during this study may explain the observation over twenty years ago of spirochaete-like objects (SLOs) identified by dark field microscopy of various tick species including *I. holocyclus* [17]. Although these SLOs were dismissed as aberrant artifacts by the authors, it is noteworthy that the SLOs shown in Figs. 1 and 2 from Russell et al. [17] bear a strong resemblance to various *Leptospira* spp., including *L. inadai*. Further work isolating and imaging *L. inadai* from *I. holocyclus* is required to confirm this possibility. Recently it was proposed that *Leptospira* spp. may also be tick-transmitted due to their high prevalence in *I. ricinus* ticks [94]. *Leptospira inadai* is pathogenic in laboratory rodents and *L. inadai* serovar Lyme was isolated from a skin biopsy of a human Lyme disease patient in North America [95]. Although in that case *L. inadai* was not thought to be associated with the patient's symptoms, its high prevalence in *I. holocyclus*, and the high prevalence of *Leptospira* spp. in *I. ricinus* warrants further investigation.

Francisella-like endosymbionts are well described in *Amblyomma* and *Dermacentor* ticks, and have recently been detected in *I. ricinus* ticks [28, 29, 96]. In this study we report the first instance of a *Francisella* sp. in the Australian paralysis tick *I. holocyclus.* Many *Francisella*-like endosymbionts infect tick species that are also capable of transmitting the zoonotic pathogen *Francisella tularensis,* making accurate identification by conventional PCR methodologies challenging due to false positive results [97]. The methodology presented here accurately identified non-tularaemia-causing *Francisella* spp. endosymbiont 16S sequences that were 6 % dissimilar from *F. tularensis* reference sequences, indicating that NGS and bioinformatics methodologies may prove useful in clinical diagnostic settings.

Conclusions

Next-generation 16S bacterial profiling is an excellent tool for the simultaneous identification of many bacterial species in arthropods. However, bacterial endosymbionts such as CMM, which are common and abundant in many arthropod vectors such as ticks and mosquitoes, can limit the effectiveness of this method by biasing PCR amplification of less abundant sequences. Here we have shown that a CMM-specific blocking primer significantly increases the amplification and detection of less abundant bacteria including pathogens. Furthermore our CMM-blocking primer is applicable to a range of arthropods that harbour CMM, and can be applied to a wide variety of disease vectors.

In this study we identified novel candidate pathogens that warrant further scrutiny in the context of investigating so-called "Lyme-like disease" in Australia. *Borrelia* relapsing fever and "*Candidatus* Neoehrlichia" pathogens are being identified in new geographic regions throughout the world and their medical importance is well recognised. The aetiological agent of Australian "Lyme-like" illness has been a source of unresolved debate for many years and the discovery of these organisms in Australian *I. holocyclus* ticks may provide insights into this medical conundrum. Given the widespread presence of endosymbionts in arthropod vectors of disease, together with the fact that such symbionts may be resident in high numbers, our findings also highlight the potential for discovering important novel arthropod-associated bacteria that are in relatively low abundance.

Additional files

Additional file 1: Cladogram of bacterial genera identified in extraction reagent blank and no-template controls.

Additional file 2: Cladogram of bacterial genera identified in *I. holocyclus* tick samples after the removal of genera found in control samples. Size of node circle represents the relative abundance of that genus between tick samples on a square-root scale.

Additional file 3: Cladogram of bacterial genera identified in *I. ricinus* tick samples after the removal of genera found in control samples. Size of node circle represents the relative abundance of that genus between tick samples on a square-root scale.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AWG performed DNA extractions, tick identification, 16S library preparations, next-generation sequencing, data analysis, drafted the manuscript and participated in the design of the study. CLO contributed to data analysis, the design of the study, and preparation of the manuscript. NL contributed to conceiving, designing and coordinating the study, tick collection and identification, and preparation of the manuscript. TB, HW, and VW contributed to tick collections and identification, and DNA extractions. DM and AP contributed to data analysis and preparation of the manuscript. TLG contributed to DNA extractions, tick identification, and preparation of the manuscript. AH, MB, and UR contributed to the design of the study and preparation of the manuscript. PI conceived, designed and coordinated the study and contributed to preparation of the manuscript. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Bacterial Profiling Reveals Novel "*Ca.* Neoehrlichia", *Ehrlichia*, and *Anaplasma* Species in Australian Human-Biting Ticks

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Abstract

In Australia, a conclusive aetiology of Lyme disease-like illness in human patients remains elusive, despite growing numbers of people presenting with symptoms attributed to tick bites. In the present study, we surveyed the microbial communities harboured by human-biting ticks from across Australia to identify bacteria that may contribute to this syndrome. Universal PCR primers were used to amplify the V1-2 hyper-variable region of bacterial 16S rRNA genes in DNA samples from individual *Ixodes holocyclus* (*n* = 279), *Amblyomma triguttatum* (*n* = 167), *Haemaphysalis bancrofti* (*n* = 7), and *H. longicornis* (*n* = 7) ticks. The 16S amplicons were sequenced on the Illumina MiSeq platform and analysed in USEARCH, QIIME, and BLAST to assign genus and species-level taxonomies. Nested PCR and Sanger sequencing were used to confirm the NGS data and further analyse novel findings. All 460 ticks were negative for *Borrelia* spp. by both NGS and nested PCR analysis. Two novel "*Candidatus* Neoehrlichia" spp. were identified in 12.9% of *I. holocyclus* ticks. A novel *Anaplasma* sp. was identified in 1.8% of *A. triguttatum* ticks, and a novel *Ehrlichia* sp. was identified in both *A. triguttatum* (1.2%) ticks and a single *I. holocyclus* (0.6%) tick. Further phylogenetic analysis of novel "*Ca*. Neoehrlichia", *Anaplasma* and *Ehrlichia* based on 1,265 bp 16S rRNA gene sequences suggests that these are new species. Determining whether these newly discovered organisms cause disease in humans and animals, like closely related bacteria do abroad, is of public health importance and requires further investigation.

Introduction

Over the last 30 years in Australia there have been reports of an illness in humans, the onset of which has been putatively associated with parasitism by ticks, most frequently the Australian paralysis tick (*Ixodes holocyclus*) [1]. This undetermined disease usually presents as acute flu-like symptoms including headache, fever, and fatigue that can persist for weeks to months, and

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may develop into a severe chronic illness that can include, but is not limited to, myalgia, arthralgia, chronic migraine, and a systemic inflammatory syndrome [1, 2]. Similarities between these symptoms and those of Lyme disease have led to the controversial diagnosis by some physicians of Lyme disease in Australian patients [3, 4].

In the northern hemisphere, Lyme disease is caused by the bacteria *Borrelia burgdorferi* sensu lato and is transmitted by several species of *Ixodes* ticks, including *I. ricinus* and *I. persulcatus* in Europe and Asia, and *I. scapularis* and *I. pacificus* in North America, none of which occur in Australia [5, 6]. *Borrelia burgdorferi* sensu lato is not considered by many physicians to occur in Australia, and over 20 years of scientific effort has failed to find sufficient evidence of *B. burgdorferi* sensu lato in Australian ticks, wildlife, or humans that did not acquire *Borrelia* infection overseas [1, 2, 7]. Consequently, there is significant public concern and medical uncertainty over the diagnosis and treatment of a Lyme disease-like illness in Australia, and there is a need for robust scientific inquiry to clarify the aetiology of this illness.

Ixodes holocyclus is the most significant Australian tick species from both a medical and veterinary perspective [8]. It is the tick most commonly found parasitising humans and domestic animals in its enzootic range, which spans coastal areas along almost the entire east coast of Australia and includes many of Australia's most densely populated regions [9]. Its natural wildlife hosts include a variety of small marsupials such as bandicoots (*Isoodon* spp. and *Perameles* spp.) and possums (*Trichosurus vulpecula* and *Pseudocheirus peregrinus*) [8]. *Ixodes holocyclus* causes life-threatening paralysis in domestic animals through envenomation, and in humans it can cause weakness, paralysis, and dermatological and allergic reactions, including mammalian meat allergies [10]. It is also a vector of the human pathogens *Rickettsia australis* and *R. honei*, agents of Queensland tick typhus and Flinders Island spotted fever, respectively [11, 12]. On the west coast of Australia, the most common human-biting tick is the ornate kangaroo tick, *Amblyomma triguttatum* [8], which is a putative host of *Coxiella burnetii*, the aetiological agent of Q fever, and the spotted fever pathogen *R. gravesii* [8, 13, 14].

Recently, a survey of bacteria harboured by *I. holocyclus* ticks from New South Wales (NSW), Australia, using bacterial 16S rRNA gene (16S) profiling, identified four novel candidate pathogens, including a relapsing fever group *Borrelia* sp., an *Anaplasma* sp., and two novel "*Candidatus* Neoehrlichia" species [7]. Phylogenetic analysis of 300 bp 16S rRNA gene sequences from these bacteria revealed that the *Borrelia* and "*Ca*. Neoehrlichia" were closely related to the known human tick-borne pathogens *B. duttonii* and "*Ca*. N. mikurensis", respectively [7], which share some clinical similarities to those described by patients suffering Lyme disease-like illness in Australia [15, 16]. The novel *Anaplasma* sp. was closely related to the tick-borne pathogen of cattle, *A. bovis* [7]. None of these candidate pathogens had been described previously in Australia.

The present study was designed in order to better understand the range and genetic diversity of microorganisms potentially transmitted to humans by ticks in Australia. As previously described [7], next-generation sequencing (NGS) and bioinformatics tools were used to profile bacterial populations within ticks removed from people around Australia. Additionally, species-specific PCR assays, Sanger sequencing, and Bayesian phylogenetic reconstructions were implemented to further analyse and confirm results obtained by NGS.

Methods

Ethics statement

This research complies with the *Australian Code for the ResponsibleConduct of Research*, 2007, and was approved by the Murdoch UniversityHuman Research Ethics Committee (Permit No. 2011–005). All tickcollections were opportunistic and were volunteered by people who

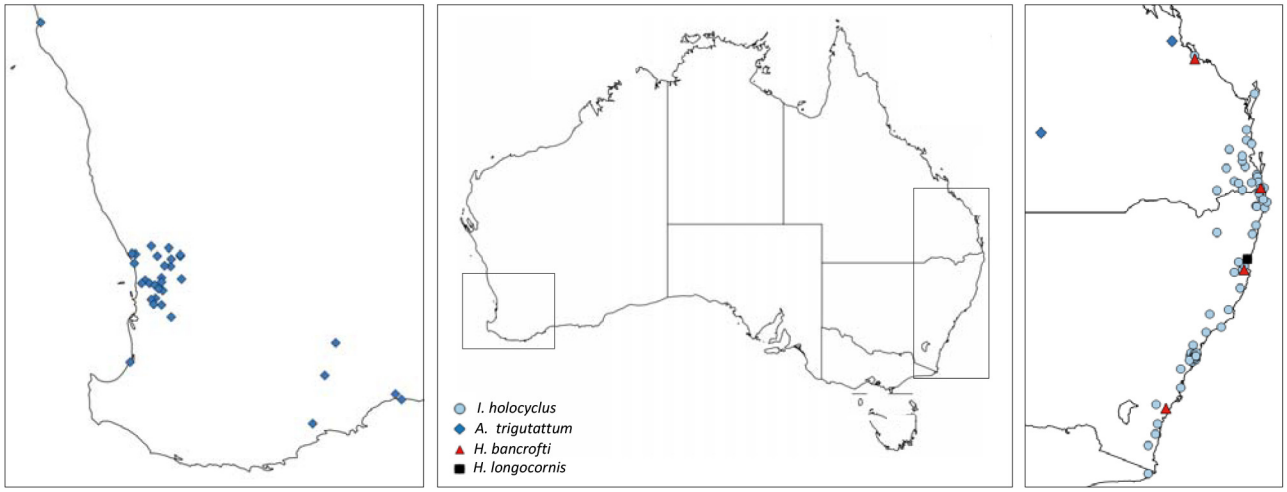

Fig 1. Geographic origin of *I. holocyclus*, *A. triguttatum*, and *Haemaphysalis* ticks used in this study. Centre, map of Australia; Left, inset of south-west Western Australia; Right, inset of Australian east coast.

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hadeither removed the ticks from themselves, or had them removed by a medicalprofessional during outpatient treatment. Participants provided writtendocumented consent to participate in this study, and the consent procedurewas approved by the Murdoch University Human Research Ethics Committee(Permit No. 2011–005).

Tick collection and identification

A total of 460 individual ticks were collected from patients attending the outpatient clinic at the Mona Vale Hospital (Mona Vale, NSW, n = 63), or solicited through media coverage and word-of-mouth (n = 397) from people experiencing tick-bite within Australia between 2013 and 2015. Information about the geographical location (Fig 1) and the date of the tick bite was obtained, and all ticks were confirmed (by medical history or questionnaire) to be actively blood feeding on humans at the time of removal. Ticks were preserved in 70% ethanol immediately after removal and morphologically identified into species, instar, and sex, at the Department of Medical Entomology, Westmead Hospital, or at Murdoch University, using standard keys [8, 17]. Tick specimens were then stored in 70% ethanol at 4°C until molecular analysis.

DNA extraction

Total genomic DNA was extracted from individual ticks using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's recommendations (QIAGEN Supplementary Protocol: Purification of total DNA from insects). Before DNA extraction, the external surface of ticks was decontaminated in 10% hypochlorite solution, washed in sterile and DNA-free PBS, and 70% ethanol, and air-dried. Ticks were then frozen in liquid nitrogen for 1 minute, and homogenised by shaking with a 5 mm steel bead at 40 Hz for 1 minute. Extraction reagent blanks (EXB) (n = 20) were performed in parallel with all DNA extractions in order to establish background bacterial populations. All DNA extractions were performed in a physical isolation hood to minimise contamination by researchers and the environment, and sterile and DNA-free equipment was used for all procedures.

Bacterial 16S rRNA gene profiling

The V1-2 hyper-variable region (250–320 bp) of bacterial 16S rRNA genes in tick DNA samples were PCR amplified using the primers 27F-Y and 338R as previously described [7]. These PCR assays for *I. holocyclus* DNA samples also included 10 μM of a "*Ca*. Midichloria mitochondrii"-specific blocking primer [7], in order to inhibit the amplification of 16S sequences from this highly abundant endosymbiotic bacterium. No-template (NT) and EXB controls were included in all PCR runs.

Amplicon library preparation was performed according to recommended protocols (Illumina Demonstrated Protocol: 16S Metagenomic Sequencing Library Preparation) with exceptions. Individual uniquely indexed libraries were normalised to equimolar concentrations with AxyPrep Mag PCR Normaliser beads (Axygen, USA) following the manufacturer's recommendations, before pooling in equimolar amounts. Up to 96 uniquely indexed libraries were pooled per sequencing run, which were performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 bp paired-end reads) following the manufacturer's recommendations. No-template and EXB controls were also sequenced to establish background bacterial populations. All pre-PCR and post-PCR procedures were performed in physically separated laboratories to minimise amplicon contamination.

Next Generation Sequencing Analysis

Sequences were first subjected to quality control procedures as previously described [7], with exceptions. Paired-end reads were merged using USEARCH v8.0.1623 [18] with a minimum overlap length of 50 bp and no gaps allowed in the merged alignments. Primer sequences and distal bases were trimmed from the ends of reads in Geneious v8.1.6 (Biomatters, New Zealand) [19] and reads shorter than the minimum previously reported length of the bacterial 16S V1-2 amplicon (< 250 bp) were removed. Singleton sequences (per sample) and sequences with a $> 1\%$ error rate were removed from the dataset using USEARCH v8.0.1623 [18]. Operational taxonomic units (OTUs) were created by clustering sequences at 97% similarity with the UPARSE algorithm [20], and taxonomy was assigned to OTUs in QIIME [21] by aligning to the GreenGenes 16S database (August 2013 release) [22] using the UCLUST algorithm [18] with default parameters. OTUs taxonomically assigned to the family or genus-level were used for further analysis. OTUs that were present in EXB and NT controls were removed from all samples in order to eliminate potentially contaminating and background bacteria.

Following OTU analysis to assign genus level taxonomy to 16S sequences, BLAST was used to resolve the species identity of families and genera that have medical or veterinary significance, or contain members that are known, or proposed, arthropod endosymbionts or pathogens. Species-level taxonomy was only inferred when the query matched 16S sequences from only one species with a $\geq 99\%$ pairwise identity over $\geq 99\%$ the length of the query sequence. Bacterial genera that were deemed not of medical or veterinary significance, known or proposed arthropod endosymbionts, or otherwise previously associated with ticks, and that were detected in less than the mean prevalence of all taxa, are herein not mentioned.

Anaplasmataceae, *Borrelia*, and *Rickettsia*-specific PCR and Sanger sequencing

In order to gain more informative phylogenetic data and to verify NGS results, species-specific PCRs were used to further confirm (or refute) the occurrence of: *Borrelia* spp., Anaplasmataceae species (except *Wolbachia* spp.), and spotted fever and typhus group *Rickettsia* species in ticks. The *Borrelia*-specific assay targeted a 441 bp region of the chromosomal flagellin gene

(*fla*B) and consisted of two nested PCRs, the primary reaction with primers *fla*B-280F and *fla*B-RL, and the nested reaction with primers *fla*B-LL and *fla*B-737R [23, 24], and verified previously in our laboratory to reliably amplify *B. burgdorferi* sensu lato, and relapsing fever group *Borrelia* spp. from tick specimens. The presence of Anaplasmataceae species in ticks was confirmed using a nested PCR assay targeting a 1.3 kb region of the 16S rRNA gene of Anaplasmataceae species (except *Wolbachia* spp.). The primary PCR contained the primers EC9 and EC12A [25, 26] and the nested reaction contained primers A17a and IS58-1345R [27]. The presence of spotted fever and typhus group *Rickettsia* species was confirmed with a qPCR assay using the primers CS-F and CS-R, and hydrolysis probe CS-P, as previously described [28].

Borrelia and Anaplasmataceae-specific primary PCRs contained 2 μl of tick DNA and the nested reaction used 1 μl of the primary PCR product as a template. PCRs contained PCR buffer, 2.5 mM $MgCl_2$, 1 mM dNTPs, 0.01 mg BSA (Fisher Biotech, Australia), 1.25 U Perfect *Taq* Polymerase (5 Prime, Germany), and 400 nM of each primer, in a total volume of 25 μl. All PCRs included NT controls and positive controls (*B. afzelii* or "*Ca.* N. mikurensis" from *I. ricinus* ticks, and *R. australis* from culture). All positive PCR products were electrophoresed in 2% agarose gels stained with GelRed (Biotium, USA), visualised under UV light, purified with the QIAquick gel extraction kit (QIAGEN, Germany), and sequenced with both forward and reverse PCR primers on an ABI 3730 96 Capillary Sequences using Big dye v3.1 terminators (Life Technologies, USA).

Anaplasmataceae 16S phylogenetic analysis

Phylogenetic analysis was conducted on 1,265bp 16S sequences obtained from the Anaplasmataceae-specific nested PCR on *I. holocyclus* and *A. triguttatum* samples, and additional Anaplasmataceae 16S sequences retrieved from GenBank. Sequences were aligned with MAFFT [29] and the gapped alignment was refined with MUSCLE [30]. The most suitable nucleotide substitution model was assessed in MEGA6 [31] and selected based on the Bayesian Information Criterion. Bayesian phylogenetic analysis was performed with the MrBayes software [32] using the HKY85 substitution model and a discrete Gamma distribution with 5 categories, a total chain length of 1,100,000, burn-in length of 100,000, and subsampling every 200 iterations.

Results

Bacterial 16S rRNA gene community profiling

The tick species collected from people while attached and feeding included *I. holocyclus* ($n = 279$), *A. triguttatum* ($n = 167$), *Haemaphysalis bancrofti* ($n = 7$), and *H. longicornis* ($n = 7$) (Table 1). *Ixodes holocyclus* ticks were received from almost the entirety of its enzootic range along the east coast of Australia from Gladstone, Queensland (QLD) to Mallacoota, Victoria (Fig 1). *Amblyomma triguttatum* ticks were primarily collected from southwest Western Australia (WA), including many semi-rural and rural areas surrounding Perth, as far north as Kalbarri, WA, and southeast at Hopetoun, WA (Fig 1). *Amblyomma triguttatum* ticks were also received from Rockhampton and Charleville, QLD (Fig 1). *Haemaphysalis longicornis* were collected from only a single location; Urunga, NSW, and *H. bancrofti* was collected from four locations, Gladstone, QLD, Currumbin, QLD, Mollymook, NSW, and Tamban, NSW (Fig 1).

After NGS quality control procedures, 30,450,159 16S sequences from 460 tick samples and 25 NT and EXB control samples were used for analysis (Table 1). A total of 41 bacterial genera that were found in NT and EXB controls were removed from the dataset as background bacteria. All of the background taxa were either ubiquitous environmental or human-associated

Table 1. Summary of sample size, NGS coverage, and taxonomic diversity of tick species and life stages.

Tick Species	Instar/ Sex	Number of samples	Total number of sequences	Mean sequences per sample	Number of bacterial genera[a]
I. holocyclus	Females	167	16,196,861	96,987.2	27
	Male	49	4,427,177	90,350.5	19
	Nymphs	63	3,188,402	50,609.5	22
A. triguttatum	Female	40	1,787,788	44,694.7	19
	Male	24	538,571	22,440.4	16
	Nymph	103	3,032,534	29,442.1	18
H. bancrofti	Male	1	13,284	13,284.0	16
	Nymph	6	145,742	24,290.3	15
H. longicornis	Female	3	72,635	24211.7	20
	Nymph	4	101,927	25,481.7	18
NT and EXB Controls		25	945,238	37,809.5	41

[a] For tick samples only genera that were found in more than the mean prevalence of all taxa are shown

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commensal bacterial genera that to the best of our knowledge have never been associated with tick-borne human or veterinary disease.

The most prevalent organisms identified in *I. holocyclus*, *A. triguttatum*, *H. bancrofti*, and *H. longicornis* ticks were environmental or commensal bacteria that included 34 genera within Actinomycetales, Bacteriodetes, Firmicutes, Rhizobiales, Burkholderiales, and Gammaproteobacteria. The genera *Propionibacterium*, *Staphylococcus*, and *Streptococcus*, which live as commensals on mammalian skin were identified in all tick species (Fig 2). Other environmental genera identified, such as *Bacillus*, *Agrobacterium*, *Corynebacterium*, *Delftia*, *Flavobacterium*, *Methylobacterium*, *Mycobacterium*, *Pseudomonas*, *Ralstonia*, and *Stenotrophomones* are considered as either ubiquitous in the environment, or associated with soil and moist leaf litter environments in which ticks spend a large proportion of their life cycle (Fig 2). No *Borrelia* sp. sequences were identified in any of the 460 ticks.

Bacterial endosymbionts in human-biting ticks. Proposed bacterial endosymbionts were highly prevalent in all ticks studied, with each tick species having one or two predominant endosymbiont species and one to three less prevalent endosymbiotic associations. As anticipated from a previous study [7], the *Ixodes* tick endosymbiont "*Ca.* Midichloria mitochondrii" (16,519 unique sequences) was found in all *I. holocyclus* ticks, however, as expected (due to the use of a blocking primer during PCR) [7], 16S sequences from this abundant bacterium only comprised 4–17% of sequences per sample. In addition, *Wolbachia*, *Francisella*, and *Rickettsiella* spp. were also identified in 1.4%, 5.4%, and 11.1% of *I. holocyclus* ticks, respectively (Fig 2). Bacteria of the genus *Rickettsia* (7,069 unique sequences) were also identified in 27.5% of females, 44.9% of males, and 27% of nymph *I. holocyclus* ticks, with a total prevalence 30.5% in this tick species. Unfortunately *Rickettsia* 16S reads were unable to be given species designation due to high sequence homology (> 99%) between many *Rickettsia* species at the 16S locus analysed.

All *A. triguttatum*, *H. bancrofti*, and *H. longicornis* ticks studied were dual-infected with *Francisella* (12,990 unique sequences) and *Rickettsia* spp. (7,069 unique sequences) (Fig 2). *Francisella* and *Rickettsia* spp. sequences from these ticks were highly abundant in the NGS results, comprised between 12%-98% and 2%-88% of sequences per sample, respectively. *Francisella* sequences from all ticks were more than 98% similar to known endosymbiotic

Francisella spp. from *A. maculatum* (GenBank: AY375407) and *Dermacentor* spp. (GenBank: AY375403, AY375401, JX101605) ticks from the northern hemisphere, and less than 94% similar to the infectious human pathogen *Francisella tularensis* (GenBank: NR074666), which has never been reported in Australia. In addition to endosymbiotic *Francisella* and *Rickettsia* spp., all *H. bancrofti* ticks also harboured a *Coxiella* sp., presumed to be an endosymbiont, as did 5% of *A. triguttatum* females. *Coxiella* sp. sequences were highly abundant in *H. bancrofti* ticks, comprising 23%-92% of sequences per sample. These *Coxiella* sp. sequences were more than 99% similar to *Coxiella* sp. endosymbionts reported previously from *H. lagrangei* and *H. longicornis* from Thailand and Korea (GenBank: KC170756, AY342036), respectively, but less than 94% similar to the infectious pathogen *C. burnetii* (GenBank: HG825990).

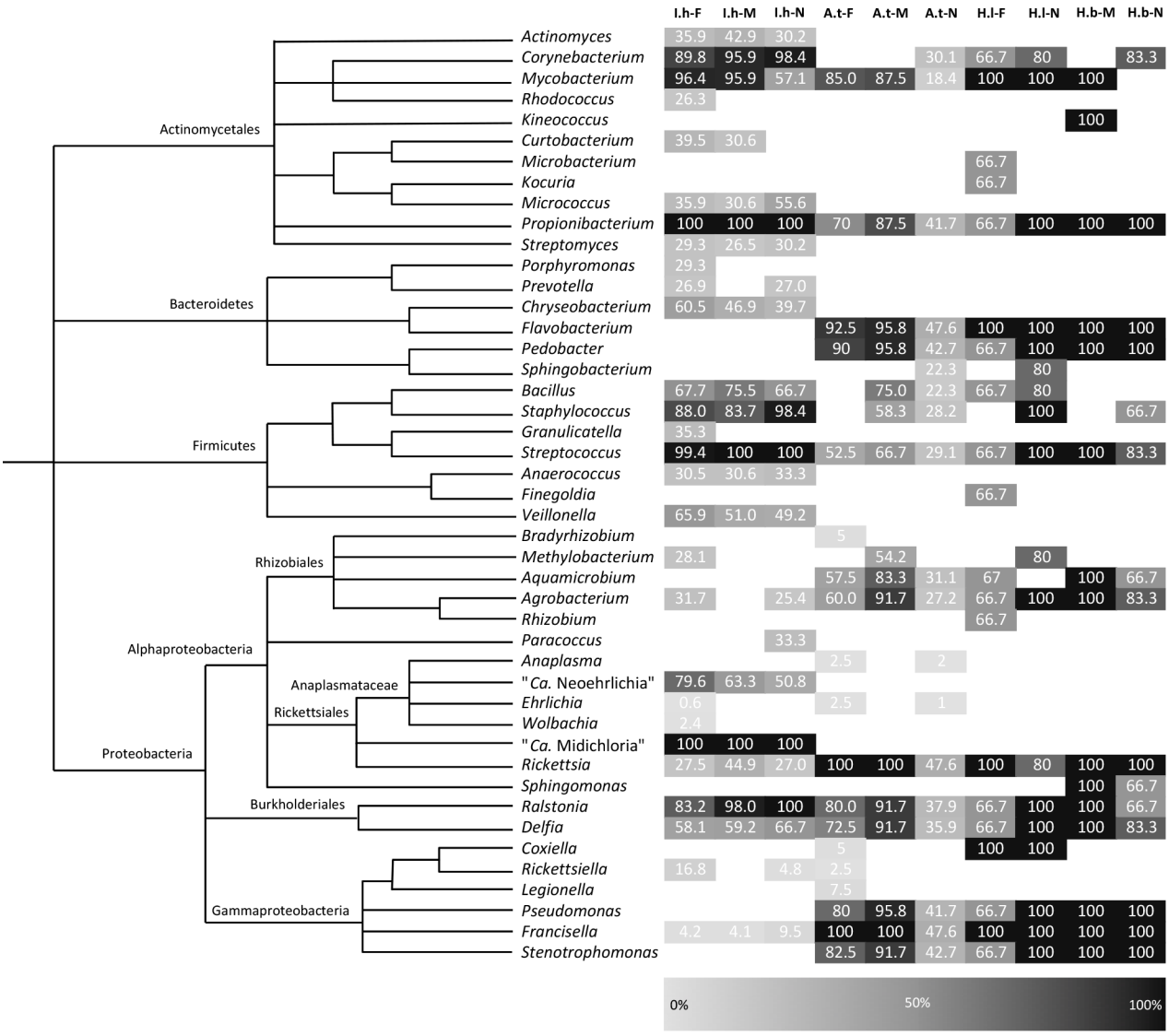

Fig 2. Cladogram and heat map showing the prevalence of bacterial genera in tick species and life stages. I.h, A.t, H.l, and H.b indicate *I. holocyclus*, *A. triguttatum*, *H. longicornis*, and *H. bancrofti* tick species, respectively. Female, Male and Nymph life stages are indicated by F, M, and N, respectively. The level of shading corresponds to the prevalence of the genera in the tick species and life stage. Blank shading indicates that bacterial genera were not detected in that tick species life stage.

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Novel Anaplasmataceae species identified in human-biting ticks. The genus "*Ca.* Neoehrlichia" (11,493 unique sequences) was identified in all *I. holocyclus* life stages studied, with a prevalence of 76.6%, 63.3%, and 50.8% in females, males, and nymphs, respectively, and a total prevalence of 88.9%. "*Candidatus* Neoehrlichia" sequences formed two distinct clusters, herein putatively named species A and B, which were 6–7% dissimilar from each other (S1 Table). The closest known relative to putative "*Ca.* Neoehrlichia" species A and B was "*Ca.* N. mikurensis" (94.6–94.9% similarity) (GenBank: AB196304) from Japan. Putative species A and B sequence were also highly similar to "*Ca.* N. lotoris" (95.9–96.3% similarity) (GenBank: EF633744), although the sequence query coverage was only 90.8%. Putative "*Ca.* Neoehrlichia" species A was most common, being found in 68.8% of "*Ca.* Neoehrlichia"-positive *I. holocyclus* ticks, compared to species B (31.2%). All sequences from both "*Ca.* Neoehrlichia" putative species A and B were more than 99% similar to "*Ca.* Neoehrlichia" spp. 16S sequences recently obtained by NGS from *I. holocyclus* ticks from NSW, Australia [7], with species A and B most similar to "*Ca.* Neoehrlichia" sp. isolates PI808 (GenBank: KT203915), and PI800 (GenBank: KT203914), respectively. Among all of the "*Ca.* Neoehrlichia"-positive *I. holocyclus* ticks, there were no cases of co-infection with both putative species A and B.

Interestingly, "*Ca.* Neoehrlichia" sequences were not detected in any *A. triguttatum* or *Haemaphysalis* ticks; however, two other Anaplasmataceae species were identified in *A. triguttatum* ticks and a single *I. holocyclus* female. Novel *Anaplasma* sp. sequences (284 unique sequences) were identified in three *A. triguttatum* ticks (1.8%), including one female (2.5%), and two nymphs (2%). These *Anaplasma* sp. sequences were most similar (98%) to an uncultured *Anaplasma* sp. (GenBank: JN862824) from southeast China, and the closest recognised species (97%) was *A. bovis* (GenBank: KJ659040). All three *A. triguttatum* ticks infected with this novel *Anaplasma* sp. originated from Yanchep National Park, Western Australia. Novel *Ehrlichia* sp. sequences (206 unique sequences) were also identified in two (1.2%) *A. triguttatum* ticks including one nymph, one female, and one *I. holocyclus* female (0.6%). These novel *Ehrlichia* sp. sequences were most similar (97%) to *E. ruminantium* (GenBank: DQ482921, CR925677), and another unresolved *Ehrlichia* sp. from *H. longicornis* ticks from Japan (GenBank: AY309970, HQ697588). The two *A. triguttatum* ticks infected with this novel *Ehrlichia* sp. both originated from Bullsbrook, Western Australia and the *I. holocyclus* tick originated from Pimpama, Queensland.

Anaplasmataceae, *Borrelia*, and *Rickettsia*-specific PCR

All 460 *I. holocyclus*, *A. triguttatum*, and *Haemaphysalis* ticks were negative for *Borrelia* spp. by nested PCR, confirming the 16S community profiling results. The spotted fever and typhus group-specific qPCR did not amplify any *Rickettsia* from *I. holocyclus* ticks. However, all *Rickettsia*-positive *A. triguttatum* and *Haemaphysalis* ticks (by NGS) were amplified with this qPCR assay, indicating the *Rickettsia* spp. in these ticks are within, or closely related to spotted fever and typhus group *Rickettsia* species. The Anaplasmataceae-specific PCR assay returned 37 positive *I. holocyclus* ticks (12.9%), including 19 females (11.4%), eight males (16.3%), and 10 nymphs (15.9%), and five positive *A. triguttatum* ticks (3%), including two females (5%), and three nymphs (2.9%). No *Haemaphysalis* ticks were positive for Anaplasmataceae species.

Anaplasmataceae Phylogenetic Analysis

Bayesian phylogenetic reconstruction of 1,265 bp 16S Anaplasmataceae sequences revealed that 36 (12.9% of all *I. holocyclus*) of the 37 positive *I. holocyclus* samples grouped with high confidence within the genus "*Ca.* Neoehrlichia". Furthermore, the 16S sequences from these ticks clustered into two distinct groups, one containing identical sequences from 25 *I. holocyclus* ticks (9%) comprising putative "*Ca.* Neoehrlichia" species A (GenBank: KT803957), and

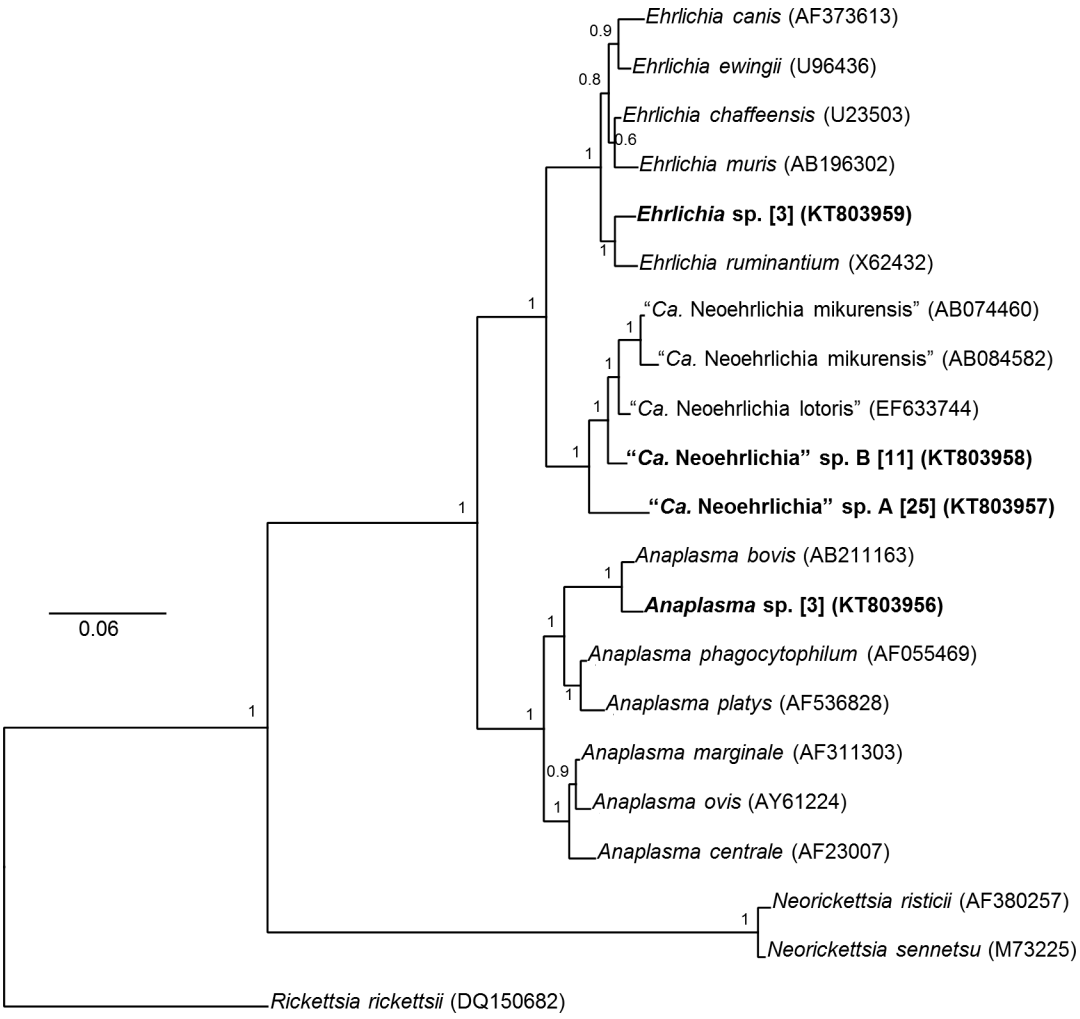

Fig 3. Bayesian phylogenetic analysis of 1,265 bp novel Anaplasmataceae 16S rRNA sequences from *I. holocyclus* and *A. triguttatum*. Bayesian posterior probabilities are displayed at each node. Bold type indicates sequences from this study. Rounded parentheses indicate GenBank accession numbers, and square parentheses indicate the number of ticks from which identical sequences were obtained.

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the other containing identical sequences from 11 *I. holocyclus* ticks (4%), comprising putative "*Ca*. Neoehrlichia" species B (GenBank: KT803958) (Fig 3). Sequence from putative "*Ca*. Neoehrlichia" species A and B shared 96.2% similarity (S2 Table). The two known members of the genus, "*Ca*. N. lotoris" (GenBank: EF633744) and "*Ca*. N. mikurensis" (GenBank: AB074460, AB084582), were 98.1–98.6% similar at the 16S loci, however, putative "*Ca*. Neoehrlichia" species A and B were only 95.7–96.2%, and 97.3–98.4% similar to these species, respectively (S2 Table).

The level of divergence at the 16S loci both between putative "*Ca*. Neoehrlichia" species A and B, and between these and known "*Ca*. *Neoehrlichia*" spp., confirms the clustering pattern observed in the NGS data, and described previously [7]. All *I. holocyclus* ticks positive here for novel "*Ca*. Neoehrlichia" spp. were also positive for "*Ca*. Neoehrlichia" spp. by NGS, although the prevalence of "*Ca*. Neoehrlichia" spp. was significantly lower as determined by nested PCR (12.9%) than by NGS (88.9%).

Three identical novel *Anaplasma* sp. 16S sequences (GenBank: KT803956) from *A. triguttatum* ticks (1.8%), including one female and two nymphs, clustered with high confidence, but were distinct (98.7% similarity) from *A. bovis* (GenBank: AB211163) (Fig 3, S2 Table). In addition, a further three identical novel *Ehrlichia* sp. sequences (GenBank: KT803959) from two *A. triguttatum* ticks (1.2%), including one female (2.5%) and one nymph (0.97%), and one *I. holocyclus* female (0.6%) clustered with high confidence, but was distinct (98.3% similarity) from *E. ruminantium* (GenBank: X62432) (Fig 3, S2 Table). The level of divergence between these novel *Anaplasma* sp. and *Ehrlichia* sp. 16S sequences, and their closest relatives, is within the range of divergence among all *Anaplasma* species (94.7–99.4%) and *Ehrlichia* species (97.3–98.9%) (S2 Table). All ticks positive by nested PCR for novel *Anaplasma* sp. and *Ehrlichia* sp. were also positive for these taxa through NGS.

Discussion

This study follows a preliminary investigation of the bacterial microbiome associated with *I. holocyclus* in a localised region of NSW, with the aim of investigating a collection of human-biting ticks over a greater geographical range, including areas of Sydney, NSW, where numerous patients have been diagnosed with a Lyme disease-like illness. In Australia, approximately eight species of hard ticks, and one species of soft tick (*Ornithodorus capensis*) are known to bite humans [8, 17, 33]. Consistent with previously published and anecdotal reports, the Australian paralysis tick (*I. holocyclus*) and the ornate kangaroo tick (*A. triguttatum*) were most frequently associated with attachment and engorgement on the skin of people in this study [8]. The introduced 'bush' tick, *H. longicornis*, normally a parasite of cattle, and the native wallaby tick (*H. bancrofti*) are also well known to bite people in Australia [8]. Curiously, we did not receive any specimens of the brown dog tick (*R. sanguineus*), the common marsupial tick (*I. tasmani*) or the southern paralysis tick (*I. cornuatus*) for analysis in this study, all of which have previously been associated with human tick bites in Australia.

Although the external cuticle of all ticks was decontaminated with ethanol and 10% hypochlorite solution prior to molecular analyses, a range of common environmental and commensal bacteria were still prevalent among all ticks surveyed. This is most likely due to remnant bacterial DNA that survived the decontamination process, perhaps in bacterial plaques that may have accumulated in less accessible places such as between leg joints or underneath the tick's palps. In future studies careful dissection of the tick's main internal tissues (midgut, salivary gland, and gonads) may prove useful in distinguishing the microbiome of the internal tissues from environmental bacteria on the tick's external surfaces. Because all ticks surveyed were collected while actively feeding on humans it must be acknowledged that some bacteria in tick samples, such as *Staphylococcus* spp. and *Propionibacterium* spp., may have been from the blood and skin of the human hosts. However, most bacteria identified in the present study have been associated previously with ticks as members of genera that contain either known tick-borne pathogens, or arthropod endosymbionts.

Consistent with previous analysis [7], endosymbiotic "*Ca.* M. mitochondrii", *Wolbachia*, *Francisella*, and *Rickettsia* spp. were identified in *I. holocyclus* ticks. All *A. triguttatum*, *H. bancrofti*, and *H. longicornis* ticks studied were dual-infected with endosymbiotic *Francisella* and *Rickettsia* spp., which comprised a large proportion of NGS sequencing output for these samples. Although *Francisella* endosymbionts have been described from northern hemisphere *Amblyomma* and *Dermacentor* ticks [34–36], and previously in *I. holocyclus* [7], this is the first description of *Francisella* spp. in a native Australian *Amblyomma* or *Haemaphysalis* tick. Species-specific blocking primers have been shown to be effective at inhibiting specific endosymbiont 16S sequences in *I. holocyclus* and *I. ricinus* [7], allowing the detection of less abundant

bacterial taxa. It is probable that the use of *Francisella* and *Rickettsia*-specific blocking primers during 16S bacterial profiling of *A. triguttatum* and *Haemaphysalis* spp. ticks may similarly reveal more information about the less abundant bacterial taxa associated with these ticks.

The very high prevalence of *Rickettsia* spp. in *A. triguttatum* and *Haemaphysalis* ticks in this study suggest these *Rickettsia* spp. are likely endosymbiotic, and either advantageous or benign to the fitness of these tick species. The fact that these species were amplified with a qPCR assay designed to amplify only spotted fever and typhus group *Rickettsia* species and not the ancestral *R. bellii* species group [28], suggests these likely bacteria are more closely related to the spotted fever and typhus group than the *R. bellii* species group [37]. However, the spotted fever and typhus group qPCR did not amplify *Rickettsia* spp. found in *I. holocyclus* ticks, suggesting that these species are more closely related to the ancestral *R. bellii* group, which are typically endosymbionts of arthropods [37]. Further studies should include species-specific PCR and Sanger sequencing of a more informative marker gene to resolve the phylogenetic identity of *Rickettsia* spp. endosymbionts in Australian ticks, and to determine the prevalence of pathogenic *Rickettsia* spp. in Australia.

The absence of *Borrelia* sp. in the ticks studied here is somewhat unexpected considering the recent description of a single relapsing fever *Borrelia* sp. isolate found in a recent survey of *I. holocyclus* ticks using the same NGS method as in the present study. In that case the *Borrelia*-infected *I. holocyclus* tick was removed from an echidna, which is not a typical host for *I. holocyclus*. Surveying the microbial communities of ticks that share a close association with echidnas, such as *Bothriocroton concolor* and *B. hydrosauri*, may reveal more Australian *Borrelia* sp. isolates.

Based on the phylogenetic inference of 1,265 bp 16S sequences, the novel "*Ca.* Neoehrlichia", *Ehrlichia*, and *Anaplasma* detected in the present study appear to be putative species, as the levels of divergence between their sequences and those of their closest relatives, is within the range of accepted species separation at the 16S rRNA gene loci [38–41]. However, formal descriptions of them as new species will require analysis at multiple loci such as the citrate synthase gene (gltA), RNA polymerase sub-unit β (*rpoB*) and heat shock operon (groESL), or whole genomes [27, 42–46].

The overall prevalence of novel "*Ca.* Neoehrlichia" species A and B across all *I. holocyclus* life stages was 88.9% by NGS but only 12.9% by nested PCR. There are several reasons that may explain this discrepancy; firstly the nested PCR amplified a large fragment (the primary amplicon was approximately 1.4 kb and the secondary amplicon was approximately 1.3 kb), which is known to reduce the efficiency of PCR [47]. For NGS, the amplicon size was much smaller (250–320 bp) and would therefore be expected to amplify with much greater efficiency [47, 48]. Secondly, NGS allows the detection of low abundant sequences, and mixed sequences that would not be detected with Sanger sequencing [48]. Further studies should include use a "*Ca.* Neoehrlichia"-specific droplet digital PCR quantitation assay targeting small amplicon sizes, as this will allow for more accurate quantitation [49, 50] and determination of the true prevalence of novel "*Ca.* Neoehrlichia" species in *I. holocyclus*.

All recognised members of the genera *Anaplasma*, *Ehrlichia*, and "*Ca.* Neoehrlichia" are obligate intracellular tick-borne mammalian pathogens that typically infect haematopoietic (mammalian) or endothelial (mammalian and tick) cells [25, 51–53]. There has been no confirmed transovarial transmission of *Anaplasma*, *Ehrlichia*, or "*Ca.* Neoehrlichia" species in vector-ticks or mammals, and therefore their persistence is attributed predominantly to infected mammalian reservoir populations [51–53]. Throughout Europe, Asia, and North America several Anaplasmataceae species are pathogens of veterinary significance (such as *E. canis* and *E. ruminantium*) and important emerging human pathogens, such as *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, and "*Ca.* N. mikurensis"

"*Ca*. Neoehrlichia" is a recently described genus that currently comprises two species, "*Ca*. N. lotoris", and "*Ca*. N. mikurensis" [27, 45]. Of these "*Ca*. N. mikurensis" is now recognised an emerging tick-borne zoonosis vectored by several tick species (*I. ricinus*, *I. ovatus*, and *I. persulcatus*), and is one of the most prevalent tick-borne infections in wildlife and ticks throughout Europe and Asia [27, 35, 54–68]. Clinical reports of human infections are steadily increasing, due in part to increased awareness and testing [53]. Infection with "*Ca*. N. mikurensis" (neoehrlichiosis) is typically severe, with a wide variety of non-specific symptoms reported [69–75]. In Europe, neoehrlichiosis usually manifests in immunocompromised patients, however in China, there are increasing reports of this infection in immunocompetent people, and asymptomatic infections in humans have also been reported [76, 77]. In contrast, "*Ca*. N. lotoris" is a tick-borne pathogen of racoons (*Procyon lotor*), and to date there are no reports of human infection [45, 78]. In the northern hemisphere treatment of patients suffering neoehrlichiosis with doxycycline (1 x 200 mg/day) for 3–6 weeks has been shown to be effective [71, 75, 79, 80], and may have implication if human or animals infections are found to occur in Australia.

The identification of four novel putative tick-borne Anaplasmataceae species in Australian human-biting ticks is of potential public health significance, especially the high prevalence of novel "*Ca*. Neoehrlichia" spp. in *I. holocyclus* ticks. Based on their phylogenetic position, as inferred here, and the disease-causing status of their close relatives, all four species are candidate human and animal pathogens, and almost certainly infective (symptomatic or asymptomatic) to Australian wildlife species. Determining whether these *Ehrlichia*, *Anaplasma* and "*Ca*. Neoehrlichia" species may cause disease in Australian humans, like their close relatives do overseas is of public health importance. Future studies should include the development of specific digital and qPCR assays to more accurately determine the prevalence and pathogen load in ticks, wildlife, and humans. In addition, the isolation and culture of these organisms, in pure culture or infected mammalian and tick cell culture, will significantly aid in understanding the biology and potential pathogenicity of these novel Anaplasmataceae, and the development of specific diagnostic serological test and therapeutic practices.

Supporting Information

S1 Table. Distance matrix of the pairwise percent similarity of the 10 most prevalent 16S V1-2 sequences from "*Ca*. Neoehrlichia" putative species A (A1-10) and B (B1-10). Shading indicated > 99% similarity between sequences in putative "*Ca*. Neoehrlichia" spp. A and B.
(PDF)

S2 Table. Pairwise percentage distance matrix of 1,265 bp Anaplasmataceae 16S sequences from this study and retrieved from GenBank used for Bayesian phylogenetic reconstruction.
(PDF)

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Author Contributions

Conceived and designed the experiments: AWG SD UR PI. Performed the experiments: AWG SD. Analyzed the data: AWG. Contributed reagents/materials/analysis tools: AWG SD AR UR CLO AP PI. Wrote the paper: AWG SD AR UR CLO AP PI.

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MICROBIOLOGY

Concordance of four commercial enzyme immunoassay and three immunoblot formats for the detection of Lyme borreliosis antibodies in human serum: the two-tier approach remains

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Summary

Serological tests show considerable variation in their ability to correctly diagnose Lyme borreliosis (LB). This study compared four commercially available screening enzyme immunoassays (EIA) for the detection of LB IgG using either whole cell lysate (WCL) antigens, purified proteins or recombinant antigens with the second-tier whole cell sonicate (WCS) western immunoblots or recombinant antigen line blots.

A consensus between three EIA results from 222 patient sera was designated as a point of comparison for each method which gave 66 positive and 156 negative results. The positive predictive values (PPV) of WCL EIA were 40% for the MarDx Diagnostics *Borrelia burgdorferi* EIA 'combined' IgG and IgM (Trinity Biotech) and 55% for the EUROIMMUN plus VlsE IgG. These were significantly lower PPVs than that produced by the recombinant antigen-based EIA NovaLisa *Borrelia burgdorferi* IgG-ELISA (NovaTec Immunodiagnostica) and the EUROIMMUN Anti-Borrelia Select ELISA IgG (90% and 100%, respectively; $p = 0.02$). The WCS western immunoblot using *B. burgdorferi* and *B. afzelii* separately showed a high PPV of 91% but its positive agreement with consensus EIA result was only 65%. Another WCL western immunoblot with purified extracts of Osp C and VlsE, the Trinity Biotech EU Lyme + VlsE IgG Western Blot had a PPV of 92% while the recombinant line blot from EUROIMMUN, the Anti-Borrelia (IgG) EUROLINE-RN-AT, demonstrated a significantly reduced PPV of 70% with some non-specific reactions in sera containing antibodies to *Leptospira* species, *Helicobacter pylori* and *Treponema pallidum*.

The use of recombinant antigens in EIA for LB IgG screening significantly improves the predictive values of serological results above those of WCL antigen EIA. Second tier WCS western immunoblots offer high PPVs, especially with added specific purified proteins, more so than in one recombinant line blot.

Key words: Lyme disease; laboratory diagnosis; serology; *Borrelia burgdorferi*.

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INTRODUCTION

Serological tests for the detection of antibodies to Lyme disease *Borrelia* show considerable variation in their ability to correctly diagnose patients with Lyme borreliosis (LB).[1,2] Reasons for variation in test performance include antigenic differences in the causative pathogen namely the spirochaete, *Borrelia burgdorferi* sensu lato (s.l.), a bacterium which encompasses a range of regionally specific genospecies.[3] *Borrelia burgdorferi* sensu stricto (s.s.) is most commonly isolated from North American patients, while in Europe *B. burgdorferi sensu stricto, B. garinii* and *B. afzelii* are associated with human disease.[4,5] Since the first serology tests were used in diagnosis, common antigenic epitopes that cross react with other bacteria or autoimmune disease proteins have been identified, especially for Lyme disease IgG assays employing whole cell lysates of *Borrelia*.[6–10] Highly variable antigenic composition of commercially available screening enzyme immunoassays (EIA) have also created difficulties in the comparison and interpretation of serological results for LB.[3,11,12] To limit such variation and cross-reactivity and to improve predictive values of serological tests, a two-tiered system of testing was introduced.[13] Specifically, immunoblots have been used as this second tier after detecting positive or equivocal results from the first tier screening EIA. Defining a confirmed case depended on the detection of at least five out of a potential 10 specific bands at 18 kDa, 21 kDa (OspC), 28 kDa, 30 kDa, 39 kDa (BmpA), 41 kDa (flagellin), 45 kDa, 58 kDa (not GroEL), 66 kDa, and 93 kDa.[13,14] A number of different assays have been introduced to optimise laboratory diagnosis of LB in the last 20 years[15,16] with gradually improving specificity due to the selection of recombinant or peptide antigens instead of the historical whole cell lysate preparations.[3,11] These different antigen preparations from various pathogenic *Borrelia* species have been utilised in various combinations in commercial assays.

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Table 1 EIA kit components and criteria for result interpretation

	MarDx (Trinity Biotech) *B. burgdorferi* EIA (IgG, IgM)	NovaLisa *Borrelia burgdorferi* IgG ELISA (recombinant)	EUROIMMUN Anti-Borrelia plus VlsE ELISA IgG	EUROIMMUN Anti-Borrelia Select ELISA IgG
LB genospecies tested	*B. burgdorferi* sensu stricto strain B31	*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*	*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*	*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*
Antigens	Whole antigen extracts from *B. burgdorferi* sensu stricto strain B31	Recombinant antigens: OspC 22kDa (*B. burgdorferi* sensu stricto strain B31) p100 (*B. afzelii PKo*) p18 (*B. afzelii PKo*) 20047 and T25 (*B. garinii* PBi) Flagellin P41i (*B. garinii* PBi)	Whole antigen extracts from *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. Recombinant VlsE	Recombinant antigens: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*
Criteria for result interpretation	Signal/cut off ratios: Positive ≥1.2 Equivocal 1.0 – <1.2 Negative <1.0	Signal/cut off ratios: Positive >1.1 Equivocal 0.9–1.1 Negative <0.9	Signal/cut off ratios: Positive ≥1.1 Equivocal 0.8 – <1.1 Negative <0.8	Signal/cut off ratios: Positive ≥1.1 Equivocal 0.8 – <1.1 Negative <0.8

This study aimed to compare four currently available screening enzyme linked immunosorbent assays (ELISA) for the detection of LB IgG antibodies with the second-tier in-house western immunoblot and two new commercial second-tier immunoblot kits using a set of samples collected in a low incidence country.

MATERIALS AND METHODS

First tier screening LB assays

Four ELISA screening kits were tested including two recombinant antigen ELISA namely the NovaLisa *Borrelia burgdorferi* IgG-ELISA (recombinant) (NovaTec Immunodiagnostica, Germany) and the EUROIMMUN Anti-Borrelia Select ELISA IgG (EUROIMMUN Medizinische Labordiagnostika, Germany). The other two ELISA kits were whole cell lysate (WCL) assays: the EUROIMMUN Anti-Borrelia plus VlsE ELISA IgG and the MarDx *Borrelia burgdorferi* EIA IgG and IgM (MarDx Diagnostics, Trinity Biotech Company, USA). The antigens and other reagents used in each assay are listed in Table 1. Testing was performed according to the manufacturer's instructions and results were expressed as signal to cut-off ratios with different equivocal or grey zones.

Second tier assays

The in-house second-tier western immunoblot for *B. burgdorferi* and *B. afzelii* IgG and two commercial immunoblot kits, namely EUROIMMUN Anti-Borrelia (IgG) EUROLINE-RN-AT and Trinity Biotech EU Lyme + VlsE IgG Western Blot were compared to the screening ELISA. The in-house immunoblot used modifications according to the method of Dressler *et al.*[14] with precast SDS PAGE gels (ExcelGel SDS homogeneous 12.5; GE Healthcare, Sweden) of 0.5 mg/mL whole cell sonicate (WCS) of *B. burgdorferi* strain 297 and separately 1.0 mg/mL WCS *B. afzelli* ATCC 51567. Each immunoblot used different antigens and different criteria, recommended by the manufacturer, for defining positive results (Table 2).

Samples

A total of 222 clinical specimens were selected to evaluate positive agreement (sensitivity) and negative agreement (specificity). The samples were collected, initially tested and archived between 2002 and 2013 and then selected from −25°C storage on the basis of previously having a MarDx and western immunoblot result. They comprised samples received from public and private pathology providers around Australia and New Zealand. All specimens were allowed to come to room temperature and were mixed well before testing. A subset of 23 of these samples was from patients with other proven infections to provide further evidence of specificity. The subset included specimens which tested positive for the following infectious diseases or autoimmune markers: syphilis, Epstein–Barr virus induced infectious mononucleosis, leptospirosis, *Helicobacter pylori* infection, anti-nuclear antibody and rheumatoid factor. In an attempt to remove the bias of selecting specimens by the result of only one EIA, a consensus of results from three of the four screening test EIAs was used to compare all kits and immunoblots. For example a specimen was considered positive if the results of three screening test EIAs were higher than their respective cut-off value.

Statistical analysis

Descriptive statistics were used to calculate agreement and predictive values and differences with *t*-test or p values <0.05 were considered statistically significant.

RESULTS

The criteria of three concurring results out of the four EIA kits revealed 66 positive and 156 negative sera in our testing set. Comparisons of the parameters of test performance of all assays are detailed in Tables 3 and 4. Using the consensus results, significant differences in agreement of results were observed comparing the WCL MarDx IgG/IgM with all other methods. Only 56% of the MarDx results agreed with the consensus while other methods had significantly higher

Table 2 Components and result interpretation criteria for immunoblot assays

	Western immunoblot IgG for *B. burgdorferi* and *B. afzelii*	EUROIMMUN Anti-Borrelia (IgG) EUROLINE-RN-AT	Trinity Biotech EU Lyme + VlsE IgG Western Blot
Test format	Western immunoblot	Line blot	Western plus line blot
LB genospecies tested	*B. burgdorferi* sensu stricto strain 297 and *B. afzelii*	*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*	*B. burgdorferi* sensu stricto strain B31, *B. garinii*, *B. afzelii*
Antigens	Whole antigen extracts from *B. burgdorferi* sensu stricto strain 297 and *B. afzelii* ATCC 51567	Recombinant VlsE from *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii*. Lipids from *B. burgdorferi* and *B. afzelii*. Recombinant proteins p83, p41, p39, p25 (Osp C), p58, p21, p20, p19 and p18	Whole antigen extracts from *B. afzelii* PKO. Purified Osp C from *B. garinii* and VlsE from *B. burgdorferi* sensu stricto
Criteria for positive immunoblot	CDC criteria for IgG: 5 or more bands from proteins at 22, 28, 30/31, 39, 41, 45, 58, 66, 83/93 kDa	Any VlsE band and/or 2 or more specific bands from: p18, p19, p20, p21, p58, OspC (p25), p39, p83, lipid Bb, lipid Ba	2 bands for Germany or 3 or more bands elsewhere from: p14, p17, OspC (p25), p30, p39, p43, p58, p100, *B. garinii* OspC, *B. burgdorferi* VlsE

ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention.

Table 3 EIA compared to screening EIA consensus

Method	Agreement n=222 Number (%) (95% CI) [p value[a]]	Positive agreement (sensitivity) n=66 Number (%) (95% CI) [p value[a]]	Negative agreement (specificity) n=156 Number (%) (95% CI) [p value[a]]	Positive predictive value (%) (95% CI) [p value]	Negative predictive value (%) (95% CI) [p value]
MarDx IgG/IgM WCL EIA	124 (56%) (49–62%)	65 (98%) (92–100%)	59 (38%) (30–46%)	40% (33–48%)	98% (91–100%)
EUROIMMUN plus VlsE IgG WCL EIA	169 (76%) (70–82%) [0.01]	66 (100%) (NA) [0.91]	103 (66%) (58–73%) [0.00]	55% (46–75%) [0.02]	100% (NA) [0.83]
NovaLisa IgG EIA recombinant Ag	214 (96%) (93–98%) [<0.001]	65 (98%) (92–100%) [1.00]	149 (96%) (91–98%) [<0.001]	90% (81–96%) [<0.001]	99% (96–100%) [0.92]
EUROIMMUN Select IgG EIA recombinant Ag	208 (94%) (90–97%) [<0.001]	52 (79%) (67–88%) [0.25]	156 (100%) (NA) [<0.001]	100% (NA) [<0.001]	92% (87–95%) [0.52]

CI, confidence interval; NA, not available as confidence intervals cannot be calculated at 100%.
[a] p value is the probability of a difference at the 0.05 level compared to the MarDx WCL EIA.

agreement at levels from 76% (p = 0.01) for the EURIMMUN plus VlsE IgG WCL EIA to 96% (p < 0.001) for the recombinant antigen NovaLisa IgG EIA. Positive and negative agreements were used to describe sensitivity and specificity, respectively, as the true state of disease of patients was not always possible to ascertain with confidence. No significant difference was observed in the capacity to accurately identify positive samples when the EIAs were compared with the WCL MarDx IgG/IgM EIA. All assays differed from the WCL MarDx IgG/IgM EIA (38%) in negative results compared to the consensus results. This is reflected in significantly improved positive predictive values (PPV) especially for the recombinant EIAs with 90% and 100% for NovaLisa and EUROIMMUN Select, respectively, while the WCL EUROIMMUN plus VlsE IgG had 55% PPV which was significantly better (p = 0.02) than the MarDx IgG/IgM WCL EIA.

Comparison of immunoblots to the screening EIA consensus results (Table 4) revealed no differences in overall agreement between the WCS western immunoblot and the commercial recombinant line blot (i.e., EUROLINE) or western immunoblot with purified proteins (Trinity Biotech) nor any difference in positive (sensitivity) and negative (specificity) agreements. These second tier tests should increase the positive and negative predictive values (NPV) of the screening test. However when the predictive value of positives was analysed a significant difference was observed in the EUROLINE recombinant line blot (70%, p = 0.01) when compared to the WCS western blot (91%). No other differences with the WCS immunoblot were observed in PPV or NPV.

Separating the specificity panel from the total number of specimens tested (Table 5) demonstrated that WCL EIA screening tests were less specific than the recombinant EIA screening assays as expected. The NovaLisa IgG showed one cross reaction with a patient serum with high anti-nuclear antibodies while the EUROIMMUN Select IgG had no false positives in this panel. The consensus of EIA results was

Table 4 Immunoblots compared to screening EIA consensus

Method	Agreement *n*=222 Number (%) (95% CI) [*p* value]	Positive agreement (sensitivity) *n*=66 Number (%) (95% CI) [*p* value]	Negative agreement (specificity) *n*=156 Number (%) (95% CI) [*p* value]	Positive predictive value (%) (95% CI) [*p* value]	Negative predictive value (%) (95% CI) [*p* value]
Western Blot WCS IgG	195 (88%) (83–92%)	43 (65%) (52–76%)	152 (97%) (94–99%)	91% (80–98%)	87% (81–91%)
EUROIMMUN Anti-Borrelia (IgG) EUROLINE-RN-AT Recombinant line blot	185 (83%) (78–88%) [0.57]	50 (76%) (64–85%) [0.17]	135 (87%) (80–91%) [0.27]	70% (58–81%) [0.01]	89% (83–94%) [0.82]
Trinity Biotech EU Lyme + VlsE IgG Western Blot	201 (91%) (86–94%) [0.74]	49 (74%) (62–84%) [0.26]	152 (97%) (94–99%) [1.00]	92% (82–98%) [0.91]	90% (84–94%) [0.74]

Table 5 Specificity panel (*n*=23)[a]

Assay	Specificity % (95% confidence interval)	Positive specimens detected
MarDx IgG/IgM WCL EIA	87% (66–97%)	1 × Leptospirosis 1 × EBV 1 × Syphilis
NovaLisa IgG recombinant Ag	96% (78–100%)	1 × Anti-nuclear factor (ANF)
EUROIMMUN plus VlsE IgG WCL EIA	83% (61–95%)	1 × Leptospirosis 1 × EBV 1 × Rheumatoid factor 1 × Syphilis
EUROIMMUN Select IgG recombinant Ag	100%	Nil
WB WCS IgG	100%	Nil
EUROIMMUN Anti-Borrelia (IgG) EUROLINE-RN-AT Recombinant line blot	87% (66–97%)	1 × Leptospirosis 1 × *H. pylori* 1 × Syphilis
Trinity Biotech EU Lyme + VlsE IgG Western Blot	96% (78–100%)	1 × Syphilis

[a] Consensus EIA results were negative for all 23 specimens.

negative for all 23 specimens. However the EUROIMMUN Anti-Borrelia (IgG) EUROLINE-RN-AT (EUROLINE) line blot suggested common antigen cross reactivity with antibodies induced in patients with leptospirosis, *Helicobacter pylori* infection and syphilis. The syphilis case is worth noting as this patient had detectable antibodies in the two WCL EIAs and in both EUROLINE and Trinity Biotech EU Lyme + VlsE IgG Western Blots.

The western immunoblot WCS IgG using the CDC (MMWR 1994) criteria of five or more specific bands to define a positive result showed different levels of agreement with the other assays depending on the number of bands observed for each specimen tested (Table 6). Forty-seven positives with five or more specific bands to either *B. burgdorferi* or *B. afzelii* antigens or both were detected by the Western immunoblot WCS IgG while only 39 positives were detected by EUROLINE, 41 by Trinity Biotech EU Lyme + VlsE IgG Western Blot and 43 by the EIA consensus. Four sera that were western immunoblot WCS IgG positive were negative by both the EUROLINE and the Trinity Biotech EU Lyme + VlsE IgG Western Blot. Of the nine specimens with four specific bands, six were reported as negative by the other immunoblots and the other three sera reported as positive by both immunoblots and the consensus EIA. The consensus EIA results were similarly split, with five agreeing with a negative result. Three specific bands were detected in 22 specimens by western immunoblot and recorded as negative which did not agree with the positive report in nine (41%) specimens by EUROLINE, six (27%) by Trinity Biotech EU Lyme + VlsE IgG Western Blot and five (23%) by the EIA consensus. Five of these 22 specimens were positive by both EUROLINE and the Trinity Biotech EU Lyme + VlsE IgG Western Blot. The number of mismatches greatly reduced when two or less bands were detected by the western immunoblot WCS IgG. The recombinant EUROLINE still reported positive blots from 15% (7/46 specimens with two bands) to 10% with no bands by the Western immunoblot WCS IgG (5/49 specimens). The Trinity Biotech EU Lyme + VlsE IgG Western Blot agreed more often to the Western immunoblot WCS IgG with only 2% positive mismatched results with two bands (1/46), 4% (2/49) with one band and none with no bands. The consensus EIA results agreed by similar amounts with mismatched positive results of 13% (6/46) with two bands, 8% (4/49) with one band and 8% (4/49) with no bands.

Table 6 CDC result criteria where the presence of five or more bands is required for a positive result applied to bands detected by Western immunoblot WCS IgG compared to other methods

Assay	EUROIMMUN Anti-Borrelia (IgG) EUROLINE-RN-AT (% mismatch)		Trinity Biotech EU Lyme + VlsE IgG Western Blot (% mismatch)		Consensus EIA (% mismatch)	
Western WCS IgG immunoblot	Positive	Negative	Positive	Negative	Positive	Negative
Wblot ≥ 5 bands n=47	39	8[a] (17%)	41	6[a] (13%)	43	4 (9%)
Wblot = 4 bands n=9	3[b] (33%)	6	3[b] (33%)	6	4 (44%)	5
Wblot = 3 bands n=22	9[c] (41%)	13	6[c] (27%)	16	5 (23%)	17
Wblot = 2 bands n=46	7 (15%)	39	1 (2%)	45	6 (13%)	40
Wblot = 1 bands n=49	8[d] (16%)	41	2[d] (4%)	47	4 (8%)	45
Wblot = 0 bands n=49	5 (10%)	44	0 (0%)	49	4 (8%)	45
Total = 222	71	151	53	169	66	156

All sera with mismatched results were retested before final analysis.
[a] Four sera with five or more bands by western blot (Wblot) were negative by both EUROLINE and Trinity Biotech.
[b] Three sera with four bands by western blot were positive by both EUROLINE and Trinity Biotech.
[c] Five sera with three bands by western blot were positive by both EUROLINE and Trinity Biotech.
[d] One serum with one band by western blot was positive by both EUROLINE and Trinity Biotech.

DISCUSSION

Our findings reconfirm the improvements in test accuracy of EIAs for LB enabled by the application of recombinant, *Borrelia*-specific antigens,[3] a trait demonstrated by both the NovaLisa and EUROIMMUN Select recombinant antigen EIA kits. For example, the NovaLisa kit utilises a combination of recombinant antigens in order to improve specificity and also utilises flagellin as an antigen which, whilst being a major constituent of the *B. burgdorferi* flagella,[3] is also known to be highly cross reactive with other bacteria.[8,17] The EUROIMMUN Select employs specific recombinant antigens from three human pathogenic *Borrelia* species. Comparing these recombinant EIAs with the consensus of three EIA results showed significant differences with the WCL assays especially in significantly improved PPV.[18] Conversely, the EUROIMMUN plus VlsE EIA relies on whole antigen extracts as does the MarDx IgG/IgM. Therefore, the presence of common bacterial antigens in the kit explains its poorer specificity due to its propensity toward cross reactivity.[8,17] Although the antigenic mix also contains recombinant VlsE, its efficacy could be overshadowed by non-specific cross reactions. However, the addition of VlsE in the EUROIMMUN EIA showed better agreement with the consensus EIA results compared to that of the MarDx IgG/IgM WCL EIA.

The conventional serological testing for LB by WCS western immunoblot with the stringent CDC criteria of five specific bands for a positive IgG makes this approach highly specific at the potential expense of losing some sensitivity. We observed lower sensitivity of WCS western immunoblot by the lower positive agreement with the consensus EIA results (65%). When used as a second tier test, WCS western immunoblot was not different to the other immunoblots. Interestingly, the positive predictive value (70%, $p = 0.01$) of the EUROLINE recombinant line blot was lower and showed less agreement with the consensus of screening EIAs (70%, $p = 0.01$). The highest agreement with the consensus EIA results was found for the Trinity Biotech EU Lyme + VlsE IgG Western Blot with 91% agreement and 92% PPV and 90% NPV but these were not significantly different to the WCS western immunoblot. The consensus EIA results were negative for all 23 specimens in the specificity panel so it seems that any positives detected from this panel by individual EIA were falsely positive. The recombinant EIAs were both highly specific with the EUROIMMUN Select having no positive results in this panel implying a greater specificity for this kit. The NovaLisa produced only one positive result for an ANF specimen, fewer in comparison with the other two kits. The two kits using whole cell lysates (MarDx and EUROIMMUN plus VlsE) both returned false positive results for leptospirosis, EBV and syphilis patient samples. The WCS western immunoblot showed no false positive results while the EUROLINE recombinant line blot showed less specificity than the Trinity Biotech Western Blot. The same syphilis case detected by these two immunoblots and the two WCL EIAs was negative according to the consensus screening result which may mean that antibodies associated with treponemal infections can still give false positive results. It must also be noted that using recombinant EIA screening tests would eliminate such false positive results and so would not go on to the second tier immunoblot.

Some potential limitations of the study should be acknowledged. The main one is the use of a retrospectively selected set of sera. However, this sample contains a significant number of samples (49 samples) from patients with disease clinically consistent with LB, history of recent travel to LB endemic regions in Northern Hemisphere with or without history of tick bites at the time of travel. Furthermore, we have relied on the consensus between several different assays to identify 'true positive' and 'true negative' samples due to the lack of 'gold standard' for LB serology. Ideally 'gold standard' positive serum samples

should be from patients with positive culture and/or reliable nucleic acid amplification test. However, the use of a 'rotating gold standard' by consensus in this study is an unavoidable compromise in an area of low prevalence. It must be noted that the consensus of 'true positive' samples included the 49 well defined LB patient samples plus a further 17 patient samples with less than five bands in the WCS western immunoblot. Using CDC criteria of five or more bands to define a positive result, these 17 patients were not followed up further. Nevertheless these 17 sera were positive by at least three different ELISA kits. Another challenge of direct comparisons of serological assays was the fact that whilst the MarDx kit detects both IgG and IgM, the NovaLisa and EUROIMMUN only detect IgG. It has been noted in the past that IgG serology offers more specific results than IgM tests,[19] which could account for some of the lack of specificity of the MarDx kit. Testing only IgG with the addition of VlsE proteins to diagnose early and late LB was advocated to minimise the risk of false positive IgM results and to streamline testing strategies.[16,20] Also antigens derived from both North American and European species of *Borrelia* were not used in the MarDx EIA kit (Table 1) even though the need to include both is now considered necessary for any testing strategy.[20] All western blot results available for this experiment were run on IgG immunoblots only.

In conclusion, EIAs for the serological diagnosis of Lyme disease that employ recombinant antigens, such as the NovaLisa *Borrelia burgdorferi* IgG ELISA (recombinant) and the EUROIMMUN Anti-Borrelia Select ELISA IgG, appear to have higher sensitivity and specificity to WCL-based EIAs like the EUROIMMUN Anti-Borrelia plus VlsE ELISA IgG and the MarDx *Borrelia burgdorferi* IgG/IgM in determining true cases of Lyme disease in a low incidence setting. Second tier testing with WCS western immunoblots can improve PPV and NPV, more so with the addition of VlsE proteins as in the Trinity Biotech EU Lyme + VlsE IgG Western Blot. However, immunoblots should not be used alone to diagnose Lyme *Borrelia* antibodies in patient sera due to risk of potential false-positive findings. The application of immunoblots as second tier tests improves the predictive value of the screening tests reinforcing the argument for the two-tier approach.

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