Diversity of the Lyme Disease Spirochetes and its Influence on Immune Responses to Infection and Vaccination

Jerilyn R. Izac, BS, Richard T. Marconi, PhD*

THE DISCOVERY OF LYME DISEASE

Lyme disease (LD), as a clinical entity, was first described in the United States in the late 1970s (reviewed by Steere¹). The path toward defining the basis of this debilitating infection began when concerned parents living in Lyme, Connecticut, contacted the Connecticut State Department of Health and reported an unusual clustering of juvenile rheumatoid arthritis cases in their area.¹ A joint investigation launched by the...
Connecticut State Department of Health and Yale University School of Medicine identified 51 cases of oligoarthritis of unknown cause in children and adults living in Lyme, Old Lyme, and East Haddam, Connecticut. Approximately 25% of the affected individuals recalled developing an enlarging rash in the weeks before disease onset. The general characteristics of the rash were similar to a rash described in 1909 in Sweden by Arvid Afzelius that was referred to as erythema migrans (EM).

Afzelius made several seminal contributions to our current day understanding of the epidemiology of LD, including the establishment of a connection between the bite of *Ixodes ricinus* ticks (a European *I.* species) and the development of EM. It was not until 1976 that a connection was made between the bite of *I. scapularis* ticks (formerly classified as *I. dammini*) and the development of EM and Lyme arthritis in patients in the United States. Shortly thereafter, researchers at the Rocky Mountain Laboratories (NIH) cultured a previously uncharacterized spirochete from *I. scapularis* ticks that was designated as *Borrelia burgdorferi*. A direct link between *B. burgdorferi* and Lyme arthritis was established with its cultivation from the blood of patients with LD. The first case of canine Lyme arthritis was diagnosed shortly thereafter.

Lyme disease is the most common arthropod-borne disease of canines and humans. The Companion Animal Parasite Council (CAPC) reported that there were 319,000 positive LD antibody tests in canines in 2018; up from 160,000 in 2012 (www.capcvet.org). As explained by CAPC, these values are underestimates because data are collected for only ~30% of the tests that are run. The Centers for Disease Control and Prevention estimates that the probable number of clinician-diagnosed cases of human LD each year in the United States is ~329,000. The incidence and endemic regions for LD and *I.* ticks are expanding in the United States, Canada, Europe, and Asia.

**CLASSIFICATION OF TICK-BORNE SPIROCHETES**

Before the identification of the LD spirochetes, the genus *Borrelia* consisted primarily of species associated with tick-borne relapsing fever (TBRF). The TBRF is a spirochetal infection transmitted by the soft-bodied *Ornithodoros* ticks. *Ornithodoros* ticks are anatomically distinct from the hard-bodied *I.* ticks that transmit LD. They also have different feeding strategies and developmental processes. Ticks that transmit TBRF are nocturnal feeders that reside in nesting materials in caves, rustic (unmaintained) cabins, and other similar structures. They feed rapidly and can transmit spirochetes within minutes. A hallmark feature of TBRF is a high-grade relapsing fever that coincides with the appearance of a remarkable number of spirochetes in the blood (10^6 to 10^8 mL⁻¹ blood) (Fig. 1A). The molecular basis of the cyclic spirochetemias can be traced to an elaborate antigenic variation system. Tick-borne relapsing fever occurs in isolated pockets in the United States but is widespread in other parts of the world. Its health consequences in parts of Africa are staggering.

Lyme disease is transmitted by tick species belonging to the genus *Ixodes*. *I. scapularis* and *I. pacificus* are the primary species that transmit LD in the United States and Canada, whereas *I. ricinus* and *I. persulcatus* are the primary vectors in Europe and Asia. *I.* ticks inhabit wooded areas, unkept brush, tall grasses, and leaf litter. They feed over the course of several days with transmission of the LD spirochetes typically requiring a feeding period of 24 to 72 hours. Transmission time can vary depending on the strain of the LD spirochete, the health of the tick and inherent variation among hosts. In contrast to TBRF, high-density spirochetemias are not a characteristic of LD. A notable exception is *B. miyamotoi*, which can cause transient spirochetemias.
Although *B. myiamotoi* causes a TBRF-like illness, this species is transmitted by *I.* ticks and is more closely related to the LD spirochetes than it is to the TBRF spirochetes. Several reviews have detailed the biology, health toll, and pathogenesis of TBRF in humans and canines.18,21,22

Soon after the discovery of *B. burgdorferi*, comparative studies of LD spirochete isolates from North America, Europe, and Asia revealed significant genetic and antigenic diversity. Based on these analyses, *B. burgdorferi* was divided into 3 distinct species: *B. burgdorferi*, *B. garinii*, and *B. afzelii*.23–25 *B. burgdorferi* is the primary species found in North America, whereas in Europe, all 3 species are present. Further exploration of the phylogenetic relationships among LD spirochete species and isolates led to the delineation of several additional species.26–29 The potential significance of these species in veterinary and human health remains to be defined.

The genus *Borrelia* has been recently divided into 2 genera: *Borrelia* and *Borreliella*.30 Consistent with taxonomic precedent, since the TBRF species were described first, they retain the *Borrelia* genus designation. The LD spirochetes and *B. myiamotoi* were assigned a new genus designation, *Borreliella*. Sharp differences of opinion exist concerning the practical implications of this reclassification.31,32 Although the use of the designation *Borreliella* is voluntary, readers should be aware of this change because it has been fully applied in public databases and is beginning to appear in the literature.

**UNIQUE FEATURES OF SPIROCHETES**

Spirochetes are distinct from other bacteria in several fundamental and fascinating ways. A feature shared by all spirochetes is their unique flat wave or spirallike ultrastructure (see Fig. 1A).33 This characteristic morphology results from the presence of endoflagella, which are found in all spirochetes. The flagella arrangement in *Treponema denticola*, the periodontal disease pathogen, is shown in Fig. 1B. The
endoflagella are organized into 2 separate flagella bundles, each of which is anchored to the inner membrane at opposite ends of the cell. The flagella bundles sit within the periplasmic space and extend ~ three-quarters of the length of the cell.34

Distinguishing features specifically of the LD and TBRF spirochetes include the composition of their cell wall and a unique genome arrangement (reviewed by Barbour and Hayes21). Like Gram-negative bacteria, they possess an inner and outer membrane, but lack lipopolysaccharide. Lipopolysaccharide is replaced by a diverse array of lipitated outer surface proteins (Osps) that play important roles in the host-pathogen interaction. Some of these Osps are described in detail below. The LD and TBRF spirochetes are distinct from all other bacteria, including other spirochetes, in that they possess a small, segmented genome consisting of a linear chromosome (0.9 Mb) and a series of linear and circular plasmids.35 Linear DNA is rare in bacteria. The plasmids range in size from 9 to 200 kb and comprise nearly 40% of the total genome.36 The total number and size of the plasmids carried by individual isolates can vary significantly.37 Plasmid variation results from plasmid loss, acquisition and genetic rearrangement.38 Some plasmids are dispensable,39 whereas others are essential for infection or survival.40 The unique properties of the LD spirochete genome are reviewed in.41

DEVELOPMENTAL STAGES OF IXODES TICKS

Lyme disease is maintained in nature in an enzootic cycle involving I. ticks and a diverse array of mammalian reservoir hosts.42 The first developmental stage of a tick is the larva. Because transovarial transmission of the LD spirochetes in ticks does not occur, on emerging from the egg, larvae do not carry the LD spirochetes. I. ticks can only become infected by feeding on an infected mammal through a process referred to as acquisition. After taking their first and only bloodmeal, the 6-legged larvae detach from their feeding source and molt into 8-legged nymphs. This anatomic change has important implications for tick biology and feeding behavior, because it allows nymphs and adult ticks to climb up into brush where they gain better access to larger and more mobile mammals. Nymphs also feed just once and then molt into sexually differentiated adults. The body weight of an adult female tick may increase by as much as 500-fold after the bloodmeal. Because male ticks do not feed, they play no significant role in transmission of LD. Images of engorged adult female I. scapularis and Amblyomma americanum (lone star tick) ticks are presented in Fig. 2. Pets and their owners are merely accidental hosts, and as such, do not play a significant role in maintaining LD in nature.

ADAPTIVE RESPONSES AND THEIR IMPORTANCE IN THE ENZOOTIC CYCLE

The acquisition of spirochetes by ticks and their transmission to mammals are active processes that are dependent on tightly regulated adaptive responses.43,44 Akins and colleagues45 conducted a creative and pivotal study that provided insight into the nature of adaptive responses. They compared the protein content of laboratory grown spirochetes with that of host-adapted spirochetes. To accomplish this, cultures of LD spirochetes were placed in dialysis membrane chambers and implanted in the peritoneal cavity of rats. Spirochetes maintained in dialysis membrane chambers become host-adapted and thus, more closely resemble spirochetes during natural infection.45 Comparison of the protein profiles of laboratory-cultivated and host-adapted spirochetes revealed significant differences in the production levels of OspA, OspB, and OspC (as well as other proteins). OspA and OspB were produced at high levels in laboratory-cultivated spirochetes but not host-adapted spirochetes.45 In contrast,
OspC production was low in laboratory spirochetes but high in host-adapted spirochetes (Fig. 3). The Akins study also proved central in shaping our understanding of humoral immune responses during infection. The Osp production patterns they reported are consistent with the development of a strong and early antibody response to OspC in mammals and the absence of a response to OspA and OspB. Low-level production of OspC during cultivation is well documented in the literature. Oliver and colleagues demonstrated that only 10% of the individual cells in a laboratory culture produce detectable amounts of OspC.

Adaptation to the distinctly different environmental conditions present in unfed ticks, fed ticks, and mammals also requires changes in Osp production. In an unfed tick, spirochetes residing in the nutrient-poor, midgut environment, produce high levels of OspA. Intake of a bloodmeal quickly changes the environment triggering a transition from OspA to OspC production. The upregulation of OspC at the tick-host interface is consistent with studies that have demonstrated that OspC is required for transmission and the establishment of an active infection in mammals. Strains that have been modified to not produce a functional OspC are unable to infect mammals.

OUTER SURFACE PROTEIN VARIATION: INFLUENCE ON VACCINE AND DIAGNOSTIC ASSAY DEVELOPMENT

The LD spirochetes produce a diverse array of Osps and the subset produced at any given time is controlled by environmental conditions. A comprehensive review of properties and functions of characterized Osps is beyond the scope of this report. Although several Osps have been investigated for use in vaccine or diagnostic assay development (reviewed by Earnhart and Marconii), the discussion here is focused on OspC. OspC is a lipoprotein that varies in molecular weight (20–24 kDa) among isolates. The ospC gene is carried by a highly stable circular plasmid of 26 kDa referred to as cp26. An individual LD isolate produces only a single OspC protein variant. The antigenic diversity of OspC among LD spirochete isolates is well documented and has been intensively studied.
Before our current understanding of OspC phylogenetics, sequence variation seemed an insurmountable hurdle to overcome in efforts to use OspC as a vaccine or diagnostic antigen.\textsuperscript{60} It was assumed that \textit{ospC} variation arises through mutation during infection with subsequent immune selection allowing for the emergence of new antigenic variants. However, OspC is genetically stable during infection.\textsuperscript{61} Numerous distinct and stable variants of OspC, referred to as OspC types, have been identified. OspC types are differentiated by a letter or other appropriate designation (reviewed by Marconi and Earnhart\textsuperscript{60}). OspC proteins of a given OspC type are conserved with percent amino acid identity values of \(~95\% \) or greater. Identity values between OspC types can be as low as 65\%. For example, Earnhart and Marconi\textsuperscript{57} compared the sequences of 55 OspC type A proteins and found that amino acid identity values among these proteins were greater than 97\%.

Studies by Brisson and colleagues\textsuperscript{62,63} have provided significant insight into the biological rationale for the existence and maintenance of multiple, stable \textit{ospC} types in nature. Although an individual LD spirochete strain produces only a single OspC type, the ability of the spirochete to express distinct OspC proteins in response to the host environment can confound efforts to use OspC as a vaccine or diagnostic antigen. The protein profiles of spirochetes cultivated in the laboratory or host-adapted spirochetes that were grown in dialysis membrane chambers implanted within the peritoneal cavity of rats are shown. The proteins were fractionated by gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie blue. Note, the significant differences in the production levels of OspA, OspB, and OspC. Molecular weight standards are shown on the left. (Courtesy of Dr. Darrin Akins, The University of Oklahoma Health Sciences Center, Oklahoma City, OK.)

![Fig. 3. Adaptive responses of Lyme disease spirochetes. The protein profiles of spirochetes cultivated in the laboratory or host-adapted spirochetes that were grown in dialysis membrane chambers implanted within the peritoneal cavity of rats are shown. The proteins were fractionated by gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie blue. Note, the significant differences in the production levels of OspA, OspB, and OspC. Molecular weight standards are shown on the left. (Courtesy of Dr. Darrin Akins, The University of Oklahoma Health Sciences Center, Oklahoma City, OK.)](image-url)
type, ticks commonly carry a heterogeneous population of strains that as a whole can produce many different OspC proteins. The existence of multiple OspC types in a given tick may help to ensure that upon feeding, at least a subset of the strains can infect an animal that has been immunologically primed by previous exposure to other OspC types. It has been hypothesized that OspC type identity may also influence mammalian host compatibility. Certain OspC types may facilitate infection of specific mammals. Rhodes and colleagues reported that the most common OspC type detected in infected canines was OspC type F. This is striking, because there have been no reports of the isolation of an OspC type F producing strain from humans. Although more research is required to address the biological rationale for the maintenance of distinct OspC types in nature, OspC diversity is critical to consider when assessing host immune responses to this immunodominant early antigen.

**ANTIBODY RESPONSES TO OspC DURING INFECTION AND ON IMMUNIZATION**

Evidence that antibody responses to OspC are type specific came from studies in which mice were inoculated with individual LD strains producing different OspC types. Immunoblot analyses of the infection serum collected from these mice revealed that IgG responses are OspC type specific. Rabbits immunized with purified recombinant OspC proteins also developed type-specific IgG responses. The lack of antibody cross-reactivity with different OspC types is intriguing because segments of sequence are shared by all OspC proteins (ie, are conserved). The specificity of the antibody response suggests that variable regions of OspC are presented to the immune system.

OspC type-specific antibody responses have also been demonstrated in naturally infected canines. Serum from dogs confirmed to be LD positive reacted with only a limited subset of OspC proteins in immunoblot analyses. The observed specificity of the OspC antibody response is consistent with epitope mapping studies that identified 2 dominant but variable epitopes of OspC. The regions corresponding to these antigenic domains were designated as the L5 and H5 epitopes. Although the sequences of these epitopes vary among OspC proteins, they are highly conserved among proteins of an individual OspC type. The immunodominance of the L5 and H5 epitopes likely explains the basis for type-specific nature of the OspC antibody response.

It has been reported in some studies that a conserved motif of OspC drives antibody responses. This suggestion is difficult to reconcile in light of the type-specific responses detailed above. This motif, referred to as either the C7, C10, or pepC10 motif, is proline rich and comprises the last 10 C-terminal residues of OspC. If a conserved sequence common to OspC types (ie, C10) constitutes a dominant epitope, then antibody to OspC should bind to all OspC proteins. In this report, potential immune responses to C10 were further investigated. All methods used in the experiments presented below have been described previously. Recombinant OspC proteins (types I, F, and T) were generated with or without the C10 motif (OspC-IΔC10, OspC-FΔC10, and OspC-TΔC10), purified and screened with serum from representative LD-positive horses (animal ID number, 1026) and dogs (TF1286). Serum from dog TF1286 bound to OspC type T but not to type F (Fig. 4). Infection serum from horse number 1026 bound to OspC type F but OspC type I. Furthermore, OspC proteins that lack the C10 motif were readily detected by antibody in infection serum. These observations support the contention that the C10 motif is not a dominant epitope and that it is the variable domains of OspC that drive antibody responses.
DOES INFECTION WITH THE LYME DISEASE SPIROCHETES ELICIT PROTECTIVE IMMUNITY?

It is common knowledge among veterinarians who practice in LD endemic areas that a significant percentage of dogs will develop repeated LD infections. This phenomenon is well documented in humans. In 1 study, 15% of patients with LD living in a Lyme endemic area developed 1 or more follow-up infections within 5 years. To add to our understanding of LD and protective immunity, we sought to determine if infection of mice with clonal populations of LD spirochetes results in broad, or strain-specific, bactericidal antibody responses. In this report, separate groups of mice were infected with *B. burgdorferi* B31, N40, and 297 and *B. afzelii* PKo using previously detailed methods. Sera harvested from the mice were then tested for bactericidal activity.

![Image](image.png)

**Fig. 4.** Specificity of the antibody response to OspC in infected dogs and horses. Recombinant OspC proteins (types I, F, and T) with or without the putative C10 epitope were produced, purified, and transferred onto membranes for immunoblot analysis. The membranes were screened with serum from an infected dog (ID number, TF1286; top panel) or an infected horse (ID number, 1026; bottom panel), and IgG binding to each protein was assessed using the appropriate secondary antibody and chemiluminescence. Note that although antibody to OspC was detected, the antibody was not cross-reactive with the different OspC types. In addition, deletion of the C10 motif from each protein had no discernible impact on the level of antibody binding.
against each strain using in vitro assays. Representative data are presented in Fig. 5. Serum from mice infected with *B. burgdorferi* B31 efficiently killed B31 but did not kill *B. burgdorferi* N40, 297, or *B. afzelii* PKo (see Fig. 5). Conversely, serum from mice infected with *B. afzelii* PKo efficiently killed PKo but not *B. burgdorferi* B31, N40, or 297. To determine if killing is complement dependent, 1 set of reactions were run with heat-inactivated complement or no complement added. Guinea pig serum served as the exogenous complement source. No killing was observed unless active complement was included in the assay. The data indicate that serum-mediated killing occurs through an antibody-mediated, complement-dependent mechanism. More importantly, it can be concluded that infection with a given LD spirochete does not induce broadly protective antibody responses.

PREVENTION: THE KEY TO TACKLING THE LYME DISEASE PROBLEM

Vaccination is widely considered to be the most cost-effective approach for prevention of infectious diseases. Concerns about accurate diagnosis and appropriate treatment strategies for LD could be alleviated to some degree through aggressive

![Fig. 5. Strain-specific bactericidal antibody responses in mice infected with Lyme disease spirochetes. As detailed in the text, sera from mice infected with *B. burgdorferi* B31 or *B. afzelii* PKo were assessed for bactericidal activity. The infection sera were incubated with each strain with or without complement preserved guinea pig serum. Bactericidal activity was measured by determining the percentage of spirochetes that were killed as a result of exposure to the infection serum (% killing). Note that the antibody-mediated killing was strain specific and occurred through a complement-dependent mechanism. It can be concluded that infection with an individual Lyme disease spirochete strain does not elicit a broadly protective antibody response.](image-url)
vaccination. Several licensed LD vaccines are available and approved for use in canines.\textsuperscript{54} These vaccines are of 2 general types: bacterin and subunit. Currently available bacterin vaccines are NovibacLyme (Merck), LymeVax (Zoetis) and Ultra Durammune Lyme (Elanco). Available subunit vaccines are VANGUARD crLyme (Zoetis) and Recombitek Lyme (Boehringer Ingleheim).

**LYME DISEASE BACTERIN VACCINES**

The composition of subunit and bacterin vaccines are inherently different. Lyme disease subunit vaccines consist of highly purified recombinant proteins (OspA and or OspC), whereas bacterin vaccines consist of lysates of 2 laboratory-cultivated LD spirochete strains.\textsuperscript{74,75} The identity of the strains that comprise each commercially available bacterin vaccine is information that is not in the public domain. Because LD bacterin vaccines are generated from cell lysates, they contain a large number of proteins and other cellular constituents. In fact, genome sequencing and proteome analyses have demonstrated that the LD spirochetes can produce in excess of 1600 different proteins.\textsuperscript{76,77} Most of these proteins are produced during laboratory cultivation.\textsuperscript{78} The precise proteins that are present in any given bacterin vaccine have not been reported. Importantly, most of the proteins produced by bacteria under any growth scenario are localized within the cell and function in metabolic pathways and other important cellular processes.\textsuperscript{79,80} Although intracellular proteins can elicit an antibody response on vaccination with a cell lysate–based bacterin formulation, they are not likely to elicit productive antibody (ie, antibody that contributes to protective immunity), because in live cells intracellular proteins are not accessible to antibody. The removal of extraneous proteins from bacterins is conceptually beneficial because it would serve to direct and focus immune responses on immunologically relevant proteins.

The differential production of LD spirochete proteins under different environmental conditions\textsuperscript{81} may also influence the composition and antigenic content of subunit vaccines. Because bacterins are made from cultivated bacteria, they may lack potentially protective antigens that are produced by the LD spirochetes only during residence in mammals.\textsuperscript{45} Similarly, there are additional proteins that are not produced during culture or in mammals that are selectively produced in ticks.\textsuperscript{82} Antigens that are produced during infection in mammals or ticks would intuitively be those that are most desirable for inclusion in an LD vaccine. In this context, subunit vaccines offer some advantages in that they are composed of carefully chosen antigens with known production patterns. In addition, subunit vaccines lack extraneous proteins that are not involved in triggering protective immunity.\textsuperscript{54}

**LYME DISEASE SUBUNIT VACCINES**

Recombitek Lyme is a subunit vaccine consisting of lipidated OspA. Anti-OspA antibody inhibits transmission from ticks to mammals by targeting spirochetes in the tick midgut.\textsuperscript{83} OspA was also the sole component of LYMErix (SmithKline Beecham), the only human vaccine to have made it to market.\textsuperscript{84} LYMErix was introduced in 1998 but then voluntarily removed in 2001. There were many factors that contributed to its demise and detailed assessments of its rise and fall can be found in several excellent reviews.\textsuperscript{85,86} Leaving the more controversial issues aside, LYMErix was compromised by low efficacy (49\%) after a 2-dose series. A 3-dose series increased efficacy to 76\%.\textsuperscript{87} The requirement for multiple boosts is because OspA-mediated protection is strictly dependent on high circulating antibody titers.\textsuperscript{87} If titers drop below a critical threshold level, spirochetes are able to transit into a vaccinated animal.\textsuperscript{88} Because
OspA is not produced by the LD spirochetes in mammals, the spirochetes cannot be targeted by anti-OspA antibody after entering an OspA-vaccinated mammal.

VANGUARD crLyme (Zoetis), the newest canine LD vaccine to be approved by the United States Department of Agriculture, is a subunit vaccine consisting of OspA and a recombinant chimeric OspC epitope-based protein referred to as a chimeritope. OspC chimeritopes consist of linear epitopes derived from several antigenically distinct OspC proteins that are joined together in a single recombinant protein. The rationale behind the development of OspC chimeritopes was to generate a protein that can elicit antibody that can target OspC proteins produced by diverse strains. The conceptual rationale for chimeritope proteins has been described in detail in earlier reviews and hence is not discussed further in this report. Antibody elicited by OspC chimeritope vaccine antigens can target spirochetes during the process of transmission and during early infection in mammals. A vaccine that induces antibody that can kill spirochetes in both ticks and mammals has the potential to use 2 synergistic mechanisms of protection and thus be less dependent on the maintenance of high circulating antibody titers.

WHERE DO WE GO FROM HERE?

The Lyme spirochetes are a fascinating and remarkably diverse group of bacteria with unique biological properties. In this report, we have focused our discussion on the importance of understanding environmentally regulated protein production, the genetic and antigenic diversity of the LD spirochetes and how that diversity influences immune responses to infection and vaccination. There are many topics not addressed here that are equally worthy of discussion. As we move forward, our ability to critically assess and interpret the results of past and future studies focused on LD will directly affect how successful we are in addressing this important veterinary and human health concern.

REFERENCES


30. Adeolu M, Gupta RS. A phylogenomic and molecular marker based proposal for the division of the genus Borrelia into two genera: the emended genus Borrelia
containing only the members of the relapsing fever Borrelia, and the genus Borreliella gen. nov. containing the members of the Lyme disease Borrelia (Borrelia burgdorferi sensu lato complex). Antonie Van Leeuwenhoek 2014;105:1049–72.


