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Development of Oral Vaccines Against Lyme Disease

Document Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Program
Biomedical Sciences

Track
Microbiology, Immunology, and Biochemistry

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DOI
10.21007/etd.cghs.2015.0209

Comments
Six month embargo expired June 2016

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Development of Oral Vaccines against Lyme Disease

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Rita Raquel dos Anjos de Carvalho e Melo
December 2015

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DEDICATION

"If we knew what it was we were doing, it would not be called research, would it?"
Albert Einstein.

This is dedicated to my family: Diogo, Joseph and Teresa. Their everlasting support, understanding and love were my daily source of ATP in the pursuit of this scientific journey.

ACKNOWLEDGEMENTS

I am grateful to everyone who helped me in the process of achieving my dream: my Ph.D. degree.

I would like to thank my advisor, Dr. Maria Gomes-Solecki, who provided me the unique opportunity to complete my graduate training in her lab. During her mentoring over the years, I have kept my passion for Science and I am very grateful for that.

I am very thankful to all the members of my graduate committee, Dr. Mark Bix, Dr. Maureen A. McGargill, Dr. Robert Belland and Dr. Susan E. Senogles for their continuous scientific advice, for their involvement and support to conduct and complete my research studies. They were the source of constant encouragement of my journey as a graduate student.

I would like to thank the faculty and staff at UTHSC who have had an instrumental role in my graduate career development. In particular, I would like to thank Dr. Michael Whitt whose support to students is an example of academic excellence.

I would like to extend my thankfulness to Dr. Gerald Byrne for his constructive comments on my ideas, rationale and work, making me even more passionate and devoted to Science.

I would like to thank Dr. Rod Hori, Dr. Ken Nashimoto and Dr. David Nelson for always welcoming me to join their friendly and funny talks about science during lunch breaks. I really hope to find such a pleasant environment in my next job.

I am grateful to my colleagues and friends at the Gomes-Solecki's lab, both past and present. Particularly, Dr. Beatriz DelRio and Ana Vieira. They made the lab a fun and a wonderful place to work.

I would like to acknowledge the funding agencies that supported this work such as CDC and NIH. Thank you for making my research possible.

Finally, on a personal level, I would like to thank all my family and friends from around the World that have been tremendously supportive throughout my Ph.D. journey. Bhargavi Manda, Hong Wang, Sharad Shrestha and Viraj Ichhaporia, my PhD student life became even more enjoyable because of them.

ABSTRACT

Lyme Disease, caused by the spirochete *Borrelia burgdorferi*, is the most common vector-borne disease in the United States and Europe. If left untreated, it can lead to permanent damage to the nervous and musculoskeletal systems. In some cases, patients that receive the recommended antibiotic therapy develop a debilitating health condition associated with substantial health care costs. Despite current preventive measures, the incidence and the geographic distribution of Lyme Disease continues to increase. Recent estimates from CDC suggest that the true number of cases of Lyme Disease in the US is approximately 300,000 per year. Yet, there is currently no vaccine available for human use, and thus novel strategies to diminish the risk of human exposure to *Borrelia burgdorferi* are of utmost importance. In an effort to address this need, we developed and tested an array of oral vaccine candidates based on recombinant *E. coli* that express *B. burgdorferi's* OspC type K, OspB, BBK32, and *Ixodes scapularis* Salp15 and Salp25. Only oral immunization with live *E. coli* expressing OspC K induced systemic immune responses characterized by high levels of OspC K-specific IgG antibodies in sera as well as IgA antibodies in mucosal secretions, obtained from C3H-HeN mice. Vaccine efficacy studies demonstrated that OspC K-vaccinated mice were not protected from infection when challenge was performed via the natural route of disease transmission using ticks infected with multiple strains of *B. burgdorferi*, as assessed by the presence of antibodies to *B. burgdorferi* coupled with positive cultures and positive q-PCR results from bladder, heart and ear. Most importantly, we have shown that antibodies specific to OspC type K do not protect mice from infection when the homologous type of *B. burgdorferi* is transmitted via ticks harboring also other types of OspCs. Our findings are both critical and relevant, and should be considered in future studies involving the design and development of OspC-based vaccines against Lyme Disease.

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LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
BAL	Bronchoalveolar Lavage
Bb	*Borrelia burgdorferi*
BBK32	*Borrelia burgdorferi* fibronectin binding protein
BmpA	*Borrelia burgdorferi* Basic Protein A
BSA	Bovine Serum Albumin
BSK-H	Barbour-Stoenner-Kelly
CDC	US Centers for Disease Control and Prevention
DbpA	Decorin-binding Protein A
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
EM	*Erythema migrans*
FDA	Food and Drug Administration
Fla	Flagellar Protein
FlaB	Flagellar Protein B
GAG	Glycosaminoglycans
HIV	Human Immunodeficiency Virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl-β-Dthiogalactopyranoside
Kb	Kilobase
LB	Luria Broth
LD	Lyme Disease
Mb	Megabase
NIH	National Institute of Health
OD	Optical Density
Osp	Outer Surface Protein
OspA	Outer Surface Protein A
OspB	Outer Surface Protein B
OspC	Outer Surface Protein C
OspC K	Outer Surface Protein C type K
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PTLDS	Post-treatment Lyme Disease Syndrome
Q-PCR	Quantitative Polymerase Chain Reaction
Salp15	Salivary Protein 15
Salp25	Salivary Protein 25
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
US	United States of America
VAL	Vaginal Lavage

CHAPTER 1. INTRODUCTION

Literature Review

Lyme Disease: a public health concern

Lyme Disease, caused by the spirochete *Borrelia burgdorferi* sensu lato, is the most common vector-borne illness in the world [1]. In Europe and Asia, *B. garinii, B. afzelii* and *B. burgdorferi* sensu stricto (hereafter referred to as *B. burgdorferi*) are responsible for most human cases, whereas in North America *B. burgdorferi* is the sole cause of Lyme Disease [2, 3]. There is no vaccine available for humans. Despite current preventive measures, the number of reported cases in the US and elsewhere continues to increase. Newly revised estimates from The Centers for Disease Control and Prevention (CDC) suggest that the true number of cases of Lyme Disease in the US is approximately 300,000 per year [4], 10 times higher than the official number reported by the CDC in 2013 **(Figure 1-1)** [5], making Lyme Disease the third most common Notifiable Disease in the nation [6]. Of all Lyme Disease cases reported to the CDC in 2013, 95% were from 14 states located in the Northeastern (New York, Connecticut, Pennsylvania, Massachusetts, Maine, Vermont, New Hampshire and Rhode Island), Mid-Atlantic (New Jersey, Maryland, Delaware and Virginia) and North Central (Wisconsin and Minnesota) regions **(Figure 1-2)** [5], where vector *Ixodes scapularis* ticks pose a high risk of infection [7]. Lyme Disease is endemic in North America, and its incidence is age-related. Children are the most affected **(Figure 1-3)**, probably because of their increased exposure to ticks [5]. Prompt diagnosis and treatment is critical to prevent disease progression. Except for the classic *Erythema migrans* (EM), the clinical manifestations of early Lyme Disease are not specific [8]. Thus, patients that do not develop EM are at risk for misdiagnosis and, consequently, for disease progression. If left untreated, patients can develop serious neurologic, cardiac and rheumatic manifestations weeks or months after infection [9]. Despite receiving the recommended antibiotic therapy, some Lyme Disease patients develop Post-treatment Lyme Disease Syndrome (PTLDS) [10-13], a debilitating condition that significantly impairs their life functioning [11]. Lyme Disease is associated with significant health care costs. Assuming that 300,000 cases occur annually [4], the total direct medical costs attributable to this disease could be approximately $890 million per year [14]. Predicted climate changes will likely promote and accelerate the expansion of *I. scapularis* to new geographic regions [15, 16], spreading the disease to areas that were previously non-endemic. As a result of northward expansion of *I. scapularis* Lyme Disease in now emerging in Canada and in some northern U.S. states [17-19].

In summary, the new disease estimates have peaked at unprecedented figures and Lyme Disease is now reaching epidemic proportions greater than ever anticipated, with annual cases estimated to be 6 times greater than the 50,000 new HIV infections in the U.S. [1, 20]. Lyme Disease represents a growing health care and economic problem that has defied our attempts to control it. Alternative preventive measures to counteract this emergent epidemic are urgently needed.

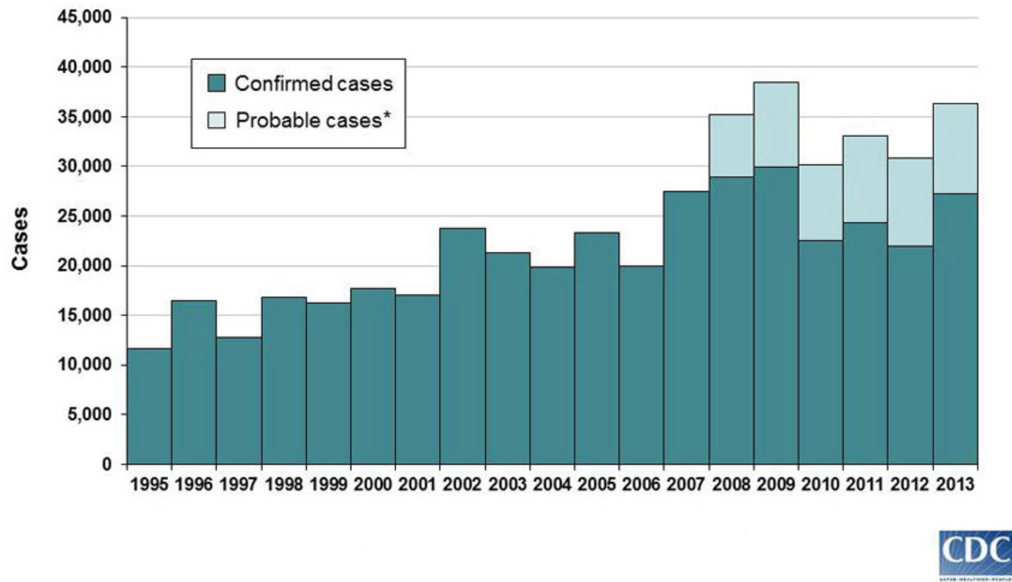

Figure 1-1. Number of reported cases of Lyme Disease in the United States, from 1995 to 2013.

During 1995 to 2013, the annual confirmed case counts increased approximately 3-fold from 11,700 in 1995 to about 30,000 in 2013. CDC currently estimates that the true number is 10 times greater than the number of confirmed cases reported in 2013 [4].
* National surveillance case definition revised in 2008 to include probable cases.
Reprinted with permission from CDC, *Reported Cases of Lyme Disease by Year, United States, 1995-2013* [5].

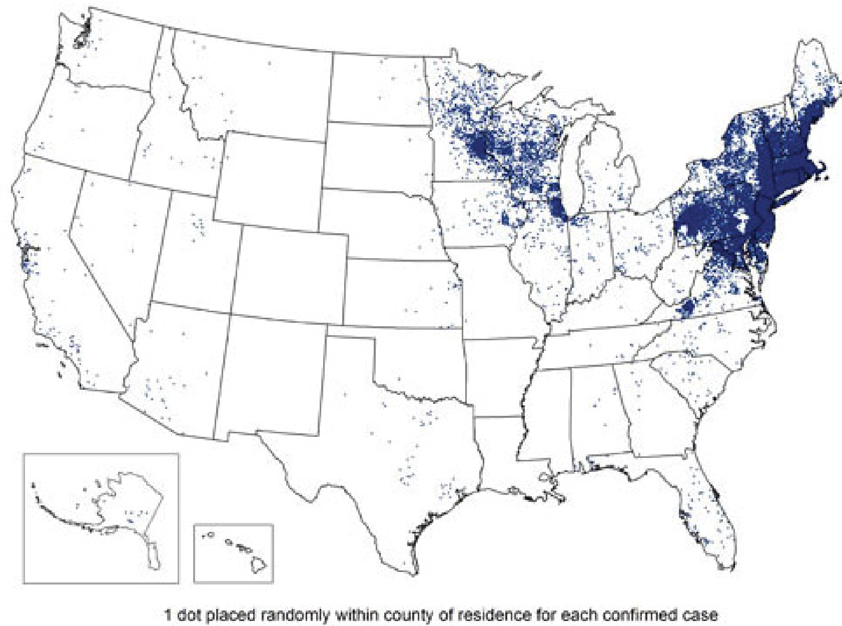

Figure 1-2. Geographic distribution of confirmed Lyme Disease cases in the United States in 2013.

In 2013, 14 states (Connecticut, Delaware, Maine, Massachusetts, Maryland, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia, and Wisconsin) accounted for 95% of confirmed US cases. One dot placed randomly within county of patient residence for each confirmed case. Reprinted with permission from CDC, *Reported cases of Lyme Disease, United States, 2013* [21].

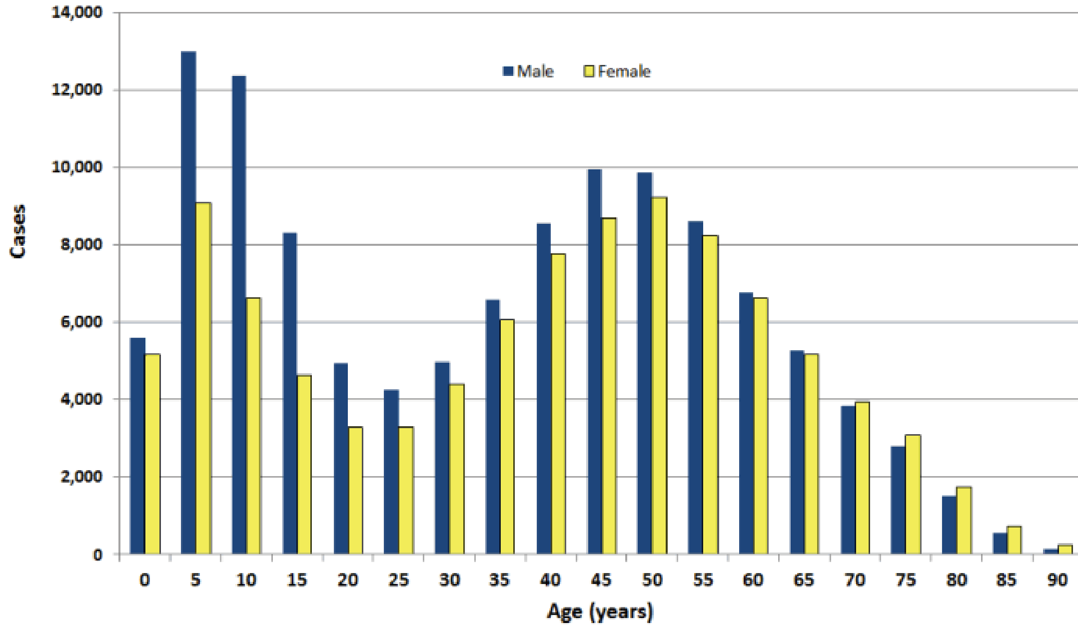

Figure 1-3. Number of confirmed cases of Lyme Disease by age and sex in the United States, from 2001 to 2010.

Incidence of Lyme Disease is higher in children, particularly in boys between 5 and 9 years old. Reprinted with permission from CDC, *Confirmed Lyme Disease cases by age and sex, United States, 2001-2010* [22].

The discovery of Lyme Disease and its causative spirochete

Lyme Disease was clinically described first by Dr. Alan Steere and colleagues in 1977. It was reported as an "epidemic form of arthritis" in the small town of Lyme, Connecticut. At that time, based on the seasonal occurrence of the symptoms (in the summer and early fall) along with a tight geographic clustering of disease cases in rural areas, it was suggested that transmission might occur via an arthropod vector [23]. A significant breakthrough occurred in 1982 when Dr. Willy Burgdorfer and coworkers discovered that *Ixodes* ticks collected in a Lyme Disease endemic area carried a spirochete, which was proposed by the authors to be the etiologic agent of Lyme Disease [24]. This spirochete was subsequently named *Borrelia burgdorferi* in recognition of Dr. Burgdorfer's discovery.

More than three decades since the discovery of *B. burgdorferi*, there has been a remarkable accumulation of knowledge on the genetics, molecular biology, host and vector interactions of this pathogen. It is now understood that the ability of the spirochete to adapt and survive in two different environments, the tick (vector) and the vertebrate (host), is largely based on changes in *B. burgdorferi* gene expression. This topic has been elegantly reviewed elsewhere [25-27].

Despite significant advances, our understanding of how *B. burgdorferi* coordinates changes in its gene expression, transcriptome, protein function, metabolism and cellular architecture during the transition between the tick and the mammalian host remains insufficient. In particular, the mechanisms by which *I. scapularis* promotes spirochetal ability to evade the host defenses need far more scrutiny.

The enzootic cycle of *B. burgdorferi*

In North America, the vast majority of Lyme Disease infections are acquired through the bites of the blacklegged or deer tick, *I. scapularis,* that carry *B. burgdorferi* [3]. The life cycle of the spirochete is depicted in **Figure 1-4** and generally lasts 2 years. *I. scapularis*, the vector of *B. burgdorferi,* undergo a three-stage life cycle: larvae, nymph, and adult. Ticks take only one blood meal per each developmental stage. The cycle of *B. burgdorferi* transmission starts when uninfected ticks feed on reservoir hosts that are already infected with the spirochete. Ticks may become infected at any stage of their life (larvae, nymph or adult). Once acquired by the vector, *B. burgdorferi* is able to persist in the gut lumen through the next developmental stage, surviving within unfed or intermoult ticks. In the early summer, when larvae hatch from eggs they are non-infected with *B. burgdorferi* as there is no transovarial transmission [25, 28, 29]. Larvae can feed on a variety of reservoir hosts usually the white-footed mouse, *Peromyscus leucopus,* the natural reservoir for *B. burgdorferi*, but also on other small mammals or birds. Uninfected larval ticks may feed on infected reservoir hosts and acquire *B. burgdorferi.* Upon blood meal, larval ticks over winter and, in the following spring, moult into the next stage: nymphs. Infected nymphs represent the highest risk for humans and they feed on a similar range of hosts to larvae. Upon feeding, nymphs moult into the adult stage, in

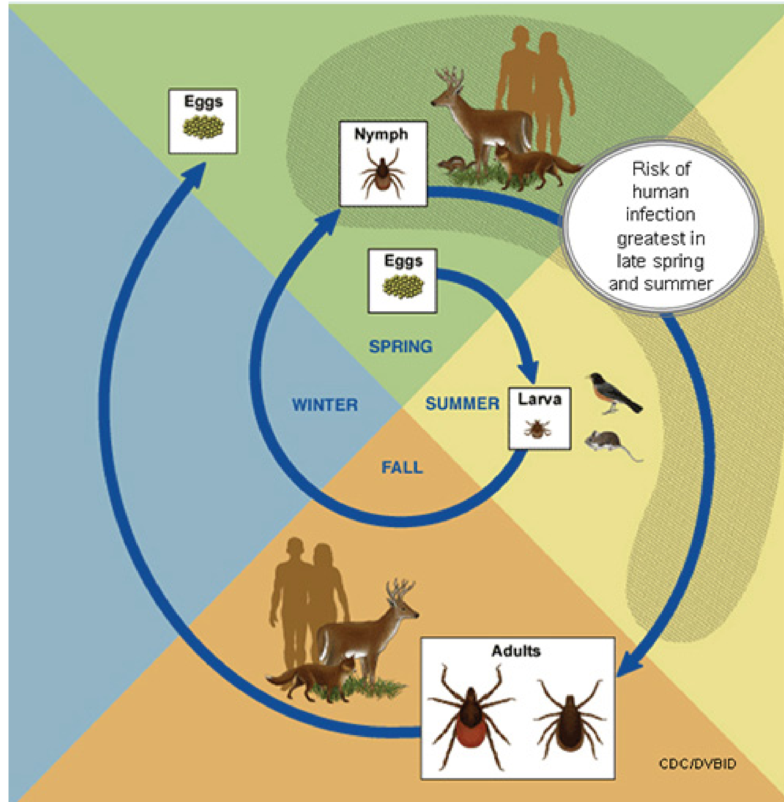

Figure 1-4. The enzootic cycle of *B. burgdorferi*.

Two-year life cycle of *I. scapularis* ticks, the vector of *B. burgdorferi*. Ticks undergo a three-stage life cycle- larvae, nymph and adult- with one blood meal per stage. In the summer, non-infected larvae hatch from the eggs. Unfed larval ticks may feed on a small mammal or bird infected with *B. burgdorferi* and become infected. Fed larvae survive the winter; they moult and emerge the following spring as infected nymphs. Nymphs are responsible for the vast majority of spirochete transmission to humans. Upon feeding, nymphs moult to become adults in the fall. During the winter, adult ticks mate, usually on large animals such as deer and feed on them. Fed female ticks lay eggs in the following spring, and the life cycle begins again. Reprinted with permission from CDC, *The lifecycle of blacklegged ticks* [28].

the fall. During the winter, adult ticks mate and feed on larger animals, preferably the deer, which are incompetent hosts for *B. burgdorferi*. After mating, female adult ticks lay eggs the following spring and, hence, the enzootic cycle begins again [8, 25]. Unlike reservoir hosts, humans are incidental or dead-end hosts. Yet, all three stages of *I. scapularis* can feed on humans [25]. Spirochaetes are deposited into the skin, at the site of the tick bite. A tick feeding period of at least 53 hours is usually needed for transmission of *B. burgdorferi* to occur [30]. Once in the dermis, *B. burgdorferi* can then disseminate to several internal organs, including the joints, heart and nervous system, and cause severe manifestations.

Clinical manifestations of Lyme Disease

The clinical manifestations of Lyme Disease varies depending on the stage of the illness and are classified into early localized disease (stage 1), early disseminated disease (stage 2), and late disease (stage 3) **(Table 1-1)** [8, 10, 13].

Erythema migrans (EM) is the clinical hallmark of the early localized stage. By its clinical definition, a primary EM is "a round or oval expanding erythematous skin lesion that develops at the site of deposition of *B. burgdorferi* by an *Ixodes* species tick" [13], usually within 7 to 14 days (can range 3-30 days) after the bite. EM can gradually expand over time and reach up to 12 inches across, and as it enlarges, parts of the center of the rash may clear, resulting in a "bull's-eye" appearance. EM is found in about 70-80% of patients with objective evidence of infection with *B. burgdorferi*. It is rarely symptomatic but might be pruritic and feel warm [10, 13, 31]. In the United States, 80% of patients with EM may experience simultaneous nonspecific systemic symptoms. Most frequently, they include malaise, headache, fever and chills, and myalgias and arthralgias whereas nausea, anorexia, dizziness, and difficulty concentrating are less common [31].

Multiple EM is the most common manifestation of the early disseminated stage and may develop days to weeks post tick bite after *B burgdorferi* spreads through the blood from the tick bite site to other areas of skin [10, 31]. Other manifestations of this stage include neurological symptoms (neuroborreliosis) such as loss of muscle tone on one or both sides of the face (facial or Bell's palsy), meningitis and radiculoneuritis as well as cardiac complications (Lyme carditis), most commonly manifested as atrioventricular block [8-10].

In the United States, arthritis is the most common manifestation of the late stage, and it affects one-third of the Lyme Disease patients [32]. Lyme arthritis occurs months to years after the initial tick bite and it causes intermittent or persistent attacks of swelling and pain in the large joints, especially the knee [10, 32]. Development of Lyme arthritis is associated with infection with *B. burgdorferi* OspC type A strain, which causes disseminated infection in humans [33], and is responsible for approximately 40% of the infections in the northeastern U.S. [32]. Although uncommon in children, encephalitis, encephalopathy, and polyneuropathy are also associated with late Lyme Disease [8].

Table 1-1. Clinical manifestations of patients with Lyme Disease.

Disease Stage	Time Post-Tick Bite	Clinical Manifestations
Early localized	3-30 days	Erythema migrans
Early disseminated	Days to weeks	Multiple erythema migrans Bell's palsy Meningitis Carditis
Late	Months to years	Arthritis Encephalitis Other neurological complications

Sources: Murray TS, Shapiro ED, *Lyme Disease.* Clin Lab med., 2010. **30**(1): p. 311-328. and Centers for Disease Control and Prevention, *Signs and Symptoms of Lyme Disease* 2015 [8, 10].

Diagnosis of Lyme Disease

According to the CDC, the diagnosis of Lyme Disease is based on signs and symptoms such as the typical EM, facial palsy, or arthritis, and a history of possible exposure to infected blacklegged ticks [34]. For serodiagnosis, the CDC recommends a two-tier test approach using an Enzyme-Linked Immunosorbent Assay (ELISA) or Immunofluorescent Assay (IFA) followed by a Western blot **(Figure 1-5)**. The first test (ELISA or an IFA) allows for quantification of antibodies against *B. burgdorferi* in the sera and, thus, is more sensitive. Samples negative by ELISA or IFA do not need to be tested further. In contrast, if the first test is positive or indeterminate (equivocal), a second step should be performed to confirm the results. The second step uses a Western Blot (WB) assay, which is more specific than the first step tests. If the WB is performed during the first 30 days of disease onset (early LD), both IgM and IgG should be assessed. A positive IgM test result alone is not recommended for use in determining active disease in individuals with illness greater than 30 days of duration given the likelihood of a false-positive result. If the WB is performed after 30 days of disease onset (early LD), only IgG should be assessed [35]. In regard to test interpretation, for results to be considered positive, IgM blots are required to have at least two of the following three bands: 23 kDa (OspC), 39 kDa (BmpA), and 41 kDa (Fla). In case of IgG blots, a result is considered positive if at least five of the following ten bands are present: 18 kDa, 23 kDa (OspC), 28 kDa, 30 kDa, 39 kDa (BmpA), 41 kDa (Fla), 45 kDa, 58 kDa, 66 kDa, and 93 kDa [36]. CDC does not recommend testing blood by WB without first testing it by ELISA or IFA [35]. Laboratory tests should be interpreted in the context of the clinical evaluation and the likelihood that the patient has Lyme Disease.

Although the sensitivity of a serodiagnosis assay likely increases with the duration of the infection, the ability to detect infection during the early phase of the disease is poor given its dependence on antibody production [37]. The two-tiered testing has a high specificity (99-99.5%) and sensitivity (100%) at later stages of Lyme Disease, whereas it shows low sensitivity (about 35%) in patients with early-stage disease [38, 39]. Another shortcoming of the current serologic assays is their inability in distinguish between active and past infection, and patients remain seropositive for years, despite absence of active infection [37].

Current antibody-based assays are the only type of diagnostic testing for Lyme Disease approved by the US Food and Drug Administration. Therefore, providing this remains unchanged, prompt recognition of the disease will be limited. If not diagnosed in a timely manner, disease will progress and patients may suffer seriously debilitating complications, which are more difficult to treat [13]. Strategies such as early diagnosis, appropriate treatment, and preventive measures are imperative to counteract sequelae associated with disease progression.

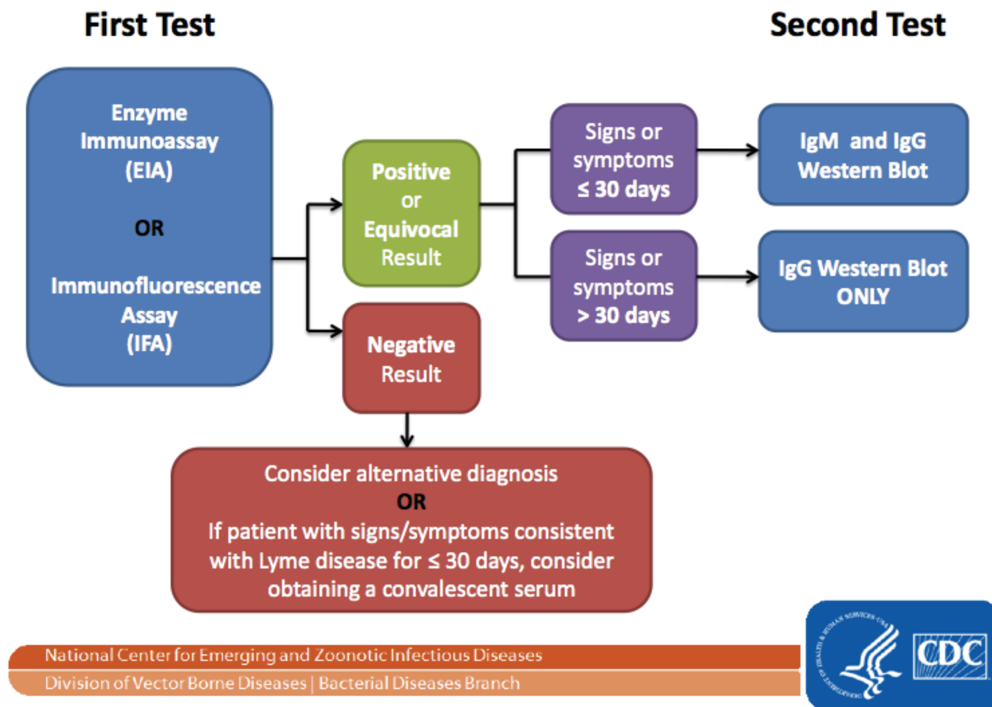

Figure 1-5. Current CDC recommendations on serologic diagnosis of Lyme Disease: 2-tier algorithm.

The CDC currently recommends a two-tier testing for the serodiagnosis of Lyme Disease as follows: the first required test is the Enzyme-Linked Immunosorbent Assay (EIA or ELISA) or the Immunofluorescence Assay (IFA). In a scenario where the first step is negative, no further testing is recommended. If the first test is positive or equivocal, the second test should be performed to confirm the results. The second test uses a Western Blot (WB) assay to detect IgGs if the patient has had symptoms for more than 30 days. Results are considered positive only if the ELISA/IFA and the WB are both positive. The IgM should not be screened if the patient has been ill for more than 30 days. Reprinted with permission from CDC, *Two-Tiered testing for Lyme Disease* [35].

Treatment of Lyme Disease

The recommended treatment of Lyme Disease is published in detail in the literature [13]. It primarily involves antibiotic therapy and is based in guidelines developed by the Infectious Diseases Society of America. The following is a brief summary of their recommendations: 1) In the absence of specific neurologic manifestations, patients with early localized or early disseminated Lyme Disease associated with EM should receive oral doxycycline, amoxicillin or cefuroxime axetil for 14 days; 2) In the presence of acute neurologic manifestations such as meningitis or radiculopathy, patients with early disseminated Lyme Disease should receive intravenous ceftriaxone (for adults) or parenteral ceftriaxone or cefotaxime (for children); 3) Adult patients with arthritis and without clinical evidence of neurologic disease should receive oral doxycycline, amoxicillin, or cefuroxime axetil for 28 days; 4) Adult patients with arthritis and objective evidence of neurologic disease should receive parenteral therapy with intravenous ceftriaxone for 2–4 weeks [13].

Although Lyme Disease is treated successfully with antibiotics in the majority of cases [13, 40], approximately 10-20% of the patients that receive an adequate course of antibiotic therapy may experience PTLDS, a set of symptoms including muscle and joint pains, cognitive difficulties, sleep disturbances or fatigue, that can last for years [10, 41]. Prolonged antibiotic therapy has failed in improving PTLDS symptoms and currently there is no Food and Drug Administration treatment approved for this condition [42]. Furthermore, joint inflammation may persist in a minority of patients for months or several years, despite receiving repeated courses of antibiotic therapy for late Lyme arthritis. This is termed Antibiotic-Refractory Lyme Arthritis [32].

B. burgdorferi

Biology. *B. burgdorferi* belongs to the eubacterial phylum *Spirochaetes*, in particular to the family *Spirochaetaceae*. Members of this phylum are identified by their distinct morphology that includes a wavelike body, a flexible cell wall, and a periplasmic flagella surrounded by an outer and inner membrane [43]. Similarly to Gram-negative bacteria, *B. burgdorferi* also contains an outer and an inner membrane. Yet, the spirochete exhibits a very distinct cellular organization and membrane composition. Unique features of its outer cell membrane include the lack of lipopolysaccharide, the presence of low density transmembrane proteins, and an extraordinary abundance of surface exposed lipoproteins [25, 27].

This extracellular organism is 10-30 μm long and 0.2-0.25 μm wide and replicates slowly as it divides every 8-12 h during log-phase growth. To be grown in the laboratory, the bacterium requires special media, Barbour-Stoenner-Kelly (BSK-H), and grows best at temperatures 30°C-34°C in microaerophilic conditions [44].

Genome. A *B. burgdorferi* genome was sequenced in 1997 by Fraser and colleagues [45]. It includes a linear chromosome of approximately 1 Mb in size and

extrachromosomal DNA elements that total about 600 kb, which contain twelve linear and nine circular plasmids [45, 46]. One of the most remarkable features of *B. burgdorferi* is a nearly complete absence of biosynthetic pathways. The spirochete is an auxotroph for all amino acids, nucleotides, enzyme cofactors and fatty acids. The metabolic capacity of *B. burgdorferi* is extremely limited as it lacks the genes encoding proteins of the tricarboxylic acid cycle or oxidative phosphorylation. Instead, the bacterium obtains energy from the fermentation of sugars to lactic acid via the Embden–Meyerhof pathway. Genome sequence analysis found that while the chromosome is fairly conserved and carries the majority of housekeeping genes, several plasmid genes exhibit much greater variability in content and encode for most of the outer-surface lipoproteins, many of which are potentially involved in infectivity and virulence [25, 45, 46].

Lipoproteins. *B. burgdorferi* is maintained in nature by alternating infections in tick and mammalian hosts, which demands spirochetal survival in two contrasting environments. To ensure success during its complex enzootic life cycle, the bacterium needs to sense the environmental cues and, accordingly, orchestrate the expression of fundamental genes [25]. The plasticity displayed in some of the spirochete's outer surface proteins (Osps) seems to be indicative of adaptations as the spirochete selectively expresses certain proteins that are essential for a specific stage of its life cycle [47]. Among Osps, lipoproteins play a pivotal role in persistence and survival of *B. burgdorferi* in the tick–host cycle. For instance, *B. burgdorferi* expresses outer surface protein A (OspA) but not OspC when residing in the midgut of unfed ticks [48, 49]. At this stage, the spirochete upregulates OspA, which binds to the tick receptor for OspA (TROSPA), enabling *Borrelia* to persist inside of ticks [50]. In contrast, when an infected tick starts feeding, *Borrelia* downregulates OspA and upregulates OspC [48, 49]. The spirochete then migrates towards the tick salivary glands, from where is transmitted to the host. Although the requirement of OspC for *Borrelia* migration from the tick midgut to the salivary gland remains debatable [30, 51-53], it is widely accepted that OspC is a *B. burgdorferi* virulence factor that is essential to establish host infection [51, 52]. OspC is a 22 kDa *B. burgdorferi* lipoprotein that is encoded by circular plasmid 26 [47]. Analyses of OspC sequences have described 25 OspC phyletic types that are differentiated by letter designation (from A to U) [54, 55]. Sequence analysis of North American isolates of *B. burgdorferi* revealed that the amino acid sequences within an OspC type are more than 99% identical, whereas a lower homology exists, approximately 80% on average, between different types of OspC [55]. Although OspC has been the focus of intense research, its function in early infection is not fully understood, neither is the mechanism by which its diversity is selectively maintained.

OspB is a 34 kDa surface-exposed lipoprotein encoded on *B. burgdorferi* linear plasmid 54. The OspB gene is highly conserved among *B. burgdorferi* isolates in the United States and exhibits high similarity in terms of protein sequence (about 50% homology) and structure to OspA [45, 47, 56]. The presence of OspB on the surface of *B. burgdorferi* within unfed ticks but not on fed ticks [57], and its similarity to OspA led to speculation that OspB could play an important role in the persistence of *B. burgdorferi* within unfed ticks. Indeed, OspB was shown not only to specifically bind to a protein or protein complex within the tick gut [58] but also to be essential for spirochetal adhesion

and survival within the tick midgut [56]. The mechanism by which *B. burgdorferi* OspB interacts with ticks and promotes survival remains to be explored.

BBK32 is a 47 kDa surface-exposed lipoprotein encoded on *B. burgdorferi* linear plasmid 36. It is upregulated during tick feeding and mammalian infection, and is involved in the attachment of spirochetes to the extracellular matrix by binding to host fibronectin and glycosaminoglycans (GAGs) [47, 59, 60]. *B. burgdorferi* mutants lacking BBK32 are significantly impaired in infecting mice, when compared with the parental wild-type strain [61]. In another study, BBK32-mediated fibronectin and GAG binding initiated the interactions between the spirochete and the host microvasculature, which led investigators to hypothesize that BBK32 may be needed for *B. burgdorferi* escape from the bloodstream, and thus spirochete dissemination [62]. More recently, a report using bioluminescent imaging of *B. burgdorferi in vivo* showed that BBK32 is required for optimal infectivity, particularly, when the infection progresses from colonization to the dissemination phase [63]. In addition, BBK32 contributes to spirochetal colonization of the joints, and this was exclusively dependent on its GAG-binding activity [64]. Taken together, these findings suggest that BBK32 is important for the spirochete pathogenicity during the early stages of infection.

Vaccines as a strategy to prevent Lyme Disease

Multiple efforts to prevent Lyme Disease using several measures have been enforced within communities [65]. However, clearly, they have not been sufficient as the number of disease cases continues to rise [4]. Moreover, a human vaccine against Lyme Disease was licensed by the FDA, but it was withdrawn from the market a few years later, for multiple reasons [66, 67]. Therefore, a powerful strategy to effectively reduce *B. burgdorferi* prevalence remains to be developed and is of utmost importance. Vaccination against infection is a highly effective means to control the spread of disease in a population and it remains a promising strategy to prevent Lyme Disease [68]. Currently, there is no vaccine available to immunize humans. Novel vaccination approaches should be explored with the ultimate goal of minimizing the risk of human exposure to Lyme Disease, and thus, detain this epidemic.

As described earlier, outer surface lipoproteins play an important role in virulence, host–pathogen interactions, and in maintaining the enzootic cycle of *B. burgdorferi*. Furthermore, antibodies directed at lipoproteins have been shown to protect animals [69-72] and humans [73] from infection with *B. burgdorferi*. In addition, several canine lipoprotein-based vaccines are currently commercially available [66]. Hence, lipoproteins have been considered important vaccine candidates to prevent Lyme Disease.

OspC. OspC has been intensively investigated as a candidate Lyme Disease vaccinogen as a DNA vaccine [74-76] and a recombinant protein-based vaccine [69, 77-80]. A disadvantage of the OspC vaccine is the heterogeneity of OspC proteins among *Borrelia* strains. Studies have been shown that OspC immunization elicits protective

immunity against challenge with homologous *B. burgdorferi* strains [69, 70, 74, 77, 79] but fails to protect from infection with heterologous *B. burgdorferi* strains [76, 78]. Yet, other authors have demonstrated that OspC antibodies fail to confer homologous protection against some *B. burgdorferi* strains [80]. One hypothesis that can be put forth to explain these inconsistencies regarding the potential of OspC as a vaccinogen relates to the fact that different studies used different strains of *B. burgdorferi,* different doses, and different routes to assess for vaccine efficacy. Nevertheless, OspC has been most promising amongst other vaccine candidates against Lyme Disease, and is included in the formulation of an OspA-based canine vaccine (Novibac® Lyme), presently being commercialized. OspC is currently considered a promising candidate for a second-generation vaccine [66].

Given the heterogeneity of OspC proteins, some authors have been working towards the development of a polyvalent chimeric OspC vaccine. Although claiming that these constructs can afford a broader protection [81, 82], these findings are built on speculation because their vaccine efficacy studies were solely based on *in vitro* experiments, and thus are distant from representing the natural context of *B. burgdorferi* transmission.

OspC type K is one of the four types (A, B, I and K) that causes disseminated Lyme Disease in humans [33], and has been shown to be highly distributed amongst important host species for *B. burgdorferi* [83]. Combined with another type, OspC type K was able to detect anti-OspC antibodies present in 96% of the Lyme Disease patients infected with several types of *B. burgdorferi* in the United States [84].

OspB. Because OspB is a tick-specific protein that is upregulated within unfed ticks and repressed during tick feeding at the time of spirochete deposition in the host [57], the great majority of Lyme Disease patients, naturally infected mice and mice experimentally infected via tick bite do not produce antibodies to OspB [85, 86]. Yet, due to its important role in colonizing the tick gut, OspB has been considered an important candidate for a transmission-blocking vaccine [56, 58]. In fact, complement-independent IgG antibodies to OspB were able to disrupt the outer membrane of *B. burgdorferi*, resulting in osmotic lysis of the spirochete *in vitro* [87]. In another study, nonbactericidal antibodies to OspB significantly inhibited the attachment of *B. burgdorferi* to the tick gut *in vivo*, and effectively prevented *I. scapularis* colonization by spirochetes [58].

BBK32. Humoral responses to BBK32 are found in the sera of *B. burgdorferi*-infected dogs [88], mice and humans [71, 86, 89] and can influence the progression of the disease. For instance, higher levels of antibodies to BBK32 during early infection have been associated with shorter and less severe Lyme arthritis [89]. Active and passive immunization with BBK32 partially protected mice against *B. burgdorferi* infection via needle challenge [71, 72]. Furthermore, antibodies against BBK32 have been shown to reduce spirochete transmission from the tick vector during feeding [72].

In addition to vaccination strategies based on *B. burgdorferi* antigens such as lipoproteins, the use of tick proteins as vaccine candidates has also been considered a promising measure to block tick–host–pathogen interactions and thus, transmission. Tick

immunity has been described and it happens when, upon repeated exposure to ticks, the host becomes resistant to subsequent tick bites [67, 90, 91]. Targeting an arthropod protein that is either required for pathogen acquisition by the vector or for transmission from the tick to the mammalian host is an attractive approach to interfere with the pathogen cycle.

Salp15. Tick salivary proteins that interact with *B. burgdorferi* are considered important targets for a vaccine against Lyme Disease [67]. Salp15 is a 15 kDa *I. scapularis* salivary protein that exhibits immunosuppressive properties, as it inhibits $CD4^+$ T-cell activation, and thus facilitates the survival of spirochetes within the infected host [92]. In addition, Salp15 is able to bind to *B. burgdorferi* OspC and protect the spirochete from innate and adaptive immune responses, promoting *Borrelia* transmission from the tick to the host [93]. Antibodies to Salp15 have been shown to neutralize the antigen-associated immunosuppressive effect, to enhance pathogen clearance, and to partially protect mice against *B. burgdorferi* via tick challenge [91].

Salp25. Salp25D (hereafter referred to as Salp25) is a 25 kDa *I. scapularis* salivary protein that functions as a potent antioxidant [94]. It plays an important role in *B. burgdorferi* acquisition by the tick during feeding given that it detoxifies reactive oxygen species produced by neutrophils in the host, promoting spirochete survival during its transition from the mammalian host to the arthropod vector. Ticks that fed on mice immunized with Salp25 were significantly impaired in acquiring spirochetes, when compared with ticks that fed on the control group [95].

Mouse model of Lyme Disease

One important landmark in the study of Lyme Disease was the development of animal models of infection. Mice are the natural reservoirs for *B. burgdorferi.* Although in nature, wild mice infected with the spirochete show no sign of disease, they develop a serological response to *B. burgdorferi* proteins [86] and become persistently infected [96]. Furthermore, specific inbred strains of the laboratory mouse *Mus musculus* infected with *B. burgdorferi* were found to exhibit features similar to those of human Lyme Disease. In particular, C3H/HeN mice develop a multi-systemic infection with reproducible arthritis and carditis, and are now a well-established model for Lyme Disease [25, 97-99]. Experimentally, mice can be infected with *B. burgdorferi* by needle inoculation or tick feeding [43], upon which the spirochete establishes a localized infection at the entry site and then quickly disseminates via the bloodstream to various tissues, including ear, joints, heart and bladder [98-100].

Scope and Objectives of Dissertation

Despite current preventive measures, the incidence and the geographic distribution of Lyme Disease continues to increase [4, 16]. Lyme Disease has become a serious and expensive public health problem, and has now reached epidemic proportions [1, 14]. Yet, there is no vaccine available to protect humans from this illness, and thus

novel vaccine strategies shall be developed. Reservoir-targeted vaccines delivered via the oral route are a well-established strategy to reduce *B. burgdorferi* prevalence and, consequently, to diminish the risk of human exposure to Lyme Disease [100-102]. In an effort to integrate this knowledge and building up on it, our laboratory took an innovative approach and proposed to deliver promising vaccine antigens either from *B. burgdorferi* or from its vector, *I. scapularis*, via the oral route. We selected OspC type K, OspB and BBK32 from *B. burgdorferi* based on evidence that these proteins induced significant IgG immune responses when administered via a parenteral route [58, 69, 71, 72, 74, 79, 87]. In addition, we elected two salivary gland proteins from *I. scapularis* -Salp15 and Salp25-, based on previous observations that they stand out as candidates for a vector-targeted vaccine [67, 91, 95]. We rationalized that Salp15 and Salp25 vector targeted vaccines would improve on current reservoir-targeted vaccines formulations.

Oral immunization has several advantages over routes of parenteral immunization. It obviates the need for sterile needles and syringes, poses less risk of disease-transmission, is easy to administer and to scale up, cost-effective, and can induce local and systemic immunity [103]. Orally delivered vaccines are currently licensed and have been shown to protect against viral and bacterial pathogens [104], including spirochetal infections [105]. In addition to the development of oral vaccines against Lyme Disease, projects in our laboratory are focused on the development of oral vaccines against other bacterial diseases such as Melioidosis (see supplemental data "Development of Oral Vaccines against Melioidosis") and Leptospirosis (see supplemental data "Development of Oral Vaccines against Leptospirosis and Design of a Mouse Model of Acute Infection with *Leptospira interrogans"*), in which ingestion is one of the most important routes of infection [106-109]. Furthermore, we have previously shown that immunized mice were protected against infection with *Leptospira interrogans* [110] and *B. burgdorferi* [100-102], when the immunogens were delivered orally via recombinant *E. coli.*

Our oral vaccine technology consisted of recombinant live *E. coli* expressing the leader sequence of *B. burgdorferi* OspA lipoprotein fused to the N-terminus of each candidate immunogen (OspC K, OspB, BBK32, Salp15 or Salp25). The leader peptide of OspA contains a tripalmitoyl-*S*-glyceryl-cysteine (Pam_3Cys) lipid moiety, the substrate for OspA lipidation, which has been associated with adjuvant activity and shown to be essential for the ability of *B. burgdorferi* lipoproteins to generate a strong humoral immune response after vaccination [111-113]. In a previous study, where we investigated the effect of the lipid modification of the leader peptide OspA in the localization of the antigen in live oral delivery vehicles, we showed that the lipidation of the leader peptide of OspA targeted the protein through the membrane and it accumulated in the cell wall. In contrast, mutation of the lipidation site generated a nonlipidated version of OspA that was localized mostly in the cell membrane [114]. We next applied this platform technology and developed *Lactobacillus*-based oral vaccines for plague [115] and *E. coli*-based oral vaccines for Leptospirosis [110] where we were able to target the immunogens for translocation across the bacterial cytoplasmic membrane to the cell envelope as a consequence of lipid modification of the leader peptide OspA. The same rationale was applied to this work. By fusing the leader peptide of OspA to the N-terminus of each

candidate immunogen, we aimed to target them across the cytoplasmic membrane to the cell envelope of *E. coli*.

Here, we proposed to develop and test five (OspC type K, OspB, BBK32, Salp15 and Salp25) oral vaccine candidates against Lyme Disease. The ultimate goal of our studies was to determine whether these candidates delivered via the oral route could induce a humoral immune response that would protect mice against infection delivered by ticks infected with heterologous strains of *B. burgdorferi.*

Amongst all the vaccine candidates that we have selected for this study, OspC is considered the most promising and thus, the most studied in the literature. Although wild ticks transmit a heterologous mixture of *B. burgdorferi* OspC strains to their host [55, 83, 116, 117], the literature lacks studies where the efficacy of OspC-based vaccines is assessed under conditions that fully mimic the natural route of *B. burgdorferi* infection. Yet, OspC is currently considered as one of the most promising second-generation Lyme Disease vaccine candidates. While this is the result of studies that failed to test OspC vaccines with the natural route of *B. burgdorferi* infection, it is essential that future studies learn from prior mistakes and investigate the potential of OspC-based vaccines in the natural context of the Lyme spirochete transmission. Aim 2 of this work intends to address this limitation, as we propose to test our most promising vaccine candidate against challenge with ticks infected with multiple strains of *B. burgdorferi*.

Specific aim 1

To develop oral vaccine constructs and understand the vaccine-induced humoral immune response. To achieve this aim we proposed to: 1) clone *B. burgdorferi* outer surface proteins (OspC K, OspB and BBK32) and *I. scapularis* proteins (Salp15 and Salp25) in *E. coli*: 2) deliver the recombinant *E. coli* to mice via the oral route and 3) assess antigen-specific total IgGs, IgG1, IgG2a in sera and IgA in mucosa.

Specific aim 2

To test the vaccine efficacy of our most promising oral vaccine candidate. To achieve this aim we proposed to immunize mice with our most promising oral vaccine candidate and challenge them with ticks carrying multiple strains of *B. burgdorferi*. Protection from infection was determined by: 1) Assessing the production of antibodies to *B. burgdorferi* proteins; and 2) Presence of *B. burgdorferi* along with spirochetal DNA quantification in target tissues and in nymphs that feed on immunized mice.

CHAPTER 2. MATERIALS AND METHODS

B. burgdorferi Strains and Cultivation

Low-passage *B. burgdorferi* strain BL204 (an OspC type K strain, kindly provided by Dr. Ira Schwartz, NYMC) and *B. burgdorferi* strain B31MI (an OspC type A strain) were used. A culture containing multiple strains of *B. burgdorferi* (MS) was also used in our studies. This culture was originally isolated from the heart of *Peromyscus leucopus* infected with field caught ticks from endemic areas for Lyme Disease (New York State). *B. burgdorferi* strains B31MI and BL204 were used as positive controls for *ospC type A* and *ospC type K*, respectively, in the PCR studies. Both cultures of *B. burgdorferi* strain BL204 and *B. burgdorferi* multiple strains were used to generate flat nymphs infected with *B. burgdorferi* OspC type K strain or with multiple strains of *B. burgdorferi*, respectively. All *B. burgdorferi* used in this study were cultured in Barbour-Stoenner-Kelly (BSK-H) medium (New York Medical College, NY) with an antibiotic mixture for *Borrelia* (Sigma-Aldrich, Saint Louis, MO), and grown at 34° C until the cells reached the mid-log-phase as previously described [118]. The number of spirochetes was determined using dark field microscopy (AxioImager, Zeiss, Germany) and q-PCR.

Oral Vaccine Candidates Construction

To construct our oral vaccine candidates **(Table 2-1)**, the genes *OspC type K* (*OspC K*), *OspB* and *BBK32* from *Borrelia burgdorferi sensu stricto,* and *Salp15* and *Salp25* from *Ixodes scapularis* were synthesized and cloned by Blue Heron (Blue Heron Biotechnology, Inc., WA, USA) into the Nde I-Bam HI restriction sites of pET9c using codons optimized for expression in *E. coli,* and fused to the nucleotide sequence encoding the leader peptide of outer surface protein A (OspA) from *B. burgdorferi*. DNA constructs were then transformed into the *E. coli* strain BL21 (DE3) pLysS. The parental *E. coli* strain transformed with the empty plasmid was used as a control construct (ctrl).

Protein Purification

To produce recombinant proteins to use in ELISA and Western blot assays, the genes *OspC K*, *OspB*, *BBK32*, *Salp15* and *Salp25* were synthesized and cloned by Blue Heron into the XhoI and BamHI restriction sites of pET28a, downstream of the 6xHis peptide tag. DNA constructs were then transformed into the *E. coli* strain BL21 (DE3) pLysS. Recombinant *E. coli* clones in pET28a vector expressing either the following proteins, OspC K, OspB, BBK32, Salp15 and Salp25 were grown in Tryptone Broth Yeast (TBY) medium supplemented with 50 µg/ml Kanamycin at 37°C, shaking at 225 rpm, until it reached an OD_{600} of 0.8. The expression of 6xHis -OspC K, -OspB, -BBK32, -Salp15 and -Salp25 recombinant proteins was induced by adding 1 mM of isopropyl-b-D-thiogalactopyranoside (IPTG, Gold Biotechnology, St. Louis, MO) followed by incubation at 37°C for 3 hrs. The cells were harvested by centrifugation at 4000 x g for

Table 2-1. List of oral vaccine constructs.

Vaccine Construct	Sequence	Product Length (bp)
OspC K	CATATGAAAAAATACCTGCTGGGTATCGGTCTGATCC TGGCGCTGATCGCGTGCAAACAGAACGTTTCTTGCAA CAACTCTGGTAAAGACGGTAACACCTCTGCGAACTCT GCGGACGAATCTGTTAAAGGTCCGAACCTGACCGAAA TCTCTAAAAAAATCACCGAATCTAACGCGGTTGTTCTG GCGGTTAAAGAAATCGAAACCCTGCTGGCGTCTATCG ACGAACTGGCGACCAAAGCGATCGGTAAAAAAATCC AGCAGAACGGTGGTCTGGCGGTTGAAGCGGGTCACAA CGGTACCCTGCTGGCGGGTGCGTACACCATCTCTAAA CTGATCACCCAGAAACTGGACGGTCTGAAAAACTCTG AAAAACTGAAAGAAAAAATCGAAAACGCGAAAAAAT GCTCTGAAGACTTCACCAAAAAACTGGAAGGTGAACA CGCGCAGCTGGGTATCGAAAACGTTACCGACGAAAAC GCGAAAAAAGCGATCCTGATCACCGACGCGGCGAAA GACAAAGGTGCGGCGGAACTGGAAAAACTGTTCAAA GCGGTTGAAAACCTGGCGAAAGCGGCGAAAGAAATG CTGGCGAACTCTGTTAAAGAACTGACCTCTCCGATCGT TGCGGAATCTCCGAAAAAACCGTAAGGATCC	657
OspB	CATATGAAAAAATACCTGCTGGGTATCGGTCTGATCC TGGCGCTGATCGCGTGCAAACAGAACGTTTCTTGCGC GCAGAAAGGTGCGGAATCTATCGGTTCTCAGAAAGAA AACGACCTGAACCTGGAAGACTCTTCTAAAAAATCTC ACCAGAACGCGAAACAGGACCTGCCGGCGGTTACCGA AGACTCTGTTTCTCTGTTCAACGGTAACAAAATCTTCG TTTCTAAAGAAAAAAACTCTTCTGGTAAATACGACCT GCGTGCGACCATCGACCAGGTTGAACTGAAAGGTACC TCTGACAAAAACAACGGTTCTGGTACCCTGGAAGGTT CTAAACCGGACAAATCTAAAGTTAAACTGACCGTTTC TGCGGACCTGAACACCGTTACCCTGGAAGCGTTCGAC GCGTCTAACCAGAAAATCTCTTCTAAAGTTACCAAAA AACAGGGTTCTATCACCGAAGAAACCCTGAAAGCGAA CAAACTGGACTCTAAAAAACTGACCCGTTCTAACGGT ACCACCCTGGAATACTCTCAGATCACCGACGCGGACA ACGCGACCAAAGCGGTTGAAACCCTGAAAAACTCTAT CAAACTGGAAGGTTCTCTGGTTGGTGGTAAAACCACC GTTGAAATCAAAGAAGGTACCGTTACCCTGAAACGTG AAATCGAAAAAGACGGTAAAGTTAAAGTTTTCCTGAA CGACACCGCGGGTTCTAACAAAAAAACCGGTAAATGG GAAGACTCTACCTCTACCCTGACCATCTCTGCGGACTC	921

Table 2-1. (Continued).

Vaccine Construct	Sequence	Product Length (bp)
	TAAAAAAACCAAAGACCTGGTTTTCCTGACCGACGGT ACCATCACCGTTCAGCAGTACAACACCGCGGGTACCT CTCTGGAAGGTTCTGCGTCTGAAATCAAAAACCTGTC TGAACTGAAAAACGCGCTGAAATAAGGATCC	
BBK32	CATATGAAAAAATACCTGCTGGGTATCGGTCTGATCC TGGCGCTGATCGCGTGCAAACAGAACGTTTCTAAAAA AGTTAAATCTAAATACCTGGCGCTGGGTCTGCTGTTCG GTTTCATCTCTTGCGACCTGTTCATCCGTTACGAAATG AAAGAAGAATCTCCGGGTCTGTTCGACAAAGGTAACT CTATCCTGGAAACCTCTGAAGAATCTATCAAAAAACC GATGAACAAAAAAGGTAAAGGTAAAATCGCGCGTAA AAAAGGTAAATCTAAAGTTTCTCGTAAAGAACCGTAC ATCCACTCTCTGAAACGTGACTCTGCGAACAAATCTA ACTTCCTGCAGAAAAACGTTATCCTGGAAGAAGAATC TCTGAAAACCGAACTGCTGAAAGAACAGTCTGAAACC CGTAAAGAAAAAATCCAGAAACAGCAGGACGAATAC AAAGGTATGACCCAGGGTTCTCTGAACTCTCTGTCTG GTGAATCTGGTGAACTGGAAGAACCGATCGAATCTAA CGAAATCGACCTGACCATCGACTCTGACCTGCGTCCG AAATCTTCTCTGCAGGGTATCGCGGGTTCTAACTCTAT CTCTTACACCGACGAAATCGAAGAAGAAGACTACGAC CAGTACTACCTGGACGAATACGACGAAGAAGACGAA GAAGAAATCCGTCTGTCTAACCGTTACCAGTCTTACCT GGAAGGTGTTAAATACAACGTTGACTCTGCGATCCAG ACCATCACCAAAATCTACAACACCTACACCCTGTTCTC TACCAAACTGACCCAGATGTACTCTACCCGTCTGGAC AACTTCGCGAAAGCGAAAGCGAAAGAAGAAGCGGCG AAATTCACCAAAGAAGACCTGGAAAAAAACTTCAAA ACCCTGCTGAACTACATCCAGGTTTCTGTTAAAACCGC GGCGAACTTCGTTTACATCAACGACACCCACGCGAAA CGTAAACTGGAAAACATCGAAGCGGAAATCAAAACC CTGATCGCGAAAATCAAAGAACAGTCTAACCTGTACG AAGCGTACAAAGCGATCGTTACCTCTATCCTGCTGAT GCGTGACTCTCTGAAAGAAGTTCAGGGTATCATCGAC AAAAACGGTGTTTGGTACTAAGGATCC	1137
Salp15	CATATGAAAAAATACCTGCTGGGTATCGGTCTGATCC TGGCGCTGATCGCGTGCAAACAGAACGTTTCTGAATC	483

Table 2-1. (Continued).

Vaccine Construct	Sequence	Product Length (bp)
	TTTCGTTGCGATGAAAGTTGTTTGCATCCTGTTCCTGG TTGGTGTTGTTGCGGCGAACGAATCTGGTCCGACCAA AGCGGACGCGTCTACCGCGGACAAAGACACCAAAAA AAACAACGTTCAGCTGCGTTTCCCGAACTACATCTCTA ACCACCAGAAACTGGCGCTGAAACTGCTGAAAATCTG CAAAGACTCTAAATCTTCTCACAACTCTCTGTCTTCTC GTTCTTCTGACGTTATCAACGACAAATACGTTGACTTC AAAAACTGCACCTTCCTGTGCAAACACGGTAACGACG TTAACGTTACCCTGAACCTGCCGGAAGACACCCCGTG CGGTCCGAACGGTCAGACCTGCGCGGAAAAAAACAA ATGCGTTGGTCACATCCCGGGTTGCTAGTAAGGATCC	
Salp25	CATATGAAAAAATACCTGCTGGGTATCGGTCTGATCC TGGCGCTGATCGCGTGCAAACAGAACGTTTCTGGTCC GCTGAACCTGGGTGACCCGTTCCCGAACTTCACCTGC GACACCACCGAAGGTAAAATCGACTTCCACGAATGGC TGGGTAACTCTTGGGGTATCCTGTTCTCTCACCCGGCG GACTACACCCCGGTTTGCACCTCTGAACTGGCGCGTG CGGCGCAGCTGCACCACGTTTTCCAGAAAAAAGGTGT TAAACTGATCGCGCTGTCTTGCGACTCTGTTGAATCTC ACCGTGGTTGGATCAAAGACATCAACGCGTTCGGTGA ACTGCCGGACGGTCCGTTCCCGTACCCGATCATCGCG GACGAAAAACGTGACATCGCGGTTAAACTGGGTATGC TGGACCCGGTTGAAAAAGACAAAGAAGGTCTGCCGCT GACCTGCCGTGCGGTTTTCATCATCGGTCCGGACAAA AAAATGAAACTGTCTATGCTGTACCCGGCGACCACCG GTCGTAACTTCGACGAAGTTCTGCGTGCGACCGACTC TCTGCTGGTTACCGAAACCCGTAAAGTTGCGACCCCG GCGGGTTGGCAGAAAGGTACCCCGTGCATGGTTCTGC CGTCTGTTACCGAAGAAGAAATCCTGAAACTGTTCCC GACCGGTATCAAACAGTACGAAGTTCCGTCTGGTAAA AACTACCTGCGTACCACCATGGACTGAGGATCC	738

10 min at 4°C. The pellet was isolated and subjected to cell wall lysis by the BugBuster Protein Extraction Reagent (Novagen, Madison, WI), and the protein extraction protocol was followed according to instructions provided by the manufacturer. The 6xHis-tagged proteins were purified using the Ni-NTA Purification System (Invitrogen, Carlsbad, CA) following instructions provided by the manufacturer. Protein concentration was determined by the Bradford protein assay (Bio-Rad, West Berkeley, CA), and samples were stored at -80°C until use.

Oral Vaccine Preparation

Recombinant *E. coli* clones in pET9c vector expressing either the following proteins: OspC K, OspB, BBK32, Salp15 and Salp25, or *E. coli* carrying the empty vector (ctrl), were cultured in TBY supplemented with 50 µg/ml Kanamycin at 37°C, shaking at 225 rpm, to an OD_{600} of 0.8. Protein expression was induced with 1 mM of isopropyl-b-D-thiogalactopyranoside (IPTG, Gold Biotechnology, St. Louis, MO) followed by incubation at 37°C for 3 hrs. The cells were harvested by centrifugation at 4000 x g for 10 min at 4°C and resuspended in 20% glycerol/phosphate buffered salt solution (PBS, Gibco, Grand Island, NY) in 1% of the initial volume. Next, serial dilutions of recombinant *E. coli* were performed and adjustments were made to reach 1×10^9 cells per 400µl of oral vaccine. Cell suspensions in aliquots of 2 ml were frozen quickly in a dry ice bath and stored at -80°C until use. Protein expression was examined by Western blot using protein-specific mouse antiserum.

E. coli Cell Fractionation

Recombinant *E. coli* clones in pET9c vector expressing either the following proteins: OspC K, OspB, BBK32, Salp15 and Salp25 were cultivated in TBY medium supplemented with 50 µg/ml Kanamycin at 37°C, 225 rpm. At an $OD_{600} = 0.8$, cells were induced with 1mM of isopropyl-b-D-thiogalactopyranoside (IPTG, Gold Biotechnology, St. Louis, MO) for 3 hrs and grown to an OD_{600} of ~1. The cells were harvested by centrifugation at 20,000 x g for 10 min at 4°C and were washed 3 times with ice-cold phosphate buffered salt solution (PBS, Gibco, Grand Island, NY). The pellet was resuspended in ice-cold PBS supplemented with protease inhibitor cocktail (complete EDTA-free, Roche Diagnostics GmBH, Germany) to an $OD_{600} \sim 1$. *E. coli* cells were disrupted with a French press (Thermo Electron Corporation, Milford, MA) and centrifuged at 20,000 x g for 10 min at 4°C to isolate the cytosol fraction (supernatant) from the cell envelope (pellet). The pellet was resuspended in 1ml of ice-cold PBS 2% Triton-X114 (Sigma Aldrich, St. Louis, MO) (v/v) and was incubated at 0°C for 1 h with frequent gentle agitation. Phase separation was performed by warming the suspension for 30 min in a 37°C water-bath, followed by centrifugation at 13 000 x g for 15 min at 25 °C. The aqueous and detergent phases were collected, separated from each other and were washed 3 times as previously described [119]. Briefly, the aqueous phase was washed by adding fresh 10% Triton X-114 to a final concentration of 2%. The aqueous phase was rewarmed for 30 min in a 37°C water-bath and centrifuged at 13 000 x g for

15 min at 25 °C. The detergent phase was washed by diluting it to 1 ml in ice-cold PBS followed by rewarming for 30 min in a 37°C water-bath and a centrifugation at 13 000 x g for 15 min at 25 °C. Total extract (TE), Supernatant (SN), Cell envelope (CE), Cell Envelope/ Detergent phase (CE/DET) and Cell Envelope / Aqueous phase (CE/AQ) were analyzed on a 10% denaturing polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (PVDF, Millipore, Billerica, MA) for analysis by Western blotting using antigen-specific- polyclonal mouse antibody (anti-OspC K,-OspB, -BBK32,-Salp15 and-Salp25). Protein was quantified by densitometry using Alpha Imager (Alpha Innotech, San Leandro, CA). Cell fractionation studies were performed in triplicates in independent experiments.

Animals

Six-eight week old female C3H-HeN mice specific-pathogen-free (Charles River, Boston) were used. Mice were housed in the Nash Research Building, room B072, at the University of Tennessee Health Science Center (UTHSC), Memphis and acclimated to housing for at least seven days prior to studies. Throughout the experiments, mice were provided food and water ad libitum. At the end point, animals were euthanized by carbon dioxide asphyxiation followed by physical euthanasia. At the time of termination, bladder and heart samples were collected, immediately weighed and individually processed for DNA extraction and culture under sterile ABSL-2 conditions. All the protocols were approved by the Institutional Animal Care and Use Committee (IACUC), UTHSC.

Antibody Production

Groups of female BALB/c mice (n=3/protein) were immunized by subcutaneous (SC) injection with 30 µg of one of the following 6xHis-tagged recombinant purified proteins: OspC K, OspB, BBK32, Salp15 or Salp25, adsorbed to aluminum hydroxide (Sigma Aldrich, St Louis, MO). Two and four weeks after immunization, mice were boosted with the same immunogenic preparation. Immune sera was collected, stored and was used to perform Western Blot assays for protein expression, lipidation and cell localization studies.

Oral Immunization

Aliquots of recombinant live *E. coli* expressing either the following proteins, OspC K, OspB, BBK32, Salp15 and Salp25 were thawed at 4°C and 400µl, a total of 1 x 10^9 cells, were placed in a ball-tipped syringe for oral gavage inoculation. Groups of female C3H/HeN mice were immunized by intragastric inoculation of induced recombinant *E. coli* or with an equivalent number of *E. coli* containing the pET9c empty vector as controls. Groups of mice received *E. coli* expressing OspC K (n=12), OspB (n=6), BBK32 (n=6), Salp15 (n=4) or Salp25 (n=4) and equal numbers of mice (n=12; n=6; n=6; n=4 and n=4) were used as controls groups for each antigen, respectively. Mice

were immunized using a protocol similar to that we previously described for OspA immunization [105]. Mice received the first immunization, twice daily, for ten days (days 1-5 and 8-12), rested for two weeks, received the first boost for five days (days 29-33), rested for another week and then received a second boost for five days (days 43-47), and were allowed to rest. On day 73, mice were terminated or challenged with infected ticks. Oral immunization studies were performed in triplicates in independent experiments.

Blood and Stool Collection, Vaginal (VAL) and Bronchoalveolar Lavage (BAL)

Blood collection: low amounts of mouse blood (up to 50μl) were obtained via tail nick on the following days: 0 (pre-immune), 28, 42, 73, 105, 135 and 170 after immunization. Stool collection, vaginal lavage (VAL) and bronchoalveolar lavage (BAL) were performed at day 73 after immunization started. Stool collection: a sample of the stool (~60mg) was collected and diluted in ELISA buffer. Vaginal Lavage (VAL): 1ml of ELISA buffer was flushed into the mouse vagina over a petri dish and the fluid was collected. Bronchoalveolar lavage (BAL): mice were euthanized and 1 ml of ELISA buffer was injected into the lungs with immediate aspiration.

Production of Infected Ticks (Flat Nymphs)

We produced laboratory *I. scapularis* ticks (flat nymphs) infected with *B. burgdorferi* with the ultimate goal of infecting OspC type K- immunized mice by the natural route of *B. burgdorferi* transmission, i.e., via tick bite, to assess for vaccine efficacy. Serial dilutions of culture of *B. burgdorferi* strain BL204 (OspC type K) and a culture containing multiple strains of *B. burgdorferi* (MS) grown to mid-log-phase were analyzed by dark field microscopy. Cells were counted by averaging eight microscope fields and the number of spirochetes was further confirmed by q-PCR. 50μl of BSK-H media containing 1 x 10^4 *B. burgdorferi* multiple strains or 1 x 10^4 *B. burgdorferi* strain BL204 was injected via intradermal route, in the back of the neck of C3H/HeN mice (n=4/group), to produce flat nymphs infected with *B. burgdorferi* multiple strains or infected with *B. burgdorferi* OspC type K, respectively. 4 weeks later (day 28), infection in mice was assessed by seroconversion to *B. burgdorferi* antigens. Mice were bled and their serum was tested for the presence of IgG anti *B. burgdorferi* antigens via immunoblotting assay (*Borrelia* B31 ViraStripe IgG, Viramed Biotech AG, Planegg, Germany). Batches of clean flat larvae (purchased from Oklahoma State University) were placed in the back of the head of anesthetized mice that seroconverted. After 1h, mice were checked to ensure larval ticks were attached, caged individually and monitored three times a day for six-seven days. Larvae were allowed to feed to repletion and engorge on mice. Engorged larvae were collected as they naturally fell off, stored at room temperature in 95% humidity and allowed to moult and harden into nymphal ticks (flat nymphs). Upon successful moulting, which took approximately three months; ten percent of the pools of flat nymphs generated in our lab were tested by q-PCR targeting the *flaB* gene to determine the prevalence of infection. All larvae were derived from a single

cohort for the experiments described here. Tick production studies were performed in duplicates in independent experiments.

Tick Challenge of Immunized Mice

All nymphal challenges described here were performed using flat nymphs infected with *B. burgdorferi* multiple strains, generated in our laboratory. Nymphal challenge was performed at day 73, by placing six flat nymphs infected with multiple strains of *B. burgdorferi* on the back of the head of individually caged OspC type K-immunized mice and respective controls, allowing them feed to repletion. Five to seven days later, nymphs that engorged after a blood meal (engorged nymphs) were collected after naturally falling off, counted, and a daily record was kept for each mouse and for each tick. Only mice where at least two fully fed nymphs were collected were included in the final readout. Hundred percent of the pools of engorged nymphs recovered after nymphal challenge, comprising nymphs that fed on OspC K-immunized mice or on control mice, were processed for DNA extraction and tested by q-PCR and PCR to assess for vaccine efficacy. Tick challenge studies were performed in duplicates in independent experiments.

Collection of Bladder, Heart and Ear Samples

Ear biopsy samples were collected from mice eight weeks (day 135) after nymphal challenge as previously described [120]. Bladder and heart samples were collected from euthanized mice four weeks (day 105) and thirteen weeks (day 170) after nymphal challenge, and harvested under a laminar flow biosafety containment hood. Collected tissues were immediately sampled in half, weighed and individually cultured into BSK-H medium for six weeks. The other half of weighed tissues was immediately stored at -20°C for further PCR and q-PCR analysis.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Measurement of IgG and IgA antibodies to OspC K, OspB, BBK32, Salp15 and Salp25 proteins

Serum, BAL, VAL and stools from mice that were orally immunized with recombinant *E. coli* clones (OspC K, OspB, BBK32, Salp15 and Salp25) or orally inoculated with *E. coli* carrying the empty vector (ctrl) were tested by indirect ELISA for the presence of IgG or IgA to OspC K, OspB, BBK32, Salp15 and Salp25. Purified recombinant 6xHis-tagged proteins (OspC K, OspB, BBK32, Salp15 and Salp25) were coated overnight at 4°C (2µg/ml) in 100mM sodium carbonate (pH 9.7) on Nunc MaxiSorp flat-bottom ELISA plates (eBioscience, San Diego, CA). Following incubation time, plates were washed 3 times with PBS (Gibco, Grand Island, NY) containing 0.05%

Tween 20 and subsequently blocked with PBS containing 1% of bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) for 1h at room temperature. Serum, BAL, VAL or stools (1:100) were incubated for 2 hrs at room temperature. Plates were washed 3 times with PBS containing 0.05% Tween 20. Goat anti-mouse IgG (1:50,00), anti-mouse IgG1 (1:50,000), anti-mouse IgG2a (1:50,000) or goat anti-mouse IgA (1:50,000) horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was incubated for 30 min at room temperature. Plates were washed 3 times and reactions were developed with SureBlue™ TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) and stopped with TMB Stop Solution (KPL, Gaithersburg, MD). Optical densities (OD) were measured at 450 nm by a Spectra MAX plus ELISA reader (Molecular Devices, Sunnyvale, CA).

Measurement of total IgG antibodies to recombinant *B. burgdorferi* proteins

Serum from mice that were orally immunized with recombinant *E. coli* expressing OspC K or inoculated with *E. coli* carrying the empty vector (ctrl) was tested by ELISA for the presence of total IgG anti-*B. burgdorferi* proteins 4 weeks (day 105) after nymphal tick challenge to assess for vaccine efficacy. A cocktail of purified recombinant proteins from *B. burgdorferi* including p41, p100, BmpA, DbpA, DbpB, FlaB (ProSpec, East Brunswick, NJ), VlsE (My BioSource, San Diego, CA) and BBK07 (kindly provided by Utpal Pal, University of Maryland) were coated overnight at 4°C (2µg/ml) in 100mM sodium carbonate (pH 9.7) on Nunc MaxiSorp flat-bottom ELISA plates (eBioscience, San Diego, CA). Indirect ELISA was performed under the same conditions described above; goat anti-mouse IgG (1:50,00) horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary antibody. Optical densities (OD) were measured at 450 nm by a Spectra MAX plus ELISA reader (Molecular Devices, Sunnyvale, CA).

Western Blot

Detection of recombinant OspC K, OspB, BBK32, Salp15 and Salp25 proteins

Total extracts (TE), supernatant (SN), pellet (cell envelope), and the aqueous and detergent fractions of the cell envelope of *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 were separated on a 10-12% denaturing polyacrylamide gel and electro transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) by semi-dry transfer. After blocking overnight at 4˚C with 5% BSA (Sigma-Aldrich, St. Louis, MO) in tris-buffered saline (Bio-Rad, West Berkeley, CA), 0.05% Tween-20 (TBST), samples were washed 3 times with 1% BSA in TBST and incubated for 1h at room temperature with specific mouse polyclonal antiserum (anti-OspC K, -OspB, -BBK32, -Salp15 or -Salp25) using a 1:100 dilution. Samples were washed 3 times and incubated for 30 min at room temperature with goat anti-mouse IgG (H+L) conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove,

PA) using a 1:1,000 dilution. Samples were washed 3 times and reaction was developed with BCIP/NBT Phosphatase Substrate (KPL, Gaithersburg, MD). Purified 6xHis-tagged recombinant proteins (rOspC K, rOspB, rBBK32, rSalp15 and rSalp25) were used as positive controls for protein identification purposes, during the characterization of protein expression of our oral vaccine constructs.

Detection of total IgG antibodies to recombinant *B. burgdorferi* proteins

The immunoblot test Borrelia B31 ViraStripe IgG (Viramed Biotech AG, Planegg, Germany) was performed according to the manufacturer's instructions and it was used to screen for anti-*B. burgdorferi* IgG antibodies in serum from OspC K-vaccinated mice 4 weeks (day 105) after nymphal tick challenge. A pattern of at least 5 out of 10 positive bands (18, 23, 28, 30, 39, 41, 45, 58, 66 and 93kDa) in control mice or a pattern of at least 5 out of 10 positive bands (18, 28, 30, 39, 41, 45, 58, 66 and 93kDa) in OspC K-vaccinated mice was considered evidence of infection, as per CDC recommendations [36].

Culture of *B. burgdorferi* from Tissues and Dark Field Microscopy

Heart, bladder and ear biopsy tissues freshly collected from mice before (day 73) or after tick challenge (days 105, 135 and 170) were individually cultured in BSK-H medium (New York Medical College, NY) with an antibiotic mixture for *Borrelia* (Sigma-Aldrich, Saint Louis, MO) for up to 6 weeks at 34°C. Cultures were checked every week by dark field microscopy (AxioImager, Zeiss, Germany) for the presence of *Borrelia burgdorferi*. Cultures were deemed positive if *Borrelia* cells were observed in any field and negative if no *Borrelia* cells were observed in 10 fields.

DNA Extraction and Preparation

DNA from *B. burgdorferi* cultures, flat and engorged nymphs, heart, bladder and ear collected from mice before (day 73) or after tick challenge (days 105, 135 and 170) was extracted with the DNeasy blood and tissue kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The extracted DNA was stored at -20°C until use. To monitor for contamination, negative controls were included in the DNA-extraction. As an additional measure to prevent contamination, DNA extractions were carried out in a separate laboratory from the PCR reaction preparations.

Real Time Quantitative Polymerase Chain Reaction (q-PCR)

DNA extracted from flat nymphs, engorged nymphs, heart, bladder and ear was quantified for the copy numbers of *flaB* by qPCR to determine the number of *B. burgdorferi* in each sample. qPCR was performed in an StepOnePlus™ Real-Time PCR

System (Applied Biosystems, Foster City, CA) using two *flaB*-specific primers designed in our lab designated as *flaB*-587F (TCTTTTCTCTGGTGAGGGAGCT) and *flaB*-635R (AGAGGGTGTTCAACAGGAAGG). Genomic copy numbers were quantitated with a 6-carboxy-fluorescein/ 6-carboxy-tetramethyl-rhodamine-labeled probe *flaB*-611 (6-FAM-AAACTGCTCAGGCTGCACCGG–TAMRA). Reactions contained a final concentration of 0.3µM of each primer (Eurofins Scientific, Huntsville, AL), 0.2µM of the specific probe (Eurofins Scientific, Huntsville, AL), 10ul of Maxima Probe qPCR Master Mix (Thermo Fisher Scientific, Grand Island, NY), 2µl of DNA solution (≤ 500 ng DNA) and nuclease-free water (Sigma-Aldrich, Saint Louis, MO) up to a total volume of 20µl. Reactions containing no DNA were also included as controls. The amplification protocol consisted of 10 min at 95ºC, followed by 40 cycles of amplification (95ºC for 15 s and 60ºC for 1 min). The *flaB* copy number was determined essentially as described previously [121]. Briefly, standard curves relating the *flaB* copy number and spirochete number were generated using a dilution series (containing $1–10^6$) of a titered culture of mid-log phase ($5×10^{7}$/ml) *B. burgdorferi* B31MI, as assayed by dark-field microscopy. The *flaB* copy number was confirmed by a PCR-based limited-dilution assay. DNA samples containing $1 –10^6$ copies of *flaB* were included in each qPCR in order to generate a standard curve. Under these conditions, an assay sensitivity of 1 spirochete was achieved. A positive result was considered when one or more copies of *flaB* were detected. Amplification was performed using MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA). Two sets of 1 well were assigned as blanks and two sets of 6 wells were assigned as DNA standards for *flaB*. Blanks, standards and samples were amplified in duplicate wells. To avoid cross-contamination and sample carry-over, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms and all fluid transfers were carried out with plugged pipette tips to eliminate aerosols. Each assay contained six negative controls containing no DNA and one sham extraction to assure no DNA contamination in the extraction reagents. These controls were randomly spaced among the experimental samples. The mean DNA copy numbers of *flaB* of each DNA sample were calculated from duplicate wells. Number of *B. burgdorferi* was expressed as the average number of *flaB* DNA copies per mg or mm of tissue or per tick.

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

B. burgdorferi ospC, *ospC type A* and *ospC type K* were amplified from DNA extracted from *B. burgdorferi* cultures, flat, engorged nymphs, heart and bladder using primers designated as OspC, OspC type A and OspC type K, respectively, that were designed in our laboratory **(Table 2-2)**. Reactions contained a final concentration of 0.6µM (*Borrelia* cultures and tick samples) or 0.4µM (tissue samples) of each primer (Eurofins Scientific, Huntsville, AL), 10ul of AmpliTaq Gold® Fast PCR Master Mix (Applied Biosystems, Foster City, CA), 5 µl (*Borrelia* culture and tick samples) or 3µl (tissue samples) of DNA solution (≤ 0.2 µg DNA) and nuclease-free water up to a total volume of 20µl. Reactions containing no DNA were also included as controls. For DNA samples extracted from cultures of *B. burgdorferi* multiple strains and from flat and engorged nymphs, DNA fragments of the loci of interest were amplified by a nested PCR

Table 2-2. List of primers used for PCR.

Name	Forward	Reverse	Product Length (bp)	Anneal. Temp. (°C)
OspC	GGAGGCACAAATTAATG	GACTTTATTTTTCCAGTTAC	705	47
OspC type A	CCGAAAATAATCACAATGGATC	CCAAGTTCTTCAGCACC	256	51
OspC type K	GAAGCGGGGCATAATGGAA	CGCATGTTCTCCTTCTAGT	165	51

procedure, comprising *ospC* amplification as the first reaction and *ospC type A* or *ospC type K* amplification as the second reaction. Here, the first round PCR product was used as template for the second round PCR. For DNA samples extracted from tissue samples (heart and bladder), DNA fragments of the loci of interest (*ospC type A* or *ospC type K*) were amplified by a single PCR reaction. *B. burgdorferi* strain B31MI (OspC type A strain), *B. burgdorferi* strain BL204 (OspC type K strain) and *E.coli* expressing OspC K (our OspC K vaccine construct) were used as positive controls for *ospC type A* and *ospC type K,* respectively. The amplification protocol of *ospC* consisted of 10 min at 95ºC, followed by 35 cycles of amplification (96ºC for 3s, 47ºC for 3s and 68 ºC for 15s). The amplification protocol of *ospC type A* and *ospC type K* consisted of 10 min at 95ºC, followed by 35 (for *Borrelia* culture samples) or 45 cycles (for nymphs and tissue samples) of amplification (96ºC for 3s, 51ºC for 3s and 68 ºC for 5s), and was performed in separate PCR reactions. In addition to standard laboratory measures to prevent contamination, negative controls at various stages of amplification were performed for each PCR reaction. After amplification, 10 µl of product were loaded on a 1% agarose gel to be separated by electrophoresis. The separated bands were visualized using ethidium bromide staining and were detected using an Alpha Image 2200 Multi-image light cabinet and alphaEase FC software (Alpha Innotech Corporation, San Leandro, CA). PCR products were further confirmed by sequencing (Genewiz, South Plainfield, NJ). In an effort to detect other types of *ospC* besides *ospC type K*, *ospC type A* was included in our studies.

Statistical Analysis

Values were expressed as means ±SD. For comparison of means between two populations of unpaired data, unpaired student's t-test was used. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) was used to perform all tests. Differences were considered significant at $p < 0.05$.

CHAPTER 3. RESULTS

Construction of Recombinant *E. coli* Expressing OspC K, OspB, BBK32, Salp15 and Salp25, and Localization and Lipidation of Target Proteins within the Cell

We cloned the outer surface protein C type K (OspC K), the outer surface protein B (OspB) and the fibronectin binding protein (BBK32) genes from *B. burgdorferi sensu stricto* as well as Salp15 and Salp25 from *I. scapularis*, downstream of the signal peptide of *Borrelia burgdorferi* outer surface protein A (OspA) into an *E. coli* expression plasmid. By doing so, we generated the oral vaccine constructs, described in **Table 2-1**, expressing recombinant immunogens anchored to the cell envelope of the bacteria via a lipidated moiety [115]. Total extracts (TE) of our five oral vaccine constructs (*E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25) along with the respective purified proteins (rOspC K, rOspB, rBBK32, rSalp15 and rSalp25) used as positive controls, were analyzed by denaturing polyacrylamide gels and protein expression was confirmed by Western blot. All recombinant proteins migrated at the expected molecular weight: OspC K at 24 kDa **(Figure 3-1A, top panel)**, OspB at 34 kDa **(Figure 3-1B, top panel)**, BBK32 at 42 kDa **(Figure 3-1C, top panel)**, Salp15 at 18 kDa **(Figure 3-1D, top panel)** and Salp25 at 27 kDa **(Figure 3-1D, top panel)** as identified by Western blot using monospecific polyclonal antibodies. We next evaluated the localization of the recombinant proteins within the cell envelope of *E. coli* by cell fractionation followed by Western Blot **(Figure 3-1, top panels)** and quantification by densitometry **(Figure 3-1, bottom panels)**. Analysis of cytosol (SN) and cell envelope (CE) fractions revealed that OspC K **(Figure 3-1A)**, OspB **(Figure 3-1B)**, BBK32 **(Figure 3-1C)**, Salp15 **(Figure 3-1D)** and Salp25 **(Figure 3-1E)** are primarily (>60%) in the cell envelope (CE) fraction. When the CE fraction was further fractionated by Triton X-114 solubilization and phase partitioning, OspC K **(Figure 3-1A)**, BBK32 **(Figure 3-1C)** and Salp15 **(Figure 3-1D)** were found primarily (76.0%, 61.2% and 82.9%, respectively) in the Triton X-114 hydrophobic, detergent phase (CE/DET) whereas OspB **(Figure 3-1B)** and Salp25 **(Figure 3-1E)** were found primarily (74.3% and 71.7%, respectively) in the aqueous phase of the cell envelope (CE/AQ).

Serological Total IgG Antibody Response to Orally Delivered *E. coli* Expressing OspC K, OspB, BBK32, Salp15 and Salp25 Antigens

We immunized C3H-HeN mice by oral gavage with live *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25. Control mice received live *E. coli* carrying the empty vector (Ctrl). Serum from immunized mice was analyzed by ELISA for the presence of antigen-specific total IgG antibodies (tIgG) on day 73, after immunization started **(Figure 3-2)**. Oral immunization with OspC K **(Figure 3-2A)** induced significant levels of antigen-specific total IgG with a mean antibody level of OD_{450}=1.108 (± 0.303 SD) when compared to mean antibody levels of OD_{450}=0.221 (± 0.127 SD) in controls, $p<0.0001$. In contrast, oral immunization with OspB **(Figure 3-2B)**, BBK32 **(Figure 3-2C)**, Salp15 **(Figure 3-2D)** and Salp25 **(Figure 3-2E)** did not induce

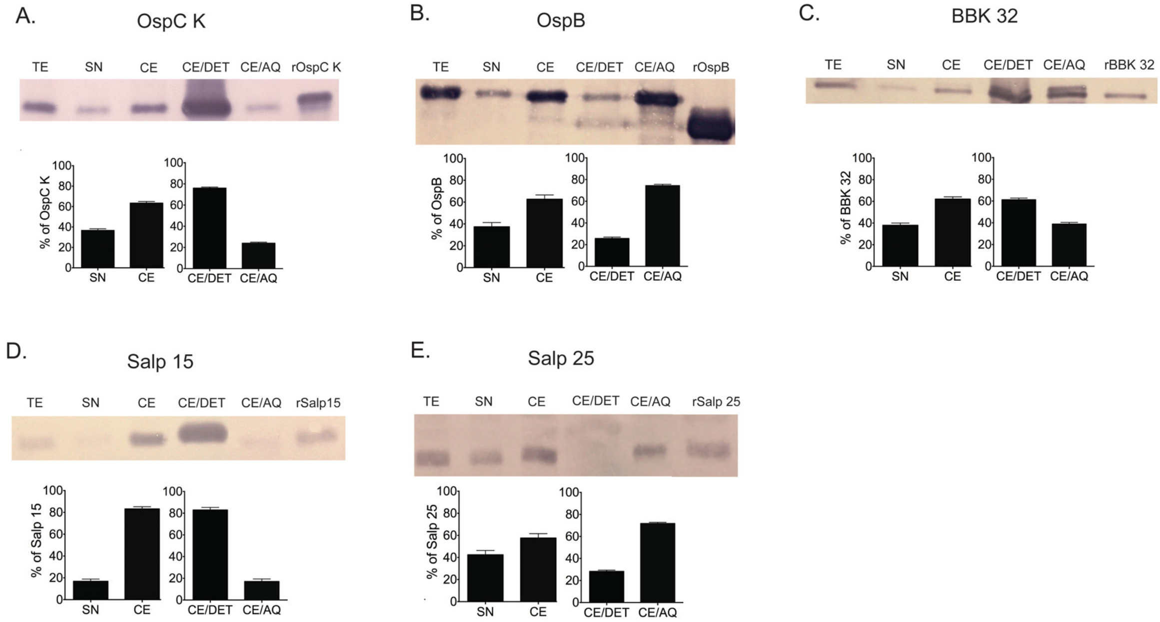

Figure 3-1. Lipidation and localization of recombinant OspC K, OspB, BBK32, Salp15 and Salp25 in *E. coli.*

E. coli expressing OspC K (A), OspB (B), BBK32 (C), Salp15 (D) and Salp25 (E) were disrupted with a French press. Total extracts (TE), supernatant (SN) and the pellet (CE = cell envelope) were collected. The cell envelope was then incubated with Triton X-114 and partitioned into detergent (CE/DET) and aqueous (CE/AQ) phases. Fractions were analyzed on a 10-12% SDS PAGE and tested by Western blot with monospecific polyclonal mouse antibody (top panels). Protein in the supernatant, cell envelope, detergent phase and aqueous phase was quantified by densitometry using Alpha Imager (Alpha Innotech, San Leandro, CA) (bottom panels). Purified recombinant 6xHis-tagged proteins: rOspC K (A), rOspB (B), rBBK32 (C), rSalp15 (D) and rSalp25 (E) were used as positive controls and showed a relative molecular mass of 24, 34, 42, 18 and 27 kDa, respectively.

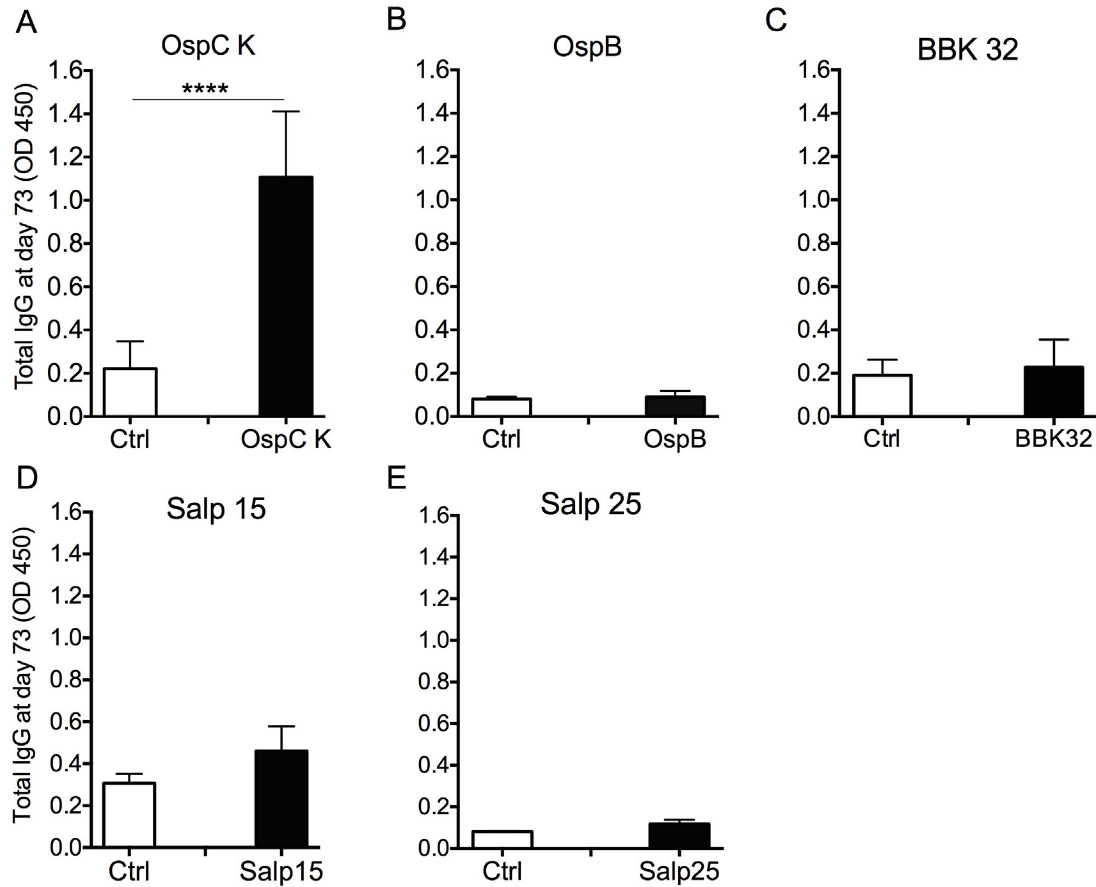

Figure 3-2. Serological total IgG antibody response to orally delivered *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 antigens.

C3H-HeN mice were immunized intragastrically with live recombinant *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 or inoculated intragastrically with live *E. coli* carrying the empty vector (Ctrl). C3H/OspC K n=12 and control n=12; C3H/OspB n=6 and control n=6; C3H/BBK32 n=6 and control n=6; C3H/Salp15 n=4 and control n=4; C3H/Salp25 n=4 and control n=4. Serum samples were collected at day 73 and anti-OspC K (A), -OspB (B), -BBK32 (C), -Salp15 (D) and -Salp25 (E) total IgG was measured by ELISA and expressed as Optical Density at 450 nm (OD_{450}). The average of triplicate readings per mouse/per group was determined and the error bar indicates standard deviation. Results are representative of one of three independent experiments. Asterisks indicate statistically significant differences, **** p<0.0001.

significant levels of antigen-specific total IgG when compared to the respective control groups, p>0.05.

Subtyping of Total IgG Antibody Response to Orally Delivered *E. coli* Expressing OspC K, OspB, BBK32, Salp15 and Salp25 Antigens

We next isotyped total IgGs into IgG1 and IgG2a subclasses in serum obtained from mice at day 73 **(Figure 3-3)**, by ELISA. Mice orally immunized with live *E. coli* expressing OspC K **(Figure 3-3A)** developed significant OspC K-specific IgG1 levels with a mean antibody level of OD_{450}=0.322 (± 0.171 SD) when compared to a mean antibody level of OD_{450}=0.056 (± 0.026 SD) in controls, p<0.0001, as well as significant OspC K-specific IgG2a levels with a mean antibody level of OD_{450}=0.375 (± 0.110 SD) when compared to a mean antibody level of OD_{450}=0.116 (± 0.040 SD) in control group, p<0.0001. Differences between anti-OspC K IgG1 and anti-OspC K IgG2a were not significant, p=0.3820 **(Figure 3-3A)**. Oral immunization with live *E. coli* expressing OspB **(Figure 3-3B)**, BBK32 **(Figure 3-3C)**, Salp15 **(Figure 3-3D)** and Salp25 **(Figure 3-3E)** did not induce significant levels of antigen-specific IgG1 neither IgG2a when compared to respective control groups, p>0.05.

Mucosal IgA Antibody Response to Orally Delivered *E. coli* Expressing OspC K, OspB, BBK32, Salp15 and Salp25 Antigens

We assessed local and distal mucosal immune responses induced by oral administration of live *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 in C3H-HeN mice. We tested levels of antigen-specific IgA in stool (GUT, local), bronchoalveolar lavage (BAL, distal) and vaginal lavage (VAL, distal) at day 73, by ELISA **(Figure 3-4)**. Mice immunized orally with OspC K **(Figure 3-4A)** produced significant levels of OspC K-specific IgA antibodies not only locally at the site of immunization (GUT), mean OD_{450}=0.425 (± 0.175 SD) compared to 0.233 (± 0.094 SD) in the control, p=0.0030; but also at mucosal sites distal to the gut, such as the lung (BAL) with a mean OD_{450}=0.163 (± 0.071 SD) compared to 0.062 (±0.008 SD) in the control, p=0.0004, and in the vagina (VAL) with a mean OD_{450}=0.385 (± 0.289 SD) compared to 0.093 (± 0.044) in the control, p=0.0022. Mice immunized orally with OspB **(Figure 3-4B)** and BBK32 **(Figure 3-4C)** produced some levels of IgA detectable in VAL, but were not significant when compared to the control, p=0.0642 and p=0.1311, respectively. We were not able to detect IgA antibodies in GUT, BAL or VAL of mice immunized orally with *E. coli* expressing Salp15 **(Figure 3-4D)** or Salp25 **(Figure 3-4E)**.

Our findings suggested that only mice immunized with *E. coli* expressing OspC K induced a significant systemic antibody immune response showed by the production of antigen-specific serological IgG **(Figure 3-2)** and mucosal IgA **(Figure 3-4)**. Therefore, studies to determine vaccine efficacy were pursued following oral immunization with *E. coli* expressing OspC K.

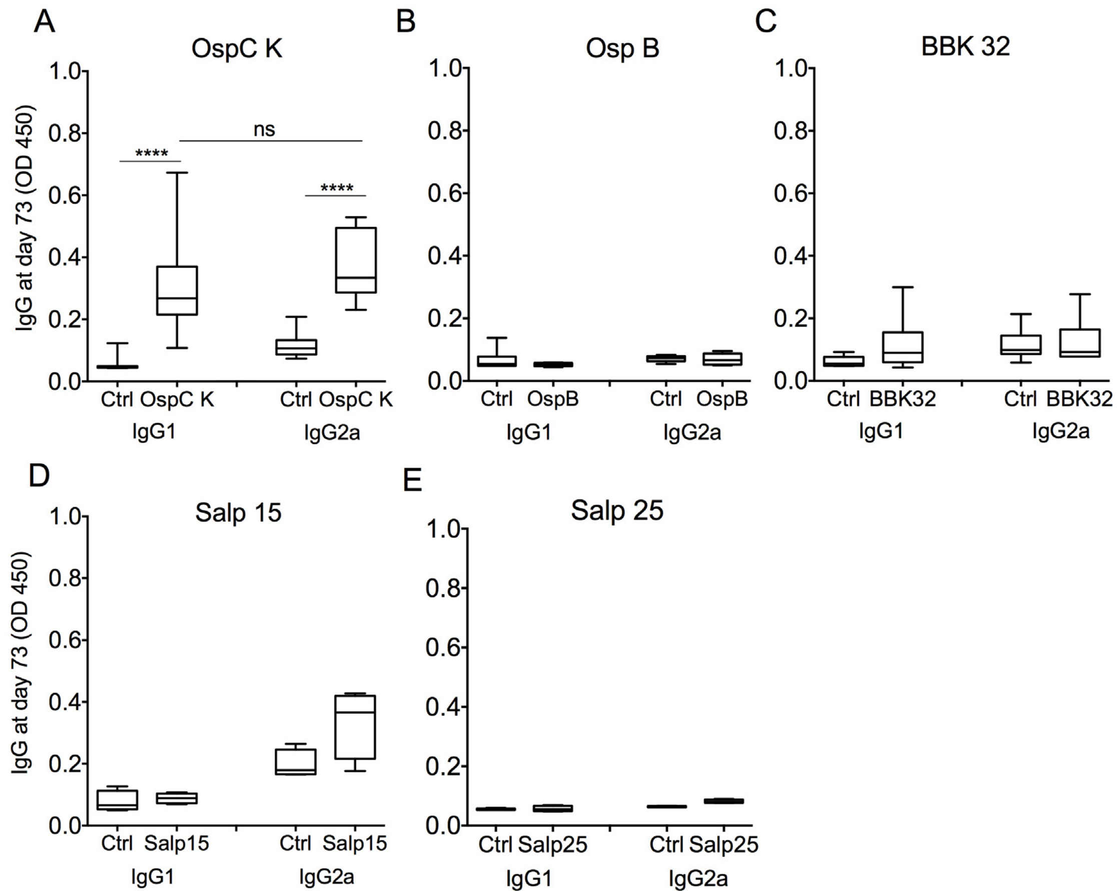

Figure 3-3. Subtyping of total IgG antibody response to orally delivered *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 antigens.

C3H-HeN mice were immunized intragastrically with live recombinant *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 or inoculated intragastrically with *E. coli* carrying the empty vector (Ctrl). C3H/OspC K n=12 and control n=12; C3H/OspB n=6 and control n=6; C3H/BBK32 n=6 and control n=6; C3H/Salp15 n=4 and control n=4; C3H/Salp25n=4 and control n=4. Serum samples were collected at day 73 and anti-OspC K (A), -OspB (B), -BBK32 (C), -Salp15 (D) and -Salp25 (E) IgG1 and IgG2a were measured by ELISA and expressed as Optical Density at 450 nm (OD_{450}). The average of triplicate readings per mouse/per group was determined and the error bar indicates standard deviation. Results are representative of one of three independent experiments. Asterisks indicate statistically significant differences, **** p<0.0001. The letters "ns" indicate nonsignificance (p>0.05).

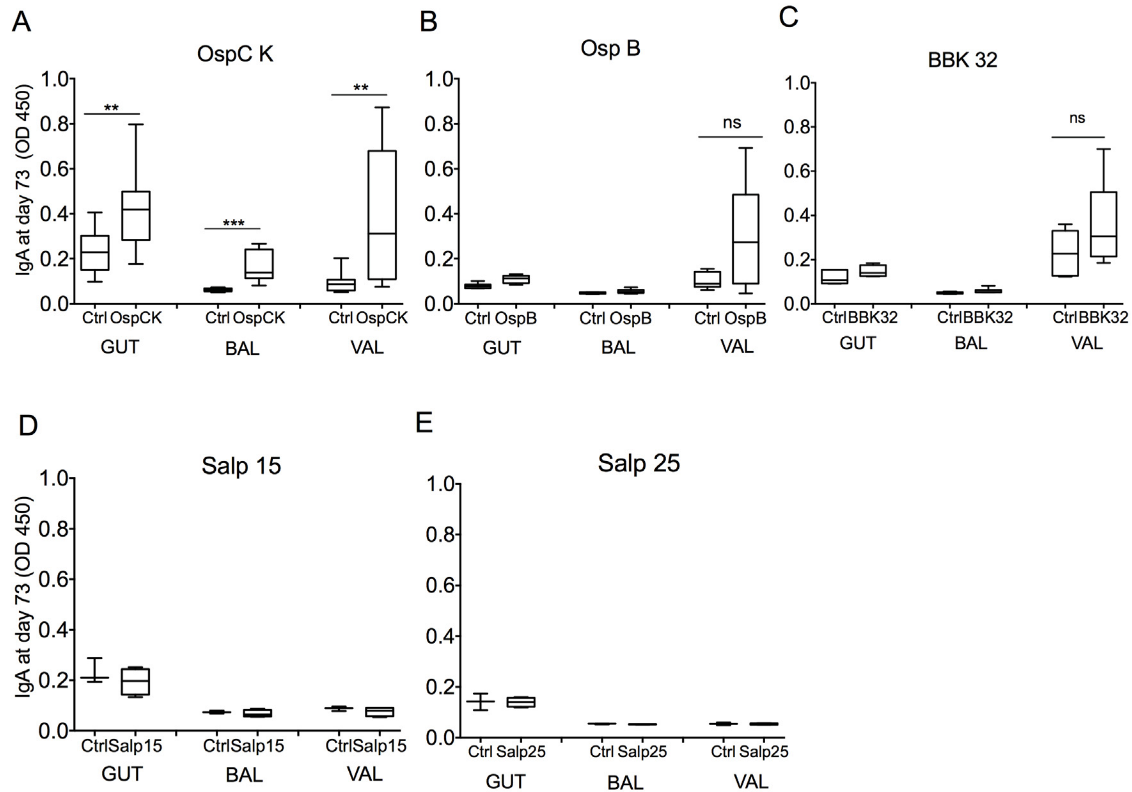

Figure 3-4. Mucosal IgA antibody response to orally delivered *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 antigens.

C3H-HeN mice were immunized intragastrically with live recombinant *E. coli* expressing OspC K, OspB, BBK32, Salp15 and Salp25 or inoculated intragastrically with *E. coli* carrying the empty vector (Ctrl). C3H/OspC K n=12 and control n=12; C3H/OspB n=6 and control n=6; C3H/BBK32 n=6; and control n=6; C3H/Salp15 n=4 and control n=3; C3H/Salp25 n=4 and control n=3. At day 73, stools (GUT), bronchoalveolar lavage (BAL) and vaginal lavage (VAL) were collected and anti-OspC K (A), -OspB (B), -BBK32 (C), -Salp15 (D) and -Salp25 (E) IgA antibodies were measured by ELISA and expressed as Optical Density at 450 nm (OD_{450}). The average of triplicate readings per mouse/per group was determined. Results are representative of one of three independent experiments. Asterisks indicate statistically significant differences. The number of asterisks indicates the significance level: ** $p<0.01$, *** $p<0.001$. The letters "ns" indicate nonsignificance ($p>0.05$).

Production of Flat Nymphs Infected with *B. burgdorferi*

To generate flat nymphs infected with multiple strains (MS) of *B. burgdorferi* or with *B. burgdorferi* OspC type K (OspC K) strain, mice (n=4/group) were inoculated intradermally with 1 x 10^4 of *B. burgdorferi* MS or of *B. burgdorferi* strain BL204, respectively. Four weeks (day 28) after inoculation, serum was collected and analyzed for the presence of total IgG anti-recombinant *B. burgdorferi* proteins by Western Blot. Seroconversion to borrelial antigens was used as a measure of infection. All mice inoculated either with *B. burgdorferi* MS (MS) or *B. burgdorferi* OspC K strain (OspC K) seroconverted as shown by the presence of 6 (six) positive bands at 66, 41, 39, 28, 23 and 18 kDa or at 93, 66, 45, 41, 23 and 18 kDa, respectively **(Figure 3-5A)**. Next, non-infected larvae were placed on seroconverted mice and allowed to feed to repletion. Fed larvae were then collected and led to moult into flat nymphs. After moulting, the infection prevalence of flat nymphs was assessed by q-PCR targeting the *flaB* gene. Flat nymphs infected with multiple strains of *B. burgdorferi* (MS) exhibited an infection prevalence of approximately 83%, with an average number of 1 x 10^5 *B. burgdorferi* per nymph (n=18) **(Figure 3-5B)**. Surprisingly, despite the fact that all mice inoculated intradermally with *B. burgdorferi* strain BL204 (OspC K) became infected **(Figure 3-5A)**, we were not able to generate infected flat nymphs from larvae that fed on any of these mice, as shown by an infection prevalence of 0% from these nymphs **(Figure 3-5B)**. A total of 20 flat nymphs (5 flat nymphs per each mouse infected with *B. burgdorferi* OspC type K strain) were screened and none of them were infected. These results were further confirmed by PCR results using primers to amplify *ospC* from *B. burgdorferi,* followed by gel electrophoresis. Studies to determine OspC K vaccine efficacy were then pursued using the flat nymphs infected with multiple strains of *B. burgdorferi* that we generated.

Characterization of the Efficacy of an *E. coli*-based Oral Vaccine Expressing Lipidated OspC K Anchored to the Cell Envelope in Preventing Infection with Multiple Strains of *B. burgdorferi*

We determined the efficacy of an *E. coli*-based oral vaccine expressing lipidated OspC K anchored to the cell envelope in preventing infection with multiple strains of *B. burgdorferi* upon tick challenge. To perform tick challenge studies, we used our laboratory produced flat nymphs infected with multiple strains of *B. burgdorferi*, which had an infection prevalence of approximately 83% **(Figure 3-5B)**. We placed six flat nymphs on each OspC K-immunized mice (n=12) and respective controls (n=12), using a total of 144 flat nymphs. 72-blinded selected nymphs were placed on immunized group while the other 72 nymphs were placed on control group. After tick challenge, we recovered 57 out of the 72 nymphs placed on the control group and 58 out of the 72 nymphs placed on the immunized group, an average of nymphal recovery rate of 80% and 81%, respectively **(Table 3-1)**.

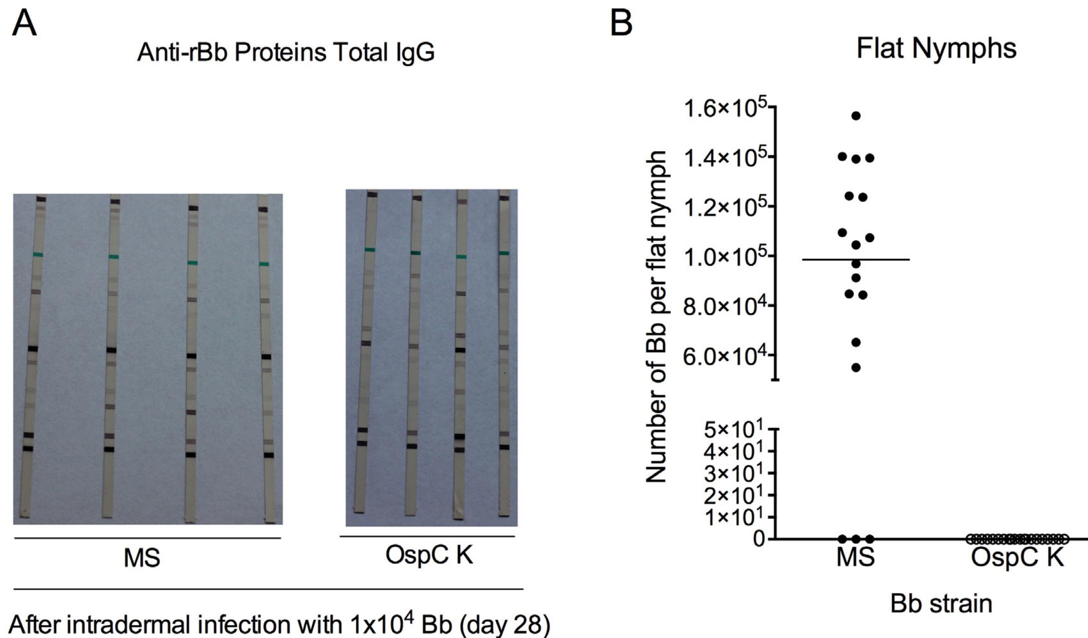

Figure 3-5. Production of flat nymphs infected with *B. burgdorferi*.

C3H-HeN mice (n=4/group) were inoculated intradermally with 1 x 10^4 of *B. burgdorferi* (Bb) multiple strains (MS) or with *B. burgdorferi* BL204, an OspC type K strain (OspC K). At day 28, once seroconversion was confirmed by Western blot (A), non-infected larvae were placed on mice infected either with MS of *B. burgdorferi* or with *B. burgdorferi* OspC K strain aiming to produce flat nymphs infected with MS of *B. burgdorferi* or with *B. burgdorferi* OspC K strain, respectively. Larvae were allowed to feed to repletion, collected and led to moult into flat nymphs. Flat nymphs were subjected to DNA extraction followed by q-PCR analysis to quantify the number of copies of *B. burgdorferi flaB* gene. Results are represented as the average of duplicate samples per tick and each dot represents the number of *B. burgdorferi* per flat nymph. Black dots depict the number of *B. burgdorferi* per flat nymphs generated from larvae that fed on mice infected with multiple strains of *B. burgdorferi* (MS). Open dots depict the number of *B. burgdorferi* per flat nymph generated from larvae that fed on mice infected with *B. burgdorferi* OspC type K (OspC K) (B). Infection was considered when five or more positive bands were present in the Western Blot. Each Western Blot stripe (n=8) represents one mouse (n=8) (A). Results are representative of one of two independent experiments.

Table 3-1. List of nymphal ticks placed and recovered per mouse.

Control[a] Mouse ID	No. Flat Nymphs Placed	No. Engorged Nymphs Recovered	Vacc[b]. Mouse ID	No. Flat Nymphs Placed	No. Engorged Nymphs Recovered
1	6	6	13	6	5
2	6	5	14	6	4
3	6	2	15	6	5
4	6	6	16	6	6
5	6	3	17	6	6
6	6	2	18	6	4
7	6	6	19	6	6
8	6	4	20	6	2
9	6	6	21	6	6
10	6	6	22	6	5
11	6	5	23	6	4
12	6	6	24	6	5
Total no.	72	57	Total no.	72	58
Average (%)	100	80	Average (%)	100	81

[a] Control mice (n=12) were inoculated intragastrically with *E. coli* carrying the empty vector.
[b] Vaccinated mice (n=12) were inoculated intragastrically with *E. coli* expressing OspC K.

Detection of *B. burgdorferi* and *B. burgdorferi* DNA as a measure of vaccine efficacy

After nymphal challenge, spirochetal infection and dissemination to target tissues was determined by culture and q-PCR **(Figure 3-6)**. Bladder, heart and ear samples were collected at specific time points after tick challenge as follows: four weeks (day 105), eight weeks (day 135) and thirteen weeks (day 170), placed in culture and checked for the presence of *B. burgdorferi* by dark field microcopy. In addition, we extracted DNA from these tissues and performed a q-PCR analysis targeting *flaB* to quantify the average number of *B. burgdorferi*. All mice orally immunized with OspC K had positive cultures of *B. burgdorferi* from bladder and heart at four (day 105) and at thirteen weeks (day 170), confirmed by positive q-PCR with an average number of 2.5×10^2 *B. burgdorferi* per mg of bladder **(Figure 3-6A)** and 3.7×10^2 *B. burgdorferi* per mg of heart **(Figure 3-6B)** tissue. In addition, we detected positive cultures of *B. burgdorferi* from ears in ten out of twelve OspC K-immunized mice, 8 weeks (day 135) after challenge. These findings were further confirmed by positive q-PCR, with an average number of 8.3×10^2 *B. burgdorferi* per mm of ear sample **(Figure 3-6C)**. Although we could not detect *flaB* copies in ears from two OspC K-immunized mice neither from two control mice **(Figure 3-6C)**, these four animals were infected with *B. burgdorferi* as shown by analysis of their bladders **(Figure 3-6A)** and hearts **(Figure 3-6B)**. We found no significant differences in the numbers of *B. burgdorferi* per mg of bladder **(Figure 3-6A)**, per mg of heart **(Figure 3-6B)**, or per mm of ear tissue **(Figure 3-6C)** between control and OspC K-immunized mice groups, $p>0.05$.

Seroconversion to *B. burgdorferi* proteins as a measure of vaccine efficacy

We also examined the spirochete specific antibody response. Four weeks after challenge (day 105), serum was collected and analyzed for the presence of total IgG anti-recombinant *B. burgdorferi* proteins by ELISA **(Figure 3-7A)** and Western Blot **(Figure 3-7B)**. Sera and tissues collected from mice before challenge (day 73) were also analyzed and used as negative control. As expected, tissue samples collected from immunized and control mice at day 73 resulted in negative q-PCR results and *B. burgdorferi* was not detected in cultures **(Figure 3-7A)**. As expected, control mice showed significantly high levels of IgG antibodies to *B. burgdorferi* proteins after challenge (day 105), when compared to the levels before challenge (day 73), $p<0.0001$. Similarly, immunized mice showed significantly high levels of IgG antibodies to *B. burgdorferi* proteins at day 105, comparing with levels day 73, $p<0.0001$ **(Figure 3-7A)**, and there were no differences in the IgG levels when compared to controls at day 105 ($p>0.05$). ELISA results were further confirmed by Western Blot **(Figure 3-7B)**. All OspC K-immunized mice developed antibodies to *B. burgdorferi* proteins after tick challenge (day 105) as shown by the presence of eight positive bands (18, 23, 28, 30, 39, 41, 66 and 93kDa) in the Western Blot **(Figure 3-7B)**. There were no significant differences between immunized and control mice, except that all immunized mice presented an additional positive band at 23 kDa, that corresponds to *B. burgdorferi* OspC antigen **(Figure 3-7B)**. The appearance of this band was expected given that all immunized mice received an OspC-based vaccine and all of them developed specific

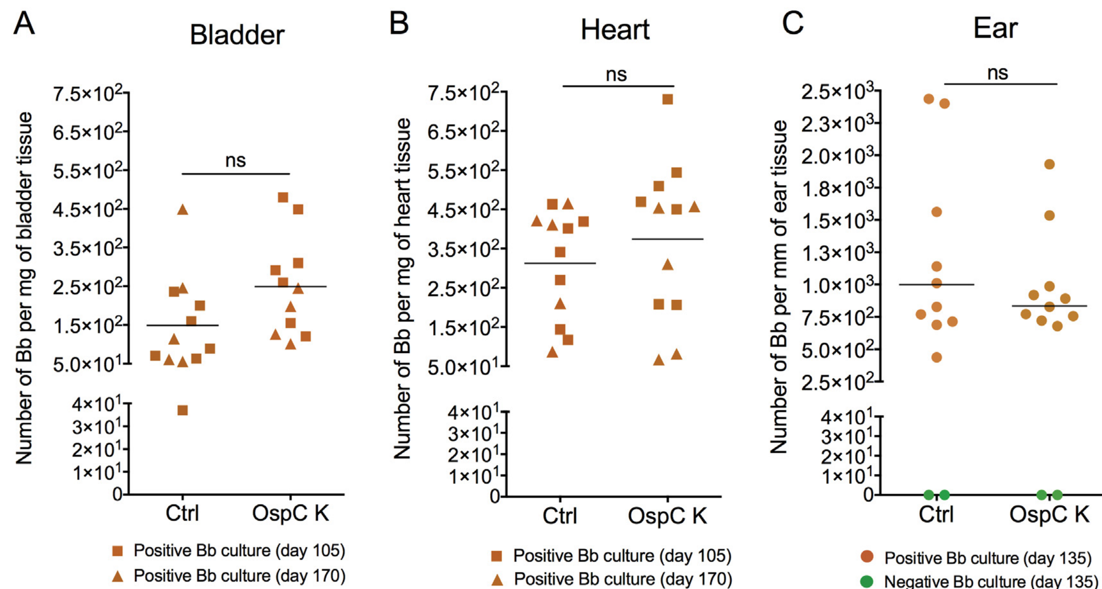

Figure 3-6. Quantification of *B. burgdorferi* in tissues from OspC K-immunized mice after tick challenge.

C3H-HeN mice (n=12/group) were immunized intragastrically with *E. coli* expressing OspC K (OspC K) or inoculated intragastrically with *E. coli* carrying the empty vector (Ctrl) and then challenged with flat nymphs infected with multiple strains of *B. burgdorferi*. To quantify infection and to assess spirochetal dissemination to target tissues, bladder, heart were collected at 4 (day 105) and 13 weeks (day 170), and ear samples were collected at 8 weeks (day 135), after tick challenge. Tissues were cultured in BSK-H medium and analyzed by dark field microscopy, and subjected to DNA extraction followed by q-PCR analysis to quantify the number of copies of *B. burgdorferi flaB*. Results are represented as the average of duplicate samples per mouse and each dot represents the number of *B. burgdorferi* per mg of bladder (A), mg of heart (B) or per mm of ear (C) tissue. Results are representative of one of two independent experiments. The letters "ns" indicate nonsignificance (p>0.05). A negative result for *Borrelia* culture was considered when *B. burgdorferi* was absent in cultures of tissues analyzed by dark field microcopy. A positive culture result was considered when *B. burgdorferi* was present in cultures of tissues analyzed by dark field microcopy.

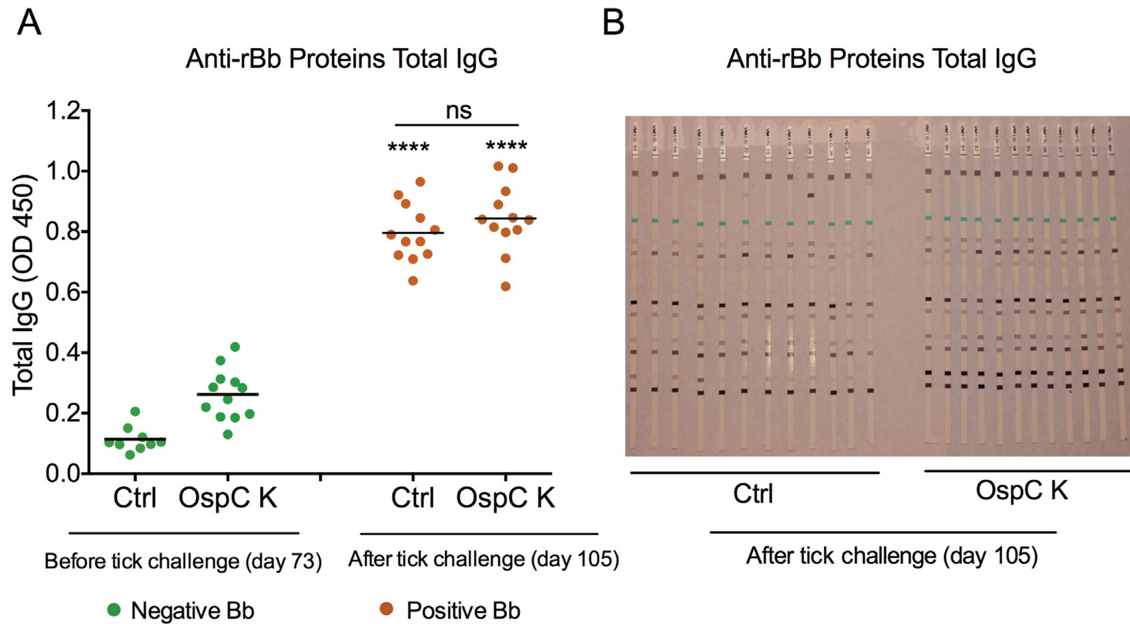

Figure 3-7. Serological total IgG antibody response to *B. burgdorferi* after tick challenge in OspC K-immunized mice.

C3H-HeN mice (n=12/group) were immunized intragastrically with *E. coli* expressing OspC K (OspC K) or inoculated intragastrically with *E. coli* carrying the empty vector (Ctrl) and then challenged with flat nymphs infected with multiple strains of *B. burgdorferi*. To assess infection, serum samples were collected before (day 73) and after challenge (day 105) and analyzed by indirect ELISA (A) and Western blot (B) for the presence of total IgG anti-recombinant *B. burgdorferi* proteins. For ELISA, the results are expressed as Optical Density at 450 nm (OD_{450}) and the average of triplicate samples per mouse was determined and plotted. Asterisks indicate statistically significant differences, **** p<0.0001. The letters "ns" indicate nonsignificance (p>0.05). A negative result was considered when tissue DNA samples were negative for *flaB* by q-PCR analysis and when *B. burgdorferi* was absent from cultures of tissues analyzed by dark field microcopy. A positive *B. burgdorferi* result was considered when tissue DNA samples were positive (one copy or more) for *flaB* by q-PCR analysis and when *B. burgdorferi* was detected in cultures of tissues analyzed by dark field microcopy (A). For Western blot, each stripe represents one mouse (n=24). A pattern of at least 5 out of 10 positive bands (18, 23 (OspC), 28, 30, 39, 41, 45, 58, 66 and 93kDa) in control mice and a pattern of at least 5 out of 10 positive bands (18, 28, 30, 39, 41, 45, 58, 66 and 93kDa) in OspC K-vaccinated mice were considered evidence of infection (B). Results are representative of one of two independent experiments.

anti-OspC (type K) total IgG antibodies, as described **(Figure 3-2A)**.

Next, we analyzed all the recovered engorged nymphs that fed on OspC K-imunized mice (n=58) and on control mice (n=57), which we had previously collected **(Table 3-1)**. All engorged nymphs (n=115) were subjected to DNA extraction followed by q-PCR analysis targeting *flaB* to quantify the average number of *B. burgdorferi* per engorged nymph. Our findings showed that engorged nymphs that fed on immunized mice had an average number of 5.2×10^5 *B. burgdorferi,* which was no different from the average number *B. burgdorferi* per engorged nymph that fed on the control group (5.2×10^5), $p>0.05$ **(Figure 3-8)**.

Taken together, our findings suggested that an OspC K-based vaccine does not protect from infection with *B. burgdorferi* via tick bite. Aiming to investigate whether an OspC K-based vaccine could, at least, afford OspC type-specific protection, we next evaluated if OspC K-immunized animals were protected from infection with *B. burgdorferi* OspC type K transmitted by tick bite carrying multiple strains of *B. burgdorferi* **(Figure 3-9)**. In addition, we investigated the level of antibodies to OspC type K after tick challenge **(Figure 3-10)**.

Evaluation of OspC K Type-Specific Protection

Spirochetal cultures, nymphs and tissues were analyzed for the presence of *B. burgdorferi* OspC, OspC type A and OspC type K strains by targeting *ospC*, *ospC type A* and *ospC type K,* respectively **(Figure 3-9)**. The band at 705bp in lanes 5 to 10 represents the general *OspC* gene analyzed by nested PCR (lanes 6, 7, 8, 9 and 10) or by single PCR (lane 5). A culture of *B. burgdorferi* BL204 strain (Bb OspC K) and our OspC type K oral vaccine construct in *E. coli* (Ec OspC K) were used as positive controls for *ospC type K.* Amplification of *ospC type K* was confirmed by the band at 165 bp in lanes 2 and 3, respectively. To identify additional types of *ospC* other than *type K*, we targeted *ospC type A.* A culture of *B. burgdorferi* B31MI strain (Bb OspC A) was used as positive control for *ospC type A* as indicated by the band at 256bp in lane 1. *B. burgdorferi* OspC type K strain was detected in the culture containing multiple strains of *B. burgdorferi* as well as in the flat nymphs that we produced from this culture, as confirmed by the band at 165bp in lanes 7, 8 and 10, respectively. Similarly, *B. burgdorferi* OspC type K strain was detected in engorged nymphs that fed on OspC K-immunized mice (lane 12) and also in the heart (lane 14) and bladder (lane 16) from these mice, as shown by band at 165bp. Furthermore, *ospC type A* was present in the culture containing multiple strains of *B. burgdorferi* (lanes 6 and 8), in flat nymphs (lane 9), in engorged nymphs (lane 11), and in the heart (lane 13) and bladder (lane 15) from OspC-K immunized mice, as indicated by the band at 256bp.

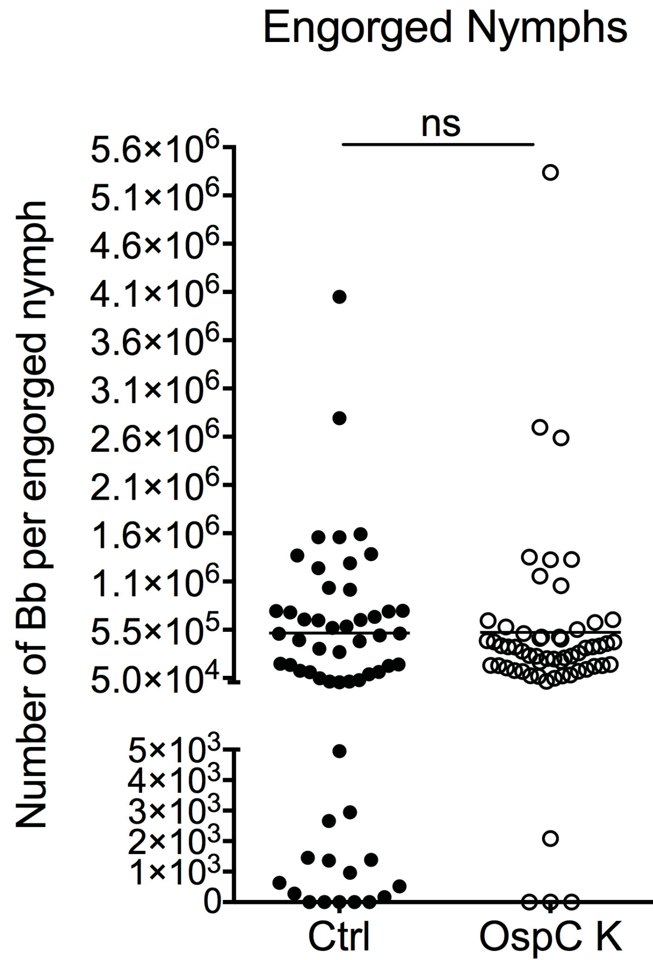

Figure 3-8. Quantification of *B. burgdorferi* in engorged nymphs that fed on OspC K-immunized mice.

C3H-HeN mice (n=12/group) were challenged with flat (unfed) nymphs (n=6/mouse) infected with multiple strains of *B. burgdorferi* (MS). A total of 144 flat nymphs (n=72/group of mice) were used to perform the challenge. Upon challenge, 57 engorged nymphs that fed on control mice (Ctrl) and 58 engorged nymphs that fed on OspC K-immunized mice (OspC K) were recovered. All recovered engorged nymphs (n=115) were then subjected to DNA extraction followed by q-PCR analysis to quantify the number of copies of *B. burgdorferi flaB* gene. Results are represented as the average of duplicate samples per tick and each dot represents the number of *B. burgdorferi* per engorged nymph. Nymphs (n=57) that fed on control mice are represented as "Ctrl" and black dots depict the number of *B. burgdorferi* per engorged nymph that fed on control. Nymphs (n=58) that fed on OspC K-immunized mice are represented as "OspC K" and open dots depict the number of *B. burgdorferi* per engorged nymph that fed on immunized mice. The letters "ns" indicate nonsignificance (p>0.05).

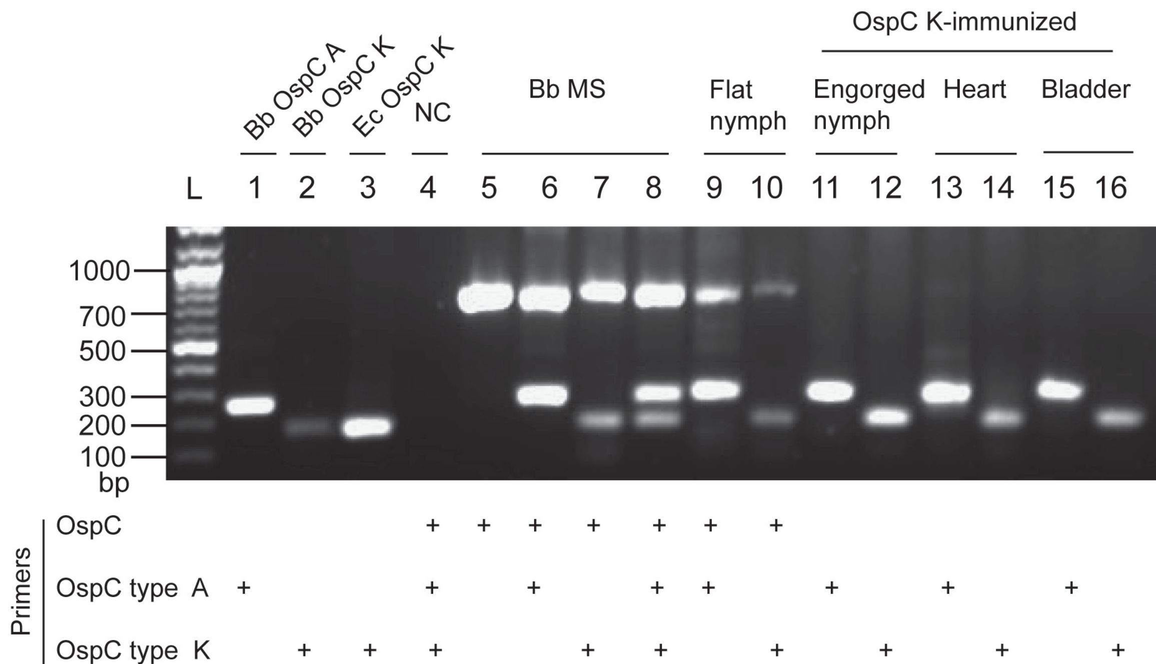

Figure 3-9. Presence of *B. burgdorferi* OspC type K in engorged nymphs that fed on OspC K-immunized mice and in mouse tissues.

1% agarose gel electrophoresis showing representative PCR products after *ospC* (705bp), *ospC type A* (256bp) and *ospC type K* (165bp) amplification. The first lane (L) shows 100bp DNA marker. Lanes 1, 2 and 3 are positive controls. Lane 1: shows *ospC type A* in a culture of *B. burgdorferi* B31MI strain, an OspC type A strain culture (Bb OspC A); lane 2: shows *ospC type K* in *B. burgdorferi* BL204 strain culture, an OspC type K strain culture (Bb OspC K); lane 3: shows *ospC type K* in the oral vaccine construct, *E. coli* expressing OspC type K (Ec OspC K); lane 4: negative control (NC); lane 5: shows *ospC* in *B. burgdorferi* multiple strains culture (Bb MS); lane 6: shows *ospC* and *ospC type A* in *B. burgdorferi* multiple strains culture (Bb MS); lane 7: shows *ospC* and *ospC type K* in culture of *B. burgdorferi* multiple strains (Bb MS); lane 8: shows *ospC type A* and *ospC type K* in *B. burgdorferi* multiple strains culture (Bb MS); lane 9: shows *ospC* and *ospC type A* in flat nymphs produced from *B. burgdorferi* multiple strains culture; lane 10: shows *ospC* and *ospC type K* in flat nymphs produced from *B. burgdorferi* multiple strains culture; lane 11: shows *ospC type A* in engorged nymph that fed on OspC K-immunized mice; lane 12: shows *ospC type K* in engorged nymph that fed on OspC K-immunized mice; lane 13: shows *ospC type A* in heart tissue from OspC K-immunized mice; lane 14: shows *ospC type K* in heart tissue from OspC K-immunized mice; lane 15: shows *ospC type A* in bladder tissue from OspC K-immunized mice; lane 16: shows *ospC type K* in bladder tissue from OspC K-immunized mice.

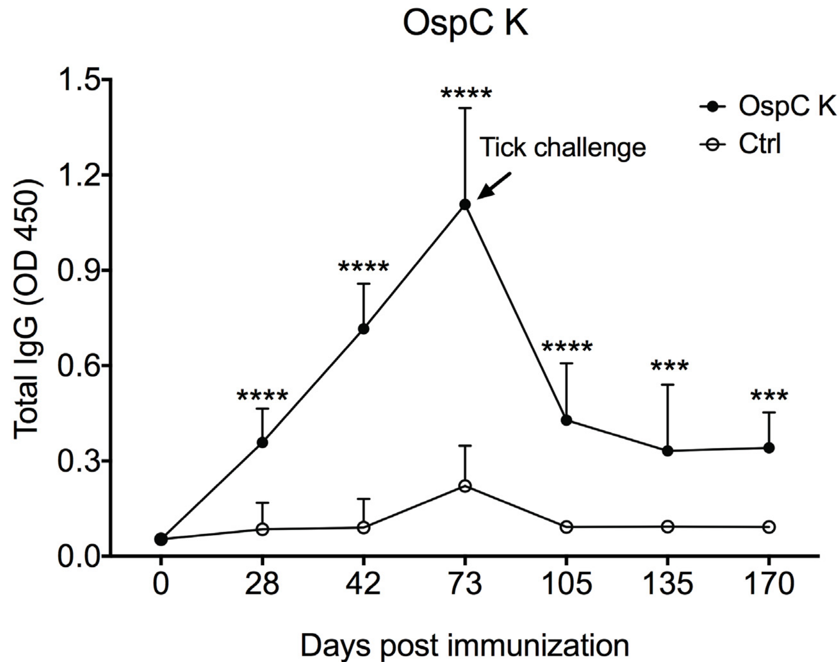

Figure 3-10. Serological total IgG response to OspC K from mice immunized orally with recombinant *E. coli* expressing OspC K before, during and after tick challenge.

C3H-HeN mice (n=12) were immunized intragastrically with *E. coli* expressing OspC K and then challenged with flat nymphs infected with multiple strains of *B. burgdorferi*, at day 73. Serum samples were collected before immunization (day 0) and after immunization, including time points before (days 0, 28, 42 and 73) and after tick challenge (days 105, 135 and 170). Serum samples were analyzed by ELISA for the presence of specific serological anti-OspC K total IgG antibodies (total IgG). The results are expressed as Optical Density at 450 nm (OD_{450}). The average of triplicate samples per mouse was determined and the error bar indicates standard deviation. Asterisks indicate statistically significant differences in total IgG levels in OspC K-immunized mice compared to the control group at the same time points. The number of asterisks indicates the significance level: *** $p<0.001$, **** $p<0.0001$. Results are representative of one of three independent experiments.

Serological Total IgG Antibody Response to OspC K in Mice Orally Immunized with *E. coli* Expressing OspC K before, during and after Tick Challenge

We compared the levels of serological total IgG antibodies to OspC K over time following oral immunization of mice with *E. coli* expressing OspC K at different time points, before (days 28, 42 and 73) and after (days 105, 135 and 170) tick challenge, by ELISA **(Figure 3-10)**. Production of total IgG antibodies to OspC K significantly increased due to a response to initial immunization (day 28), which kept increasing as a consequence of the first (day 42) and then the second (day 73) vaccine boosts. Tick challenge was performed at day 73, when total IgG antibodies to OspC K reached the highest level, compared to control group, $p<0.0001$. Although after tick challenge, the antibody levels dropped they continued to be significantly higher compared to the levels in control group, $p<0.001$, and remained constant showing no differences between days 135 and 170. Interestingly, we found no increase in the antibody levels to OspC in the control group after four (day 105), eight (day 135) or thirteen weeks (day 170) after tick challenge.

CHAPTER 4. DISCUSSION AND CONCLUDING REMARKS

Discussion

This study examined whether recombinant *E. coli* –based oral vaccines expressing OspC K, OspB and BBK32 from *B. burgdorferi* as well as Salp15 and Salp25 from *Ixodes scapularis* could induce a humoral antibody response that protects against infection with multiple strains of *B. burgdorferi* transmitted via tick bite. Our vaccine technology consisted of recombinant live *E. coli* expressing the leader sequence of *B. burgdorferi* OspA lipoprotein fused to the N-terminus of each candidate immunogen (OspC K, OspB, BBK32, Salp15 or Salp25), aiming to target them across the cytoplasmic membrane to the cell envelope as previously shown [110, 114, 115]. Analysis of localization and lipidation of the five vaccine constructs used in this study revealed that the OspA signal peptide tagged OspC K, OspB, BBK32, Salp15 and Salp25 for translocation across the cytoplasmic membrane, given that these proteins were primarily found in the envelope fraction (>60%) as opposed to the cytosolic fraction of the cell. Furthermore, OspC K, BBK32 and Salp15 constructs partitioned mostly into the detergent phase **(Figure 3-1)**, the hydrophobic fraction of the *E. coli* cell envelope, consistent with protein lipidation and with our previous findings [110, 114, 115].

Amongst all tested oral vaccine constructs, only mice immunized with *E. coli* expressing OspC K induced a significant systemic humoral antibody immune response as showed by serological IgG **(Figure 3-2A)** and mucosal IgA **(Figure 3-4A)** immune responses. Given that this oral construct was our most promising candidate, vaccine efficacy studies following oral immunization with *E. coli* expressing OspC K were further pursued.

A multitude of studies have previously shown that OspC vaccination affords protection not only via needle infection [74, 77, 79] but also via tick challenge [69, 70] against *B. burgdorferi* strains with a single and a homologous OspC type. However, these conditions do not represent what happens in the natural setting. In nature, *B. burgdorferi* infection is acquired by tick bite that does not simply transmit a single OspC strain to the host, as reproduced by these experiments [69, 70, 74, 77, 79], but transmits simultaneously a mixture of heterologous strains of *B. burgdorferi,* including multiple OspC types [33, 55, 83, 116, 117]. Therefore, the aforementioned studies [69, 70, 74, 77, 79] have failed to evaluate the potential of protection conferred by OspC vaccination in the natural context of infection, and thus their findings should not be taken as definitive evidence for OspC immunoprotection. In an effort to address this need, we next sought to investigate the ability of an OspC-based vaccine to confer protection upon co-infection with heterologous *B. burgdorferi* OspC populations after tick challenge.

Intending to perform our OspC K vaccine efficacy studies using the natural route of *B. burgdorferi* transmission, we attempted to produce flat nymphs infected with multiple strains (MS) of *B. burgdorferi,* harboring multiple types of OspC, as well as flat nymphs infected with a single type of OspC, *B. burgdorferi* OspC type K (OspC K)

strain. We generated flat nymphs infected with multiple strains of *B. burgdorferi,* with an infection prevalence of 83% **(Figure 3-5B)**. Despite the fact that all mice inoculated intradermally with *B. burgdorferi* OspC type K strain became infected **(Figure 3-5A)**, we were not able to generate infected flat nymphs from larvae that fed on these mice, as shown by an infection prevalence of 0% **(Figure 3-5B)**. One reason that might explain our results is that larval *I. scapularis* could not acquire *B. burgdorferi* OspC type K strain upon feeding. Another possibility is that larvae acquired spirochetes during feeding, but these were not able to survive in the engorged larvae and were, eventually, shed by the vector. Once acquired, spirochetes must adjust to the physiology of the arthropod and endure this new environment. Colonization, persistence and survival of *B. burgdorferi* in the tick are tightly regulated by differential gene expression [50, 122-125]. For example, spirochetes that fail to adhere to midgut tissue are unable to survive within the tick as early as 48 hours postfeeding [125]. Furthermore, production of laboratory-infected ticks using *B. burgdorferi* OspC type K strain is not described in the literature. In fact, in the natural context of *B. burgdorferi* transmission, ticks in the wild harbor multiple strains of *B. burgdorferi,* including multiple types of OspC and not single types [55, 83, 116, 117]. Therefore, it is tempting to speculate that the reason why we were not able to produce ticks infected with *B. burgdorferi* OspC type K strain was because this strain, although it efficiently infected mice by needle inoculation, might lack ecological fitness to be acquired and/or to persist within the tick, by itself. Yet, we cannot exclude the fact that this isolate was grown *in vitro*, a method prone to induce loss of plasmids in *B. burgdorferi* [126-129] that encode for genes required for the establishment and persistence of tick infection [124, 130, 131]. Persistence of *B. burgdorferi* in an unfed tick gut represents one of the most challenging stages of the spirochete lifecycle since the bacterium has to survive during prolonged periods of nutrient deprivation. As such, during this stage, the spirochetes adapt a dormant metabolic state that still remains poorly understood [132]. Therefore, despite speculation, further studies are needed to proper address our failure in generating flat nymphs infected with *B. burgdorferi* OspC type K strain.

Vaccine efficacy of our OspC K oral construct was next assessed by challenge performed with laboratory-produced nymphs infected with multiple strains of *B. burgdorferi*. We found that upon challenge, similarly to controls, OspC K–immunized mice had spirochetal dissemination to their tissues **(Figure 3-6)** and developed IgG antibodies to the spirochete **(Figure 3-7)**, showing that OspC K immunization did not prevent infection with multiple strains of *B. burgdorferi* via tick challenge.

Induction of OspC on *B. burgdorferi* begins upon tick feeding [48, 49] and anti-OspC antibodies have shown to be borreliacidal in mammals [133-135]. Although the role of OspC during tick transmission remains debatable [30, 51-53, 136], we investigated whether anti-OspC K total IgG antibodies present in the serum of OspC K-immunized mice **(Figure 3-2A)** could have a borreliacidal effect within fed ticks. Upon analysis of engorged nymphs that fed on OspC K- immunized mice and on control mice, we found that there were no differences between the number of *B. burgdorferi* harbored in engorged nymphs that fed on OspC K-immunized mice and the number of *B. burgdorferi* harbored in engorged nymphs that fed on control mice (**Figure 3-8)**. These

results indicate that antibodies to OspC in the serum of mice do not have a borreliacidal activity within the ticks upon blood meal intake, which is consistent with previous findings [69]. One could infer that a higher level of host serum OspC antibody might be necessary for protection. Still, this seems to be unlikely considering that at the day of the tick challenge (day 73), OspC K antibodies reached the highest level. In addition, between eight (day 135) and thirteen weeks (day 170) after the tick challenge, the antibody levels in the immunized mice remained constant and were still significantly higher when compared to the levels in control mice **(Figure 3-10)**. Surprisingly, we did not detect any increase in the antibodies to OspC after tick challenge as shown by the levels in the control group **(Figure 3-10)**. Yet, our observations support a recent study where only 13% of the dogs infected via tick bite developed IgM or IgG anti-OspC antibodies [137]. Antigenic variation of OspC does not seem to occur during infection [138, 139]. Therefore, the lack of an immunological response to OspC following infection with *B. burgdorferi* by tick challenge suggests that this protein is not being expressed on the spirochetes surface as they enter the host. In fact, *B. burgdorferi* is able to negatively regulate OspC expression in presence of anti-OspC antibodies as a mechanism of immune evasion [140-142]. Thus, if spirochetes do not express OspC on their surface shortly after they infect the host, the ability of circulating OspC antibodies to clear the pathogen is compromised. It is described that protective immunity to spirochetal infection can only be achieved if antigens are exposed on the spirochetal surface [143]. This could partially justify why our vaccine failed.

More importantly, our findings showed that, despite significant levels of antigen-specific antibodies **(Figure 3-2A** and **Figure 3-4A)**, mice immunized with *E. coli* expressing OspC K were not protected from infection with a homologous *Borrelia* strain as shown by the presence of *B. burgdorferi* OspC type K strain in their tissues **(Figure 3-9)**. This was consistent with a previous study where, despite the development of a strong humoral immune response, mice actively immunized with OspC isolated from *B. burgdorferi* strain N40 were not protected from infection via tick-borne challenge carrying that same (homologous) spirochetal strain [80].

Here, we have demonstrated that an OspC-based vaccine did not protect from infection with multiple strains of *B. burgdorferi* via the natural route of infection. Most importantly, we have shown for the first time that an OspC K-based vaccine does not afford protection to the homologous *B. burgdorferi* OspC isolate (type K) if transmission occurs via ticks infected with a diversity of types of *B. burgdorferi* OspC strains. Our findings are both critical and relevant, and should be considered in future studies involving the design and development of OspC-based vaccines against Lyme Disease.

Concluding Remarks

B. burgdorferi undergoes dramatic adaptive changes in gene expression and protein composition to persist at different stages of the mouse-tick-mouse infectious cycle [123, 144]. The bacteria has the ability to regulate the expression of several lipoproteins in response to environmental cues such as changes in temperature [145], pH

[146] or cell density [147]. For example, as the spirochete migrates from the tick to the host, it differentially expresses lipoproteins. OspC is induced during tick feeding [30, 48, 49, 51], and antibodies to OspC have been shown to protect mammals against infection with *B. burgdorferi* [69, 74, 77, 80]. Despite these attributes that make OspC a promising vaccine candidate, its function in mammalian infection has remained undefined. Moreover, discrepancies regarding the potential of OspC as a vaccinogen exist in the current literature [69, 70, 74, 76-80] and should be further elucidated. Aiming to expand our understanding of this antigen, it is pivotal that we revisit important literature findings, reassess their significance and integrate them in future studies of development and testing of OspC-based vaccines.

It is described that *B. burgdorferi* shuts down *ospC* expression in a reversible manner in the presence of anti-OspC antibodies in the host [140-142]. Moreover, despite the fact that spirochetes up regulate OspC once the tick starts feeding [48, 49], they down-regulate OspC production while they move from the gut to the salivary glands and enter the host [30], and the ones that fail to repress OspC are unable to establish infection in mice [148]. Taken together, these findings suggest that the ability of *B. burgdorferi* to shut down *ospC* expression allows the pathogen to avoid clearance, and thus to succeed during mammalian infection and persist throughout its enzootic life cycle. It is tempting to speculate that, from an evolutionary standpoint, a population of spirochetes that retains this ability might be favorably selected in the natural setting. Wild ticks transmit, simultaneously, a mixture of heterologous strains of *B. burgdorferi,* including multiple OspC types [55, 83, 116, 117] to their host in nature. Not surprisingly, the coexistence of heterogeneous *B. burgdorferi* strains favors the maintenance of the organism in reservoir mammals in nature [117]. In addition, tick-transmitted and culture-grown spirochetes have shown to have different antigenic and genetic structure. For instance, immune sera protected mice challenged with *B. burgdorferi* cultured *in vitro* but did not protect them from infection if the same spirochete was tick-transmitted [149]. Despite this knowledge, the majority of studies that assessed protection conferred by vaccination with OspC used conditions that do not mimic the natural context of spirochete transmission, undervaluing the tick-spirochete interaction. Evaluation of efficacy of OspC-based vaccines by tick challenge is quite scarce [69, 70, 80], as most authors have been assessing vaccine efficacy using the non-natural route of *B. burgdorferi* infection [74, 76, 77, 79]. The few who tested their vaccines against tick challenge [69, 70, 80] used a single strain of *B. burgdorferi*, neglecting the fact that wild ticks are infected with and transmit multiple heterologous OspC strains to the host [33, 55, 83, 116, 117]. Familiar with these limitations, we designed a study where we applied our expertise in tick production and mimicked the natural route of *B. burgdorferi* infection, aiming to extend our current knowledge about OspC vaccine efficacy.

Here, we developed and tested an immunogenic OspC-based oral vaccine *in vivo* against challenge with multiple strains of *B. burgdorferi* isolated from heart of *Peromyscus leucopus,* the natural reservoir host*,* infected with field caught ticks from endemic areas for Lyme Disease. We showed that, despite the development of a strong humoral response to OspC K, mice were not even protected from infection with the homologous type of *B. burgdorferi* (OspC type K) when the spirochete was presented to

the host in the natural context of transmission. Protective antibody responses can be circumvented by *B. burgdorferi* during natural transmission [149, 150]. For instance, immunization with *B. burgdorferi* lipoprotein decorin-binding protein A (DbpA) elicited a strong protective antibody response *B. burgdorferi* in mice. However, this protective response was only seen in mice that were challenged by needle inoculation with *in vitro*-cultivated spirochetes [151]. When spirochetes were transmitted through *I. scapularis* bite this protective response was no longer apparent, which led the authors to hypothesize that antibodies to DbpA are unable to kill *B. burgdorferi* if the spirochete is transmitted by tick bite [150].

Changes in *B. burgdorferi* gene expression during tick feeding are well described, they promote effective spirochete transmission and survival in the host [93, 123, 152], and should not be underestimated. Although far beyond our current understanding, one plausible explanation for our results is that if transmission occurs via tick bite, OspC is not shuttled to the surface of *B. burgdorferi* when the spirochete is being transmitted to the host. As a result, OspC was antibody-inaccessible in the host, which might have precluded specific antibodies from clearing the spirochete. It is described that OspC can be shuttled to and from the surface of the spirochete. OspC was barely detectable on the cell surface and it was mostly found in the periplasm of motile *B. burgdorferi* at different points during infection [153, 154].

There is strong evidence that the antigenic composition of the Lyme Disease spirochete profoundly differs if the spirochete is tick-transmitted or culture-grown [132, 149]. Reports where OspC conferred protective immunity are highly associated with challenges using *in vitro* cultivated spirochetes [74, 76, 77, 79], which led some authors to speculate that, under these conditions, the OspC antigen might be associated with a greater degree of surface exposure [153]. Interestingly, and along these lines, a study has shown that the majority of spirochetes (about 89%) present in the salivary glands of ticks that have fed on animals for more than 53 hours, a time period that coincides with the time required for infection of mammals, did not express OspC [30]. This suggests that if transmission of *B. burgdorferi* occurs via tick bite, only a minor population of OspC-producing bacteria enters the host, which supports our results. It is possible that when *B. burgdorferi* is carried in wild ticks and co-exists with multiple and heterogeneous strains of OspC, the regulation, expression and surface localization of OspC within the spirochete are controlled by a complex cross-talk network, involving conditionally regulated and stochastic elements. This would favor spirochetal survival in the tick-borne parasites as well as evasion of the specific humoral immunity in the host. In addition, it would allow *B. burgdorferi* to preserve the integrity of the ospC gene during immune evasion [138] for subsequent need during its life cycle. Another reason that supports this hypothesis is that the temporal synthesis of OspC by spirochetes is involved exclusively in transmission from tick to mammal and not from mammal to tick [48]. Moreover, expression of OspC plays an essential part in the establishment of infection in a mammalian host [51, 52]. These findings suggest that the tick environment is solely responsible for modulation of OspC expression before *B. burgdorferi* is transmitted to the host, and therefore is imperative that OspC-based vaccines be tested via tick challenge. Failing to do so can have profound implications for vaccine testing and development.

Despite a nearly complete absence of biosynthetic pathways [45], *B. burgdorferi* has an extraordinary zoonotic success, as it continues to be a public health threat [1, 4]. The transmission, survival and the pathogenicity of *B. burgdorferi* depend on the bacterium's ability to adapt to markedly divergent conditions as it transits between the arthropod vector and the reservoir host. This requires a tightly orchestrated modulation of the bacterium transcriptome in response to different environmental signals [132], which can be largely explained by the co-evolution with its arthropod and mammalian hosts [25]. Yet, this intricate topic only recently began to be intensely investigated.

Future studies should focus on further elucidating the interactions between the tick and *B. burgdorferi* as this would help in developing new and superior vaccination strategies to combat this arthropod-borne disease. We should start with tick challenges becoming the mainstay of Lyme Disease vaccine research. Furthermore, the identification of novel candidates for a second generation vaccine against Lyme Disease is of utmost importance and must focus on antigens produced within the tick at early stages of the blood meal that are important for spirochete transmission or vector colonization.

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VITA

Rita Raquel dos Anjos de Carvalho e Melo was born in Lisbon, Portugal in the summer of 1985. Rita graduated from Dr. Antonio Carvalho Figueiredo High School in May of 2003. In August of that same year, she was admitted to the College of Pharmacy at the University of Lisbon, where she began her Doctor of Pharmacy Degree (Pharm.D.) studies. In 2008, Rita received an honor award from the Dean of the University of Lisbon, Dr. António Sampaio da Nóvoa, for her outstanding performance as a student. In 2009, Rita graduated with a Pharm.D. and started her professional career as a Pharmacist in Charge in a community pharmacy in Lisbon downtown. Aiming to pursue her passion for scientific research, Rita moved to the United States in early 2010 to join the Department of Microbiology, Immunology and Biochemistry at the University of Tennessee Heath Science Center (UTHSC) as a Research Scholar. During this time, she also worked as a Research Assistant for Biopeptides Corporation, and she became involved in the development of oral vaccines against Melioidosis and peanut allergies. Given her positive experience at UTHSC, Rita selected this institution to pursue a higher education. As such, in August of 2011 Rita began her Ph.D. studies in the Program of Biomedical Sciences, with a concentration in Microbiology, Immunology and Biochemistry. Since then, Rita has been working on Lyme Disease, Melioidosis and Leptospirosis and published three manuscripts. She is expected to obtain her Ph.D. in December of 2015 and her goal is to continue to advance science.

Publications

1. Richer LM, Brisson D, **Melo R**, Ostfeld RS, Zeidner N, Gomes-Solecki M, *Reservoir targeted vaccine against Borrelia burgdorferi: a new strategy to prevent Lyme disease transmission.* J Infec Dis., 2014. **209**(12): p. 1972-80.

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