5-26-2021

# Development of improved diagnostic tests for Lyme disease

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**A thesis in the program in Microbiology and Immunology**
**submitted to the faculty of the**
**Graduate School of Basic Medical Sciences**
**in partial fulfillment of the requirement**
**for the degree of Master of Science**
**at New York Medical College**

May 2021

# Development of improved diagnostic tests for Lyme disease

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March 31, 2021

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# ACKNOWLEDGMENT

All thanks and gratitude to the principal investigator of this project, Dr. Paul Arnaboldi for the valuable guidance and advice, the cooperation and support he provided all the way.

Also many thanks to the Ministry of Education in the Kingdom of Saudi Arabia for granting me a full academic scholarship, and to the Saudi Arabian Cultural Mission to the US for the continuous support throughout my study and during the hard time of COVID-19 pandemic.

All the love and thanks to my family for being a huge support in my life.

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| List of Abbreviations | |
|---|---|
| **AA** | Amino acid |
| **ACA** | Acrodermatitis chronica atrophicans |
| **AUC** | Area under the curve |
| **BL** | Borrelial lymphocytoma |
| **BSK** | Barbour-Stoenner-Kelly medium |
| **CDC** | Centers for Disease Control and Prevention |
| **Cps** | Circular plasmids |
| **CSF** | Cerebrospinal fluid |
| **d.$H_2O$** | Distilled water |
| **DMSO** | Dimethyl sulfoxide |
| **EIAs** | Enzyme Immunoassays |
| **ELISA** | Enzyme Linked Immunosorbent Assay |
| **EM** | Erythema Migrans |
| **FDA** | The US Food and Drug Administration |
| **LD** | Lyme Disease |
| **LDBB** | Lyme Disease BioBank |
| **LFAs** | Paper-based lateral flow assays |
| **Lp54** | 54-kb linear plasmid |
| **lps** | Linear plasmids |
| **$MgCl_2.6H_2O$** | *Magnesium Chloride*, Hexahydrate |
| **MTTT** | Modified two tiered-testing |
| **NYMC** | New York Medical College |
| **Osp** | Outer surface protein |
| **PBST** | Phosphate-buffered Saline containing 0.1% Tween-20 |
| **PCR** | Polymerase Chain Reaction |
| **pgf 54** | Paralogous gene family 54 |
| **pNPP** | para-Nitrophenyl Phosphate |
| **POC** | Point-of-care |
| **RA** | Rheumatoid Arthritis |
| **ROC curve** | Receiver operating characteristic curve |
| **RPLs** | Large random peptide libraries |
| **RPR** | Rapid plasma reagin |
| **RT** | *Room Temperature* |
| **sl** | *Sensu lato* |
| **STTT** | Standard two tiered-testing |
| **TBRF** | Tick-borne Relapsing Fever |
| **WB** | Western blot |
| **xVFA** | Multiplexed vertical flow assay |

## Abstract

Laboratory diagnosis is very critical for early treatment of Lyme disease, as Lyme disease could be difficult to diagnose because of vague symptoms or the confusion with symptoms from other illnesses. Previously, the standard two tiered-testing (STTT) was the traditional testing for Lyme disease with two different lab testing methods, Enzyme Immunoassay (EIA) as the first-tier test and immunoblot as the second-tier test to confirm the diagnosis. However, western immunoblotting had a poor sensitivity of <50% for early Lyme disease and high costs. Recently, the Centers for Disease Control and Prevention (CDC) recommended the use of a modified two tiered-testing (MTTT) which was cleared by The US Food and Drug Administration (FDA) in which EIA can be used instead of western immunoblotting.

In this study we followed a recent multiplexed paper-based immunoassay study for early Lyme disease serodiagnostic testing by using an Enzyme Linked Immunosorbent Assay (ELISA) technique to detect specific antibodies for different *Borrelia* antigens. These include BBA64-7 and BBA65-94 peptides of *B. burgdorferi* that showed promising results with high seropositivity against IgM antibody in early Lyme disease samples provided by CDC with 50%, and 83% positivity, respectively.

CHAPTER ONE

# INTRODUCTION

### 1.1 The genus Borrelia

The genus *Borrelia* includes several disease-causing spirochetes, that are a members of the Spirochaetaceae family. With the exception of louse-borne *Borrelia* that causes a relapsing fever, borrelia infections are considered zoonoses (Johnson and Hughes, 1992). *Borrelia* was named after the French biologist Amédée Borrel. The major known species of *Borrelia* to cause Lyme disease or borrelioses are: *Borrelia burgdorferi, Borrelia garinii,* and *Borrelia afzelii,* cumulatively called *Borrelia burgdorferi* sensu lato (sl), transmitted by different "hard" ticks. *Borrelia burgdorferi* is the predominant species causing Lyme disease in the United States is specifically named as *Borrelia burgdorferi sensu stricto* that contains several different strains (Gern, 2008). In addition, there are other *Borrelia* species that do not cause Lyme borreliosis such as, *Borrelia* species causing the Tick-borne Relapsing Fever (TBRF), including *B. recurrentis* and *B. duttoni.* Most members of relapsing fever borreliae are transmitted by species of soft ticks (Dworkin *et al*., 2008).

### 1.2 Borrelia burgdorferi

*B. burgdorferi sensu stricto* was first isolated in 1982 by William Burgdorfer from a deer tick in Long Island NY. *Borrelia burgdorferi* sensu lato can be found in Europe, North America, and Asia. *B. burgdorferi sl* are tick-borne spirochetes that are 10-25μm long. It has a number of flagella located in the periplasmic space, which also contains a layer of peptidoglycan between the inner and the outer membranes. Flagella allow the spirochete to be highly invasive to the tissues of the infected host and tick vector (Tilly *et al*., 2008). The outer membrane of *B. burgdorferi* contains an LPS-like substance, that makes the organisms weak gram-negative if gram-stained. *B. burgdorferi sl* are extracellular bacteria that even though do not have an intracellular niche, they can be internalized by

the host cells as a defense mechanism (Petnicki-Ocwieja and Kern, 2014). *B. burgdorferi* is nutritionally a very fastidious bacterium when cultivated in vitro. The Barbour-Stoenner-Kelly (BSK) medium is used to grow *B. burgdorferi sensu stricto* in a microaerobic environment with an optimal temperature of 32$^{o}$C. Generation time is about 12-24 hours (Todar, 2006). The *B. burgdorferi* genome is very complex. It consists of a linear chromosome of a 950kb, together with circular plasmids (cps) and linear plasmids (lps) that constitute a variable complement. *B. burgdorferi* is a scavenger with transporters and/or binding proteins encoded on about 52 genes to bind carbohydrates, amino acids, and peptides as a way to compensate for the loss of genes encoding the enzymes needed for the synthesis of these substances, which occurs during coevolution with its vector and host (Brisson *et al*., 2012).

### 1.2.1 Transmission and source of infection

*B. burgdorferi sensu stricto* is transmitted through the bite of infected ticks. The hard tick *Ixodes scapularis* or deer tick is the vector of Lyme disease in the northeastern, mid-Atlantic, and north-central United States, while the *Ixodes pacificus* western blacklegged tick spreads the disease on the Pacific coast. During blood feeding, spirochetes migrate from the midgut of the tick to the salivary gland with consequent transmission to the host via saliva (Crippa *et al*., 2002). Although these ticks often take blood meals from humans, humans are considered incidental hosts, which means that humans are not required for the continuance of existence of either spirochetes or ticks in nature. *Ixodes* ticks have four life stages in a two-year life cycle: egg, larva, nymph, and adult. The larvae feed on infected reservoir hosts. The white-footed mouse is thought to be the natural reservoir for *B. burgdorferi.* Larvae acquire the spirochetes and moult to the nymphal stage where they feed on either uninfected reservoirs or incidental hosts and transmit the pathogen. There

is a wide host range for larvae and nymphs, including birds, rodents, and insectivores. Adult stage ticks are not as important as immature ticks in maintaining *B. burgdorferi* in the wild; however, adult ticks mate on deer which makes deer important in maintaining the tick population. Adult male ticks do not feed, while adult females feed on large animals a third time converting the blood meal to eggs which makes the mature ticks less significant vectors of the disease than nymphs. Moreover, adult ticks are more active in cold weather when humans are most likely to be indoors and less apt to encounter ticks. Humans and dogs are considered dead-end hosts for the tick (Radolf *et al*., 2021).

### 1.2.2 Pathogenesis and disease

One of the main cellular components of *B. burgdorferi* in causing Lyme disease are the outer surface proteins (Osps) that play an important role in bacterial transmission and possibly survival inside the host. OspA and OspC are the most well studied surface proteins, with OspA being expressed mostly in the midgut of unfed ticks, and OspC is expressed when the bacteria are inside of the infected host (Schwan *et al*., 1995). A previous mouse study demonstrated that OspA was expressed by all spirochetes residing in the midgut of ticks prior to transmission to mice, and was greatly decreased during tick attachment and feeding when spirochetes migrate to the salivary gland of ticks (Schwan and Piesman, 2000). OspA was not detected in spirochetes during mammalian infection, which suggests that it may be downregulated by the spirochetes. This finding was supported further by the fact that OspA did not provide protection to mice immunized with OspA and challenged by the infection (Schwan and Piesman, 2000). OspC was found to be downregulated in spirochetes inside the midgut of ticks before transfer to mice and prior to feeding. OspC was then upregulated by *B. burgdorferi* following transmission to mice. Observations of OspC expression by the spirochetes during tick

feeding suggested that OspC is essential in the transmission of spirochetes from ticks to the mammal. Immunological responses observed in humans and mice to OspC following a tick bite infection challenge, was also suggestive of OspC expression by spirochetes during their transmission to new hosts (Fung *et al*., 1994). Thus, differential regulation of OspA and OspC is a significant factor for the alternation of spirochetes between ticks and mammals. The majority of Lyme disease cases in humans are caused by *B. burgdorferi* sensu stricto in the US but not in Europe, and *B. garinii*, and *B. afzelii* and the closely related *B. bavariensis* are more prevalent pathogenic species.

Lyme disease can be divided into three different stages. Stage one is Erythema Migrans (EM), a primary skin lesion which occurs within 1 to 30 days following an infected tick bite, where spirochetes inoculate into the skin causing an initiation of the immune system in mammalian hosts (Petzke and Schwartz, 2015). Primary EM in adults is located most commonly below the waist, but it could be located anywhere on the body. EM is often described as a classic "bull's eye rash": an expanding, erythematous lesion with a median of 15 cm in diameter, and possibly a central clearing. Symptoms associated with EM can vary from none to moderate, including low-grade fever and chills, fatigue, headache, malaise, and arthralgia (Radolf *et al*., 2021). Stage two or early disseminated infection occurs if Lyme disease was not treated early. Multiple EM appear within 3 to 5 weeks after tick bite, and are usually smaller than the primary lesion. This is a common manifestation of stage two disseminated LD as well as meningitis and facial nerve palsy. Systemic symptoms include, myalgia, fatigue, arthralgia, fever, and headache. Carditis is considered a rare manifestation of the disease where patients may present with fatigue and dizziness. Borrelial lymphocytoma (BL) is a dermatologic disorder that is associated with Lyme disease in Europe and usually occurs with EM, or a few days before, or several weeks after EM. BL appears as a bluish-red nodule on the nipple area or the ear

lobe. The most common manifestation of late Lyme disease in US is arthritis, which occurs weeks to months after initial infections. Arthritis mainly affects large joints causing the affected joint to be swollen and tender. Other manifestations of stage three LD that are rare in children include encephalopathy, polyneuropathy, and encephalitis. Acrodermatitis chronica atrophicans (ACA) is another dermatologic disorder that is a common manifestation of LD in Europe, but is unknown in the United States. ACA lesion typically develops extremely late, within months to years after untreated infection, and involves distal parts of extremities. Small and large joint arthritis, polyneuropathy, and thickening of bones may arise in the same extremity as ACA (Murray and Shapiro, 2010).

### 1.2.3 Clinical diagnosis

Lyme disease patients may be asymptomatic, which may not require treatment as prognosis for asymptomatic patients is usually good. Most cases of LD are presented at one of the three known stages, occurring sequentially when an earlier stage was left untreated. EM is a characteristic clinical feature of early Lyme occurring less than 30 days from exposure to tick bite, which can sometimes be unnoticed if the tick bite was in an area that is difficult to see. EM is usually more than 5cm in diameter, painless and expanding in size slowly. As mentioned earlier in disease pathogenesis, early LD can be associated with fever, arthralgia, and headache. Early disseminated Lyme disease usually occurs in less than 3 months after tick bite exposure. Symptoms of the LD second stage may include the development of multiple secondary lesions, fever, headache, and other systemic symptoms that usually occur several weeks after the primary EM. Other manifestations include Lyme carditis and neuroborreliosis which commonly presents together with cranial nerve (Bell's palsy) in North America. Unexplained conduction abnormalities should be considered by physicians as signs of LD in patients living in or

having recently visited Lyme-endemic areas, as suggested by US data from 1996 to 2006 showing that conduction abnormalities have been reported in 0.8% of Lyme disease patients. However, with no history of EM, serological confirmation is required (Hatchette *et al*., 2014). Lyme arthritis is a common clinical feature of the late stage LD, involving joints, mostly the knees. Serology is very sensitive and specific in diagnosing Lyme arthritis, and it can rule out the infection with a negative test. However, results of serology testing take time, which is why clinical diagnosis is required for immediate treatment. Lyme arthritis can be clinically differentiated from septic arthritis which is helpful in Lyme-endemic areas. Lyme arthritis usually involves knees, and is generally less accompanied by signs of inflammation and fever (Hatchette *et al*., 2014).

### 1.2.4 Laboratory diagnosis

Serology is the only FDA-approved *in vitro* diagnostic test and standardized laboratory test to support the clinical diagnosis of Lyme borreliosis in the US by detecting the immunological responses, particularly IgM and IgG to *B. burgdorferi* antigens. Developing antibodies to antigens takes time which delays the reliability of serology in diagnosing an early acute disease. Different direct tests for Lyme disease have been developed, including culture of bacterial agent from skin, blood, or CSF; also the use of molecular techniques including polymerase chain reaction (PCR), or quantitative real-time PCR, to detect the genetic material in skin, synovial fluid, blood, and CSF. These direct tests are used mainly in reference and in research and academic laboratories, but not in routine use as both culture and PCR show relatively low sensitivity. Spirochetes are present in low amount in the body as they replicate slowly. Spirochetes can be rapidly cleared from the site of tick bite inoculation of the skin, which means their recovery from an EM skin biopsy sample is inversely related to the EM duration. The hematogenous

dissemination of *B. burgdorferi* spirochetes occurs within several weeks, which is considered a brief period, and is another reason for the low sensitivity of culture. Blood cultures are usually negative in the late stage of LD. Culture test also requires a special media and expertise, and long incubation. PCR low sensitivity could also be related to the small volume of the sample tested (Johnson, 2011). Spirochete transmission from ticks to hosts has shown to change the expression of some of the proteins; for example, the switch in the outer surface protein from OspA to OspC after a blood meal by a tick which continues to be expressed for 2 weeks following the infection of a mammalian host. Similarly, in vitro culture of spirochetes showed an upregulation of OspC expression during spirochetes growth from logarithmic to stationary phase (Ramamoorthy and Philipp, 1998). Expression of OspC in vitro appears to be regulated by temperature and pH. OspC was induced in vitro at pH less than 7.5, which resembles pH in the midgut of a feeding tick, and at a temperature of about 34°C resembling the upshift in temperature during tick feeding. Expression of other proteins under the same conditions of OspC induction in in vitro culture may follow a similar role of these proteins as OspC in the transmission of spirochetes from tick to host during feeding and in establishing infection in mammalian hosts (Ramamoorthy and Scholl-Meeker, 2001). Histopathology is mainly used to evaluate BL and ACA suspected cases. Focus-floating microscopy, Warthin-Starry and modified Dieterle silver stains, and direct and indirect immunofluorescence assays have been used in the diagnosis of *B. burgdorferi* infection but have proven to be difficult in result interpretation and require special expertise. Currently, antigen assays are not recommended for the diagnosis of LD (Marques, 2015). The conventional serodiagnosis standard in North America is the two-tiered serology (STTT) in patients with indications other than acute EM. The first tier is a sensitive serological test that detects IgG and IgM antibodies, either together or separately. Enzyme immunoassays

(EIAs) such as ELISA could be used as a first-tier test. If the result is negative, the sample is not tested further and is reported as negative for *B. burgdorferi* antibodies (CDC, 1995). If the result is positive or borderline, a supplemental immunoblot, such as Western Blot WB is performed for confirmation. Using WBs in two-tiered tests provides specificity and sensitivity in the late stage of LD. However, they are considered insensitive during early infection, because of the delay in humoral immune response. Therefore, the diagnosis of early LD is not ruled out by a negative result, if symptoms are present for <30 days. Also, immunoblots results take days and are more expensive than EIAs (Branda *et al*., 2017). To overcome these limitations, a modified two tiered-testing (MTTT) was developed and it was cleared by the FDA to be used as a base methodology for LD serological assays (Mead *et al*, CDC., 2019). MTTT first tier-test is EIA followed by a next-generation EIA assay (2-EIA). Compared to STTT, MTTT or 2-EIA protocol was found to have equal sensitivity and specificity to STTT in LD later stages; however, it showed higher sensitivity in diagnosing early LD, as well as cost-effectiveness (Lipsett *et al*., 2019). Paper-based lateral flow assays (LFAs), are rapid, easy to use, cost-effective tests for point-of-care (POC) serological analysis. LFAs detect antibodies specific to disease using colorimetric, or fluorescent conjugates that are embedded in single-use cassettes. LFAs have an in-line geometry allowing them to only measure one or two antibodies in a single test, which limits their ability to detect multiple antigens. Measuring a single antibody exposes tests to false positive results due to highly cross-reactive epitopes of antigens with epitopes in other bacteria. A recent study (Joung *et al*., 2020) developed a multiplexed vertical flow assay (xVFA), composed of a number of functional paper layers which allows for the measurement of multiple LD-specific antibodies for a multiantigen panel on a single sensing membrane. The sensing membrane of xVFA contains 13 immunoreaction spots with *B. burgdorferi*-specific antigens (BmpA, P41,

OspC, Crasp1, P35, DbpB, Erpd/Arp37) and a peptide (Mod-C6) which is a C6-like epitope joined to a specific p41 epitope. After about 15 min of operation, the assay cassette of xVFA is opened and the sensing membrane is visualized by a reader. Colorimetric signals generated on the membrane are then quantified using computational analysis, which eventually draw a diagnosis from the immunoreaction. There is no current FDA-approved POC test that can replace the two-tier method for LD diagnosis. However, xVFA testing approach provides a cost-effective, rapid assay that is promising for early LD testing at the POC (Joung *et al.*, 2020).

Identifying peptide epitopes as individual diagnostic antigens is an approach that allows for a chemically simple, controllable format for ELISA and other reactions. They are also of high quality, and cost-effective in production. Common methods used to identify peptide epitopes are limited to known antigens. A panel of eight peptide epitopes was identified by Yu and coworkers, which synthesized dodecamers to span the amino acid sequences of *B. burgdorferi* four immunodominant surface proteins and screened them with LD patients, sera. Using these peptides for a diagnostic test resulted in a specificity of 71% and sensitivity of 75% (Yu *et al*., 1996). A new approach in identifying diagnostic peptides is epitope discovery. In this strategy, a large random peptide libraries (RPLs) panel is used as the source of peptides in a phage display format. Each peptide in a library is linked to a surface protein of a carrier phage, and called a “guest” peptide. The carrier phage with the peptide coding sequences in the viral DNA will then infect bacterial cells, and the guest peptides are cloned. The peptide ligands are selected from libraries using a panel of antibodies from human sera, and screened for reactivity. This strategy can be used in research studies on pathogens to purify antipathogen antibodies in patient sera using peptide mimics, and antibodies can then be used as probes in identifying the pathogen (Kouzmitcheva *et al*., 2001).

### 1.3 Paralogous proteins

Paralogy concept was originally introduced in 1970 by Walter Fitch. Paralogs are genes in the same organism that developed by gene duplication. These homologous genes code for paralogous proteins, which are similar proteins with possibly different functions. Paralogous genes with a large population size and high mutation rate, and before becoming functionally distinct can mask inactivating mutations of each other, causing their maintenance by natural selection (Dandage and Landry, 2019). Human and yeast studies demonstrated that paralog compensations for loss-of-function (LOF) mutations of each other at the molecular level is allowed by genetic redundancy. Observations in humans showed that genes with paralogs are less involved in diseases, and sequence similarity of a gene with its closest homolog in a genome decreases the probability of disease association for this gene (Hsiao and Vitkup, 2008). A series of gene products of *B. burgdorferi* induce antibody responses in human and experimental animals during *in vivo* infection, i.e., BBA64, BBA65, BBA66, BBA68, BBA69, BBA70, and BBA73. These proteins are encoded by paralogous genes located on the 54-kb linear plasmid (lp54) and are known to be expressed in ticks and/or mammalian hosts. Proteins BBA64, BBA65, and BBA66 are members of the *Borrelia burgdorferi* paralogous gene family 54 (pgf 54). They were assessed in a previous study by (Gilmore *et al*., 2007) for genetic stability, host antibody response, and gene expression kinetic in mice. Results of the study demonstrated that pgf 54 are stable genes that do not produce variant antigens during persistent infections. BBA64 and BBA66 antibodies were detected soon after infection suggesting early induction of these genes. The expression profile of BBA64 suggested an important role in early infection and tick-to-host transmission, while the expression profile for BBA65 and BBA66 genes suggested a role in persistent infection indicating a significant role of the plasmid containing the paralogs in establishing infection in the host, and that it hinting an essential

reason for spirochetes to maintain the lp54 plasmid. Paralogous proteins could be used to improve the serological testing of early Lyme disease through their incorporation into the current IgM/IgG immunoblotting tier assay (Weiner *et al*., 2015).

### 1.4 Aim of the project

A recent study developed a multiplexed paper-based immunoassay for the serodiagnosis of early LD. This allows a simultaneous detection of LD-specific antibodies against multiple *B. burgdorferi*-specific antigens concentrated on a single membrane. Following a 15min manipulation, the membrane is imaged by a reader, followed by quantification of the colorimetric signals on the sensing membrane using a computational analysis. This approach showed 96.3% specificity, and 85.7% sensitivity in relation to the standard two-tier testing that showed poor sensitivity of <50% (Joung *et al*., 2020). In this study, we identified linear epitopes from *B. burgdorferi* peptides using B-cell epitope mapping. After the use of an ELISA to detect specific antibodies against these *B. burgdorferi* antigens (BBA64-3-5, BBA64-4-7, BBA64-7, BBA65 62-63, BBA65 101-02, BBA65-94, BBA66-117+, and BBA73 196-199), and we evaluated the use of these antigens in the paper-based assay, we also tested for other potentially serodiagnostic assays for LD diagnosis.

CHAPTER TWO

# MATERIALS & METHODS

## 2.1 Peptides preparation

Peptides of *B. burgdorferi*, BBA64-3-5, BBA64-4-7, BBA64-7, BBA65 62-63, BBA65 101-02, BBA65-94, BBA66-117+, and BBA73 196-199 were commercially synthesized to >90% purity with an N-terminal biotin tag. Lyophilized peptides were reconstituted in 10% acetic acid, or dimethyl sulfoxide DMSO based on solubility chart as determined by the biochemical properties of the individual amino acids in each sequence. (**Table 2.1**).

**Table 2.1:** Peptides of *B. burgdorferi* and their solubility

| No. | Peptide | Sequence | Solubility |
|---|---|---|---|
| **1** | B-BBA64-3-5 | TVLILISCSLEVKDSNESKK | DMSO |
| **2** | B-BBA64-7 | NESKKHKKEKRKGKV | 10% acetic acid |
| **3** | B-BBA64 4-7 | TVLILISCSLEVKDSNESKKHKKEKRKGKV | 10% acetic acid |
| **4** | B-BBA65 62-63 | TLGITTFFSCDLNNKDNKDK | DMSO |
| **5** | B-BBA65 94 | TIKISGGIRIQQGFEVA | DMSO |
| **6** | B-BBA65 101-02 | KNTLEEIYNLIVDLTLIKKEW | DMSO |
| **7** | B-BBA66-117+ | KLLGLFLFSCTIDANLNEDYKNK | DMSO |
| **8** | B-BBA73 196-199 | MKRNKIWKTLKLFQITLLFSCSFYSKSNNT | 10% acetic acid |

## 2.2 Plates

NUNC Immobilizer Streptavidin F96 clear plates from ThermoFisher Scientific were used. Theses plate are treated with streptavidin, a biotin-binding protein that binds four biotin per molecule for greater sensitivity and increased signal output.

## 2.3 Serum samples

This study was conducted using serum/plasma stocks previously collected from patients with a confirmed diagnosis of Lyme disease with a characteristic skin rash, erythema migrans (EM), or with a well-defined disseminated disease symptoms. Commercially purchased sera were used as controls. Some samples were also collected at New York Medical College (NYMC), some at Gundersen Lutheran in Wisconsin, and some at Stony

Brook University NY. All samples were collected under approved IRB protocols from the relevant institutions and informed consent/assent. Negative control samples for LD were disease controls (Rheumatoid Arthritis (RA), Syphilis, and Fibromyalgia) as well as, healthy non-endemic controls. All controls were purchased from BioIVT. CDC-defined CDC and Lyme disease Biobank LDBB samples were used to confirm the results. LDBB samples were obtained from the Bay Area Lyme foundation (**Table 2.2**).

**Table 2.2:** Collection of samples by location and disease state

| Sample (location) | No. of Samples |
|---|---|
| Early Lyme disease | |
| NYMC | 64 |
| Stony Brook University | 26 |
| Wisconsin | 43 |
| Early neurologic Lyme disease (Gundersen Lutheran,Wisconsin) | 17 |
| Late Lyme disease (Wisconsin) | 12 |
| All early Lyme disease | 150 |
| Total Lyme disease | 162 |
| | |
| Other diseases (BioIVT, LLC) | |
| Fibromyalgia | 15 |
| Rheumatoid Arthritis | 38 |
| Syphilis | 29 |
| Healthy Controls (BioIVT, LLC) | |
| Healthy; non-endemic | 41 |
| LD CDC samples | 31 |
| LD Biobank samples | 118 |

## 2.4 Reagents preparation

In-lab prepared phosphate-buffered saline PBST, 0.1% TWEEN was used as the wash buffer. For the secondary antibody, goat anti-human IgG and goat anti-human IgM with alkaline phosphatase as an enzyme conjugates (recommended with streptavidin-coated plates) from Southernbiotech company were used, diluted in 1:4000 PBST. The substrate used was para-nitrophenyl phosphate pNPP from SIGMA-Aldrich; 5mg tablet:5ml

substrate buffer. Substrate buffer was prepared in the lab with 400ml d.$H_2O$, 24.5mg $MgCl_2.6H_2O$, and 48ml diethanolamine.

### 2.5 Technique

The standard technique of indirect ELISA was used to detect antibodies in early LD samples. Experimental steps are as follows: each plate was pre-washed three times x3 with wash buffer, then coated with 100µl peptide diluted to concentration of 2µg/ml in PBST and incubated for one hour at room temperature RT with agitation, or stored overnight at 4°C. Next, each plate was washed x3 with wash buffer, and 100µl of serum diluted 1:100 in PBST was added per well and incubated for two hours at RT with agitation. Each plate was washed again x3 with wash buffer and 100µl of detecting antibody diluted 1:4000 in PBST was added per well and incubated for one hour at RT with agitation. In the last step, after washing the plate x3 with wash buffer we added 100µl substrate (5mg tablet:5ml substrate buffer) and incubated for 15 minute at RT. The plate was then read at 405nm using SpectraMax plus microplate reader.

### 2.6 Data analysis

Statistical analysis was performed using Prism 9.0 (GraphPad software, LLC). The sensitivity and specificity of each peptide were calculated via receiver operating characteristic (ROC) analysis comparing IgM binding in Lyme patient sera with negative controls. The cutoff value used for comparing sensitivity and specificity was 2 standard deviations (SD) from the mean of the healthy controls. The statistical analysis of the categorical data presented in the tables and text was performed using a chi-square analysis.

# CHAPTER THREE
# **RESULTS**

### 3.1 Epitope mapping and peptide synthesis

Previous work in the lab identified the individual epitopes from four paralogous proteins, BBA64, BBA65, BBA66, BBA73. Briefly, overlapping peptide libraries for each of the proteins was generated consisting of 15AA-long peptides overlapping by 10-AA (5-AA offset). These peptides were hybridized to glass slides and incubated with different dilutions of sera from 8 patients with high seropositive Lyme disease (9-10 distinct positive bands on a second tier western blot). This was performed by ProImmune, LLC. The data for antibody binding to peptides were evaluated. Epitope sequences (**Table 2.1**) were considered for further evaluation if they bound antibody in a minimum of 6 of 8 patient sera at more than one serum dilution. Epitopes were synthesized as individual peptide antigens with the exception of BBA64-3-5, BBA64-4-7, BBA64-7 each of which had overlapping sequences. Initial screening of the peptide antigens did not reproduce results of epitope mapping (data not shown), leading us to hypothesize that the short peptides were not binding to the surface of the plate. The peptides were re-synthesized with a biotin tag on the N-terminus, and were used in the present study.

### 3.2 ELISA results

The affinity of peptide binding of both IgM and IgG antibodies were evaluated independently in a panel of patient sera using ELISA. At the beginning of this study, the following 8 peptides were used, BBA64-3-5, BBA64-4-7, BBA64-7, BBA65 62-63, BBA65 101-02, BBA65-94, BBA66-117+, and BBA73 196-199. As the study progressed, 6 peptides were excluded and only 2 peptides (BBA64-7, BBA65-94) remained for the rest of the study. BBA64-3-5, BBA64-4-7, BBA65 62-63, BBA73 196-199 peptides were used to evaluate IgM and IgG binding in samples of early Lyme disease patient sera using the cutoff calculated from the mean absorbance of IgG or IgM

binding in sera of healthy control samples (number of healthy control samples used for IgG and IgM is determined in the corresponding figure of each peptide, **Figure 3.1, 3.2, 3.3, and 3.4**), and positive cutoff values were determined as the mean values calculated plus 2X the standard deviation. The positive binding of IgM to all four peptides was poor, with BBA64-3-5 detecting antibodies in 25% (5/20) of ($EM^+$) early Lyme patient sera (**Figure 3.1a and Table 3.1**), 15% (3/20) for BBA64-4-7 (**Figure 3.2a and Table 3.1**), 35% (7/20) for BBA65 62-63 (**Figure 3.3a and Table 3.1**), and 13% (2/16) for BBA73 196-199 (**Figure 3.4a and Table 3.1)**. The positive binding of IgG in ($EM^+$) early Lyme patient sera to all four peptides was very low, with 6% (2/36) for BBA64-3-5 (**Figure 3.1b**), 5% (2/43) for BBA64-4-7 (**Figure 3.2b**), 2% (1/43) for BBA65 62-63 (**Figure 3.3b**), and 3% (1/31) for BBA73 196-199 (**Figure 3.4b)**. These peptides (BBA64-3-5, BBA64-4-7, BBA65 62-63, BBA73 196-199) were excluded from the rest of this study due to low IgM and IgG binding efficacy in early Lyme disease patient screening sera.

**Table 3.1:** Initial IgM screening

| **Serum (total)[c]** | **No. of IgM-reactive[a] serum samples (%)[b]** | | | |
|---|---|---|---|---|
| | BBA64-3-5 | BBA64-4-7 | BBA65 62-63 | BBA73 196-199 |
| Lyme disease | | | | |
| Early Lyme disease[e] (20) | 5 (25) | 3 (15) | 7 (35) | 2/16[g] (13) |
| Late Lyme disease[f] (12) | 0 (0) | 1 (8) | 1 (8) | - |
| Total Lyme disease (32) | 5 (16) | 4 (13) | 8 (25) | 2/16 (13) |
| Other diseases | | | | |
| Rheumatoid Arthritis (16) | 1 (6) | 0 (0) | 1 (6) | 0 (0) |
| Healthy Controls | | | | |
| Healthy; non-endemic[d] (16) | 1 (6) | 1 (6) | 2 (13) | - |

a Reactive samples, positive and equivocal (total of 3SD and 2SD from the mean of the healthy controls)
b Results are presented as number (percent) of samples testing positive
c Total of each group of samples
d Non-endemic, serum samples were collected from patients in regions where Lyme cases are not increasing constantly
e Early Lyme, erythema migrans positive
f Late Lyme, Lyme arthritis
g Total of early Lyme samples for BBA73 196-199 was 16 samples

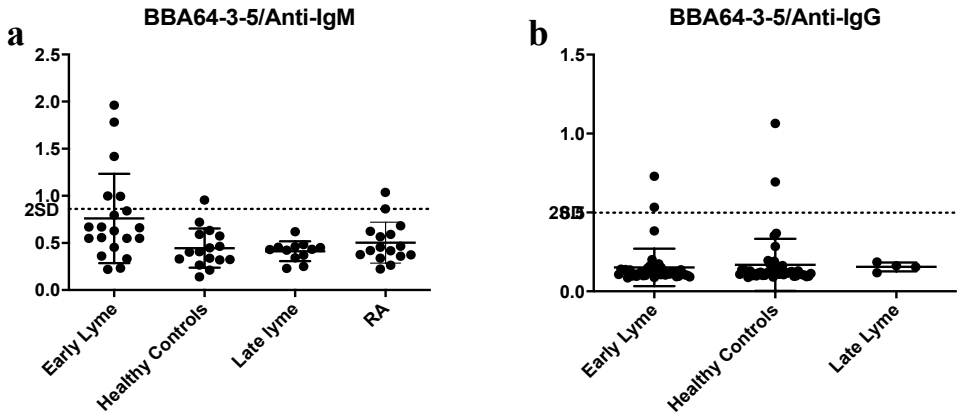


**Figure 3.1:** IgM (a) binding of BBA64-3-5 peptide in serum samples from patients with early Lyme disease (*n* = 20), late Lyme disease (*n* = 12), healthy controls (*n* = 16), rheumatoid arthritis (RA) (*n* = 16). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls. IgG (b) binding of BBA64-3-5 peptide in serum samples from patients with early Lyme disease (*n* = 36), late Lyme disease (*n* = 4), healthy controls (*n* = 48). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls.

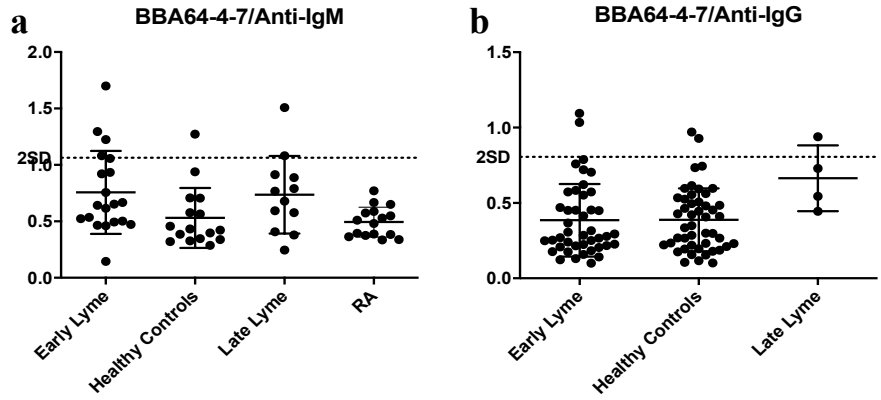


**Figure 3.2:** IgM (a) binding of BBA64-4-7 peptide in serum samples from patients with early Lyme disease (*n* = 20), late Lyme disease (*n* = 12), healthy controls (*n* = 16), rheumatoid arthritis (RA) (*n* = 16). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls. IgG (b) binding of BBA64-4-7 peptide in serum samples from patients with early Lyme disease (*n* = 43), late Lyme disease (*n* = 4), healthy controls (*n* = 47). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls.

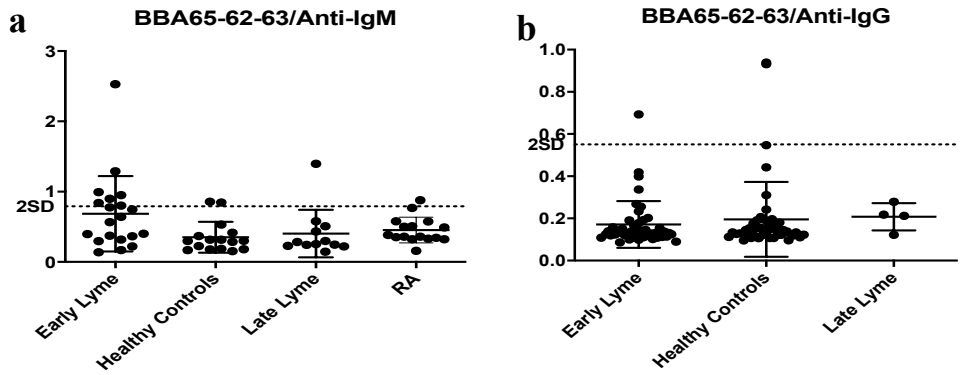


**Figure 3.3:** IgM (a) binding of BBA65-62-63 peptide in serum samples from patients with early Lyme disease (*n* = 20), late Lyme disease (*n* = 12), healthy controls (*n* = 16), rheumatoid arthritis (RA) (*n* = 16). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls. IgG (b) binding of BBA65-62-63 peptide in serum samples from patients with early Lyme disease (*n* = 43), late Lyme disease (*n* = 4), healthy controls (*n* = 47). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls.

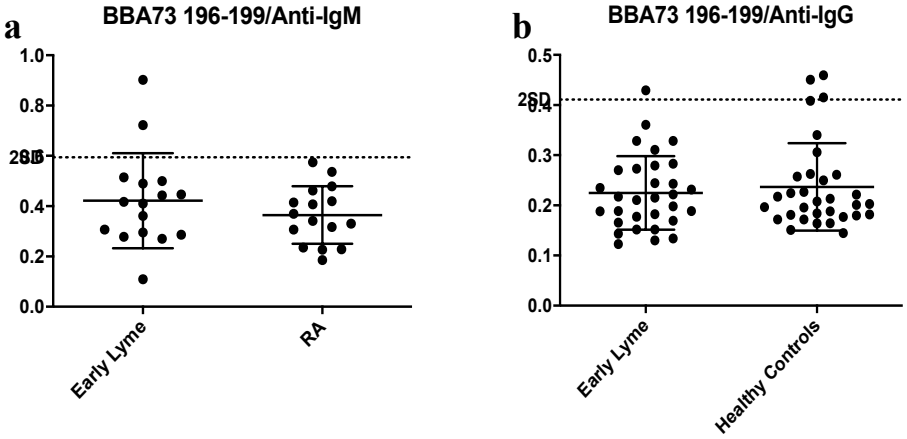


**Figure 3.4:** IgM (a) binding of BBA73 196-199 peptide in serum samples from patients with early Lyme disease (*n* = 16), rheumatoid arthritis (RA) (*n* = 16). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls. IgG (b) binding of BBA73 196-199 peptide in serum samples from patients with early Lyme disease (*n* = 31), healthy controls (*n* = 32). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls.

The four remaining (BBA64-7, BBA65-94, BBA65 101-02 and BBA66-117+) peptides had better performance characteristics and were further evaluated with a larger number of samples. BBA65 101-02 and BBA66-117+ peptides were evaluated as targets for the detection of both IgG and IgM binding in samples of early Lyme disease patient sera ($EM^+$ early Lyme and early disseminated Lyme samples), using the cutoff calculated from the mean absorbance of IgG or IgM binding in sera of healthy control samples (number of healthy control samples used for IgG and IgM is determined in the corresponding figure of each peptide, **Figure 3.5 and 3.6**). Positive cutoff values were determined as the mean values calculated plus 2X the standard deviation. IgM positive binding to both peptides was ultimately very poor, with BBA65 101-02 detecting antibodies in 14% (14/101) of early Lyme patient sera (**Figure 3.5a and Table 3.2**), and 1% (1/101) for BBA66-117+ (**Figure 3.6a and Table 3.2**). The IgG binding efficacy to BBA65 101-02 and BBA66-117+ peptides in patient sera with early Lyme disease was low, as the percentage of antibody detection was 10% (10/101) for BBA65 101-02 (**Figure 3.5b**), and 7% (7/101) for BBA66-117+ (**Figure 3.6b**).

**Table 3.2:** IgM screening of BBA65 101-02 and BBA66-117+ peptides

| Serum | No. of samples | No. of IgM-reactive[a] serum samples (%)[b] | |
|---|---|---|---|
| | | BBA65 101-02 | BBA66-117+ |
| Lyme disease | | | |
| Early Lyme disease[d] | 101 | 14 (14) | 1 (1) |
| Other diseases | | | |
| Rheumatoid Arthritis | 32 | 0 (0) | 0 (0) |
| Fibromyalgia | 14 | 0 (0) | 0 (0) |
| Healthy Controls | | | |
| Healthy; non-endemic[c] | 25 | 1 (4) | 2 (8) |

a Reactive samples, positive and equivocal (total of 3SD and 2SD from the mean of the healthy controls)
b Results are presented as number (percent) of samples testing positive
c Non-endemic, serum samples were collected from patients in regions where Lyme cases are not increasing constantly
d Early Lyme, erythema migrans positive and early disseminated Lyme samples added together

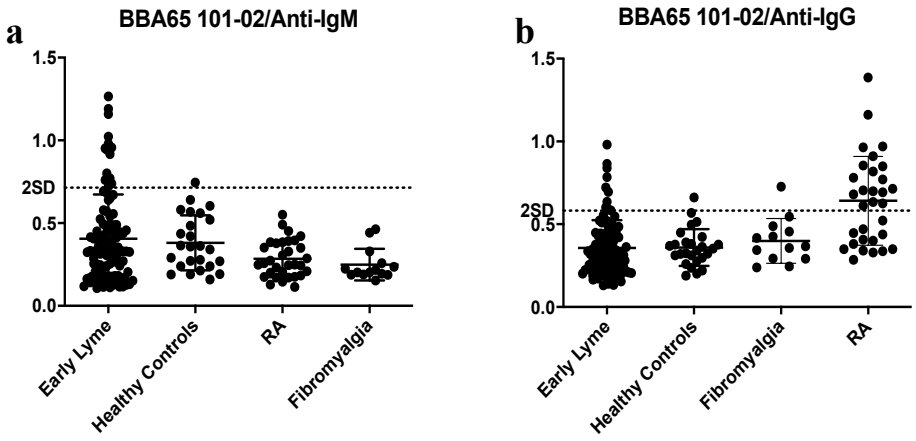


**Figure 3.5:** IgM (a) binding of BBA65 101-02 peptide in serum samples from patients with early Lyme disease (*n* = 101), healthy controls (*n* = 25), rheumatoid arthritis (RA) (*n* = 32), fibromyalgia (*n* = 14). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls. IgG (b) binding of BBA65 101-02 peptide in serum samples from patients with early Lyme disease (*n* = 101), healthy controls (*n* = 26), rheumatoid arthritis (RA) (*n* = 31), fibromyalgia (*n* = 13). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls.

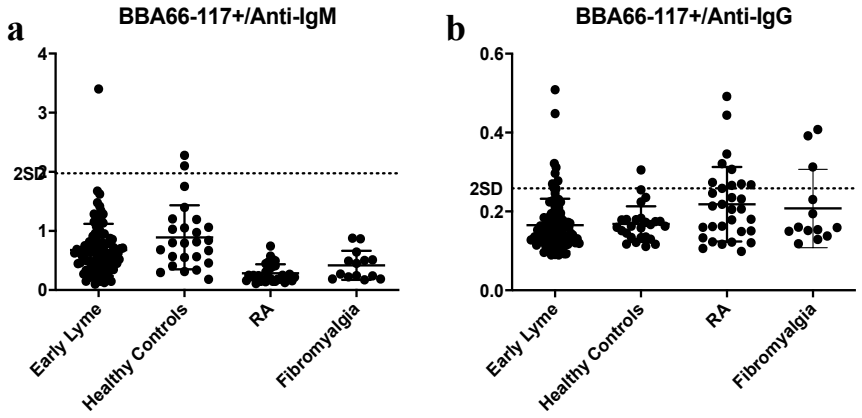


**Figure 3.6:** IgM (a) binding of BBA66-117+ peptide in serum samples from patients with early Lyme disease (*n* = 101), healthy controls (*n* = 25), rheumatoid arthritis (RA) (*n* = 32), fibromyalgia (*n* = 14). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls. IgG (b) binding of BBA66-117+ peptide in serum samples from patients with early Lyme disease (*n* = 101), healthy controls (*n* = 26), rheumatoid arthritis (RA) (*n* = 31), fibromyalgia (*n* = 13). The dotted lines represent the cutoff values for positive binding, 2SD from the mean of healthy controls.

BBA65 101-02 and BBA66-117+ peptides were excluded from the rest of this study because of the low IgM and IgG binding efficacy in early Lyme disease patient sera. Decisions to exclude peptides from further study were made to conserve limited quantities of sera available for study. The remaining BBA64-7, and BBA65-94 peptides were incubated with a larger number of samples, including serum from patients with early localized and disseminated Lyme disease ($EM^+$, and early neurologic Lyme disease), late Lyme disease with Lyme arthritis, syphilis ($RPR^+$), rheumatoid arthritis (RA), Fibromyalgia, and healthy individuals. The rapid plasma reagin $RPR^+$ sera were considered negative controls for possible cross-reactivity of antibodies against related bacteria, *Treponema pallidum*. RA sera were considered negative controls for chronic inflammation with high levels of antibody and joint damage. Fibromyalgia sera were used as negative controls for some non-specific symptoms common with LD. All controls sera used in the study were negative for Lyme disease by WB. Cutoff values were determined for each peptide using the mean absorbance of IgG or IgM binding in sera of healthy control (number of healthy control samples used for IgG and IgM is determined in the corresponding table of each peptide, **Table 3.3 and 3.4**), positive cutoff values were determined as the mean values calculated plus 2X the standard deviation. For BBA64-7 peptide, 31% (42/135) early Lyme samples were positive for IgM binding to the peptide (**Figure 3.7a and Table 3.3**). While for BBA65-94, 32% (42/131) early Lyme samples were positive for IgM binding to the peptide (**Figure 3.8a and Table 3.4**). The total number of early Lyme samples positive for IgM binding was high when the sera were incubated with either BBA64-7, or BBA65-94 peptides, and results (values of early Lyme compared to healthy controls, RA, fibromyalgia, and syphilis) from both peptides reached statistical significance with a *P* value of 0.0002, 0.0104, 0.0109, and 0.0021, respectively for BBA64-7, and 0.004, 0.0007, 0.0094, and 0.0004, respectively for BBA65-94 (*P*

<0.05, statistically significant). For BBA64-7 peptide, 1% (2/144) early Lyme samples were positive for IgG binding to the peptide (**Figure 3.7b and Table 3.3**), and 4% (5/132) early Lyme samples were positive for IgG binding to BBA65-94 peptide (**Figure 3.8b and Table 3.4**).

**Table 3.3:** IgM and IgG screening of BBA64-7 peptide

| Serum | No. of samples for IgM[c]/IgG[d] | No. of IgM-reactive[a] serum samples (%)[b] | No. of IgG-reactive[a] serum samples (%)[b] |
|---|---|---|---|
| Lyme disease | | | |
| Early Lyme disease[f] | 135/144 | 42 (31) | 2 (1) |
| Late Lyme disease[g] | 12/4 | 2 (17) | 0 (0) |
| Total Lyme disease | 147/148 | 44 (30) | 2 (1) |
| Other diseases | | | |
| Rheumatoid Arthritis | 33/11 | 3 (9) | 0 (0) |
| Fibromyalgia | 15/15 | 0 (0) | 0 (0) |
| Syphilis | 29/29 | 1 (3) | 0 (0) |
| Healthy Controls | | | |
| Healthy; non-endemic[e] | 41/61 | 1 (2) | 4 (7) |

a Reactive samples, positive and equivocal (total of 3SD and 2SD from the mean of the healthy controls)
b Results are presented as number (percent) of samples testing positive
c Total number of IgM samples
d Total number of IgG samples
e Non-endemic, serum samples were collected from patients in regions where Lyme cases are not increasing constantly
f Early Lyme, erythema migrans positive and early disseminated Lyme samples added together
g Late Lyme, Lyme arthritis

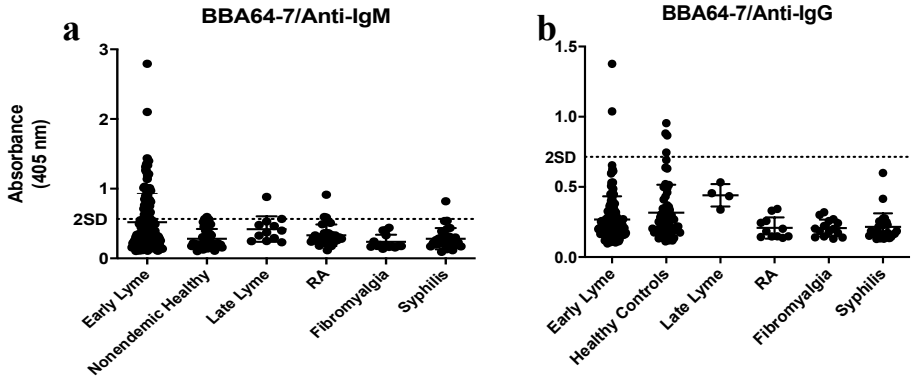


**Figure 3.7:** IgM (a) binding of BBA64-7 peptide in serum samples from patients with early Lyme disease (*n* = 135), late Lyme (n = 12), healthy controls (*n* = 41), rheumatoid arthritis (RA) (*n* = 33), fibromyalgia (*n* = 15), syphilis (*n* = 29). The dotted lines represent the cutoff for positive binding, 2SD (>0.56) from the mean of healthy controls, and negative (<0.56). IgG (b) binding of BBA64-7 peptide in serum samples from patients with early Lyme disease (*n* = 144), late Lyme (n = 4), healthy controls (*n* = 61), rheumatoid arthritis (RA) (*n* = 11), fibromyalgia (*n* = 15), syphilis (*n* = 29). The dotted lines represent the cutoff values for positive binding, 2SD (>0.71) from the mean of healthy controls, and negative (<0.71).

**Table 3.4:** IgM and IgG screening of BBA65-94 peptide

| Serum | No. of samples for IgM[c]/IgG[d] | No. of IgM-reactive[a] serum samples (%)[b] | No. of IgG-reactive[a] serum samples (%)[b] |
|---|---|---|---|
| Lyme disease | | | |
| Early Lyme disease[f] | 131/132 | 42 (32) | 5 (4) |
| Other diseases | | | |
| Rheumatoid Arthritis | 33/11 | 1 (3) | 0 (0) |
| Fibromyalgia | 15/15 | 0 (0) | 1 (7) |
| Syphilis | 29/29 | 0 (0) | 0 (0) |
| Healthy Controls | | | |
| Healthy; non-endemic[e] | 25/45 | 1 (4) | 2 (4) |

a Reactive samples, positive and equivocal (total of 3SD and 2SD from the mean of the healthy controls)
b Results are presented as number (percent) of samples testing positive
c Total number of IgM samples
d Total number of IgG samples
e Non-endemic, serum samples were collected from patients in regions where Lyme cases are not increasing constantly
f Early Lyme, erythema migrans positive and early disseminated Lyme samples added together

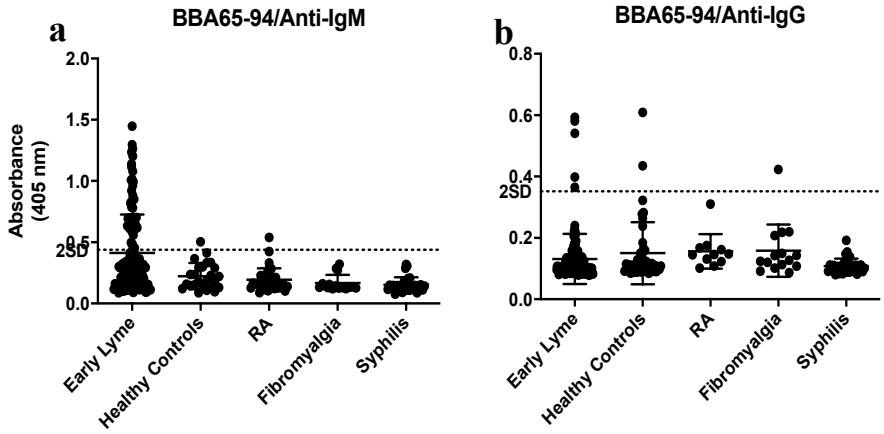


**Figure 3.8:** IgM (a) binding of BBA65-94 peptide in serum samples from patients with early Lyme disease (*n* = 131), healthy controls (*n* = 25), rheumatoid arthritis (RA) (*n* = 33), fibromyalgia (*n* = 15), syphilis (*n* = 29). The dotted lines represent the cutoff for positive binding, 2SD (>0.44) from the mean of healthy controls, and negative (<0.44). IgG (b) binding of BBA65-94 peptide in serum samples from patients with early Lyme disease (*n* = 132), healthy controls (*n* = 45), rheumatoid arthritis (RA) (*n* = 11), fibromyalgia (*n* = 15), syphilis (*n* = 29). The dotted lines represent the cutoff values for positive binding, 2SD (>0.35) from the mean of healthy controls, and negative (<0.35).

Receiver-operating-characteristic (ROC) analysis was used to test the diagnostic ability of the peptides (BBA64-7 and BBA65-94) analyzed. IgM binding to peptides in early Lyme disease sera was compared to binding of all negative control samples. The area under the curve (AUC) for (healthy controls, RA, Fibromyalgia, and syphilis) were 0.6934, 0.6186, 0.7600, and 0.6973, respectively for BBA64-7, and 0.6834, 0.7398, 0.7837, and 0.8192, respectively for BBA65-94. At the cutoff we calculated for BBA64-7 peptide positivity, the sensitivity and specificity determined from the ROC analysis curve were 31.11% and 95.12%, respectively for healthy controls, 31.11% and 90.91%, respectively for RA, 31.11% and 100%, respectively for fibromyalgia, and 31.11% and 96.55%, respectively for syphilis samples (**Figure 3.13**). For BBA65-94 peptide, the sensitivity and specificity determined from the ROC analysis curve were 31.30% and 96%, respectively for healthy controls, 31.30% and 96.97%, respectively for RA, 31.30% and 100%, respectively for fibromyalgia, and 31.30% and 100%, respectively for syphilis samples (**Figure 3.14**).

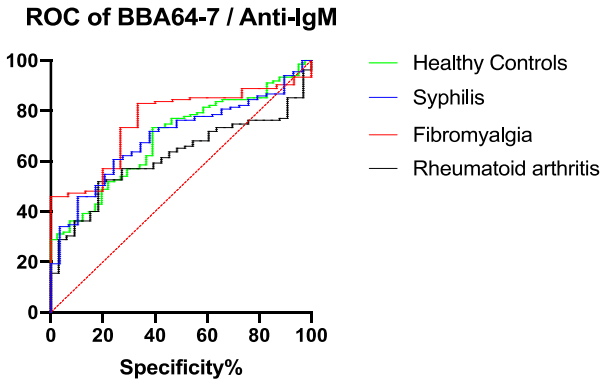


**Figure 3.13:** ROC curve for IgM binding to BBA64-7 peptide in early Lyme disease vs binding of all negative controls.

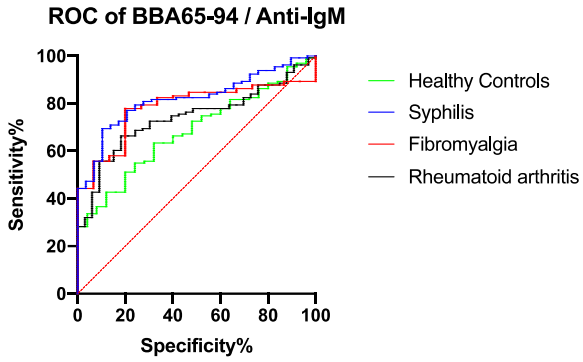


**Figure 3.14:** ROC curve for IgM binding to BBA65-94 peptide in early Lyme disease vs binding of all negative controls.

### 3.3 Peptide results of patient samples compared to reference samples

BBA64-7, and BBA65-94 peptides showed consistent moderate seropositivity for IgM in early Lyme patients samples. BBA64-7, and BBA65-94 were evaluated in duplicate for both IgG and IgM antibodies against a blinded panel of 31 serum samples (12 early-stage LD, 5 diseased controls, 10 non-endemic controls, and 4 endemic controls) collected by the CDC and provided to investigators for diagnostic assay development, using the cutoff values calculated from the mean absorbance of each peptide panel of patients serum of either IgG or IgM binding in sera of healthy control (number of healthy control samples used for IgG and IgM is determined in the table of each peptide, **Table 3.3 and 3.4**). Positive cutoff values were determined as the mean values calculated plus 2X the standard deviation. 50% (6/12) $EM^{+}$ early Lyme samples were positive for IgM binding to BBA64-7 peptide (**Figure 3.9a and Table 3.5**), and 83% (10/12) for BBA65-94 peptide (**Figure 3.10a and Table 3.5**). BBA64-7 IgM results (values of early Lyme compared to diseased controls) were statistically significant with a *P* value of 0.0493, and BBA65-94 IgM results (values of early Lyme compared to healthy non-endemic controls, diseased controls, and healthy endemic controls) were statistically significant with a *P*

value of 0.0113, 0.0015, and 0.0293, respectively. The positive binding of IgG in ($EM^+$) early Lyme patient sera to BBA64-7 peptide was 0% (0/12) (**Figure 3.9b and Table 3.5**), and 8% (1/12) for BBA65-94 (**Figure 3.10b and Table 3.5**).

**Table 3.5:** Blinded samples testing results for CDC LD samples

| CDC Serum | No. of samples | No. of IgM-reactive[a] serum samples (%)[b] | | No. of IgG-reactive[a] serum samples (%)[b] | |
|---|---|---|---|---|---|
| | | BBA64-7 | BBA65-94 | BBA64-7 | BBA65-94 |
| Lyme disease | | | | | |
| Early Lyme disease[e] | 12 | 6 (50) | 10 (83) | 0 (0) | 1 (8) |
| Diseased Controls[f] | 5 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Healthy Controls | | | | | |
| Healthy; non-endemic[c] | 10 | 2 (20) | 3 (30) | 0 (0) | 1 (10) |
| Healthy; endemic[d] | 4 | 1 (25) | 1 (25) | 0 (0) | 0 (0) |

a Reactive samples, positive and equivocal (total of 3SD and 2SD from the mean of the healthy controls)
b Results are presented as number (percent) of samples testing positive
c Non-endemic, serum samples were collected from patients in areas where Lyme cases are not increasing constantly.
d Endemic, serum samples were collected from patients in areas where Lyme cases are increasing constantly
e Early Lyme, erythema migrans positive
f Diseased controls, RA, syphilis, and fibromyalgia samples

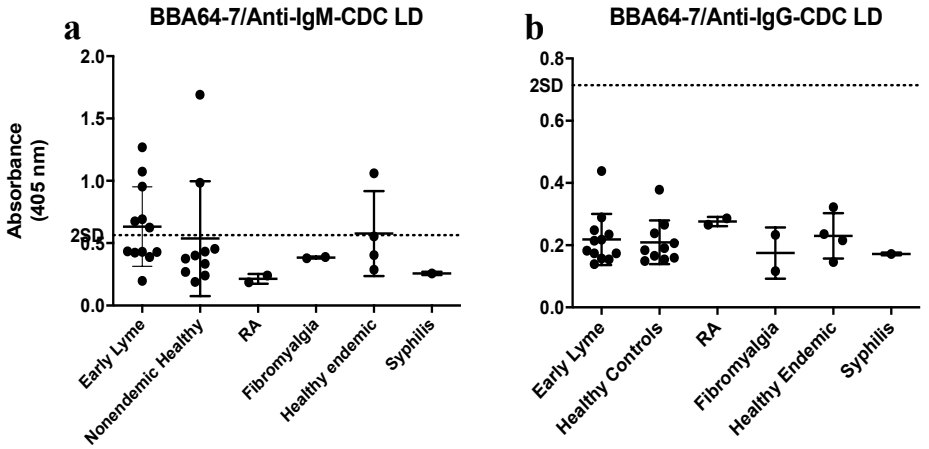


**Figure 3.9:** IgM (a) binding of BBA64-7 peptide in serum samples from CDC LD, early Lyme disease ($n$ = 12), healthy non-endemic controls ($n$ = 10), diseased controls ($n$ = 5), endemic controls ($n$ = 4). The dotted lines represent the cutoff for positive binding, 2SD (>0.564) from the mean of healthy controls of BBA64-7 IgM binding main panel, and negative (<0.564). IgG (b) binding of BBA64-7 peptide in serum samples from CDC LD, early Lyme disease ($n$ = 12), healthy non-endemic controls ($n$ = 10), diseased controls ($n$ = 5), endemic controls ($n$ = 4). The dotted lines represent the cutoff values for positive binding, 2SD (>0. 714) from the mean of healthy controls of BBA64-7 IgG binding main panel, and negative (<0.714).

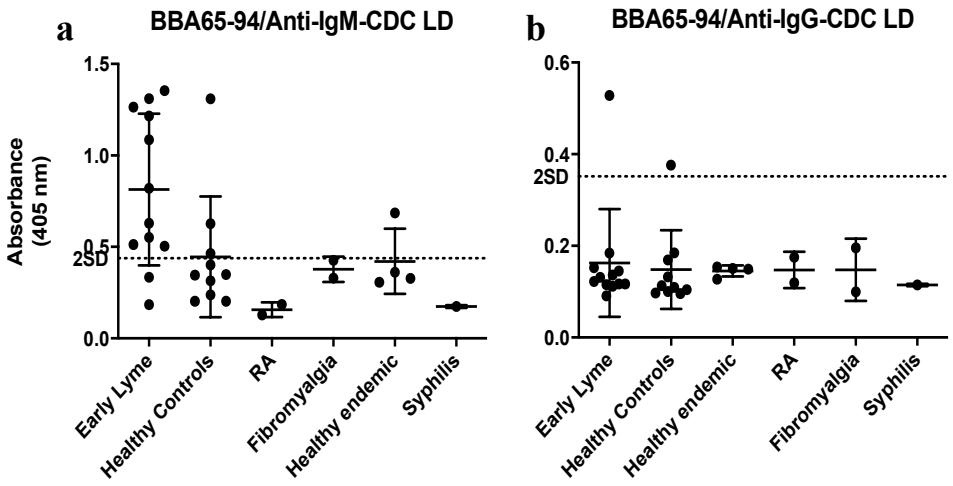


**Figure 3.10:** IgM (a) binding of BBA65-94 peptide in serum samples from CDC LD, early Lyme disease (*n* = 12), healthy non-endemic controls (*n* = 10), diseased controls (*n* = 5), endemic controls (*n* = 4). The dotted lines represent the cutoff for positive binding, 2SD (>0.4381) from the mean of healthy controls of BBA65-94 IgM binding main panel, and negative (<0.4381). IgG (b) binding of BBA65-94 peptide in serum samples from CDC LD, early Lyme disease (*n* = 12), healthy non-endemic controls (*n* = 10), diseased controls (*n* = 5), endemic controls (*n* = 4). The dotted lines represent the cutoff values for positive binding, 2SD (>0.3516) from the mean of healthy controls of BBA65-94 IgG binding main panel, and negative (<0.3516).

Both peptides were also evaluated through testing of a panel consisting of an additional 118 human serum samples (25 probable Lyme, 25 confirmed Lyme, 43 control (+ve), and 25 control (-ve)) obtained by the LD Biobank samples. Cutoff values of LD Biobank samples were determined for each peptide using the mean absorbance of IgG or IgM binding in sera of 25 control (-ve) samples, and positive cutoff values were determined as the mean values calculated plus 2X the standard deviation (**Table 3.7**). When unblinded the LDBB samples consisted of 25 probable Lyme samples, 25 confirmed Lyme samples, 43 false positive control samples (positive by one or more standard serological assays), and 25 true negative control samples, 8% (2/25) probable Lyme samples, 20% (5/25) confirmed Lyme samples, 9% (4/43) control (+ve) samples, and 8% (2/25) control (-ve) samples were positive for IgM binding to BBA64-7 peptide (**Figure 3.11a and Table 3.7**). For IgM binding to BBA65-94 peptide in LDBB samples, 0% (0/25) probable Lyme

samples, 16% (4/25) confirmed Lyme samples, 5% (2/43) control (+ve) samples, and 8% (2/25) control (-ve) samples showed seropositivity (**Figure 3.12a and Table 3.7**). Both BBA64-7 and BBA65-94 IgM results (values of confirmed Lyme compared to control (+ve), and control (-ve)) showed no statistical significance with a *P* value <0.05. The positive binding of IgG to BBA64-7 peptide in LD Biobank samples were 0% (0/25) in probable Lyme samples, 4% (1/25) in confirmed Lyme samples, 9% (4/43) in control (+ve) samples, and 4% (1/25) in control (-ve) samples (**Figure 3.11b and Table 3.7**). For IgG binding to BBA65-94 peptide in LD Biobank samples, 4% (1/25) probable Lyme samples, 20% (5/25) confirmed Lyme samples, 19% (8/43) control (+ve) samples, and 4% (1/25) control (-ve) samples showed seropositivity (**Figure 3.12b and Table 3.7**).

**Table 3.7:** Blinded samples testing results for Lyme disease Biobank samples

| BioBank Serum | No. of samples | No. of IgM-reactive serum samples (%)[a] | | No. of IgG-reactive serum samples (%)[a] | |
|---|---|---|---|---|---|
| | | BBA64-7 | BBA65-94 | BBA64-7 | BBA65-94 |
| Probable Lyme disease[b] | 25 | 2 (8) | 0 (0) | 0 (0) | 1 (4) |
| Confirmed Lyme disease[c] | 25 | 5 (20) | 4 (16) | 1 (4) | 5 (20) |
| Controls | | | | | |
| Control (+ve)[d] | 43 | 4 (9) | 2 (5) | 4 (9) | 8 (19) |
| Control (-ve)[e] | 25 | 2 (8) | 2 (8) | 1 (4) | 1 (4) |

a Results are presented as number (percent) of samples testing positive
b Probable Lyme, physician-diagnosed Lyme disease that has laboratory evidence of infection
c Confirmed Lyme, $EM^{+}$ with laboratory evidence of infection, or at least one late manifestation that has laboratory evidence of infection.
d Control (+ve), serum samples with antibody cross-reactivity
e Control (-ve), no cross-reactive antibodies

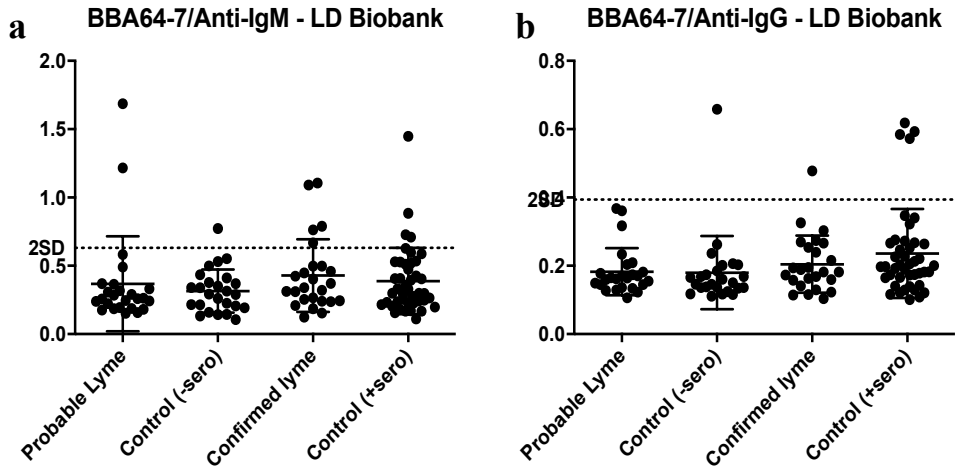


**Figure 3.11:** IgM (a) binding of BBA64-7 peptide in serum samples from LD Biobank, probable Lyme (*n* = 25), confirmed Lyme (*n* = 25), control +ve (*n* = 43), control -ve (*n* = 25). The dotted lines represent the cutoff for positive binding, 2SD (>0.6304) from the mean of healthy controls, and negative (<0.6304). IgG (b) binding of BBA64-7 peptide in serum samples from LD Biobank, probable Lyme (*n* = 25), confirmed Lyme (*n* = 25), control +ve (*n* = 43), control -ve (*n* = 25). The dotted lines represent the cutoff values for positive binding, 2SD (>0.3937) from the mean of healthy controls, and negative (<0.3937).

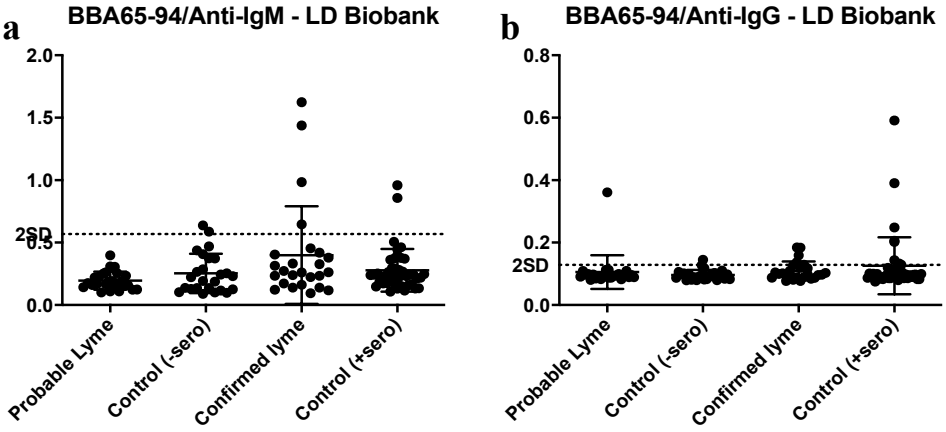


**Figure 3.12:** IgM (a) binding of BBA65-94 peptide in serum samples from LD Biobank, probable Lyme (*n* = 25), confirmed Lyme (*n* = 25), control +ve (*n* = 43), control -ve (*n* = 25). The dotted lines represent the cutoff for positive binding, 2SD (>0.5676) from the mean of healthy controls, and negative (<0.5676). IgG (b) binding of BBA65-94 peptide in serum samples from LD Biobank, probable Lyme (*n* = 25), confirmed Lyme (*n* = 25), control +ve (*n* = 43), control -ve (*n* = 25). The dotted lines represent the cutoff values for positive binding, 2SD (>0.12826) from the mean of healthy controls, and negative (<0.12826).

**Table 3.6:** Conventional testing results for CDC Lyme Disease Samples

| CDC Serum (total)[b] | EIA Interpretation Pos (%)[a] | Western blot IgM (%)[a] | | Western blot IgG (%)[a] | | Two-tier Positive (%)[a] |
|---|---|---|---|---|---|---|
| | | Pos | Neg | Pos | Neg | |
| Early Lyme[c] (12) | 8 (67) | 7 (58) | 5 (42) | 5 (42) | 7 (58) | 8 (67) |
| Diseased controls[d] (5) | 0 (0) | 0 (0) | 5 (100) | 0 (0) | 5 (100) | 0 (0) |
| Healthy, non-endemic[e] (10) | 2 (20) | 0 (0) | 10 (100) | 0 (0) | 10 (100) | 0 (0) |
| Healthy, endemic[f] (4) | 1 (25) | 0 (0) | 4 (100) | 0 (0) | 4 (100) | 0 (0) |

a Results are presented as number (percent) of samples testing positive or negative
b Total number of samples in each group
c Early Lyme, erythema migrans positive
d Diseased controls, RA, syphilis, and fibromyalgia samples
e Non-endemic, serum samples were collected from patients in areas where Lyme cases are not increasing constantly.
f Endemic, serum samples were collected from patients in areas where Lyme cases are increasing constantly

**Table 3.8:** Conventional testing results for Lyme Disease Biobank Samples

| BioBank Serum (total)[b] | Western blot IgM (%)[a] | | Western blot IgG(%)[a] | | Two-tier Positive | Bb[g] Culture | Bb Culture + PCR | Bb PCR |
|---|---|---|---|---|---|---|---|---|
| | Pos | Neg | Pos | Neg | | | | |
| Probable[c] LD (25) | 2 (8) | 16 (64) | 0 (0) | 14 (56) | No (25) | Pos=0 | Pos=0 | Pos=0 |
| Confirmed[d] LD (25) | 17 (68) | 3 (12) | 5 (20) | 9 (36) | Yes (19) | Pos=1 | Pos=1, Equiv=1 | Pos=1 |
| | | | | | No (6) | | | |
| Control +ve[e] (43) | 13 (30) | 14 (33) | 4 (9) | 20 (47) | Yes (6) | Pos=0 | Equiv=1 | Pos=0 |
| | | | | | No (37) | | | |
| Control -ve[f] (25) | 0 (0) | 19 (76) | 0 (0) | 16 (64) | No (25) | Pos=0 | Pos=0 | Pos=0 |

a Results are presented as number (percent) of samples testing either positive or negative, the rest of samples were reported as IT
b Total number of samples in each group
c Probable Lyme, physician-diagnosed Lyme disease that has laboratory evidence of infection
d Confirmed Lyme, $EM^{+}$ with laboratory evidence of infection, or at least one late manifestation that has laboratory evidence of infection.
e Control (+ve), serum samples with antibody cross-reactivity
f Control (-ve), no cross-reactive antibodies
g Bb, *Borrelia burgdorferi*
*Results for PCR and culture other than Pos=positive and Equiv=equivocal, were reported as NA=Not-applicable, Neg=negative, No, and Yes

# CHAPTER FOUR
# DISCUSSION

### 4.1 Results interpretation

Peptides were first screened against a bank of Lyme disease serum that had been obtained by our lab over a large number of years. This consisted of a number of samples from different stages of disease, and from different regions. We initially screened peptides against a subset of these sera for potential sensitivity. Those that produced intriguing results were continued with additional sera, and were evaluated again. In the early results of peptides testing, a smaller number of normal controls was used to calculate cutoffs and a smaller number of Lyme disease samples showed better results. However, as more healthy controls were run through the study, a greater variability was shown. This had led to an increased SD, which increased the cutoff, lowering sensitivity of the peptide for positive detection. The positivity in negative control samples might be due to cross-reactivity of antibodies with similar antigens. In the end, only two of the peptides were screened against the full sample set. The results of the sensitivity and specificity obtained for BBA64-7 peptide which were, 31.11% and 95.12%, respectively for healthy controls, 31.11% and 90.91%, respectively for RA, 31.11% and 100%, respectively for fibromyalgia, and 31.11% and 96.55%, respectively for syphilis samples (**Figure 3.13**), and for BBA65-94 peptide were, 31.30% and 96%, respectively for healthy controls, 31.30% and 96.97%, respectively for RA, 31.30% and 100%, respectively for fibromyalgia, and 31.30% and 100%, respectively for syphilis samples (**Figure 3.14**) suggested a moderate sensitivity and decent specificity for IgM binding in Lyme patient serum, but not IgG. This could indicate that these antigens are rapidly downregulated following transmission and do not persist for long enough to generate an IgG response. Additionally, this could be the result of early treatment, which can sometimes prevent continued antigen expression and seroconversion. It could also be a combination of the two, and additional as of yet unknown factors. ROC analysis demonstrated that BBA65-

94 was a moderately better antigen than BBA64-7 at distinguishing Lyme disease patients from control samples, with AUCs of generally 0.7 or better. An AUC of 1.0 would be found in an assay with 100% sensitivity and 100% specificity.

Following analysis of antibody binding to our in-house serum panel, we evaluated binding in two panels that were received blinded from the CDC and the Bay Area Lyme Foundation, Lyme Disease biobank. First evaluating binding against the CDC panel. Early Lyme sample results for conventional testing results of CDC LD samples showed 67% (8/12) positivity in EIA, 58% (7/12) in IgM WB, 42% (5/12) in IgG WB, and 67% (8/12) in two-tier testing (**Table 3.6**). When compared to the unblinded EIA and IgM WB results from the CDC LD samples, both BBA64-7 and BBA65-94 peptides showed high seropositivity against IgM antibody in early Lyme samples with 50%, and 83% positivity, respectively. Therefore, for one peptide we saw superior sensitivity compared to conventional 2-tier testing. Results for the LDBB samples were less promising. However, it represents a more 'challenging' sample set. Probable Lyme sample results for conventional testing results of LD Biobank samples showed 8% (2/25) positivity in IgM WB, 0% (0/25) in IgG WB, and no positivity in two-tier testing. Confirmed Lyme samples demonstrated 68% (17/25) positivity in IgM WB, 20% (5/25) in IgG WB, and 76% (19/25) positive two-tier test. Control (+ve) samples resulted in 30% (13/43) positivity in IgM WB, 9% (4/43) in IgG WB, and 14% (6/43) positive two-tier test. Control (-ve) samples had 0% (0/25) positivity in IgM WB, 0% (0/25) in IgG WB, and no positive two-tier test (**Table 3.8**). In comparison to the unblinded 68% positivity of 25 confirmed Lyme samples in IgM WB, BBA64-7 had 20%, and BBA65-94 had only 16% positivity. Both peptides did not show high seropositivity against IgM in LD Biobank confirmed Lyme sera. Variation in positivity is likely due to differences in antibody specificity for different antigens.

### 4.2 Study limitations and future work

Though clearly not sensitive enough for use as peptide antigens in a single antigen assay, the initial results for both antibody binding and ROC analysis suggest that BBA64-7 and BBA65-94 peptides were promising results as targets for the detection of IgM antibody in early Lyme disease sera. There is potential for these antigens to have value as a component of a multiantigen, possibly multiplex assay as diagnostic targets in Lyme serologic assays, particularly in North America where *B. burgdorferi sensu stricto* is the predominant species causing Lyme disease.

# REFERENCES

**Branda JA, Strle K, Nigrovic LE, Lantos PM, Lepore TJ, Damle NS, Ferraro MJ, Steere AC (2017).** Evaluation of Modified 2-Tiered Serodiagnostic Testing Algorithms for Early Lyme Disease. *Clinical Infectious Diseases*. 64(8):1074–1080.

**Brisson D, Drecktrah D, Eggers CH, Samuels DS (2012).** Genetics of *Borrelia burgdorferi*. *Annu Rev Genet*. 46:515-536.

**Centers for Disease Control and Prevention (CDC) (1995).** Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Dis. *MMWR Morb. Mortal. Wkly. Rep.* 44:590–591.

**Crippa M, Rais O, Gern L (2002).** Investigations on the Mode and Dynamics of Transmission and Infectivity of *Borrelia burgdorferi* Sensu Stricto and *Borrelia afzelii* in *Ixodes ricinus* Ticks. *Vector Borne Zoonotic Dis*. 2(1):3-9.

**Dandage R, Landry CR (2019).** Paralog dependency indirectly affects the robustness of human cells. *Mol Syst Biol*. 15:e8871

**Dworkin MS, Schwan TG, Anderson DE Jr, Borchardt SM (2008).** Tick-borne relapsing fever. *Infect Dis Clin North Am*. 22(3):449-468, viii.

**Fung BP, McHugh GL, Leong JM, Steere AC (1994).** Humoral immune response to outer surface protein C of Borrelia burgdorferi in Lyme disease: role of the immunoglobulin M response in the serodiagnosis of early infection. *Infection and Immunity*. 62(8) 3213-3221.

**Gern L (2008).** Borrelia burgdorferi *sensu lato*, the agent of Lyme borreliosis: life in the wilds. *Parasite*. 15(3):244-247.

**Gilmore RD, Howison RR, Schmit VL, Nowalk AJ, Clifton DR, Nolder C, Hughes JL, Carroll JA (2007).** Temporal Expression Analysis of the *Borrelia burgdorferi* Paralogous Gene Family 54 Genes BBA64, BBA65, and BBA66 during Persistent Infection in Mice. *Infection and Immunity*. 75 (6) 2753-2764.

**Hatchette TF, Davis I, Johnston BL (2014).** Lyme disease: clinical diagnosis and treatment. *Can Commun Dis Rep*. 40(11):194-208.

**Hsiao TL, Vitkup D (2008).** Role of duplicate genes in robustness against deleterious human mutations. *PLoS Genet*. 4(3):e1000014.

**Institute of Medicine (US) Committee on Lyme Disease and Other Tick-Borne Diseases: The State of the Science (2011).** Critical Needs and Gaps in Understanding Prevention, Amelioration, and Resolution of Lyme and Other Tick-Borne Diseases: The Short-Term and Long-Term Outcomes: Workshop Report. *Washington (DC): National Academies Press (US)*. 6, Pathogenesis.

**Johnson RC and Hughes CA (1992).** The Genus *Borrelia*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH. (eds) The Prokaryotes. (pp 3560-3567). Springer, New York, NY.

**Johnson BJ (2011)**. Laboratory diagnostic testing for Borrelia burgdorferi Infection. In: Halperin JJ. (ed) Lyme disease: an Evidence-based Approach. CAB international

**Joung H, Ballard ZS, Wu J, Tseng DK, Teshome H, Zhang L, Horn EJ, Arnaboldi PM, Dattwyler RJ, Garner OB, Carlo DD, and Ozcan A (2020).** Point-of-Care Serodiagnostic Test for Early-Stage Lyme Disease Using a Multiplexed Paper-Based Immunoassay and Machine Learning. *ACS Nano.* 14:229−240.

**Kouzmitcheva GA, Petrenko VA, Smith GP (2001).** Identifying diagnostic peptides for Lyme disease through epitope discovery. *Clin Diagn Lab Immunol*. 8(1):150-160.

**Lipsett SC, Branda JA, Nigrovic LE (2019).** Evaluation of the Modified Two-Tiered Testing Method for Diagnosis of Lyme Disease in Children. *Journal of Clinical Microbiology.* 57(10): e00547-19.

**Marques AR (2015).** Laboratory diagnosis of Lyme disease: advances and challenges. *Infect Dis Clin North Am*. 29(2):295-307.

**Mead P, Petersen J, Hinckley A (2019).** Updated CDC Recommendation For Serologic Diagnosis of Lyme Disease. *CDC*. 68(32):703.

**Murray TS, Shapiro ED (2010).** Lyme disease. *Clin Lab Med*. 30(1):311-328.

**Petnicki-Ocwieja T, and Kern A (2014).** Mechanisms of *Borrelia burgdorferi* internalization and intracellular innate immune signaling. *Front. Cell. Infect. Microbiol.* 4, 175.

**Petzke M, Schwartz I (2015).** Borrelia burgdorferi Pathogenesis and the Immune Response. *Clinics in Laboratory Medicine. 35(4), 745–764.*

**Radolf JD, Strle K, Lemieux JE, and Strle F (2021).** Lyme Disease in Humans. *Curr Issues Mol Biol*. 42:333-384.

**Ramamoorthy R, Philipp MT (1998).** Differential Expression of *Borrelia burgdorferi* Proteins during Growth In Vitro. *Infection and Immunity*. 66(11) 5119-5124.

**Ramamoorthy R, Scholl-Meeker D (2001).** Borrelia burgdorferi proteins whose expression is similarly affected by culture temperature and pH. *Infect Immun*. 69(4):2739-2742.

**Schwan TG, Piesman J, Golde WT, Dolan MC, and Rosa PA (1995).** Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. *Proc Natl Acad Sci USA.* 92(7): 2909–2913.

**Schwan TG, Piesman J (2000).** Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, Borrelia burgdorferi, during the chain of infection in ticks and mice. *J Clin Microbiol*. 38(1):382-388.

**Tilly K, Rosa PA, Stewart PE (2008).** Biology of Infection with *Borrelia burgdorferi*. *Infect Dis Clin North Am*. 22(2):217-234.

**Todar K, and University of Wisconsin--Madison (2006).** *Borrelia burgdorferi* and Lyme Disease. *Todar's Online textbook of bacteriology*. Madison, WI: Kenneth Todar, University of Wisconsin-Madison Dept. of Bacteriology.

**Weiner ZP, Crew RM, Brandt KS, Ullmann AJ, Schriefer ME, Molins CR, Gilmore RD (2015).** Evaluation of Selected *Borrelia burgdorferi* lp54 Plasmid-Encoded Gene Products Expressed during Mammalian Infection as Antigens To Improve Serodiagnostic Testing for Early Lyme Disease. *Clin Vaccine Immunol*. 22:1176 –1186.

**Yu Z, Carter JM, Sigal LH, and Stein S (1996).** Multi-well ELISA based on Independent peptide antigens for antibody capture Application to Lyme disease serodiagnosis. *Journal of Immunological Methods*. 198(1), 25–33.