CORRELATING BORRELIA BURGDORFERI PLASMIDS WITH SURVIVAL IN DIFFERENT ANIMAL SERA

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CORRELATING *BORRELIA BURGDORFERI* PLASMIDS WITH SURVIVAL IN DIFFERENT ANIMAL SERA.

A Thesis

Presented to the College of Graduate Studies

In Partial Fulfillment of the Requirements for

Master's Degree

Jillian Kay

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I am submitting herewith a thesis written by Jillian B. Kay entitled "CORRELATING *BORRELIA BURGDORFERI* PLASMIDS WITH SURVIVAL IN DIFFERENT ANIMAL SERA". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Dr. Chad S. Brooks, Major Professor

We have read this thesis
and recommend its acceptance:

Dr. Don C. Dailey, Second Committee Member

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Acceptance for the Council:

Dean of the Graduate School

8-20-09

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Abstract

Borrelia burgdorferi is well known to the medical community as the etiological agent of Lyme disease and it also well known to the research community as being a unique and complex spirochete. *B. burgdorferi* is maintained in nature by a complex enzootic cycle which is reliant on *Ixodes* ticks and many different animal hosts. Adding to the complexity of *B. burgdorferi* is a multi-component genome consisting of a linear chromosome as well as up to twenty one different extra-chromosomal elements; twelve of which are linear and nine are circular in structure. In fact, *B. burgdorferi* has the most extra-chromosomal plasmids of any prokaryotic organism known. This thesis hypothesizes a dependent relationship between the complex enzootic cycle of *B. burgdorferi* and the maintenance of its complex genome. Data described herein supports that specific, but unknown, animal factors induce retention of specific plasmids in a host-dependent manner. These data indicate that specific plasmids have possible necessary role(s) during the infection of particular animal species. In particular, the linear plasmid of 17 kilobases in length (lp17) appears to be necessary in all animals examined. The data generated in this research supports the hypothesis that *B. burgdorferi* plasmids are maintained in an animal host-dependent manner and lays the ground work to focus on more laborious gene-by-gene studies to better understand the biology of *B. burgdorferi*.

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Introduction

Overview of Lyme Disease

Lyme disease is an illness caused by the pathogenic spirochete *Borrelia burgdorferi* which has a broad range of effects on the infected human host. The disease sequelea include minimal symptoms such as acute arthritis or more serious disease states such as cardiac infarction (24,30,37,44,55). The importance of understanding how *B. burgdorferi* infects and lives in each host is part of finding a relief to its epidemic.

Lyme disease has been found all over the world, including numerous locations in the United States (10,18,34,35,40). Currently, Lyme disease is the most prevalent tick-borne disease in the United States (46). However, humans are considered an accidental host for the bacterium and not part of its life cycle. Therefore, *B. burgdorferi* must infect several other animals then return to the tick vector to be maintained in nature. It appears that *B. burgdorferi* is able to infect a seemingly large array of other animal hosts including various mammals, birds, and reptiles (48).

The phases of Lyme disease infection can be categorized in three different stages: early localized, early disseminated and late or chronic disease (44). The early localized stage is coupled with the appearance of erythema migrans, or EM, (Figure 1) since there are no other early methods to distinguish the disease without blood tests. EM is a rash that occurs around the site of the

tick bite which usually resembles a bull's-eye, and is commonly referred to as a bull's-eye rash. The actual redness of the rash is caused by the immune response as the spirochetes begin disseminating through the tissue of the host. Concurrent with EM is the most common symptoms of the early localized stage of *B. burgdorferi* infection resembling those of flu-like symptoms, which lead to a higher risk of misdiagnosis (44).

The early disseminated phase is associated with multiple EM rashes. As well, there is a continuation of flu-like symptoms with the possibility of acute neurological problems in addition to the onset of the arthritis-like symptoms including joint and muscle pain (38). Acute cardiac problems have also been observed to include blockage of the blood in arteries, which can result in palpitations and dizziness (45).

After many months of untreated Lyme disease, also known as Lyme borreliosis, the late persistent infection stage begins. This stage is associated with the impairment of many organs and organ systems such as the nervous system, to include the brain and nerves, as well as the eyes, joints and the heart (43). By far, the most common symptom of chronic infection is Lyme arthritis, however in some rare cases, death can occur (41).

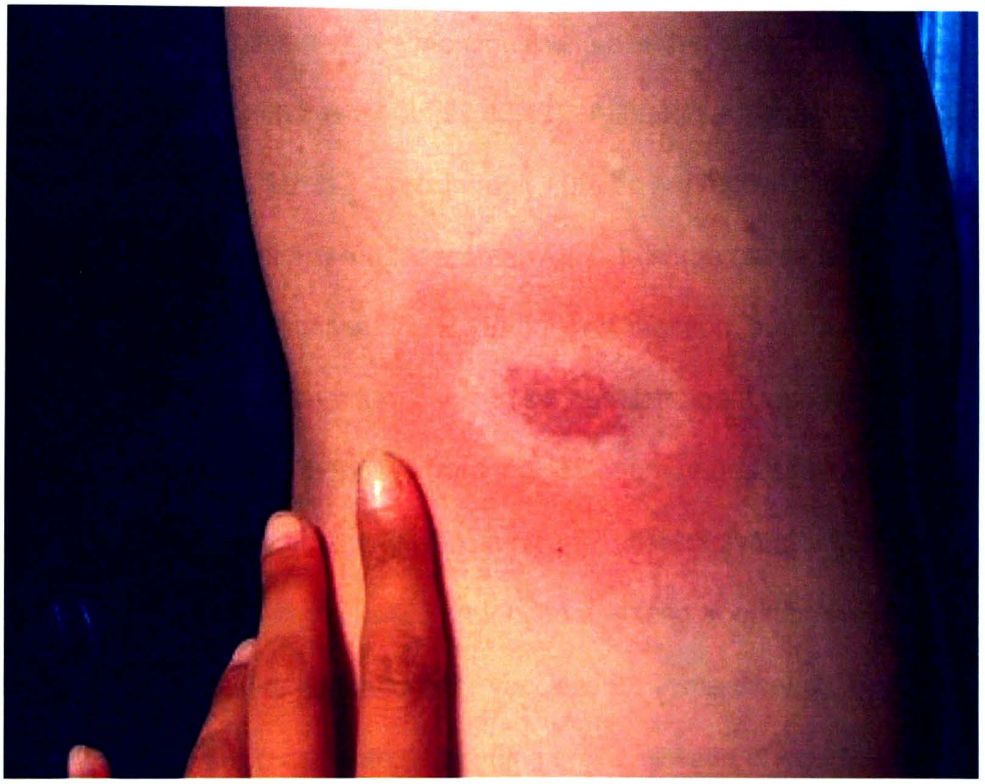


Figure 1. An illustration of the erythema migrans, or EM rash, also known as the bull's eye rash.

Treatment

Treatment for those with Lyme disease infection today is usually an extensive antibiotic treatment regime, consisting of either doxycycline, amoxicillin, or cefuroxime axetil (25,29,43,47). Although antibiotic treatment is successful for most patients, approximately 10% do not recover from the infection and the symptoms tend to worsen (12). In some cases the antibiotics are able to effectively eliminate the *B. burgdorferi* infection, but it is usually not in time to prevent the infection from causing serious damage. Despite being cleared of infection, the immune response from the bacterium appears to causes tissue damage in the infected host, and while new and different symptoms may stop appearing, the other symptoms are prolonged and tend to continue (12). Unfortunately, there are no marketable vaccines for Lyme disease, but on the bright side, there are many patents pending on the release of new therapeutics.

Life Cycle

The enzootic life cycle of *B. burgdorferi* is inextricably combined with the life cycle of the *Ixodes* tick, illustrated in figure 2 (20). Upon the larval tick's, also known as a seed tick's, first blood meal from an infected host, *B. burgdorferi* is taken into the midgut of the tick (46). The transcription and translation of *B. burgdorferi* DNA has been shown to drastically change during this period; presumably to prepare for infection of the next mammalian host (3,31). While the next host could be another wild animal, it could also be a hiker or camper in the

woods. It is important to recognize that only after a tick's first blood meal from an infected host that humans and other animal hosts can then become infected by an infected tick bite.

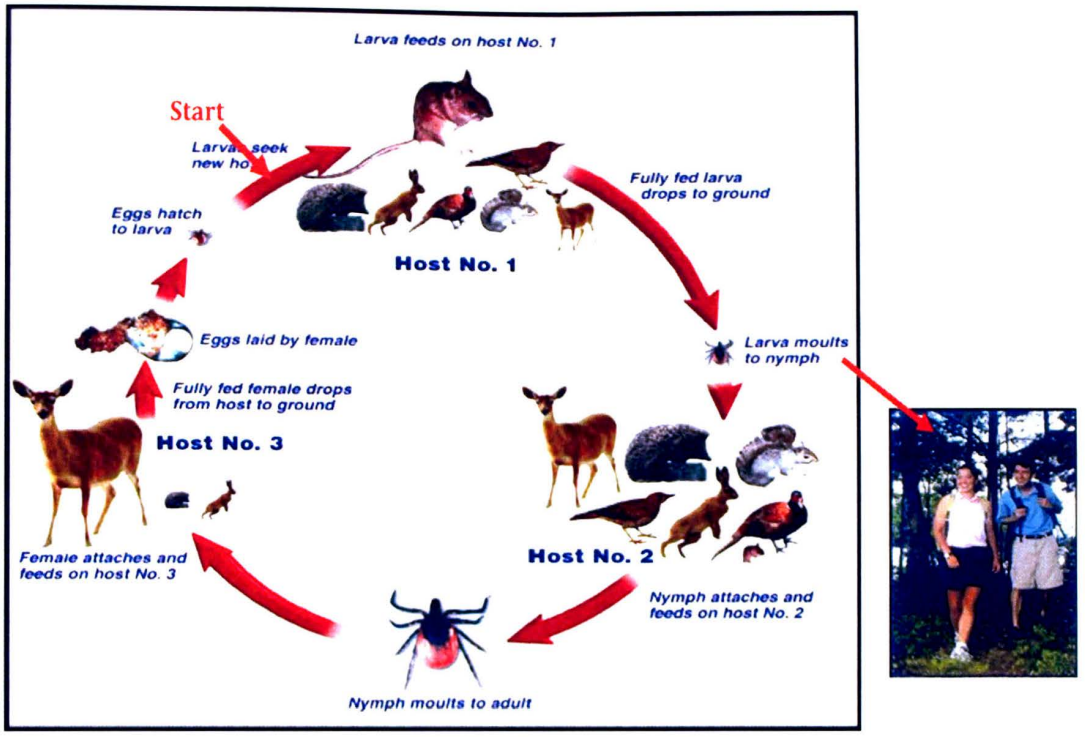


Figure 2. The enzootic life cycle of *Borrelia burgdorferi* is congruent with the life cycle of the *Ixodes* tick.

Epidemiology of *Borrelia burgdorferi*

Lyme disease is a wide spread ailment that has been reported on every continent except Antarctica (10,18,34,35,40). In fact, it is the most wide-spread arthropod-borne disease in the United States and Europe (9). In the United States, Lyme disease is most prevalent in the northeast, higher Midwest and west coast regions as seen in figure 3 (9). Fortunately, humans are not a suitable vector for successful transfer for *B. burgdorferi* back to the tick. The principle reason for this assumption is that human *B. burgdorferi* infections are considered a paucibacillary infection; meaning there are very few organisms causing the disease and therefore the likelihood of a spirochete to return to the tick and complete its enzootic cycle from the human host is remote (5,27). Consistent with this notion, humans are considered a dead end host for *B. burgdorferi*'s enzootic cycle.

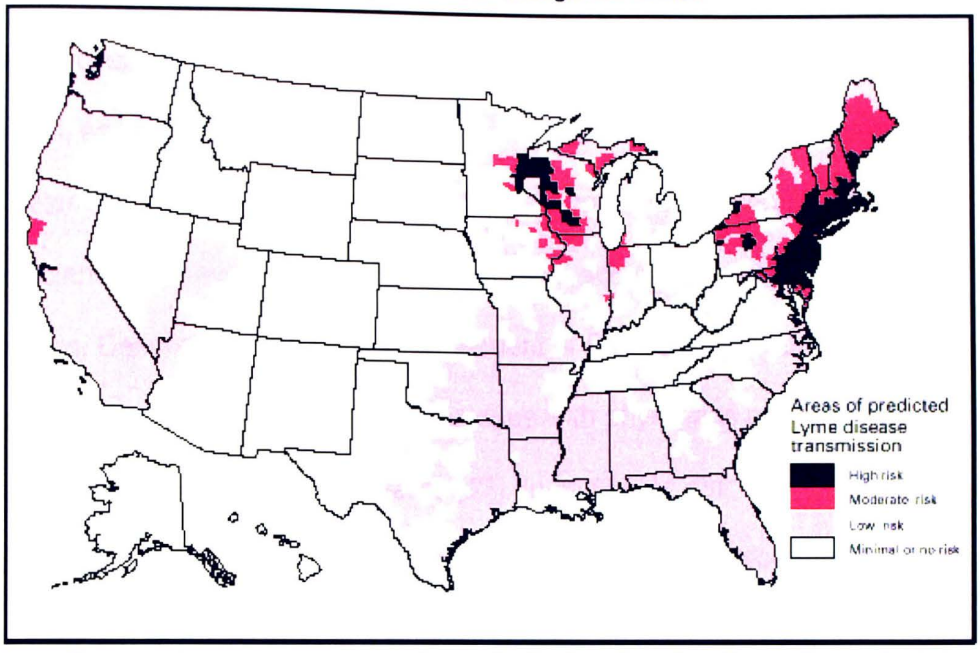
The bacterium *B. burgdorferi* appears to be successful at infecting a large array of different animals (48). The relative infection prevalence of *B. burgdorferi* appears to vary tremendously across the world (1,49,51). For example, two recent studies centered in middle Tennessee showed *B. burgdorferi* to infect approximately 11% to 25% of several different rodent species (22,28). In stark contrast, Barandika and coworkers in Spain showed approximately 68% of one particular rodent species to be infected (1).

Avid outdoorsman, such as hunters, hikers, and soldiers are most at risk for contracting Lyme disease due to their increased risk of tick bites (15).

However, any person who is vulnerable to being bit by a tick is just as much at risk. Humans that are most at risk for contracting Lyme disease are those who are frequently outdoors. Nymphal ticks are the ticks most likely to effectively transfer. This believed to be the case because, nymphal ticks are much smaller than their adult counterparts, and it is due to their small size that they are far more difficult to detect and remove. It has been shown that *B. burgdorferi* requires at between 60 to 72 hours to translocate from the gut of the tick to the salivary glands (23). It is from the salivary glands that the bacteria is then able to enter into the host's bloodstream. Therefore, if the infected tick is removed before 48 hours after exposure, then it is unlikely that *B. burgdorferi* has had the chance to effectively translocate (23). Most cases of Lyme disease are reported in the summer months which are most likely due to an increased chance of outside activity giving the pathogen a route of infection. While ticks remain in their environments year-round, it is during the more temperate months of summer, that humans participate in more outdoor activities. The presence of more humans outside ultimately leads to an increase in reports of Lyme disease.

Interestingly, the record of Lyme disease reports in the United States has not been consistent over the years (9). Since the Centers for Disease Control first listed Lyme disease as a reportable disease in 1982, there has been a consistent rise in the number of Lyme disease cases (figure 4). Unlike past years, the annual report in 2002 indicated a noticeable increase in the number of reported Lyme disease cases. Since there is not any evidence proving that there

National Lyme disease risk map with four categories of risk



Note: This map demonstrates an approximate distribution of predicted Lyme disease risk in the United States. The true relative risk in any given county compared with other counties might differ from that shown here and might change from year to year. Risk categories are defined in the accompanying text. Information on risk distribution within states and counties is best obtained from state and local public health authorities.

Figure 3. A Centers for Disease Control and Prevention map of the national Lyme disease risk for the United States.

has been an unexpected influx of ticks, or tick bites, the increase may be due to the improvement of *B. burgdorferi* detection techniques and procedures.

As well, since more research has been conducted over the years to identify what symptoms are specifically consistent with *B. burgdorferi* infection, doctors and physicians may have finally come to terms with accurately diagnosing the infection. Despite this continual improvement of *B. burgdorferi* detection techniques, there is still an undeniable issue with physicians misdiagnosing Lyme Disease with more common ailments (42). In particular, since Lyme disease is a multisystem infection, it is prematurely misdiagnosed as the common flu during the early stage of infection, or it is misdiagnosed as arthritis during the late stage of infection (46). The symptom most contingent upon this misdiagnosis is the presence of the EM rash, however as research shows, this notable rash has only been present with approximately 70% of the reported cases. In other words, the victim may not exhibit the EM rash, but may still be infected (44). The physicians' standard for diagnosing Lyme disease is the presence of three main symptoms: 1. EM rash, 2. flu like symptoms, and 3. the exposure to tick bites, however, laboratory tests of the patients' blood remains the most accurate technique for a reliable diagnosis of Lyme Disease (42).

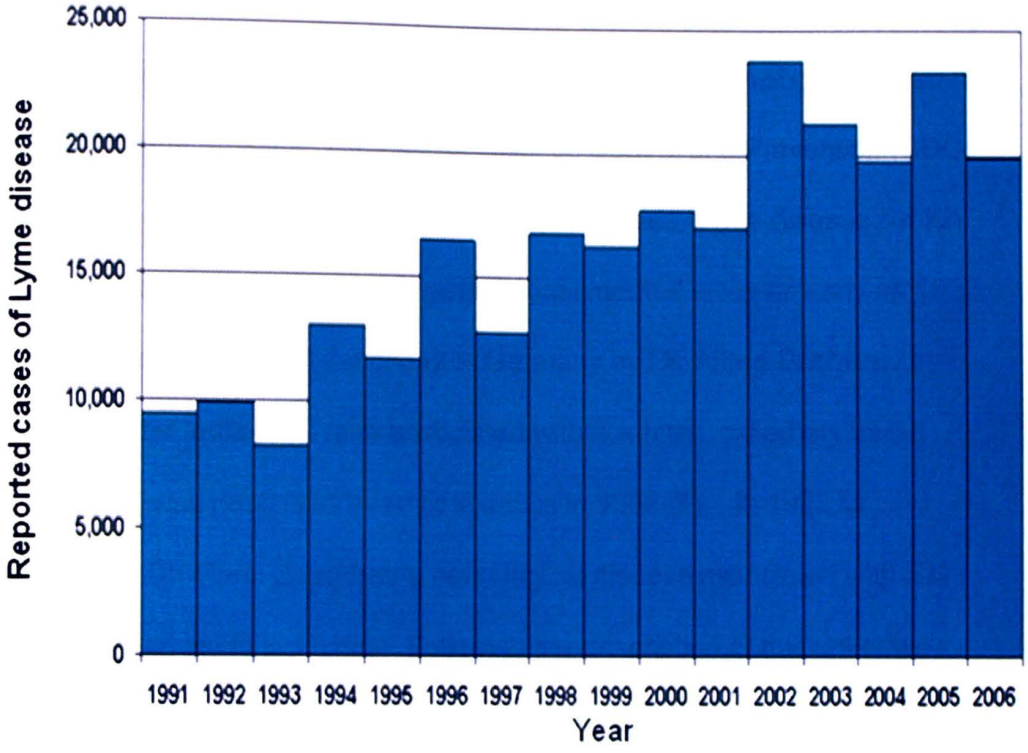


Figure 4. A Centers for Disease Control and Prevention bar graph illustrating the number of Lyme disease cases reported from the year 1991 to 2006.

A historical perspective of *B. burgdorferi*

Lyme disease is a relatively new reportable medical condition that was not recognized by the American Centers for Disease Control and Prevention (CDC) until 1982 (9). Although that is true for the Unites States, Lyme disease, which has several other names, has been globally documented since as early as 1922 (6). Lyme disease was first described in Germany by Dr. Alfred Buchwald in 1883. Later, the "bulls-eye" rash associated with tick bites, called erythema migrans (EM), was described by Arvid Afzelius in 1909 (6). In 1922 Dr. Bujadoux and Dr. Garin described a neurological disease associated with EM based on a case study in Austria. Following the description of this unknown disease, many physicians, to include Dr. Binder and Dr. Hollstrom, concluded that the EM rashes could be successfully treated with antibiotics and therefore deducing that the infections were of a microbial source (6).

Before 1975, there was a collection of unknown diseases that caused neurological disorders in Europe as well as no records of Lyme disease in the United States. Dr. Allen Steere, who lead the research at Yale University, described an unusual outbreak of clustered cases symptomatic of rheumatoid arthritis, so they named the disease after that area of Connecticut (6). The area of Connecticut described included the cities of Lyme and Old Lyme. Dr. Steere put all of the previous documented cases from Europe together and concluded that this was all the dawn of a new disease (46). Based on Dr. Steere's extensive

study of this region's outbreak, he summarily described the disorder as Lyme Arthritis, which is now known as Lyme Disease.

While the disease symptoms were well documented, the particular pathogen, or source, of the disease was still unknown. Dr. William Burgdorfer and Jorge Benach lead the research and discovered that a spirochete bacterium, now referred to as *Borrelia burgdorferi*, was the causative agent of Lyme disease (6). By virtue of their background in tick-borne illnesses they were able to isolate the bacterium and prove that the patients with "Lyme arthritis" all had antibodies against this specific strain of bacterium. Dr. Barbour did not only isolate *B. burgdorferi,* but he also created novel ways to culture it. Many of his methods are still used in *B. burgdorferi* research today (2). In fact, the spirochete was later named after Dr. William Burgdorfer for his contributions to the discovery and study of the spirochete.

Genomic characteristics of *B. burgdorferi*

The spirochete, *B. burgdorferi,* has and one linear chromosome of approximately 910 kbp, and 21 extra-chromosomal plasmids, summing up to approximately 600 kbp, which is the most plasmids of any known bacterium (7,8). Interestingly, each of these plasmids have been shown to be maintained at a one to one ratio within each spirochete (7,8). Therefore, it appears that these plasmids have essential roles in the life style of *B. burgdorferi.* However, when *B. burgdorferi* is cultivated *in vitro*, most of the plasmid repertoire can be

spontaneously lost. The hypothesis of this study is to show that the plasmid content of wildtype *B. burgdorferi* is dependent on unknown animal host factors found in serum which necessitate the maintenance of plasmid content.

Despite the large amounts of extra-chromosomal DNA in *B. burgdorferi's* genome, sequencing has been successful completed (14). A major goal of sequencing data is to identify, albeit presumptively at times, the specific roles of the genes on these plasmids. The roles of several genes have been elucidated and given roles either in infecting the tick, infecting the animal or evading host immune responses, for examples (4,32,56). But, these are short gains, although important, in understanding the biology of *B. burgdorferi*. The maintenance of the plasmid content still remains a mystery to the scientific community.

Mechanisms of persistence

The ability of *Borrelia burgdorferi* to maintain its virulence, regardless of immune response activation is an interesting topic of discussion in the research community. Every day the routes of pathogenesis become a little clearer, but there are still many unanswered questions. Once the tick transfers the bacterium into the tissue of a human, there is an instant immune response, like what most antigens induce (38). As discussed earlier, this is why the EM rash resembles a bulls-eye. Some confusion remains in the bacterium's ability to evade that initial immune response. The immune cells responding to the presence of the spirochetes are unable to clear the bacteria, which does not create any real

barrier from dissemination into the surrounding tissue and the blood stream (39,44).

The sprirochete, *B. burgdorferi's,* motility appears to enhance its virulence *in vivo* (11). *B. burgdorferi* has two alternate endoflagella located between the outer membrane casing and the peptidoglycan helix. Rotation of these filaments causes the spirochete to have a cork-screw like motility (11). *B. burgdorferi*'s effective pattern of motility, which is very similar to human sperm cell movement, enables the spirochete to rapidly traverse through viscous types of liquids. *B. burgdorferi* has a maximum speed of 2800 μm/min, which is approximately twice as fast as the fastest cell in the human body, the neutrophil (26). Out running the immune response is just one of many other ways that *B. burgdorferi* evades the immune system (26) .

Another fascinating method *B. burgdorferi* continues infection is through the many outer surface proteins that many have yet to be fully identified or categorized (3,17,31). Since *B. burgdorferi* has the largest amount of extra-chromosomal DNA out of any other prokaryote and approximately 30% of the genes appear to be outer surface proteins, *B. burgdorferi* has great potential for creating many quantities of diverse proteins (31). Some of these outer surface proteins have been discovered and researchers conclude that the functions are usually associated with the protection of the spirochete from host defenses (4,17,19). Though, there is a downfall to these outer surface proteins; outer surface proteins are antigenic determinants which could result in bactericidal

actively induced by the immune system of the host. Therefore, unsurprisingly, people have studied these outer surface proteins in the hopes of generating a vaccine against Lyme disease (50,52-54). The most often mentioned example for Lyme disease vaccines is the OspA vaccine which was voluntarily taken off the market after about three years of use. The major failing of this particular vaccine was that it only killed the spirochetes found within the tick and not animal host disseminating spirochetes (36).

Methods and Materials

Cultivation of *B. burgdorferi*

B. burgdorferi strain B31MI as well as Clone F were used in this study. B31MI is the naturally virulent strain of *B. burgdorferi*, originally collected form a Lyme disease patient, contains all 21 plasmids, whereas Clone F has been serially cultivated to lose all but eight plasmids, those of which appear are only important to its minimal survival. Each strain of *B. burgdorferi* was first cultivated in BSK (Barbour-Stoenner-Kelly) media, the most common and effective media for cultivation of *B. burgdorferi*. After approximately $5x10^6$/ml *B. burgdorferi* bacteria were enumerated using dark field microscopy, $5x10^4$/ml from each sample grown was passed as well as electroporated, which is a means of artificial transformation that is commonly used (21). Passing of *B. burgdorferi*, which entails serially culturing the bacteria multiple times, was the preferred method in creating an environment in which the plasmids unnecessary for survival in sera would be lost.

The passed samples were then placed in new BSK media that consists of 40% of the specific host sera in question. The *B. burgdorferi* was then allowed to grow in the new media for approximately one week and cultivated again as described above. Bacterial DNA from each of the sample's final pass, which was pass 24, were then isolated and Real Time PCR (polymerase chain reaction, which amplifies a target set of DNA) analysis of these isolates were completed to assay which plasmids were present in each isolate that was grown in each host

sera. Alongside the experimental group, there was a control group that is only grown in BSK media. All data collected was completed in triplicate for statistical purposes.

Collection of mammalian host sera.

Four mammalian host blood sera were collected. Mammalian blood samples were taken from the Canines, Rattus, and Equus families. The amount of animals used in this study was one canine, two rats and one horse. The only animals that were euthanized were the rats, in accordance with IACUC protocol.

Blood samples taken from each animal type were placed in serum separator tubes and immediately centrifuged at 3500 rpm for 15 minutes to separate the sera from the cells in the blood. The supernatant, or sera was then removed and allocated into 100 μl volumes and frozen to -80°C. Once the sera samples were ready to be used, they were individually thawed and added to 60% of BSK media for *B. burgdorferi* mutants for further study.

Preparation of DNA samples

Once the final passed and electroporated samples were allowed to grow in each animal sera type for 3 days. The samples were then centrifuged at 3500 rpm for 15 minutes to pellet the bacterium. The supernatant was removed and 100μl of molecular water was added and mixed with the pellet. Each sample was

then boiled in water for 10 minutes to lyse the bacteria, which allowed the plasmids and chromosomal DNA to be accessible for Real Time-PCR.

Sample data collection using Real Time Polymerase Chain Reaction

After the samples were boiled, the DNA from each sample was assayed using RT-PCR. All 21 plasmids as well as the chromosomal DNA were assayed for each sample. Real time polymerase chain reaction is a molecular technique, which utilizes traditional polymerase chain reaction amplification of a target strand of DNA while simultaneously quantifying the presence and amount of the target DNA in the sample. This is an extremely useful process in quantifying relative amounts of target DNA strands against a control, which in this case was a sample of B31MI *B. burgdorferi* that had never been cured of its plasmid content.

The reagents used to conduct RT-PCR in this study were SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif.) and oligonucleotide reverse and forward primers (Integrated DNA Technologies, Coralville, IA). The 21 primers corresponding to the extra-chromosomal plasmids in *B. burgdorferi* were designed using PRIMER EXPRESS software (PE Biosystems, Foster City, Calif.) for RT-PCR use as shown in Table 1.

All sample DNA was quantified using a Nanodrop spectrophotometer and diluted to >20ng/µl<50ng/µl. Then, 2µl of DNA, or approximately 70ng/µl of DNA were placed in each sample well along with 1µl of primer and 22µl of molecular

water with SYBR Green master mix for a total volume of 25µl reactions in each sample well. Table 2 shows the exact reagents for each RT-PCR well.

Once the plates were prepared, using the template shown in figure 5, they were assayed using ABI Prism 7500 sequence detection system software. The results of each cycle were recorded and analyzed using an unpaired standard t-test to determine if each plasmid's content was significantly different from that of the control.

Table 1. List of primers used in this study

Target gene	Plasmid	Primer Direction	Primer
BBA65	lp54	Forward	CCCAAAAGCCCACGATACA
		Reverse	CAAGTGCAACTTCAAATCCTTGTT
BBB19	cp26	Forward	AATCAGTAGAGGTCTTGTCAAAAGCA
		Reverse	CCACAACAGGGCTTGTAAGCT
BBC10	cp9	Forward	CAAACTCAAAATACGCTCAATAGCTCTA
		Reverse	TGTTAATAGCATGGAGTGCAATAGG
BBD11	lp17	Forward	CAACGAATAGAATGTTGCTAAATCTAAGC
		Reverse	CAACCCATTCTCATTCTCAATAAAAG
BBE19	lp25	Forward	ACGCCGATCCACAAGCAT
		Reverse	AACATCTACCCCTTGTTCTTCCAA
BBF18	lp28-1	Forward	CGAAGGGAATCCTCTTGAAGAA
		Reverse	TTTGCAGTAAATAAATAGGGATTGGA
BBG13	lp28-2	Forward	TCAAGATCAAAGAACCAAATTCATG
		Reverse	CCTGGCTTGGGTTGTAAGTTG
BBH18	lp28-3	Forward	TGGACCTGCTAATAGATGGGATAAA
		Reverse	TGCTGAATGTTCTGCCTTATACTTTG
BBI28	lp28-4	Forward	AAGAAGAACCCGAAGATCAATACG
		Reverse	CCCGGCCCCCAATTC
BBJ23	lp38	Forward	ACCATTCAAAGCGATATTTGCA
		Reverse	CCCGCATACCTCAAAAGCAT
BBK23	lp36	Forward	CCGATTTTTATACTCTTGCAGGAGAA
		Reverse	CCAGCATGTCCTGAAAAGCTAGT
BBL40	cp32-8	Forward	GGGTGATGATCCTAATAGTGGTGTAA
		Reverse	CACTTTGTTCCGCTGCTTTTAA
BBM38	cp32-6	Forward	GGGTCAAGTTGAATCTGCAATTG
		Reverse	CGCAAGGCTTCTATTCCAACTTT
BBN32	cp32-9	Forward	GCGGTGTTGGTAAAAGCACAA
		Reverse	GCCTGTGGATCACTATCAATTATCA
BBO40	cp32-7	Forward	GCAGGGCGATGATCCTAATAAC
		Reverse	ACTGGTGGATTGTCATGACTATTTTC
BBP32	cp32-1	Forward	CGTCAATTAAGGGCGGTGTT
		Reverse	GTTGTCTTTTGATAATAGCGTTGCTAA
BBQ05	lp56	Forward	AAAGAATGCAAGAAGAACCTTCAGA
		Reverse	TTCCAACGGACAAGTCTAATTCC
BBR41	cp32-4	Forward	GAAAGACCGTACCCCAAATGG
		Reverse	CAACTAAAGCGCACCTTCTGAA
BBS35	cp32-3	Forward	ACCAGCCTGGGAACTTTGTATTAT
		Reverse	CAGCCGTCATTGGAATTATTACAT
BBT03	lp5	Forward	AGCTAAAACGACTCAACACGAAGTT
		Reverse	GGGACATTTTACGATTACTTTTGGA
BBU05	lp21	Forward	GCGGTGTTGGAAAAAGTACGTTA
		Reverse	TTTGAGGGTCCATATCAACGATT

Table 3. Table of Reaction Mixtures for PCR Analysis

Label	Ingredients	Total Reaction Volume
Primer ID	12.5µL of Sybr Green polymerase 1µL of *each plasmid* primer 9.5µL of molecular grade water 2µL of Sample DNA	25 µL
+C	12.5µL of Sybr Green polymerase 1µL of *each plasmid* primer 9.5µL of molecular grade water 2µL of positive control DNA	25 µL

	1	2	3	4	5	6	7	8	9	10	11	12
A	Tec1 +C	Tec1 +C	Tec1 +C	C-	G	G	G	C-	O	O	O	C-
B	Tec1	Tec1	Tec1	C-	H	H	H	C-	P	P	P	C-
C	A	A	A	C-	I	I	I	C-	Q	Q	Q	C-
D	B	B	B	C-	J	J	J	C-	R	R	R	C-
E	C	C	C	C-	K	K	K	C-	S	S	S	C-
F	D	D	D	C-	L	L	L	C-	T	T	T	C-
G	E	E	E	C-	M	M	M	C-	U	U	U	C-
H	F	F	F	C-	N	N	N	C-				

Figure 5. RT-PCR plate template used throughout the study. Lettered wells correspond to the primers (as shown in table 4) used for the study and c- is the negative control for each triplicate primer set.

Table 3. Plasmid key with primer code, plasmid identifier and the corresponding gene.

Primer code	Plasmid	Corresponding Gene
A	lp54	BBA65
B	cp26	BBB19
C	cp9	BBC10
D	lp17	BBD11
E	lp25	BBE19
F	lp28-1	BBF18
G	lp28-2	BBG13
H	lp28-3	BBH18
I	lp28-4	BBI28
J	lp38	BBJ23
K	lp36	BBK23
L	lp32-8	BBL40
M	cp32-6	BBM38
N	cp32-9	BBN32
O	cp32-7	BBO40
P	cp32-1	BBP32
Q	lp56	BBQ05
R	cp32-4	BBR41
S	cp32-3	BBS35
T	lp5	BBT03
U	lp21	BBU05

Results

The goal of this study is to determine if plasmids can be cured from samples containing various animal sera over numerous pass cycles inferring that certain plasmids must be maintained for survival within a specific animal species. The results demonstrate that plasmids can be lost, and that certain plasmids are maintained, which supports the hypothesis. The plasmids that were lost statistically significantly due to animal sera alone were cp32-3, cp32-1, cp32-8, cp28-2 and lp21 as shown in Figure 6. This is shown by the sum of plasmid content change for B31MI Pass 24 compared to all passed samples grown in animal sera.

Each different animal sera sample was compared to B31MI Pass 24 control as well as to the B31MI control (zero passes). Horse sera pass 24 sample compared to B31MI Pass 24 sample demonstrated a significant retention of only the plasmid lp17 and no significant change in the plasmids cp26, cp9, lp28-1, lp28-3, lp28-4, lp36 and cp32-8. All other plasmids were significantly lost and therefore considered not necessary for survival in horse sera (figure 7).

Dog sera pass 24 sample compared to B31MI Pass 24 sample demonstrated a significant retention of only the plasmid lp17 and no significant change in the plasmids lp28-3, and lp28-4. All other plasmids were significantly lost and therefore considered not necessary for survival in Dog sera (figure 8).

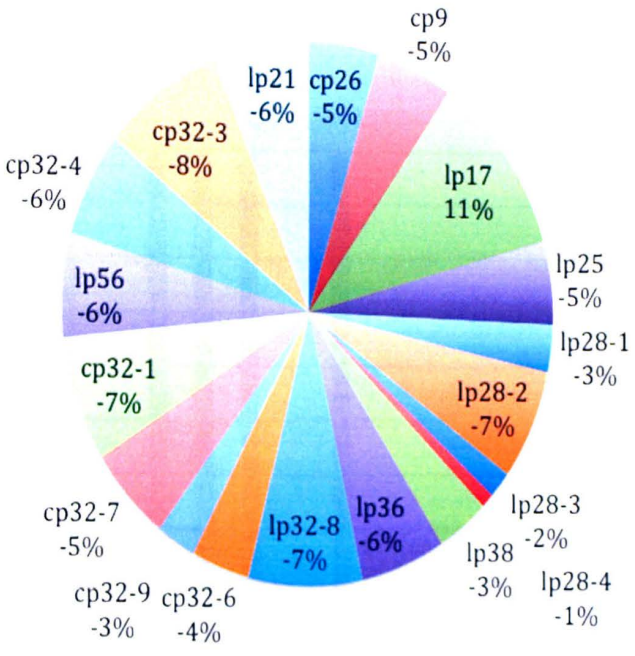


Figure 6: Pie chart illustrating the sum of plasmid content change for B31MI Pass 24 compared to all passed samples grown in animal sera.

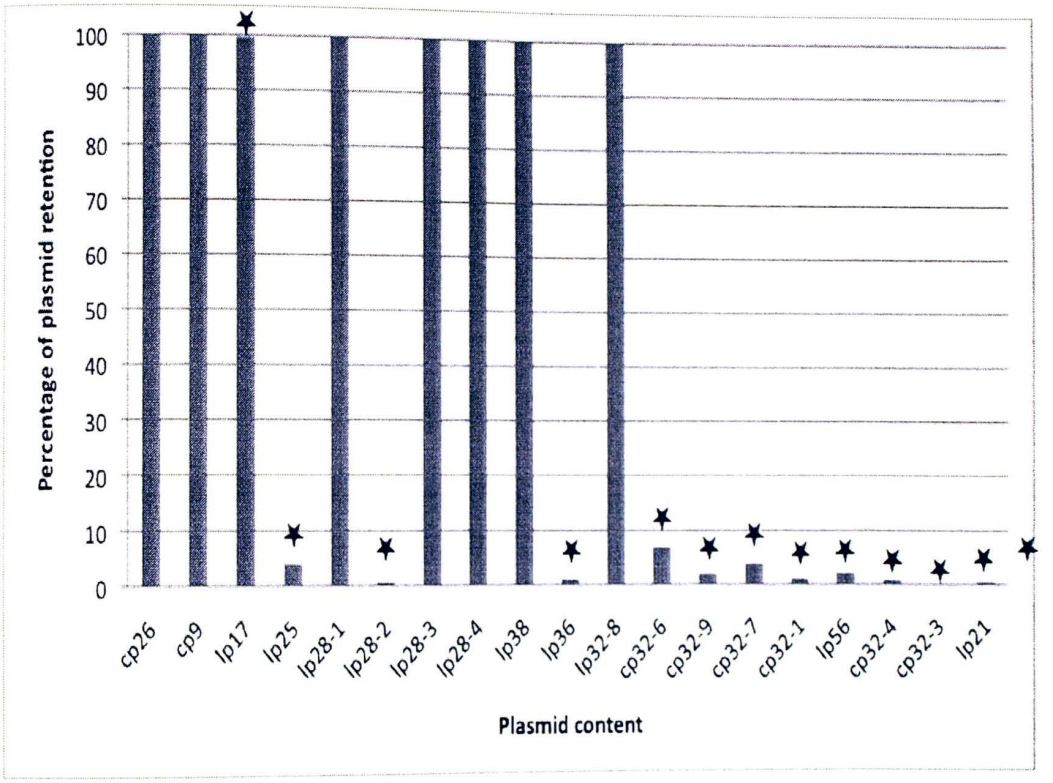


Figure 7. Horse sera Pass 24 compared to B31MI Pass 24 control. ★ indicates plasmids that were significantly different ($p<0.01$).

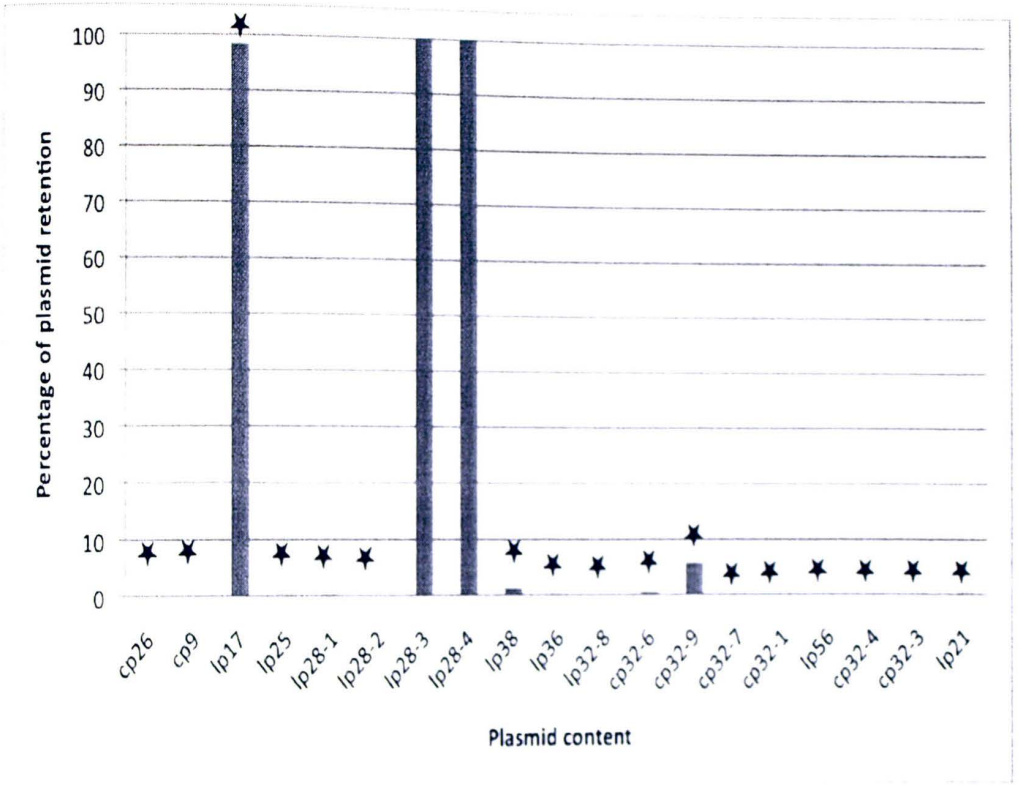


Figure 8. Dog sera Pass 24 compared to B31MI Pass 24 control. ★ indicates plasmids that were significantly different ($p<0.01$).

Rat sera pass 24 sample compared to B31MI Pass 24 sample demonstrated a significant retention of the plasmids lp17, cp9, and cp32-9 and no significant change in the plasmids cp26, lp25, lp28-1, lp28-2, lp28-3, lp28-4, lp38, lp36, cp32-6, cp32-1, lp56 and lp21. All other plasmids were significantly lost and therefore considered not necessary for survival in Rat sera (figure 9).

Each animal sera pass 24 was also compared to B31MI as a control. The data for this comparison was compiled into one large figure (figure 10). The plasmids that were most significantly retained were lp28-3, and lp56 while the plasmids that consistently had no significant change were lp28-4, lp32-8, cp26, cp32-1 and lp21.

Another comparison made between samples were B31MI pass 0 control compared to B31MI Pass 13 sample as well as B32MI Pass 13 compared to B31MI Pass 24 (figure 11). Figure 11a shows B31MI control to B31MI Pass 13 while graph B is comparing B31MI Pass 13 to Pass 24. The data illustrated in this figure shows which plasmids were lost most frequently between Pass 0 and Pass 13 as well as what plasmids were lost between Pass 13 and Pass 24.

The last comparison made was between Horse sera B31MI Pass 24 with Horse sera B31MI electroporated. The data collected from this comparison showed the differences between the two types of plasmid curing to tell which method may be more advantageous. Passing, instead of electroporating had a greater significant loss in overall plasmid content, creating greater diversity.

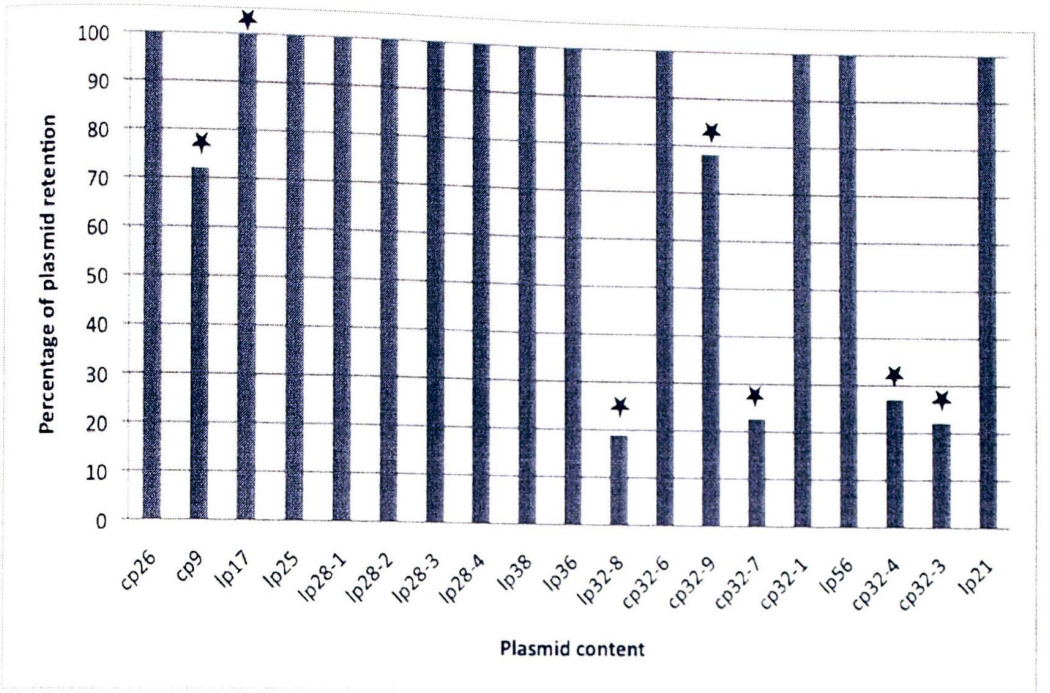


Figure 9. Rat sera Pass 24 compared to B31MI Pass 24 control. ★ indicates plasmids that were significantly different ($p<0.01$).

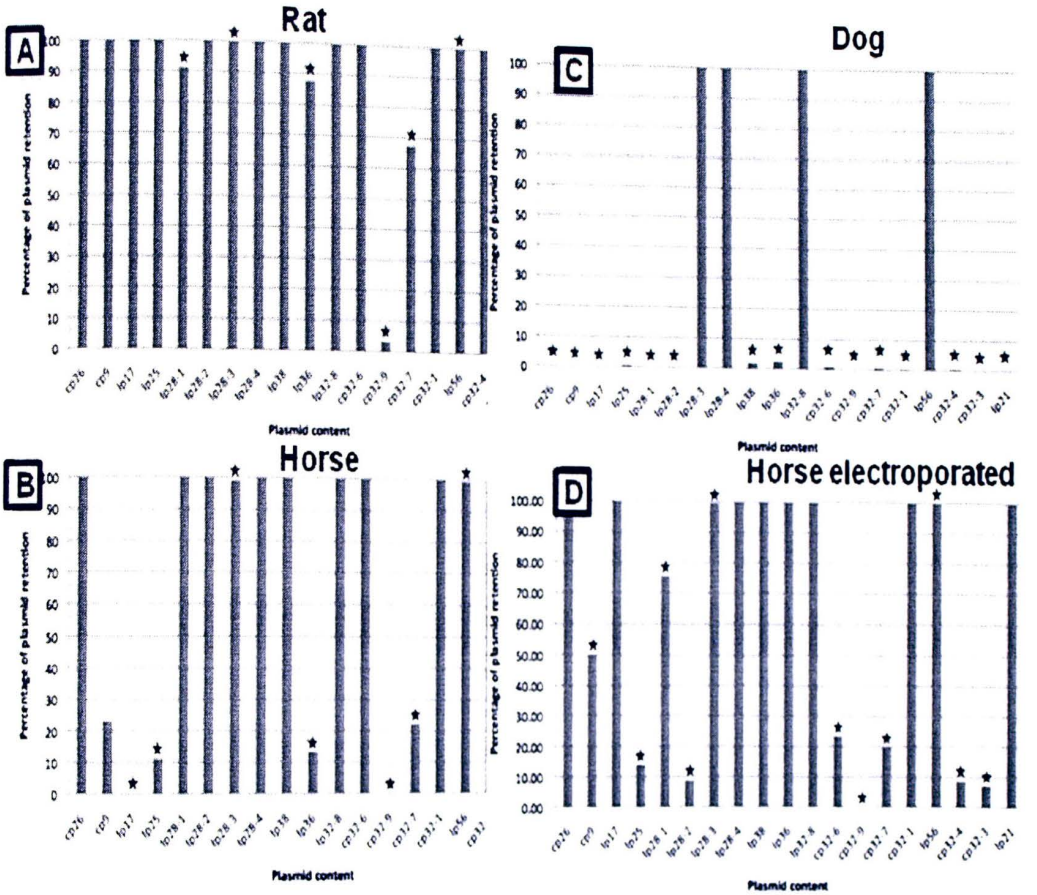


Figure 10. Each animal sera pass 24 compared to B31MI as a control. A- Rat sera B31MI Pass 24, B- Horse sera B31MI Pass 24, C- Dog sera B31MI Pass 24 and D- Horse B31MI electroporated. ★ indicates plasmids that were significantly different ($p<0.01$).

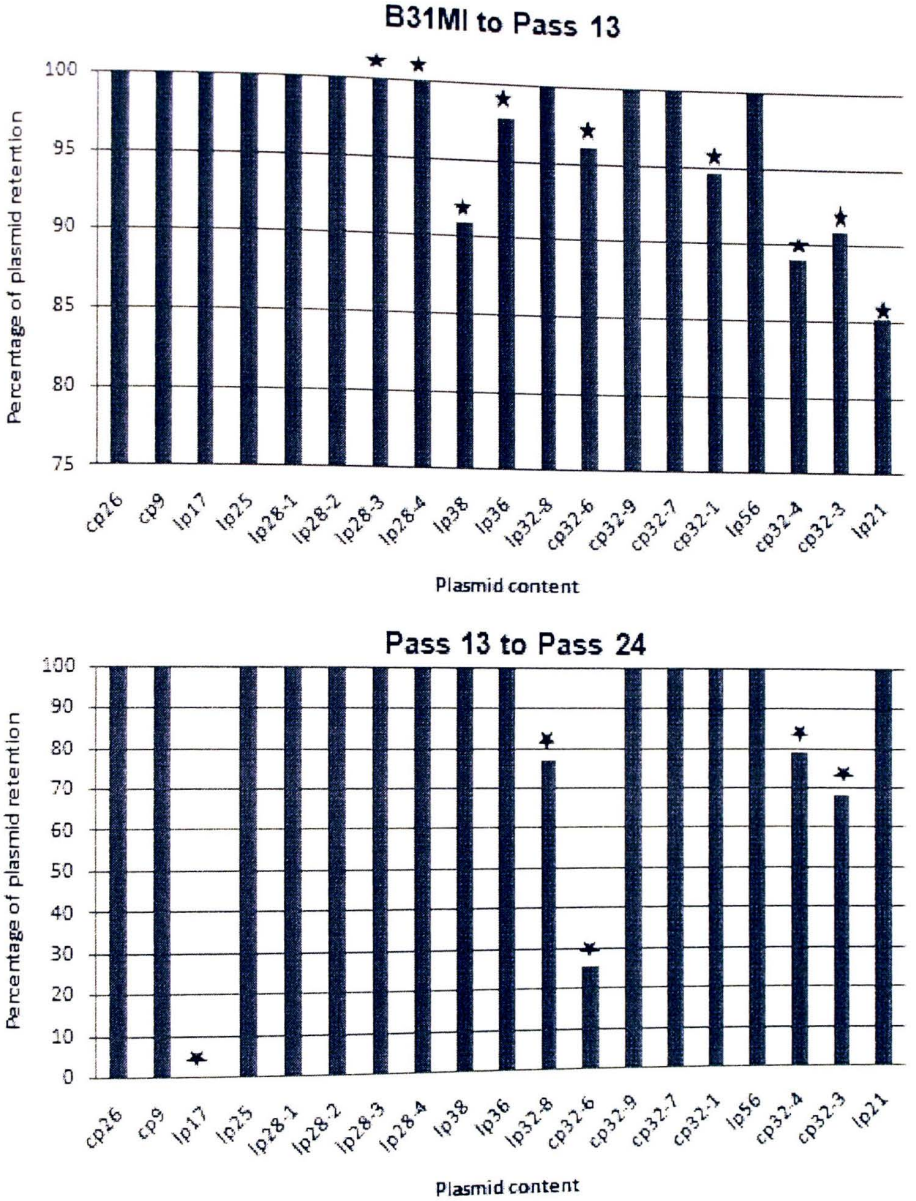


Figure 11. Graphs comparing B31MI control to B31MI Pass 13 sample as well as B32MI Pass 13 compared to B31MI Pass 24. Graph A is comparing B31MI control to B31MI Pass 13 while graph B is comparing B31MI Pass 13 to Pass 24. ★ indicates plasmids that were significantly different ($p<0.01$).

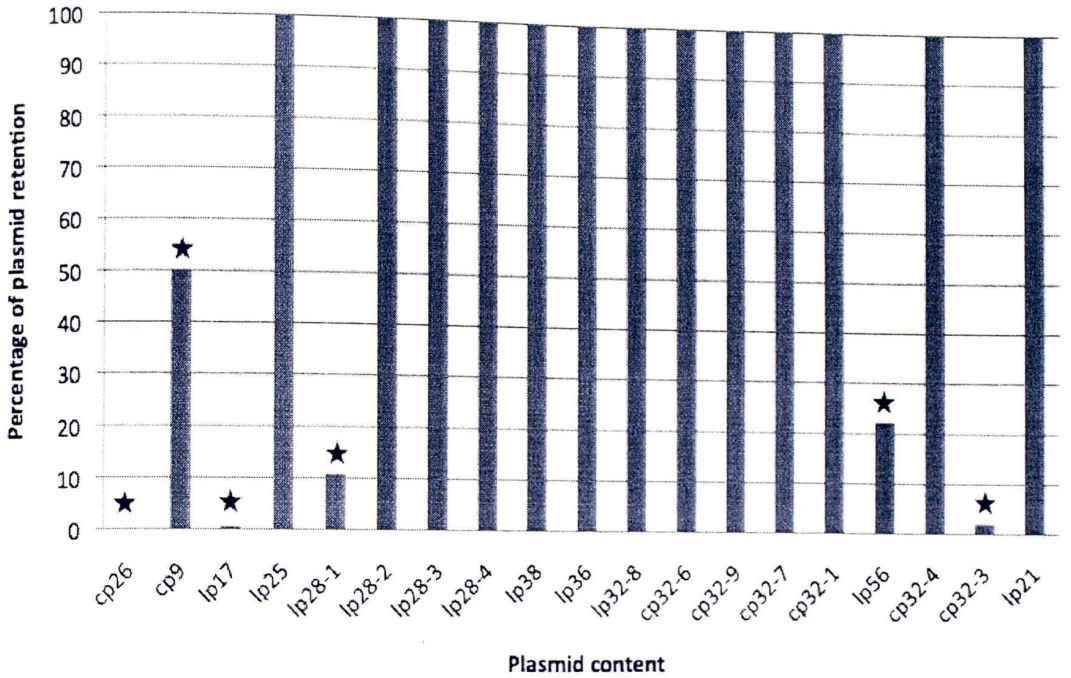


Figure 12. Horse sera B31MI Pass 24 compared to Horse sera B31MI electroporated. ★ indicates plasmids that were significantly different ($p<0.01$).

Discussion

During the 18 months of serial culturing of B31MI, a total of 24 passes were made which accounts for approximately 1.3 $x10^{325}$ spirochete replication events for B31MI; the generation time for CloneF is about four times faster. This appears to be the most extensive growth study of *B. burgdorferi* of its kind. However, since only three different animal sera samples were collected, the scope of this study is recognizably limited.

Interestingly, the RT-PCR assays used in this study detected significant plasmid loss and retention in each animal sera analyzed. Not only were there significant retentions of plasmids, there were also a correlation between the plasmids maintained which implies a possible necessity for maintenance of those plasmids retained in a host-dependent manner. This observation is the tool used to determine "plasmid fitness" in each of the three animal sera examined and to conclude that overall plasmid retention is possibly host-specific. This type of analysis regarding the necessity of plasmids of *B. burgdorferi* in various animal host sera has not been documented. The novelty of this research lays the foundation for more laborious-gene-by-gene research to better understand the host dependency of some *B. burgdorferi* plasmids.

Since there is a great deal of data supporting the retention and loss of certain plasmids, it is possible to speculate the reasons why those plasmids are vital, or not vital for survival in a certain animal host. For example, horse sera

demonstrated a significant retention of only plasmid lp17 and no significant change in plasmids cp26, cp9, lp28-1, lp28-3, lp28-4, lp36 and cp32-8, while all other plasmids were significantly lost, or not necessary for survival in horse sera. Also inferred from these data is that lp25, lp28-2, lp36, cp32-6, cp32-9, cp32-7, cp32-1, lp56, cp32-4, cp32-3 and lp21 are not essential for the survival of *B. burgdorferi* in horse sera. Therefore, all other plasmids are either vital for the survival of *B. burgdorferi*, contain vital function(s) independent of horse sera, or were not yet cured from the B31MI Pass 24 sample. Interestingly, lp17 saw a statistical increase in frequency (i.e., 99% of the bacteria retained it) and therefore suggests lp17 to be necessary for survival in horse sera compared to pass 24 wildtype strain grown *in vitro*. Since, lp17 is almost completely lost from B31MI Pass 23 (conserved at only 2% of the bacteria), but maintained at a 99% in horse sera Pass 24, it appears that lp17 is vital for the survival of *B. burgdorferi* in horse sera alone.

What gene on lp17 is necessitating its maintenance in horse sera? Interestingly, with exception to two genes, lp17 is composed of almost entirely uncharacterized and therefore unknown genes. Two of these genes which have been initially characterized in a 2008 report by Deneke and Chaconas shows *bbd14* and *bbd21* have roles in plasmid replication initiation and possibly maintenance (13). In their study, they cloned *bbd14* and *bbd21* into mutant *E. coli* and showed *bbd14* and *bbd21* could restore the faulty protein plasmid replication machinery, albeit partially, of mutant *E. coli*. The role that horse sera

and gene expression from lp17 is still a mystery, but these data strongly suggest a link to host dependency.

Similarly, dog sera demonstrated a significant retention of only the plasmid lp17 and no significant change in the plasmids lp28-3, and lp28-4. All other plasmids were significantly lost and therefore considered not necessary for survival in dog serum (figure 8). From this, it appears that almost all 19 sampled plasmids, with the exception of lp17, lp28-3 and lp28-4, are not retained in *B. burgdorferi* and therefore are not vital for its survival in dog sera. Interestingly, dog sera lost the most plasmids as compared to all other animal sera samples used in this study. The reasons for such a phenomenon are guesses at best. Perhaps the single dog sampled had been in contact with *Borrelia* before, causing an extremely restrictive environment for growth; meaning specific antibodies are killing those bacteria which harbor plasmids that produce specific antigens and therefore reduce the overall heterogeneity of plasmid content. It is important to mention that while growing *B. burgdorferi* in dog serum, it was observed that there was a significant lack of growth and presumably death compared to the other animal sera used. Dog sera generated the fewest countable bacteria in the same culture conditions as the others. Due to limited reagents, the dog sera concentration had to be decreased to 20% instead of 40% which was used in all other animal sera experiments. Due to this observation, more data should be collected from other dog species to determine if the sera is inherently prohibiting growth, or if it just one dog's immunological history.

However, given the latter possibility that the dog has preexisting antibody against *B. burgdorferi* and given the data generated, it is tempting to speculate that immunologically "aware" sera could induce greater selective pressure to lose plasmids which encode antigenically harmful products.

Not only was lp17 retained in horse and dog sera, lp17 was also retained in rat sera. Rat sera demonstrated a significant retention of the plasmids lp17, cp9, and cp32-9 and no significant change in the plasmids cp26, lp25, lp28-1, lp28-2, lp28-3, lp28-4, lp38, lp36, cp32-6, cp32-1, lp56 and lp21. All other plasmids were significantly lost and presumably not necessary for survival in rat serum. Interestingly, the plasmids that were considered lost, were still retained at levels between 17% and 22% which could be indicative of some trace utility and possibly a satellite effect could be occurring that supplies nearby plasmid-null bacteria with necessary exo-proteins for survival. It is interesting to note that of the plasmids "lost" by spirochetes grown in rat sera, they never were truly lost beyond approximately 17% of the population of bacteria retaining a particular plasmid compared to the other sera which did have bacteria populations which appeared to completely lose some plasmids. Meaning, all the plasmids were retained with at least 17% of the population of bacteria retaining a particular plasmid. These data support the notion that it is likely that *B. burgdorferi* requires nearly all of its plasmids to survive in rat sera which could underscore why passing *B. burgdorferi* through a mouse animal model has consistently produced bacteria containing all the plasmids (21,33). Although, it also should be noted

that cp9 can be lost and the resulting cp9 deficient bacteria infect a mouse. With this in mind, it is interesting that cp9 was retained in 72% of the bacteria grown in rat sera, since cp9 is not necessary for growth or infection in the mouse model (16). It was essentially lost from the overall population (~28% lost), but the majority of cp9 was retained for a reason in rat sera, unlike the mouse model.

Another deduction from this study was in the comparison of two methods of culturing to lose plasmids: serial passes and electroporation. Illustrated in figure 12, the data collected from this comparison showed the differences between the two types of plasmid curing to tell which method may be more advantageous. Serial passing, instead of electroporating had a greater significant loss in overall plasmid content, creating greater diversity. While serial culturing of *B. burgdorferi* did create more plasmid loss than electroporating. It is important to note that to get the samples to lose plasmids, it took 24 passes over an 18 month time span, whereas electroporating takes a mere 5 days; this includes growth of *B. burgdorferi*. To best determine which method is more advantageous, the time constraints and plasmid loss potential must be considered.

The last comparisons made between samples were B31MI pass 0 control compared to B31MI Pass 13 sample as well as B32MI Pass 13 compared to B31MI Pass 24 (figure 11). Figure 11 A shows B31MI control to B31MI Pass 13 while graph B is comparing B31MI Pass 13 to Pass 24. The data illustrated in this figure shows which plasmids were lost most frequently between Pass 0 and Pass 13 as well as what plasmids were lost between Pass 13 and Pass 24. The

plasmids lost most frequently between the pass cycles reveals how many serial passes are necessary to lose each plasmid, if it is able to be lost. For instance, lp21 and cp32-4 were lost at the highest frequencies in the first 13 passes, but lp17 and cp32-6 were lost at the highest frequencies between the 13^{th} and 24^{th} passes, while lp21 and cp32-4 were maintained with no significant change. Data such as this can give insight to the particular plasmid loss in the serial passing of B31MI.

Overall, the data generated from this study provides evidence for the retention of specific plasmids in three different animal sera models and suggests a host-dependent maintenance of some plasmids. Furthermore, this study compared two different methods in which to cure plasmids which may offer assistance to future studies involving plasmid curing. Lastly, information gathered from the comparison of passes from two different pass stages can give insight to the explicit plasmid loss in the serial passing of B31MI.

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Vita

Jillian Kay graduated from Okeechobee High School in the town of Okeechobee, Florida in 2004. She started her undergraduate degree at the University of Florida in 2004 where she attended for 2 and a half years and majored in Dietetics. She later moved to Clarksville, TN where she finished her undergraduate degree and majored in Biology and minored in Education at Austin Peay State University in May of 2008. During her studies, she also taught as a substitute teacher in Clarksville, TN as well as tutored for Upward Bound. She began her teaching career at Northeast High School in January 2009 teaching science. In May of 2008, she began her graduate degree in Biology at Austin Peay State University where she was awarded a graduate tuition assistantship grant. After graduating with her Master's in Biology, she will continue teaching at West Creek High School and apply for Ph.D programs in Florida in 2010.