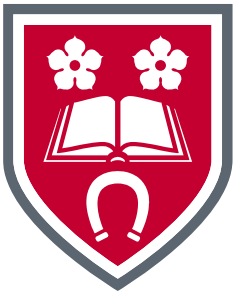

UNIVERSITY OF LEICESTER

ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES INFECTING *BORRELIA BURGDORFERI* SENSU LATO

Thesis submitted for the degree of

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LAMIAA FINGAN NASHI AL-MALIKI

DEPARTMENT OF INFECTION, IMMUNITY AND INFLAMMATION

UNIVERSITY OF LEICESTER

United Kingdom

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Abstract

ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES INFECTING *BORRELIA BURGDORFERI* SENSU LATO

by Lamiaa Fingan Nashi Al- Maliki

Bacterial species of the *Borrelia burgdorferi* sensu lato (s.l.) complex are the main causative agents of Lyme disease. The disease is transmitted to humans by infected ticks. There are currently no vaccines available. Lyme disease can be effectively managed with antibiotics, such as Amoxicillin and Doxycycline, if it is diagnosed early. Phages (viruses that infect bacteria) may complement antibiotics to combat disease. Until now, no *B. burgdorferi* s.l. lytic phages have been identified, and only one temperate phage induced from *B. burgdorferi* CA-11.2A has been morphologically studied. The aim of this project is to isolate and characterise phages of *B. burgdorferi* s.l in order to investigate potential future therapeutic exploitation. Thus, ticks were targeted for *B. burgdorferi* s.l. and phage isolation. Subsequently, induction of prophages from several *B. burgdorferi* s.l. strains was carried out. The induced phages were characterized according to their morphological types, 'potential' virulent activities and genomes.

B. burgdorferi s.l. carriage rate in ticks collected throughout UK was estimated to be 3.2% (6/187), as determined according to a published *16S rRNA* PCR. However, using a novel PCR targeting the terminase gene developed in this thesis, the positive rate was 17% (31/187). This indicates that this method was more than five times more sensitive than the *16S rRNA*-based PCR. Single colony isolation coupled with the whole genome sequencing demonstrated that the dominant *B. burgdorferi* s.l. genotypes in UK ticks were *B. garinii, B. afzelii* and *B. burgdorferi* B31. Transmission Electron Microscopy (TEM) analysis of *B. burgdorferi* s.l. cultures treated with Mitomycin C, Norfloxacin, and UV light revealed the presence of putative phages particles in twelve samples. Specifically, five myoviruses were induced from five *B. burgdorferi* s.l. cultures of *B. afzelii* ACA-1, *B. garinii* S18, *B. burgdorferi* S19, *B. burgdorferi* S21, and *B. garinii* S90. Six podoviruses were induced from six *B. burgdorferi* s.l. cultures of *B. afzelii* ACA-1, *B. garinii* 190P91, *B. valaisiana* NE218, *B. burgdorferi* UK, *B. burgdorferi* Vs185p9, and *B. garinii* S18 respectively. A further myovirus was morphologically identified in the *B. burgdorferi* B31 culture without any treatment,

which represents a spontaneous phage release. In addition, some of the induced phage samples showed potential 'anti- *B. burgdorferi* s.l.' activity according to a fluorescence live/dead assay. Using of bioinformatic analysis such as PHAge Search Tool Enhanced Release (PHASTER) analysis revealed the presence of "incomplete" prophages in the studied strains. However, the presence of phage particles in the induced cultures indicates that these prophages may be complete and functional. Although progress was made to detect lytic *B. burgdorferi* s.l. phages, further research effort is needed to optimize the processes. *B. burgdorferi* s.l. phage purification was attempted in this project; the bottleneck lies in the low phage titer and a lack of 'plaque assay' detection method. This study opens a new door for further *B. burgdorferi* s.l. phage researches; and it presents evidence of *B. burgdorferi* s.l. phage presence and provides practical considerations for future 'breakthrough' in detecting *B. burgdorferi* s.l. phages which could be used in potential therapeutic applications to treat Lyme disease.

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List of Abbreviations

General Abbreviations:

MNNG: 1-methyl-3-nitro nitroso guanidine

BA: bacteriological agar

BSA: Bovine serum albumin

BSKII: Barbour-Stonner-Kelly II

CBSKII: Complete Barbour-Stonner-Kelly

CI: Ciprofloxacin

cp32: Circular plasmid 32

CRML: CMRL-1066 Medium (10X).

dNTPs: deoxyribonucleotide triphosphate

EDTA: Ethylene diaminetetra acetic acid

ENA: European Nucleotide Archive

HCl: hydrogen chloride

Hyp: Hypothetical proteins

ICTV: International Committee of Viral Taxonomy

LB: Lyme Borreliosis

LPS - Lipopolysaccharide

M: Mitomycin C

$MgCl_2$: Magnesium chloride

$MgSO_4$ Magnesium sulphate

NaCl: Sodium Chloride

NCBI: National centre for biotechnology information

N: Norfloxacin

P: P value

PBS: Phosphate-Buffered Saline

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

PI: Propidium iodide

PLP: phage like protein

SDS: sodium dodecyl sulphate

SM: SM buffer

Spp: Species

TAE: Tris-acetate-EDTA

TBE: Tris-Borate-EDTA

TE: Tris-EDTA

TEM: Transmission electron microscopy

TM: Primer Melting Temperature

Tris HCl: tris (hydroxymethyl) aminomethane hydrochloride

UK: United Kingdom

UPH_20: Ultra-pure water

USA: Unites State of America

UV: Ultra violate light

ϕ BB-1: Phage BB-1

Bioinformatic abbreviations

BLAST: Basic local alignment search tool

bp: base pairs

CDs: coding DNA sequences

DNA: Deoxyribonucleic acid

dsDNA: double stranded DNA

dsRNA: double stranded RNA

Fwd: Forward

GC: Guanine-cytosine

Kbp: kilo base pairs

NCBI: National Centre for Biotechnological Information

NGS: next generation sequencing

ORF: open reading frame

RNA: Ribonucleic acid

rRNA: ribosomal RNA

RS: Reverse

ssDNA: single stranded DNA

ssRNA: single stranded RNA

List of Units:

%: Percentage

°C: degree centigrade

cm: centimetre

g: gram

h: hour

M: Molar

mg: Milligram

min: mintues

ml: Millilitre

Mm: Milli molar

nm: nanometer

RPM: Revolutions per minute

U: Units

V: Volume

W: weight

X g: x gravity

µg : Microgram

µl: Microliter

CHAPTER ONE

General Introduction

Chapter 1 General Introduction

1.1 *Borrelia burgdorferi* s.l. and Lyme disease

Lyme disease or Lyme borreliosis (LB) is recognized as the most common tick-borne disease in the Northern Hemisphere (Mysterud *et al*., 2019). The disease was first described in the 1970s in association with an epidemic of arthritis in young children in Old Lyme, Connecticut, USA (Steere *et al*., 1983). LB is a bacterial infection caused by a group of spirochetes called *Borrelia burgdorferi* sensu lato (s.l.). The etiological agent of LB, *B. burgdorferi* sensu stricto (s.s.) was firstly discovered by Professor Willy Burgdorfer in 1982 (Barbour, 1984, Johnson *et al.*, 1984). LB is spread to humans via bites from *B. burgdorferi* s.l.-infected ticks belonging to the genus *Ixodes*. It was estimated that approximately 300,000 cases are reported every year according to the Centers for Disease Control and Prevention (CDC) in the United States (Kuehn, 2013, Mysterud *et al*., 2019). In Europe, recent estimation showed that as many as 85,000 cases occurred annually (Pritt *et al*., 2016, Mysterud *et al.*, 2019). In the UK, approximately 3,000 new cases of LB are reported annually, while a study in 2019 suggested that there are three times higher than previous estimates of cases of LB (Mysterud *et al*., 2019). The majority of LB cases were reported during the early spring and late summer. The seasonality of LB has been suggested to be driven particularly by the temporal pattern of tick distribution, which peaks in May through August (Clover and Lane, 1995). The rise in vector-borne disease cases including LB globally has been suggested to be linked to climate change (Medlock and Leach, 2015). For example, warmer winters could have promoted the spread of ticks, i.e. *Ixodes ricinus*, in urban areas and consequently led to an increased change of *B. burgdorferi* s.l. survival, hence increased number of LB cases (Gray, 2008). Another environmental factor that could contribute to the rise of LB was the landscape, for example, the correlation between the increased numbers of LB cases to the increase of tick density was found to be much significant in the forest than the suburbs (Dobson *et al*., 2011).

The clinical manifestations of LB range from early localized to early disseminated, and late disseminated stages. However, three stages can overlap in a single case and not all patients go through all three stages mentioned above (Hansen and Lebech, 1992). In the early stage of LB, a distinctive circular (bull's-eye, clinically known as "erythema migrans") rash develops at the site of the tick bite, usually approximately 2 to 30 days post-tick bite. Although the

presence of the typical rash is usually considered to have diagnostic significance, only approximately 75-80% of patients develop the rash, and some patients never develop the rash (Wormser *et al*., 2006). In most cases, early-stage LB starts with flu-like symptoms and if untreated, the symptoms can progress and worsen into a long-term debilitating illness manifested as fatigue, muscle, and joint pain, headaches, mental fog causing difficulty with memory or finding words, irritability, and sleeplessness (Skar and Simonsen, 2018). For early disseminated LB, bacteria spread to different parts of the body and in addition to flu-like symptoms, this stage is often characterized by an increase in symptoms such as chills, fever, headaches, fatigue, pain, weakness or numbness in the arms, legs, heart problems and facial paralysis (Decker *et al*., 2012). However, if LB is left untreated or not treated earlier, symptoms may develop to late LB with symptoms such as swelling in joints (arthritis), problems with the central nervous system, paralysis of facial muscles, memory problems and difficulty in concentrating, meningitis and heart block and heart failure, could linger for years (Cook, 2015). If LB goes untreated in the first two stages, Many patients would suffer at the late disseminated stage would suffer from 'chronic symptoms' including arthritis as well as an increase in neurological and cardiac symptoms (Decker *et al*., 2012). The treatment for late LB is complicated and sometimes antibiotics are not working in eradicating LB symptoms, consequently, patients (about 10%) may go on to develop into musculoskeletal pain and cognitive dysfunction even after recommended antibiotic treatment. This condition is sometimes referred to as post-Lyme disease syndrome (PLDS) or post-treatment Lyme disease syndrome. PLDS is a controversial topic due to variable definitions and the lack of reliable diagnostics. Some experts suggested that *B. burgdorferi* s.l. can activate " auto-immune'' response causing symptoms that last long time even after the infection was gone. Others suggested that PLDS results from a persistent and difficult to detect infection. Some believed that PLDS symptoms were due to causes unrelated to patient's *B. burgdorferi* s.l. infections (Goodlet and Fairman, 2018). Patients with PLDS are completely get better and feel well after many months of infection (Strizova *et al*., 2019).

1.1.1 The causative agent of LB: Direct detection and different genotypes

The current diagnosis of LB is primarily clinical: relying on the evaluation of patient's symptoms, area of residence and recent travel history, etc. Since symptoms of LB (known as "The Great Imitator") mimic a range of diseases including Fibromyalgia, Chronic Fatigue Syndrome, Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ALS), Parkinson's and Alzheimer's, etc. There is a significant difficulty in clinical diagnosis of LB (Horowitz and Freeman, 2019). Additionally, there is an ongoing debate on the need for a 'standard' diagnostic criterion to confirm clinical findings. The current laboratory-based diagnostic is a two-tier serological test consisting of an initial ELISA screen to detect IgG and IgM *B. burgdorferi* s.l. antibodies, followed by the standard immuno-blots laboratory test to assess the reactivity to a range of *B. burgdorferi* s.l. antigens (Dillon *et al*., 2010, Borchers *et al*., 2015). Within UK, the national incidence of laboratory confirmed that LB cases have been raised from 0.38 per 100,000 population in 1997 (Public Health England, 2013) to 1.95 per 100,000 population in 2016 (Public health England, 2017). LB is associated with a range of clinical symptoms which can vary in infection progress, erythema migrans combined with the flu-like symptoms consider the first sign of infection (National Institute for Health and Care Excellence (NICE), 2018). Other symptoms such as borrelial lymphocytoma, Lyme neruroborreliosis, carditis, arthritis and acrodermatitis chronica atrophicans (ACA) are also included (Steere *et al*., 2016). This result in various signs which make it more problematic. However, UK and European case definitions agreed that erythema migrans alone confirm the disease infection (Stanek *et al*., 2011).

Microscopic analysis, direct culturing and Polymerase Chain Reaction (PCR)-based methods have been developed and may also be used to assist serological tests. However, the non-serological methods were described in the literature as 'unreliable and no diagnostic values' due to low sensitivity (Kanjilal *et al*., 2019). This intrinsic low sensitivity could be attributed to low numbers of *B. burgdorferi* s.l. present in patient samples (Biesiada *et al*., 2012). In addition, it usually takes up to 12 weeks to produce identifiable *B. burgdorferi* s.l. culture from patient samples, consequently resulting in delayed treatment and progression of LB (Biesiada *et al*., 2012). Due to the inherent difficulties associated with diagnosis LB, the numbers of LB cases tend to be under-estimated (Murphree Bacon *et al*., 2008). Therefore, sensitive methods in LB diagnosis are urgently needed to increase accuracy in LB diagnosis and reduce

suffering among patients. If LB is diagnosed early, a short course of antibiotic treatment, such as Doxycycline for 21 days, has shown to be successful for curing the disease (Cruickshank *et al*., 2018).

The non-serological methods in direct detection of *B. burgdorferi* s.l. DNA in body fluids (blood or serum) and tissues, can demonstrate the presence or absence of *B. burgdorferi* s.l. and sometimes provide genotyping information but can't distinguish an active infection from infection from the past (a dormant infection) (Lebech *et al*., 1991, Stanek *et al*., 2002). Much sought-after LB diagnostic methods are those that have the ability to distinguish between active and inactive *B. burgdorferi* s.l. infection (Piesman and Gern, 2004).

Identification and differentiation of *B. burgdorferi* s.l. species and strains depend on their genetic characteristics (Wang *et al*., 2014). Research has identified 42 *B. burgdorferi* s.l. species. Analysis has identified two distinct clades, informally known as "relapsing fever" clade and the "Lyme disease" or "*B. burgdorferi* s.l." clade (Wang *et al*., 2014b). Adeolu and Gupta 2014 propose that these would better accommodated in to their own genus. However, this suggestion is contested (Barbour *et al*., 2017, Margos *et al*., 2017). Twenty of the species are considered to belong to *B. burgdorferi* s.l. clade (http://www.bacterio.net/borrelia.html; (Wang *et al*., 2015). The clade now contains a number of *B. burgdorferi* s.l. species that are associated with LB and others that have not (Wang *et al*., 2014).

1.1.2 Microbiology of *B. burgorferi* s.l.

B. burgdorferi s.l. are microaerobic spirochetes. They have a 'Gram-negative-like' double layer cell envelope which is made of the outer membrane, peptidoglycan layer, and inner membrane, and no lipopolysaccharide (LPS). The characteristic components of the Gram-negative cell wall is identified from the outer member. Therefore, *B. burgdorferi* s.l. is not regarded as Gram-negative, and can have varied gram staining results (Takayama *et al*., 1987, Ben-Menachem *et al*., 2003). Giemsa or Wright stains are more appropriate for visualising *B. burgdorferi* s.l. (Zückert, 2007). As depicted in Figure 1-1, *B. burgdorferi* s.l. is a helical cell with dimensions of 0.2-0.5 µm in width and 10-30 µm in length. The bacterium has seven to eleven flagella attached at each end of the cell cylinder. Unlike other bacteria, the flagella carried by *B. burgdorferi* s.l. is inserted in the cytoplasmic membrane and is located in the

periplasm. These flagella enable *B. burgdorferi* s.l. to be highly motile in both low and high viscosity media (Motaleb *et al*., 2000).

The cultivation of *B. burgdorferi* s.l. has been a significant challenge for researchers (Zückert, 2007). The current BSK-II medium which is based on a recipe developed originally by Kelly (1971). Then 'fortified' by Stoenner, and finalised by Barbour (he modified the medium composition twice), hence the name of Barbour-Stoenner-Kelly II medium (BSKII) (Stoenner, 1974). The complete BSKII media was able to achieve high spirochetes densities of 1 to 4x 10^8 bacteria per ml (Barbour, 1984), with the generation times of 11 to 12 hr with a starting inoculum of one to two microorganisms (Barbour, 1984). *B. burgdorferi* s.l. can grow on solid BSKII medium (with the addition of agarose into BSKII) into colonies, which are opaque- white in color, regularly shaped, measure up to 3mm in diameter, and have a dense center and a diffuse border. Colony size and morphology can vary from strain to strain or even within the same isolate (Elias *et al*., 2002).

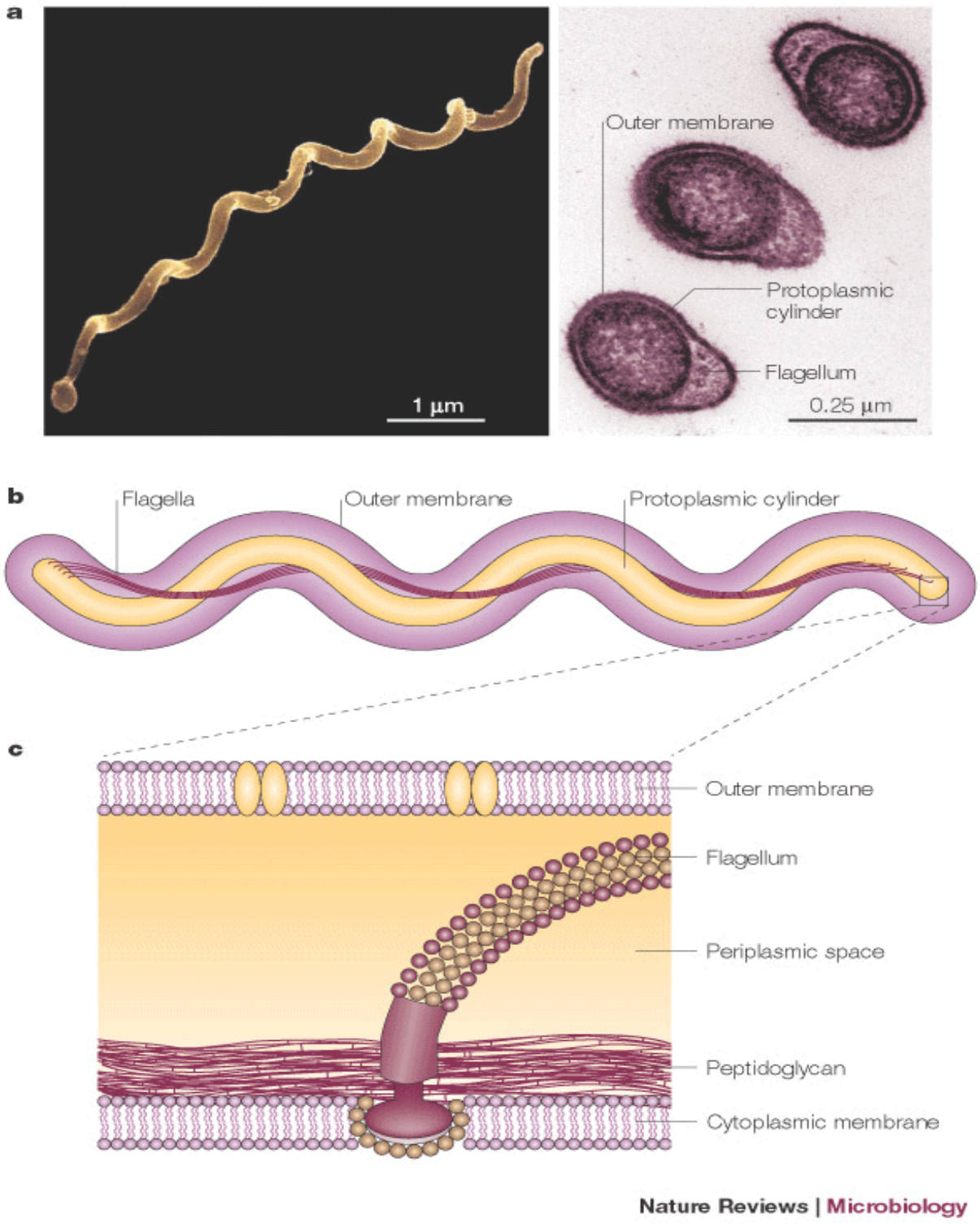


Figure 1-1 Morphological and structural characteristics of *B. burgdorferi* s.l.

(a) Left (scanning) and right (transmission) electron micrograph of *B. burgdorferi* s.l. (b) Diagram showing the flagella insertion points at the end of spirochetes and the bundle of flagella winds around the flexible protoplasmic cylinder shape of *B. burgdorferi* s.l. (c) Diagram showing flagella insertion site and its surrounding structures. Image adapted from (Rosa *et al*., 2005).

B. burgdorferi s.l. is known to form different pleomorphic variants in different culture conditions at physiological temperature (Rosa *et al*., 2005). In response to changes in temperature, salts, nutrient content, acidity fluctuation, multiple host, *B. burgdorferi* s.l. can also form dormant sub-populations or round bodies (persisters) and survive the hostile environment (Meriläinen *et al*., 2015). The ability of *B. burgdorferi* s.l. to develop into different pleomorphic forms, including a 'biofilm-like' structures made of aggregated cells covered by a polysaccharide layer, has been a hot topic in recent years. Different pleomorphic variants have been implicated in different conditions, morphology, cell envelope architecture and metabolic activity as well as biochemical features may influence the development of novel diagnostics and treatment (Meriläinen *et al*., 2015). *B. burgdorferi* s.l. 'biofilm' has also been suggested to be an alternative mechanism of antibiotic resistance (Hoyle and Costerton, 1991, MacDonald, 2006) . In addition to biofilm formation, *B. burgdorferi* s.l. appeared to be able to establish a 'cystic' form under stressful conditions, such as a change of temperature (Murgia and Cinco, 2004, Miklossy *et al*., 2008). The term 'cysts' has been used in *B. burgdorferi* s.l. literatures, ambiguously and generated debate about whether it was a correct use because the formation of *B. burgdorferi* s.l. cysts conveyed an impression that the 'cyst-form' are able to evade destruction by antibiotic treatment and host immune system, thereby allowing persistent infection or built immunity to antibiotics. More solid experimental and clinical evidence is needed to elucidate the potential role of 'cysts' in *B. burgdorferi* s.l. biological development. *B. burgdorferi* s.l. also showed the ability to 'grow back' to the active spirochete form from their 'non-active' pleomorphic forms. There were also some reports about *B. burgdorferi* s.l. formation of cell wall deficient (CWD) or L form or spheroplast as a result of a complete or partial loss of peptidoglycan layer (Sapi *et al*., 2011, Meriläinen *et al*., 2015). However, a paper published in 2015 by Meriläinen et al rejected the presence of CWD or L form *B. burgdorferi* s.l. on the basis of microscopic and biochemical analysis of *in vitro B. burgdorferi* s.l. cultures. Apart from forming 'dormant forms', *B. burgdorferi* s.l. can also develop into 'persisters', which displayed typical antibiotic-tolerance, although antibiotic resistance has not been observed for *B. burgdorferi* infections (Meriläinen *et al*., 2015).

1.1.3 Genetics of *B. burgdorferi* s.l.

The genetic makeup of *B. burgdorferi* s.l. species is unique among prokaryotes (Wang *et al*., 2014). One characteristic feature of this microorganism is its unusual genome which consists of one linear chromosome with an approximate size of one mega base and several circular and linear plasmids (Casjens *et al*., 2000). Specifically, *B. burgdorferi* B31 genome consists of a 910,725 bp linear chromosome. Approximately 60% of B31 genes are predicted to be similar to known genes in other organisms, 10% of unknown genes were found similar to other bacteria, and 30% of genes have an unknown role and are unique to *B. burgdorferi* s.l. (Brisson *et al*., 2012). The type strain *B. burgdorferi* B31 carries 12 linear plasmids (lp) and 9 circular plasmids (cp), ranging from 5kb to 56kb in size, which make up of 40% of the bacterial genome. The lp plasmids do not encode genes which are homologous to genes in other bacteria. However, all the lp contain many non-coding genes (non- protein coding DNA) and a large number of pseudogenes, with the exception of the lp54 and lp28-2 plasmids that are known to contain a few prophage genes thought to be acquired from the cp (Brisson *et al*., 2012) *B. burgdorferi* s.l. plasmids have been suggested to encode some 'essential' genes for bacterial survival (Wang *et al*., 2015). For example, some plasmids, such as cp26, carry lipoprotein-encoding genes that provide functions required for bacterial viability (Byram *et al*., 2005), and are important in detection, prevension and pathogensis of *B. burgdorferi* s.l. (Dowdell *et al*., 2017).

The 9 cps encode highly paralogous sequences genes of a similar nature suggesting they have diverged from a common ancestor. The cps do not encode as many pseudogenes as the lps do. Interestingly the cp26 is reported to be the only plasmid required to culture *B. burgdorferi* B31. This is because cp26 encodes for many essential proteins involved in key metabolic processes of the *B. burgdorferi* s.l., including the telomere resolvase (Stewart *et al*., 2003). The cp plasmids includes cp32-1, cp32-2, cp32-7, cp32-4, cp32-6, cp32-8, and cp32-9. These plasmids are highly homologous to each other and encode many bacterial surface proteins and putative prophages associated genes. A prophage is an inactive phage genome either integrated as part of bacterial DNA or as free-living plasmids (Radolf and Samuels, 2010). There is bioinformatics and experimental evidence to suggest that the cp32 plasmids are prophages because cp32 plasmids carry essential phage structural genes encoding phage capsid, tail, holin and endolysin-like and portal proteins (Casjens *et al.*, 2000). Plasmids such

as cp18, lp-54, lp56, and lp28-2 contain DNA that is homologous to many cp32 putative prophages associated genes (including the putative terminase and portal genes) that are responsible for the replication and maintenance of the phage DNA (Brisson *et al*., 2012). The genes, terminase and portal gene, are highly conserved in tailed phages and no homologs can be found that have different functions, the presence of these two genes gives indications that the cp32 plasmids are prophages (Casjens *et al*., 2000, Wang *et al*., 2015, Wang *et al*., 2016). It has been reported that a large number of plasmid DNA is in a state of evolutionary decay and that many of the plasmid genes have had recent DNA rearrangement (Wang *et al*., 2015). The differentiation among *B. burgdorferi* s.l. species was traditionally carried out using *16S rRNA* gene (Casjens *et al*., 2000). It is worth noticing that *B. burgdorferi* s.l. only has a single copy of *16S rRNA* gene whereas it contains many tandem repeats located between the *23S* and *5S rRNA* genes (Wang *et al*., 2015). The 23S and 5S rRNA genes are tandemly duplicated in the order *23S-5S-23S-5S* and apparently not linked to the *16S rRNA* gene which is situated over 2 kb upstream from the *23S-5S* duplication (Schwartz *et al*., 1992).

One of the characteristics of *B. burgdorferi* s.l. cells is the presence of many polypeptides called outer surface proteins (Osps) on the outer membranes (Hirschfeld *et al.*, 1999). The expression of the *osp* genes was differentially regulated both temporally and in different hosts. For example, *ospA* and *ospB* genes are constitutively expressed in the midgut of unfed ticks and were downregulated upon tick feeding, while *ospC* is necessary for infectivity or persistence within vertebrate and it is an important factor in tick-mediated transmission (Grimm *et al*., 2004, Tilly *et al*., 2008). *In vivo* experiment carried out in mice demonstrated that proteins from OspF family played an important role in the adherence of bacteria to vascular endothelium (Antonara *et al*., 2007). During the initial stages of infection of *B. burgdorferi* s.l., OspF-family may serve as adhesion factors. OspF has not been fully characterized at the functional level, and it was identified as a potential adhesion to heart tissue using an *in vivo* phage display system (Schwan, 1996, Kenedy *et al*., 2012). Outer surface proteins (*osp)* gene family members have been demonstrated to undergo mutation and rearrangement during infection, which leads to the generation of antigenically distinct variants that may contribute to immune evasion (Sung *et al*., 2000). It was reported that OspE functions via binding to the H factor, a factor in complement regulation, and this binding deactivates antimicrobial activities of the complement system (Kenedy *et al*., 2012). As a

result, *B. burgdorferi* s.l. gains the ability to evade the host's immune system (Glöckner *et al*., 2006).

One of the other ways for *B. burgdorferi* s.l. to evade the immune response is antigenic variation of the variable major protein (VMP)-like sequence (Vls) E lipoprotein (VlsE) (Bankhead and Chaconas, 2007, Tilly *et al*., 2008). VlsE includes two invariable domains at the N and C terminals consisting of six variable areas and six invariable areas (Liang *et al*., 1999). The invariable areas and regions are not exposed on the intact spirochete surface and therefore cannot be used as antibody objectives when the bacterium has been alive (Eicken *et al*., 2002). The C-terminal domain and two invariable areas are immuno-dominant and can serve as decoy epitopes to distract immune responses from variable areas (Stanek, 1991). The synthesis of VlsE start after OspC and both are required for persistent mammalian infections (Glöckner *et al*., 2006).

1.1.4 Ecology of *B. burgdorferi* s.l.

B. burgdorferi s.l. are zoonotic tick-borne pathogens. They are transmitted to the host by ticks. Small birds and small mammals are essential for maintaining transmission of tick-borne diseases for the completion of their life-cycle (Rizzoli *et al*., 2014). As shown in Figure 1-2 *B. burgdorferi* s.l. have the enzootic life cycle, they are transmitted between ticks and hosts. Tick's mid-gut is the site at which the *B. burgdorferi* s.l. resides and multiplies; during the tick feeding process, *B. burgdorferi* s.l. can migrate to the salivary glands via the tick hemolymph. Therefore in the case of LB transmission, feeding of an infected *Ixodes* would result in the deposition of *B. burgdorferi* s.l. via the infected tick saliva (Shapiro, 2014). The larger hosts or the second hosts include cats, dogs, deer, and humans if accidentally come in contact with ticks. Ticks maintain their population by drinking the blood from their hosts. During this process, ticks transfer any pathogens into the hosts or vice versa, i.e. the hosts will pass any pathogens to ticks (Shapiro, 2014). *B. burgdorferi* s.l. are carried mainly by ticks belonging to *Ixodes* genus from the *Ixodidae* family (Parola and Raoult, 2001). Currently there are four predominant species in the world such as *I. scapularis*, *I. pacificus, I. ricinus*, and *I. persulcatus* (Piesman and Gern, 2004). The most frequent ticks in the UK, where *B. burgdorferi* s.l. was identified, are *I. ricinus, I. hexagonus, I. trianguliceps* and *I. canisuga* (Toutoungi and Gern, 1993). Many, if not all, species of this complex are vectors of other pathogens causing illnesses

to humans and animals, including tick-borne encephalitis, anaplasmosis, and babesiosis (Stanek *et al.*, 2012). Changes in the tick- born disease prevalence were also reported to be related to the changes in the vector distribution (Daniel *et al*., 2008).

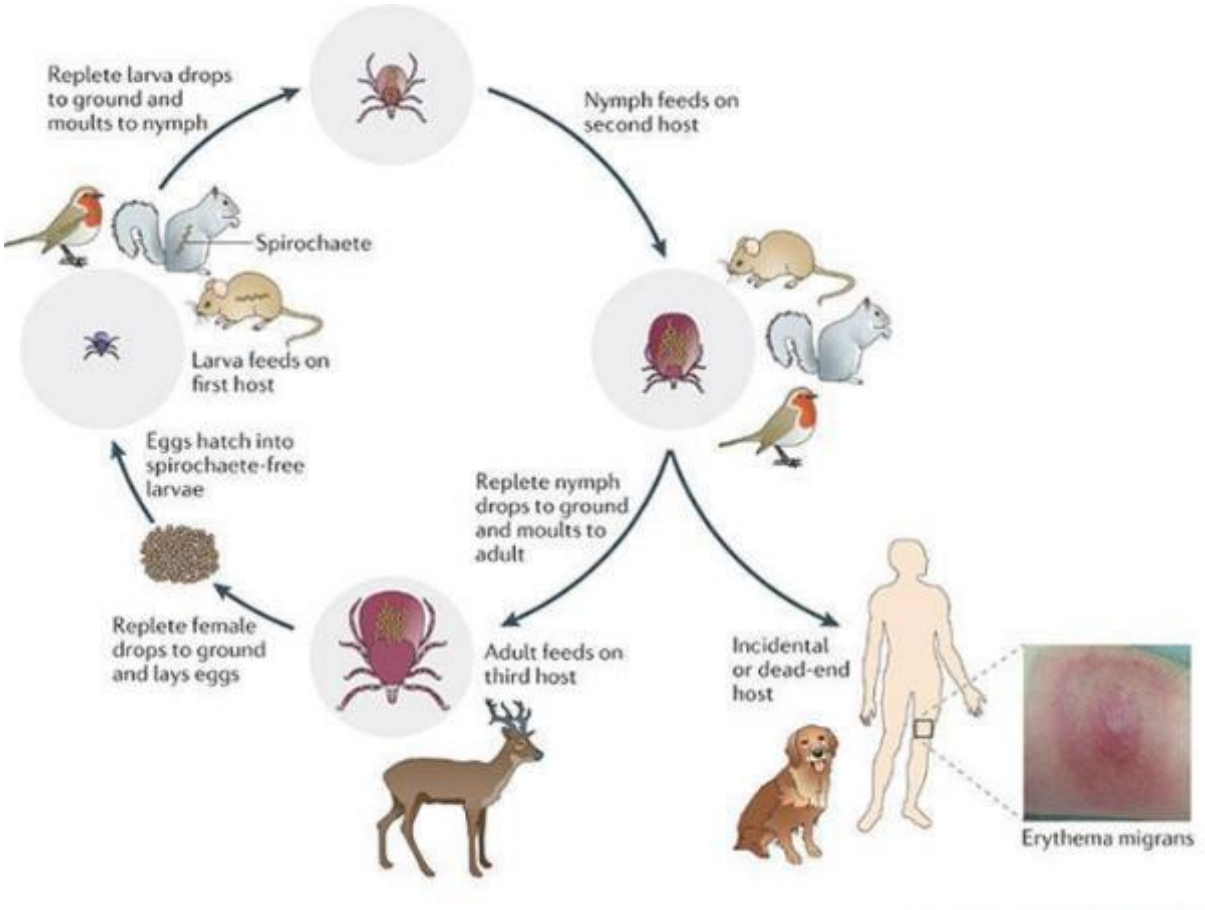


Figure 1-2 Diagram shows the enzootic cycle of *B. burgdorferi* s.l.

Ixodes tick egg first hatch into six-legged larvae. Then the larva starts to search for food. The larva will first feed on small mammals as a first host such as birds, mice, and squirrels or any host which has the *B. burgdorferi* s.l. *,* and transfer it. In this case the larva will be infected with *B. burgdorferi* s.l. and can transfer *B. burgdorferi* s.l. to other hosts. The fed larvae will drop off from the first host and start to grow up or molt to eight legs nymph. The nymph will start to find other hosts as a second host for feeding. During feeding stage, the nymph will transfer *B. burgdorferi* s.l. during the infected saliva, this results in infecting other hosts or creating reservoirs. A nymph can separate and disseminate a large amount of the disease to humans, which consider the last stage. Adult ticks try to find blood meals from different mammals such as dogs, deer. Female ticks will lay eggs and the new tick life cycle will start (Radolf *et al.*, 2012).

1.2 Introduction to Phages

Phages are viruses that infect bacterial hosts. They were discovered in 1915 by William Twort and two years later by Felix d'Herelle, they observed that phages had bacterial killing activity (Clokie *et al*., 2011). Phages are the most abundant biological entity present on earth and have been isolated from different environmental samples such as soil, seawater, sewage or any environments where their hosts are found (Chatain-LY, 2014). It is estimated that there are 10^{31} phages on earth and only a small proportion of them have been studied (Hendrix, 2003).

Phages consist of a nucleic acid genome (DNA or RNA) surrounded by a protein coat called a capsid. Phages, like viruses, are varied in their morphology. They can be icosahedral in shape, filamentous or complex structures consisting of icosahedral heads with helical tails (Ackermann, 2003). For example, a typical T4-like phage consists of an icosahedral head with nucleic acid double-stranded DNA inside, a tail of various lengths which has the ability to contract and tail fibres which bind to the surface of their target bacteria (Ackermann, 2003, Ackermann, 2005). In addition to phages that may possess a lipid-containing envelope or contain lipids in the particle shell (Ackermann, 2009).

Phages are closely interacting with their bacterial hosts (Chibani-Chennoufi *et al*., 2004). Phage adsorption is determined by specific interaction between phage tail fibers and bacterial surface receptors called phage-binding protein receptors (Moldovan *et al*., 2007). This process is highly specific to the receptors present on the bacterial surface (Kasman *et al.*, 2002, Moldovan et al., 2007, Kasman and Whitten, 2018). Tail fibers are fundamental in dictating phage host range or the bacterial strains that lysed by specific phage (Dowah and Clokie, 2018). Phage binding starts randomly by adsorption of phages to the receptors. Then phage binds reversibly and finally binds irreversibly to the bacterial cell receptors where it binds to the same receptors or different receptors (Dowah and Clokie, 2018). The successful binding of phages reversibly and irreversibly is important in phage bacterial interaction (Thanki *et al*., 2018).

1.2.1 Phage Classification

Phages are classified according to nucleic acid properties and their morphology. The majority of phages contain double-stranded DNA (ds DNA). Whilst other groups of phages contain either single-stranded RNA (ss RNA) or double-stranded RNA (ds RNA) or single-stranded DNA (ssDNA) (Ackermann, 2009).

According to the International Committee on Taxonomy of Viruses (ICTV), phages were classified into one order, thirteen families and 30 genera based on their morphology. Most of the phages fell into the order of *Caudavirales*, which are tailed phages and represent 96 % of known phages (Ackermann, 2009). They are the most studied group of phages and can be recognised by their double-stranded DNA and a tail ranging from approximately 24-400 nm in length. Of the 5568 phages that have been examined by electron microscope since 1959, 5360 (96.2%) are tailed phages (Clokie *et al*., 2011). A typical example of a tailed phage, as shown in Figure 1-3, consists of head, tail and tail fibers.

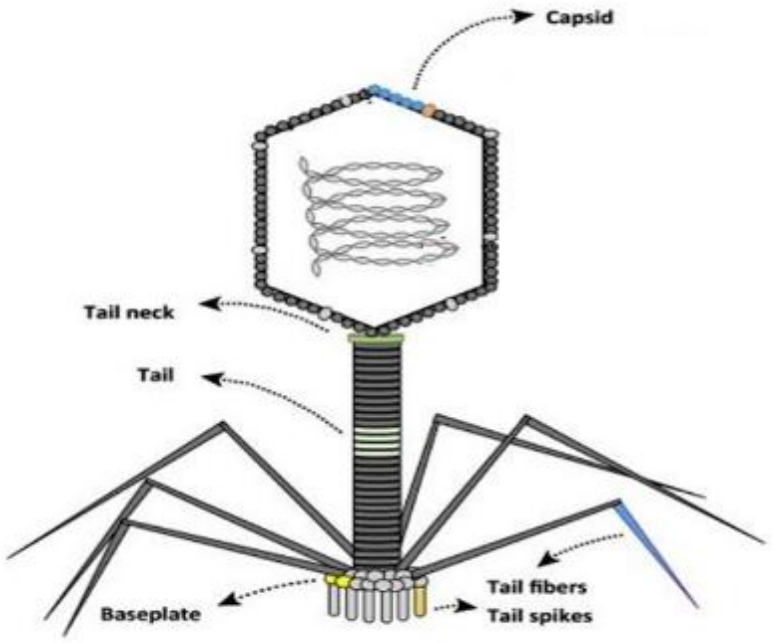


Figure 1-3 A typical structure of a tailed phage of the *Caudovirales* (Nobrega *et al.*, 2015).

The head contains nucleic acid. The phage capsid is attached to the tail through a connector which plays a vital role during the phage infection cycle including packaging dsDNA into the capsid and participating in the release of phage DNA into the host bacterium. The phage DNA is delivered to the host bacterial cell through the tail which has tail fibers at its end so it can bind to its specific bacterial receptor.

The *Caudovirales* are divided into three families according to the tail morphology as *Myoviridae*, *Siphoviridae*, and *Podoviridae* as shown in Figure 1-4. The structure of the phage head remains similar between the three families. The *Siphoviridae* possess long, flexible non-contractile tails and they are the most common of the three types. *Siphoviridae* constitutes ~ 61% of tailed phages and *Podoviridae* make up approximately 14% with a very short and non-contractile tail (Ackermann, 2009). The *Myoviridae* is the second most common of the three types of tailed bacteriophages. Phages in the family of *Myoviridae* compromise ~ 25% of phages reported so far with contractile, more or less rigid, long and relatively thick tails, and a central tube surrounded by a helical contractile sheath, and a tail neck separating the head from the tails, The *Podoviridae* is the least common of the *Caudovirales*, and possess a short, no contractile tail (Ackermann, 2009).

The genome sizes of phages can also be quite varied, such as those of *C. difficile* 027 which are often as small as 30kb (Nale *et al*., 2012), to the larger phages of *P. aeruginosa* which can frequently be between 100kb and 300kb (Ceyssens *et al*., 2010).

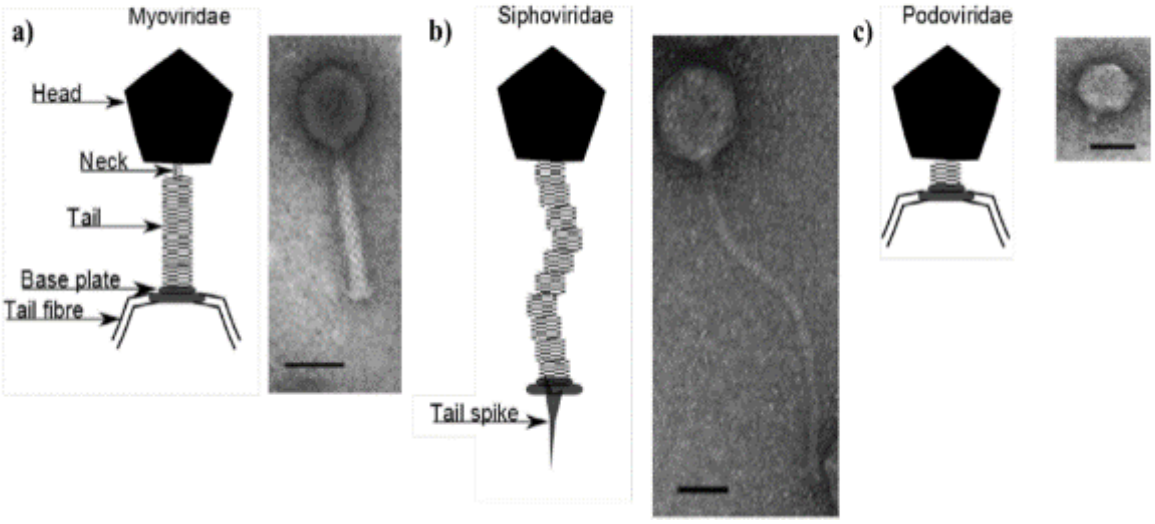


Figure 1-4 Common bacteriophages structure.

The most common structures of bacteriophages. All of which are *Caudovirales*. A) *Myoviridae*, with their contractile tails, b) *Siphoviridae*, with their long, non-contractile tails, and c) *Podoviridae*, with short tails with bars representing 50 nm. The image was adapted from (Sahota, 2016).

Apart from tailed phages, there are pleomorphic and filamentous phages as shown in Figure 1-5, which are further classified into ten small families and accounted for make up approximately 4 % of the phage population (Ackermann, 2009).

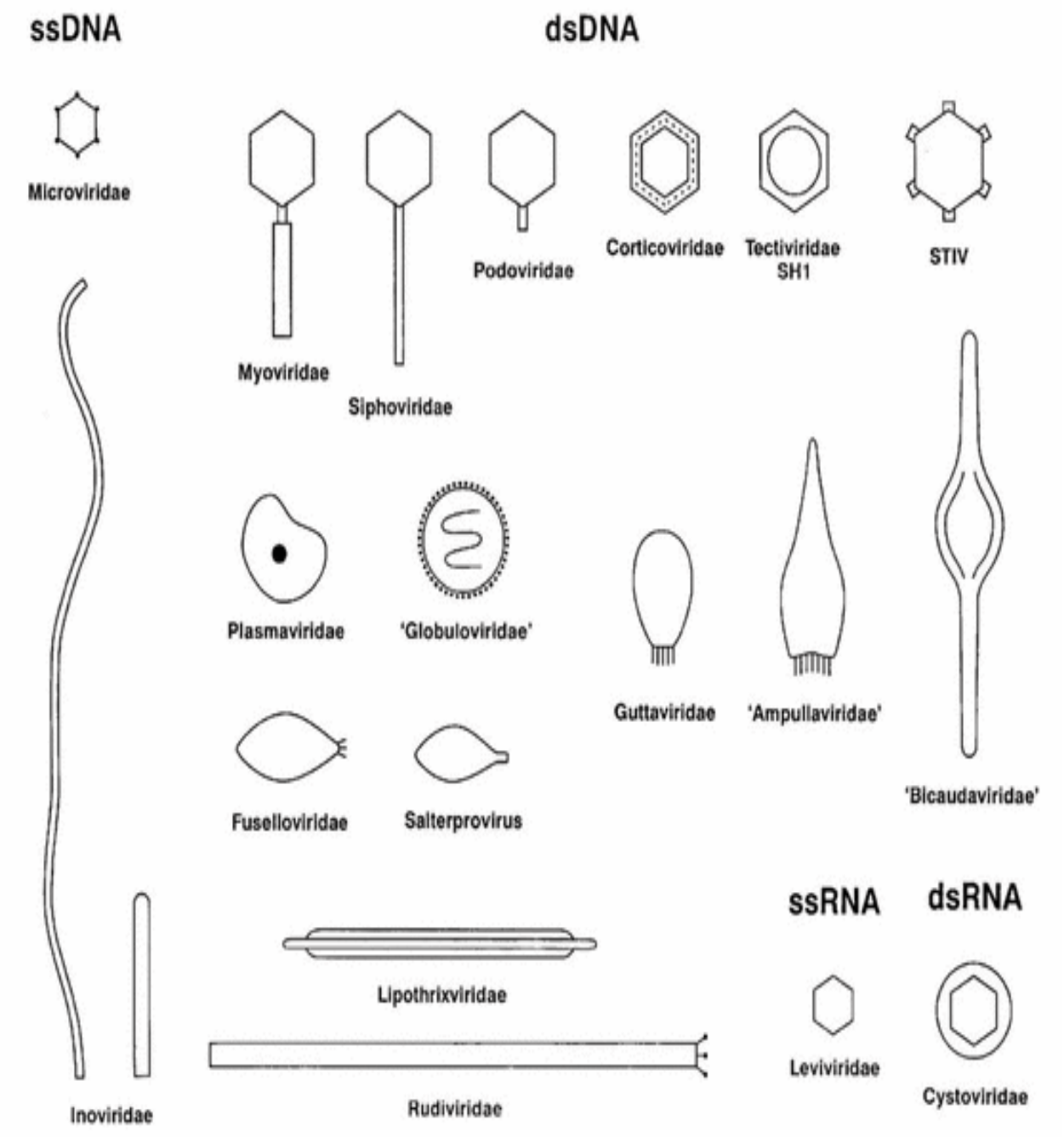


Figure 1-5 Schematic diagram representing various types of phage morphologies (Ackermann, 2009).

1.2.2 Phage life cycle

Phages can be broadly classified into two groups either lytic or lysogenic/temperate phages. A Lytic phage is a virus that infects the host and causes cell lysis. A Lysogenic phage is a virus that infects a host and integrates the genetic material into the host genome. The phage infection cycle starts by phage binding to the host cell through interaction between phage receptor-binding protein located on the phage tails and phage receptors located on the bacterial surface. Phage receptors can be protein, glycoprotein, phospholipids or lipopolysaccharide (Rakhuba *et al*., 2010). The Life cycle of the typical temperate and lytic phages is shown in Figure 1-6. Following phage adsorption, phage DNA will be injected into the host cell. For lytic phages, other phage DNA will replicate and take over the bacterial protein machinery toward producing phage structural proteins and enzymes followed by phage assembly and the nucleic acid packing into the capsid. This process is mediated by two proteins; holins that make pores within the cytoplasmic membrane and endolysins that pass through the holes and cleave the peptidoglycan layer of the bacterial cell wall. Mature phage particles can burst out of the bacterial cells; generally 50-200 new phages could be produced from a single burst of the bacterial cell (burst size), then new progenies are released into the extracellular space (Campbell, 2003).

During the lysogenic cycle, phages will usually integrate into bacterial genomes and remain as part of the bacterial genome (The genetic materials of the phage is called prophages) for generations without lysis of the bacterial host. Under certain conditions such as UV light and antibiotic treatment, the prophages could be induced out of the bacterial genomes and enter the lytic cycle. There is an intermediate phase between the lytic and lysogenic cycle known as pseudolysogeny. In this stage, the phage does not immediately initiate a lytic cycle and does not integrate its genome into the host genome. Instead, it remains as a circular DNA structure (plasmid-like) within the cytoplasm. This phenomenon is thought to occur during conditions such as starvation that make the cell unready for efficient phage infection and cell lysis (Campbell, 2003).

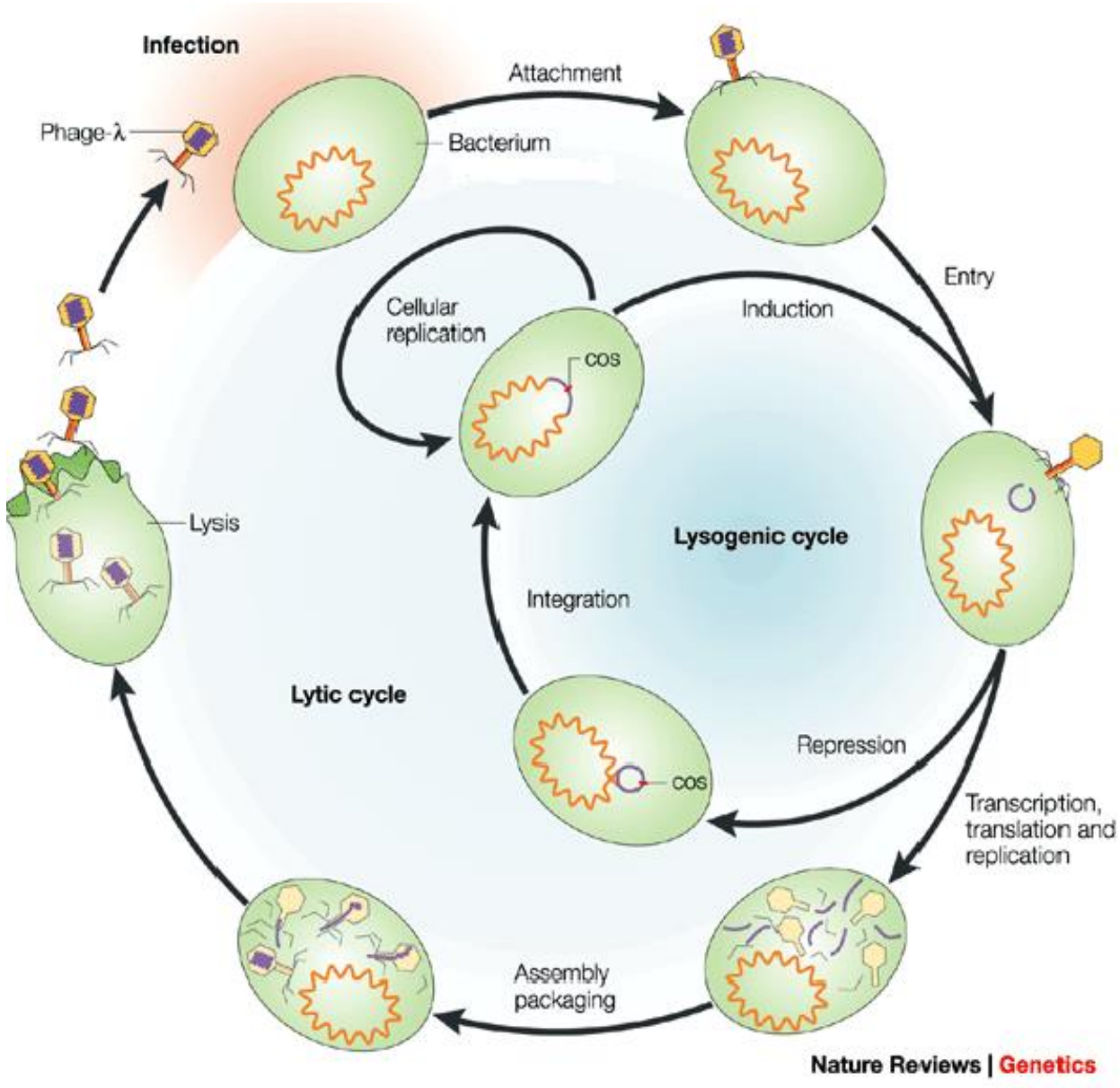


Figure 1-6 Life cycle of the typical temperate and lytic phages.

The phage attaches to the cell surface. Phage starts to integrate the genetic material inside the cell, the DNA starts to circulate to form a circular shape called cos site. In some infected cells, the DNA is transcribed, translated and replicated. Then the phage progeny starts to form the new phages. The new phages start to lysis the cell. This life cycle is called lytic cycle. In other infected cells, phage development is repressed and phage DNA integrates into the bacterial chromosome. This results in formation of lysogenic cells. Lysogenic phage can induce to return to the lytic cycle with the excision of phage DNA from the chromosome. Then, the phage starts to inter to lytic cycle (Campbell, 2003).

1.2.3 Phages associated with spirochetes and particularly with *B. burgdorferi* s.l.

Phage-like particles have been observed under the transmission electron microscope (TEM) in association with a number of spirochetes, including *Leptonema*, *Leptospira*, *Brachyspia*, *Treponema*, and *B. burgdorferi* s.l. (Eggers *et al*., 2000). Most of the TEM images showed typical phages that have polyhedral heads and tails. The majority of them seemed to have a contracted tail, so they appeared to be members of *Myoviridae* family. Additionally, prophage-like gene transfer agent (GTA), named VSH-1, has been identified from Mitomycin C-inducible prophage of the anaerobic spirochete *Brachyspira hyodysenteriae* (Matson et al., 2005). Purified VSH-1 is non-infectious, contains random 7.5-kb fragments of the bacterial genome, and mediates generalized transduction of *B. hyodysenteriae* cells. TEM revealed that VSH-1 has a 45nm head with a 64nm short non-contract tail (Humphrey *et al.*, 1995, Humphrey *et al*., 1997, Eggers and Samuels, 1999, Eggers *et al.*, 2000). Up-to-date, the only confirmed lytic phages isolated from spirochetes were phages of LE3, and LE4 isolated from *Leptospira biflexa* As shown in Figure 1-7**,** phage LE1 is a lysogenic phage that was specific to the *Leptospira biflexa* (Schiettekatte *et al.*, 2018). It has polyhedral head and approximately 85nm in diameter and 100nm long tails. LE3 and LE4 have a hexahedral capsid of 65 nm in diameter, and a contractile tail of 65 to 80 nm in length (Schiettekatte *et al.*, 2018).

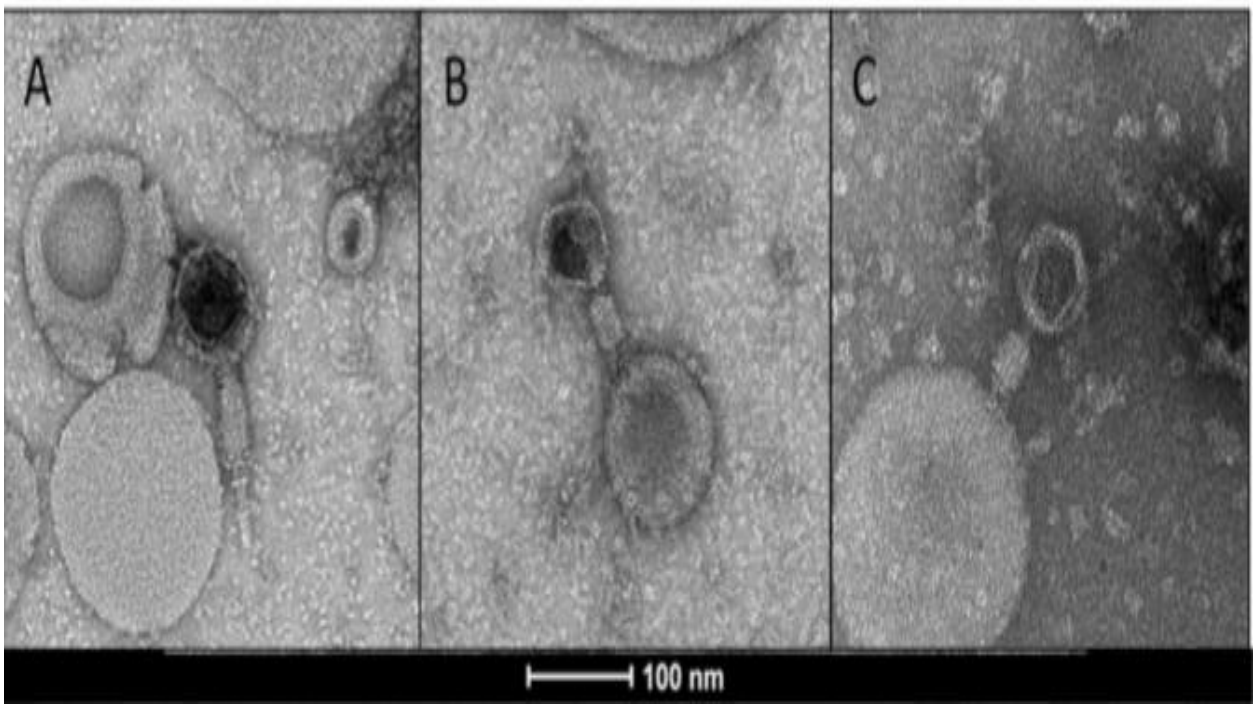


Figure 1-7 Phages of spirochetes (Morphology of phages in *Leptospira bilfexa*).

A) LE1, B) LE3, C) LE4 Image adapted from (Schiettekatte *et al.*, 2018).

The first phage was observed using TEM analysis of *B. burgdorferi* s.l. isolated from *Ixodes dammini* (Hayes *et al.*, 1983). It showed 40 to a 50-nm elongated head and a 50 to 70 nm tail. In addition, bacteriophage like particles were induced from *B. burgdorferi* s.l. culture after treatment with a low dose of ciprofloxacin (Neubert *et al*., 1993). There were two seemingly different phage types morphologically, both showing non-contract tails. DNA alkylating agent 1-methyl-3-nitro nitrosoguanidine (MNNG) has also been successfully used in inducing phages from *B. burgdorferi* s.l. species. For example, phage ϕBB-1 was induced using MNNG from *B. burgdorferi* strain CA- 11.2A (Eggers and Samuels, 1999, Eggers *et al*., 2000). It has an isometric head of 45 to 50 nm in diameter and a contractile tail in a 90 nm length as shown in Figure **1-8**. The origin of ϕBB-1 was determined to be a circular plasmid named cp32 (32-kb in size) (Zhang and Marconi, 2005). Cp32 can spontaneously release and can produce after chemical induction with MitomycinC (Zhang and Marconi, 2005).

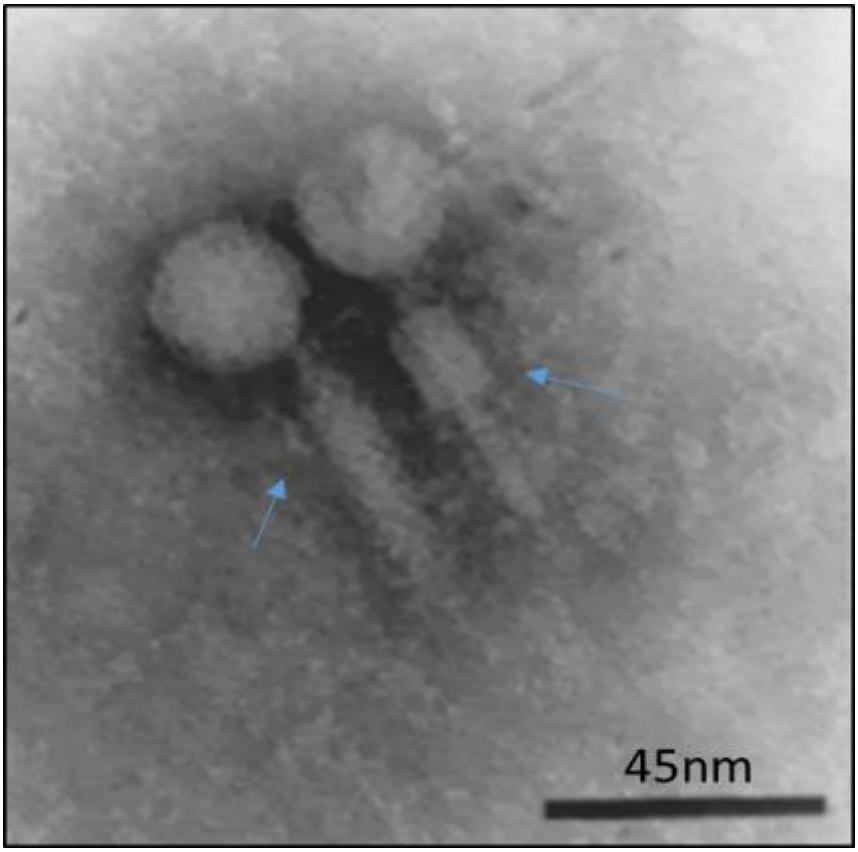


Figure 1-8 TEM image of phage particles (ϕBB-1) induced from *B. burgdorferi* s.l.

Image adapted from (Eggers *et al.* 2001). Left is a phage with a non-contracted tail and right is a phage with a contracted tail were emphasised by blue arrows.

Further analysis of the genome of phage ϕBB-1 revealed two genes, *blyA* and *blyB* coding for a holin and endolysin-like phage lysis cassette. Initial characterisation of the two proteins encoded by *blyA* and *blyB* showed that they had a lytic effect on *E.coli*, leading to their proposed role as a *B. burgdorferi* s.l. encoded hemolysis system (Guina and Oliver, 1997). However, a further investigation by the same research group demonstrated that both genes were, in fact, located on a prophage (in the form of a plasmid cp32) which can be induced out of its bacterial hosts. The bioinformatics analysis also predicted as BlyA to be holin and BlyB to be 'endolysin-like' (Eggers and Samuels, 1999, Eggers *et al*., 2000).

There is no current experimental evidence to confirm the 'holin' nature of BlyA, however, BlyB has not been biochemically proven to be an endolysin- related protein. Interestingly, both genes were up-regulated when the *B. burgdorferi* s.l. subjected to prophage induction treatment (Eggers *et al.*, 2000).

1.2.4 Phage therapy

Research studies exploring the therapeutic application of bacteriophages has been conducted since their discovery at the beginning of the 20th century due to their nature of specifically killing susceptible bacterial hosts (Merril *et al*., 2003). The initial interest in the therapeutic application of phages at the time of their discovery dwindled with the rising popularity of antibiotics (Keen, 2012). However, phage therapy has been continuously studied and practiced in countries such as Russia, Georgia and Poland (Abedon *et al*., 2011) With the rise of bacterial resistance to antibiotics, alternative therapies are being sought after, thus the interest in the phage therapy has been re-kindled globally (Abedon *et al*., 2011).

Phage therapy has been developed for human and non-human applications; in this thesis, I will discuss phage therapy in the context of human applications. Phage therapy involves using single phages, phage cocktails or phage-encoded proteins to treat bacterial infections (Sulakvelidze et al., 2001, Chan *et al*., 2013).

There are many advantages associated with the use of phages in comparison to antibiotics. Phages act in a bactericidal manner, whereas some antibiotics act only in bacteriostatic manner. This can contribute to bacterial resistance as bacteria may persist after treatment; resistant bacteria can survive and further multiply and spread (Abedon *et al*., 2011).

The nature of phage life cycle results in the release of progeny phages; therefore initially phages can be applied in low dose and can be self-sustaining (Abedon *et al*., 2011). Whereas multiple dosages are required in antibiotics therapy. Unlike antibiotics, phages only target a susceptible bacterial host, i.e. show specificity to bacterial genus and species. Phages can target specific bacterial subpopulations, thus maintain the balance of microbiota and avoid the possibility of dysbiosis (imbalances of microbiota). While antibiotics have been shown to affect the microbiota of the host, and lead to antibiotic-associated diarrhoea (Loc-Carrillo and Abedon, 2011). Phages are considered as GRAS (Generally recognized as safe). Few side effects have been reported today for phage therapy. Therefore phages could be useful substitutes to treat patients who are allergic to antibiotics (Borysowski and Górski 2008; Housby and Mann 2009). Finally, production of phages can be relatively inexpensive, therefore having an advantage for application in developing countries. Despite positive results in experimental infections, there are limitations and hurdles associated with the 'live' nature of phages. For example, bacteria are able to evolve phage resistance. The property

and perceived advantage of phages being specific is also a disadvantage; it is difficult to isolate phages that have a broad host range. However, both issues could be overcome by using different phage combinations to target resistant bacteria (Labrie *et al*., 2010). Additionally, the isolation of phages from bacteria that need specific culture conditions can be challenging; therefore for such bacterial species, traditional therapies are more effective (Loc-Carrillo and Abedon, 2011). It is also worth noting that the question remains to be answered whether phages can be effective in treating intracellular bacterial infections, such as tuberculosis and brucellosis.

1.3 Rationale of the study

Currently, no vaccines are available to prevent LB. However, antibiotics therapies against LB can work effectively if the disease is diagnosed early. Despite successful antibiotic therapies being available, patients may suffer from persistent long-term infections or reinfections. This would require long term antibiotic use. The associated negative effects of long-term antibiotic administration and increasing antibiotic resistance; warrants the need for alternative LB therapies. Therapies such as phage therapy can be used to either complement or potentially replace current LB therapies. In term of phage therapy to treat LB is theoretically depend on the idea based on, that there is a phage for every bacteria. If *B. burgdorferi* s.l. phages can find, and then technically modified in order to change their behavior or to find the way to create delivery system where phages be able to kill and lyse *B. burgdorferi* s.l. However, so far phages infecting *B. burgdorferi* s.l., their biology and potential applications are under studied. This study opens promising ways in *B. burgdorferi* s.l. phage research. It investigates the presence of *B. burgdorferi* s.l. from ticks using a novel diagnosis way. It provides practical consideration for future plans to detect *B. burgdorferi* s.l. phages which could be used further for application of phage therapy to treat LB.

1.4 Hypotheses

1. Phages specific for *B. burgdorferi* s.l. can be isolated from ticks.
2. Prophages encoded in *B. burgdorferi* s.l. genomes can be induced and may provide insights into the biology of *B. burgdorferi* s.l.
3. Phages isolated and characterised can be exploited as potential therapeutic agents and used to treat *B. burgdorferi* s.l.

1.5 Aim

In order to explore the therapeutic use of phages to combat *B. burgdorferi* s.l. infection, the fundamental biology of *B. burgdorferi* s.l. phages needs to be investigated. To date, no *B. burgdorferi* s.l. lytic phages have been identified and only one temperate phage from *B. burgdorferi* s.l has been studied following induction. However whole-genome bacterial sequencing revealed a number of free plasmids resemble 'putative phage DNA' in all *B. burgdorferi* s.l. isolates and nearly all of closely related *B. burgdorferi* s.l. species. In this project, the systematic effort will be made to study phages that infect the Lyme *B. burgdorferi* s.l. species. The aim of the study is to isolate and characterise a set of phages associate with *B. burgdorferi* s.l.

1.6 Objectives

1. To induce and study prophages from various *B. burgdorferi* s.l. strains, using Mitomycin C, Norfloxacin and UV light.
2. To ascertain the presence of induced *B. burgdorferi* s.l. phages, using SYBR Green I/PI - based live and dead assay in addition to the traditional methods such as spot test and plaque assay.
3. To characterise the both induced and lytic phages using TEM, whole-genome sequencing and PCR.

4. To identify prophages encoded within the genomes of *B. burgdorferi* s.l. genomes using bioinformatic tools such as PHAST (PHAge Search Tool) and PHASTER (PHAge Search Tool Enhanced Release).

CHAPTER TWO

Materials and Methods

Chapter 2 Materials and Methods

2.1 Ethical issues

This project of isolation and characterisation of bacteriophages infecting *B. burgdorferi* s.l. was approved by University of Leicester Ethics Sub-Committee for Medicine and Biological Sciences (Ethics Reference: 5988-js401-infection immunityinflamm).

2.2 BSK II media

One liter of BSKII medium was prepared using MilliQ Ultra-Pure water. Briefly, BSA Fraction V was firstly dissolved in 900 ml Ultrapure water followed by the addition of the following ingredients as shown in Table 2-1.

Table 2-1 Ingredients of BSKII (Zückert, 2007).

Weight	Contents	Company	Batch number	size
50g	Bovine Serum Albumin Fraction V	GE healthcare	K51-001	1Kg
5g	Neopeptone	Difco BD	211681	500 g
6 g	HEPES sodium salt	Sigma	H-3784	100g
0.7g	Citric acid(trisodium	Sigma	C-8532	100g
	Salt Dihydrate)			
5g	D (+) glucose	Sigma	G-7021	1Kg
2g	TC Yeastolate	Difco BD	255752	100g
0.8g	Pyruvic acid	Sigma	P-8574	25 g
0.4g	N-Acetyl-D-Glucosamine	Sigma	A-3286	25g
2.2g	Sodium Bicarbonate	Sigma	S-5761	500g
100ml	CMRL-1066 Medium(10X)	Gibco BRL	21540	500ml
6%	Rabbit serum	Sigma	R-4505	500ml

The ingredients were mixed together (except Rabbit serum) then sterilised by filtration using 0.22 µm Stericup® Filter vacuum (Merck Millipore, UK) and stored in aliquots at 4°C until needed. The complete BSKII medium (C-BSKII) was prepared by the addition of filtered rabbit serum.

2.3 Plating BSKII (P-BSKII) medium

P-BSKII (yellow in colour) medium was prepared according to the following Table 2-2 (kindly provided by Dr. Ingela Nilsson based at Professor Sven Bergström group, Sweden).

Table 2-2 P-BSKII ingredients (Zückert, 2007).

Weight	Contents	Company	Batch number	size
75 g	Bovine Serum Albumin fraction V	GE healthcare	K51-001	1Kg
7 g	Neopeptone	Difco BD	211681	500 g
9 g	HEPES sodium salt	Sigma	H-3784	100 g
1.1 g	Citric acid(trisodium	Sigma	C-8532	100 g
	Salt Dihydrate)			
7.5 g	D (+) glucose	Sigma	G-7021	1Kg
3.8 g	TC Yeastolate	Difco BD	255752	100 g
1.2 g	Pyruvic acid	Sigma	P-8574	25 g
0.6 g	N-Acetyl-D-Glucosamine	Sigma	A-3286	25g
3.3 g	Sodium Bicarbonate	Sigma	S-5761	500 g
6%	Rabbit serum	Sigma	R-4505	500 ml
100 ml	CMRL-1066 Medium(10X)	Gibco BRL	21540	500ml

All ingredients (except rabbit serum) were mixed together in 1 liter of MilliQ Ultra-Pure water (adjust pH to 7.5 if needed) then sterile using 0.22 µm millipore filter. P-BSKII was aliquoted into bottles (120 or 240 ml), and were stored at -20°C. Sodium bicarbonate solution needs to be freshly prepared every time.

2.4 *B. burgdorferi* s.l. strains

The strains of *B. burgdorferi* s.l. Table 2-3 in the lab were kindly provided by Prof. Sven Bergström, Department of Molecular Biology Umea University, Sweden. All strains were stored in 25% glycerol at - 80^{o} C.

Table 2-3 *B. burgdorferi* s.l. strains used in this study.

Lab serial number	Scientific name	Isolate name
3	*Borrelia burgdorferi* s.s.	VS185 P9
4	*Borrelia valaisiana*	NE218
5	*Borrelia afzelii*	ACA1
6	*Borrelia burgdorferi* s.s.	UK filtered
7	*Borrelia garinii*	190 P9
8	*Borrelia burgdorferi* s.s.	China23
9	*Borrelia burgdorferi* s.s.	UK isolate B31

2.5 Selective BSK II Medium

Selective BSK II medium used for the enrichment of tick samples was prepared by supplementing the BSK II medium with two low concentrations of antibiotics that can inhibit the growth of other bacteria but don't affect the growth of *B. burgdorferi* s.l.. To do that, rifampicin and phosphomycin were added into c-BSKII at a final concentration of 1 µg/mL and 100 µg/ml, respectively (Zückert, 2007, Sapi *et al*., 2013). Rifampicin was prepared as a stock solution of 5 mg/ml in DMSO, while phosphomycin was prepared as 100 mg/ml in sterile distilled water.

2.6 Culturing *B. burgdorferi* s.l.

To prepare *B. burgdorferi* s.l. culture, glycerol stocks of *B. burgdorferi* s.l. in -80ºC were cultured in selective c-BSK II medium at 34ºC. These cultures were then incubated for up to three weeks and examined by phase-contrast microscopy regularly (daily). Subcultures were performed by inoculating 1% of actively growing *B. burgdorferi* s.l. into fresh c-BSKII. (Zückert, 2007).

2.7 Microscopy analysis

Visualisation of *B. burgdorferi* s.l. was carried out using phase-contrast microscopy (CETI magnum-T, scientific limited, UK) under 200X and 400X of magnification. The density of *B. burgdorferi* s.l. was counted using a DHC-N01-Chip Haemocytometer (NanoEn Tek, Korea) The *B. burgdorferi* s.l. concentration was calculated according to the method as follows: cell per ml=average count per 5 large squares x dilution factor x 10000 (volume factor). For a rough estimation, every single *B. burgdorferi* s.l. appeared under 400 magnification was equivalent to the *B. burgdorferi* s.l. concentration 2×10^5 cell per ml (Zückert, 2007).

2.8 Tick enrichment

In this study, 'pooled ticks' were defined as ticks collected from different geographical location in UK as illustrated in Figure 2-1 including Bournemouth, Thetford forest, Woburn, Suffolk, Cambria, Lincolnshire, Lancaster, Nottingham, Yorkshire, Leicestershire, Rushden, Attenborough, Exeter, Essex, Southampton, Dorchester, Bristol, Devon, Dorset, Northland, and other region in Scotland such as Scottish Highlands, Angus, Dundee, Argy II, Orkney, Ayrshire, Glasgow, Moray, Stranraer, and Perth . Tick were collected using our 'tick collection kit' consisting of an O'tom Tick Twister, 15 ml Falcon tubes (to store ticks once collected), clear plastic zipper bags (to hold the Falcon tubes), a paper towel inserted into the Falcon tubes, as well as pre-paid and addressed padded envelopes. Upon the delivery of the collected samples to the lab, the ticks were kept in the fridge for up to one month. The ticks were safely stored in the fridge, and once taken out to room temperature, began moving as usual. Generally, Ticks were collected from August 2015 to November 2016. The peak season for tick activity was during June to September. There were 187 tube of pooled ticks. To increase the chances of obtaining *B. burgdorferi* s.l. and *B. burgdorferi* s.l. phages from ticks, an 'enrichment' procedure was carried out as described below. Ticks were sterilised (washed) in 70% ethanol for 3 mins, dried (to evaporate the alcohol), and crushed manually in 0.5 ml of PBS using sterilised mortar and pestle. The resulting liquid fraction was referred to as 'tick homogenates` that were then inoculated into complete BSKII (C-BSKII) media with both 1 µg/ml of rifampicin and 100 µg/ml of phosphomycin and incubated at 34°C up to four months (enrichment). The enrichment cultures were examined by phase-contrast microscopy after two weeks. Subcultures for tick enrichment cultures were performed every month by inoculating 1-10 % of tick enrichment cultures into fresh C-BSKII broth.

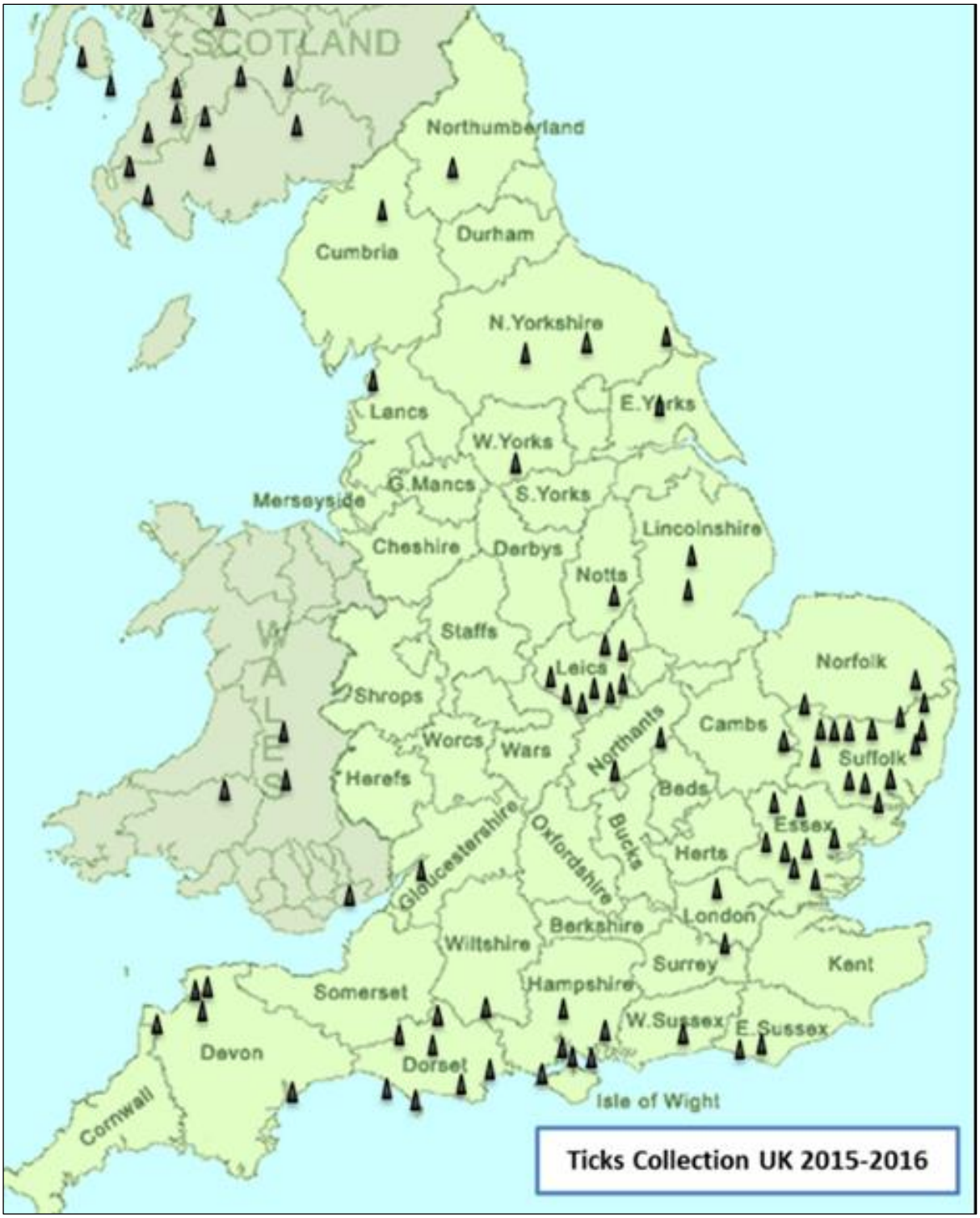


Figure 2-1 Geographical locations showing ticks collection within the UK from August 2015 to November 2016. Black triangles represent the areas where ticks were collected.

2.9 Ticks dissection

Paraplast X-TRA® wax (Sigma-Aldrich Co.) was used to prepare tick embedding plates. The dissection process is illustrated in Figure 2-2. A glue gun was used to melt a small area of wax on the embedding wax plates. Then, forceps were used to place the tick with its scutum (back) facing up in the wax. The tick head and legs were embedded into the melting wax. Once wax solidified the tick became fully immobilised. A drop of PBS solution was added to cover the tick to prevent any tissue desiccation. Then a micro-scalpel was used to remove the scutum by fully cutting around the edges of the tick scutum. Tweezers were used to remove the scutum by exposing the internal organs of the tick. Once the scutum was removed, a dark red, spider-shaped structure (midgut) was revealed. Tick dissection was only possible for large adult ticks or fully-fed nymphal ticks. Larva and un-fed nymph ticks were too small to be dissected, instead, they were grounded in a sterilised mortar and pestle (Edwards *et al*., 2009). Ticks midguts and grounded ticks were placed in Eppendorf tubes contain 500 µl of PBS and homogenised with an inoculation loop. Tick homogenates were either stored at 4°C or inoculated into selective BSK II media at 35°C for up to 4 months to enrich for *B. burgdorferi* s.l. (Edwards *et al*., 2009). The subculture of enrichment culture was carried out every month by inoculating 1-10% of tick enrichment culture into fresh cBSKII broth. The enriched sample was examined for presence of spirochetes using a Microscope.

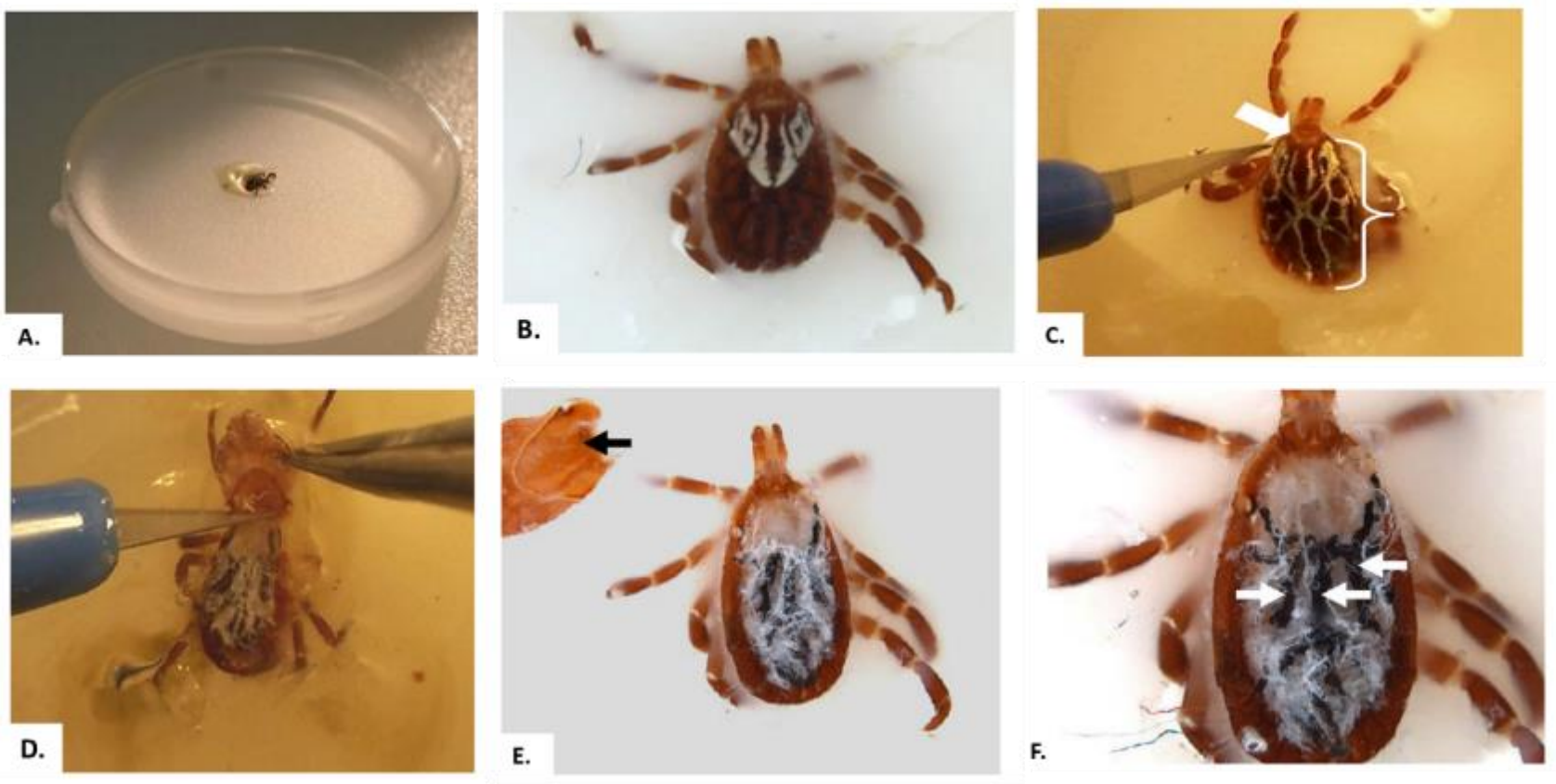


Figure 2-2 Pictures showing tick dissection procedure by (Edwards *et al*., 2009).

A) A glue gun was used to melt a small area on the wax to embed the tick. B) The tick legs were immersed into wax in order to fully immobilise the tick. C)The white bracket indicates the Scutum. D) Using a micro scalpel to remove the Scutum by the cutting around the Scutum dge, E) a black arrow determines Scutum removed, F) the white arrows indicate the internal organs of the tick, (black organs). It surrounded by other organs of the tick. The tick gut was picked up and placed in either enrichment media or PBS. Images were adapted from (Edwards *et al*., 2009).

2.10 Culturing *B. burgdorferi* s.l. in solid media

Before growing *B. burgdorferi* s.l. on petri dishes, the liquid *B. burgdorferi* s.l. culture was examined under a phase-contrast microscope. A healthy and active *B. burgdorferi* s.l. culture will be dominated by typical corkscrew-shaped spirochetes displaying 'snake-like' movement. To culture *B. burgdorferi* s.l. on solid surface, P-BSKII media were prepared according to Section 2.3. 2% Agar was prepared by dissolving 4 grams of Bacteriological agar (VWR BDH Chemicals, UK), in 200 ml of ultrapure water, and autoclaving for 15 mins at 121 °C and equilibrating to 55°C. Then 200 ml of P-BSK medium was mixed with 32 ml of 10 x CMRL 1066, 20 ml rabbit serum, and 17 ml fresh 5% sodium bicarbonate (freshly prepared by dissolving 1 g in 20 ml dH2O) were mixed and filtered through 0.2 µm Millipore syringe filter. The filtrate was then mixed with 200 ml of 2% agar prepared above (mix gently to prevent the formation of air bubbles) and was referred to as 'agar mixture' (equilibrated to 55°C). To prepare the bottom layer, 25 ml of agar mixture was poured into a 90 ml petri dish and left for 20 mins for the agar to solidify. The top layer was prepared by inoculating 300 µl of active growing *B. burgdorferi* s.l. culture in 5 ml of 0.2% agar P-BSKII in (10 ml polystyrene tube) followed by pouring carefully onto the bottom layer. Once the medium solidified, plates were sealed with Parafilm and incubated with the lid facing up at 34°C for two weeks. Single colonies (compact and round) start to appear after 5-7 days.

2.11 DNA isolation

Total DNA was extracted from *B. burgdorferi* s.l. cultures, tick homogenate and the tick enrichment culture using the QIAGEN DNA Mini kit according to the manufacturer's protocol. DNA was quantified by Nanodrop® ND-1000 spectrophotometer and Qubit™ 4 Fluorometer.

2.12 *B. burgdorferi* s.l. DNA isolation using Chelex-100 solution

DNA was extracted from *B. burgdorferi* s.l. colonies using Chelex® 100 (Molecular Biology Grade Resin Bio-Rad Laboratories, California, USA) (Rauter *et al*., 2002). To do this, 5% (w/v) Chelex-100 was prepared in ultra-pure water. One single colony from a C-BSKII plate was added into 200 µl of 5% Chelex. Eppendorf tubes were incubated at 56°C for 30 mins. The

bacterial mixtures were vortexed and heated at 100°C for 10 mins. To prevent accidental opening of the lids due to mounting pressure, the Eppendorf tubes were secured with fasteners (lock) during the heating process. After cooling for 5 mins, the mixtures were centrifuged at 6000 × g for 10 mins. The supernatants containing the DNA templates were collected into sterile Eppendorf tubes. DNA checked for quantity and quality and used immediately for PCR.

2.13 Polymerase chain reaction (PCR)

Different primers in Table 2-4 were used to amplify *B. burgdorferi* s.l./phage DNAs. 50 µl PCR mixtures was prepared by mixing the following ingredients: 25 µl of Dream Taq Green PCR Master Mix (2X), 1 µl of each of the forward and reverse primers, 2 µl of template DNA and nuclease-free water. The negative control was prepared by replacing the DNA template by an equal volume of ultra-pure water. 1 KB DNA Ladder (10 µl) (Bioline, London, UK) was used as a molecular standard. PCR was prepared in a sterile environment such as a class II biological cabinet. Sterile pipettes, tips, Ultra-pure water, and PCR tubes were used. Master mix was prepared according to the number of samples. In each PCR assay *B. burgdorferi* s.l. genomic was used as a PCR positive control. It has been shown that the use of the Thermo Scientific DreamTaq PCR master mix (2X) had many advantages for this project. It was found to be straight forward and easy to use when setting up the PCR, thus avoiding technical errors and ensuring repeatability. As mentioned on the Thermo Scientific website (https:/www.thermofisher.com). The dreamTaq PCR results in a high yield and highly sensitive PCR. The PCR reaction was clean and accurate, according to the negative control. There were no bands for the negative control, indicating that the PCR reaction was carried out under sterile conditions.

2.14 *B. burgdorferi* s.l.-specific PCR targeting *16S rRNA* gene

The primers details are shown in Table 2-4 a with an expected amplicon size of 288 bp (Bugrysheva *et al*., 2003).

2.15 Universal *16SrRNA*

Primers targeting universal *16SrRNA* gene were designed by (Lane, 1991). The amplicon size was 1500 bp. Primer sequences are shown in Table 2-4.

Table 2-4 PCR primers for amplification of target genes with expected fragment sizes.

Primer	Sequence (5'-3')	Target Fragment	Size	References
16SrRNA f	GGC CCG AGA ACG TAT TCA CC	*16SrRNA* gene	288 bp	Bugrysheva *et al*., 2003
16SrRNA r	CGA GCG CAA CCC TTG TTA TC	*16SrRNA* gene	288 bp	Bugrysheva *et al*., 2003
Universal *16SrRNA* f	AGA GTT TGA TCA TGG CTC AG	*16SrRNA* gene	1500 bp	(Lane, 1991)
Universal *16SrRNA* r	GGT TAC CTT GTT ACG ACT T	*16SrRNA* gene	1500 bp	(Lane, 1991)

PCR thermal cycle conditions were as following:

1- *B. burgdorferi* s.l.-specific *16S rRNA* PCR thermal cycles determined in Table 2-5.

Table 2-5 Thermal cycle conditions for *B. burgdorferi* s.l. specific *16SrRNA* PCR.

Primary DNA denaturation	94 °C	2 mins	1 cycle
DNA denaturation	94 °C	30 s	30 cycles
DNA annealing	50 °C	30 s	30 cycles
DNA extending	72 °C	40 s	30 cycles
Final extending step	72 °C	10 mins	1cycle

2- Universal *16SrRNA* thermal cycles is determined in Table 2-6.

Table 2-6 Thermal cycle conditions for Universal *16SrRNA* PCR.

Primary DNA denaturation	94 °C	5 mins	1 cycle
DNA denaturation	94 °C	45 s	30 cycles
DNA annealing	55 °C	1 min	30 cycles
DNA extending	72 °C	1 min	30 cycles
Final extending step	72 °C	10 mins	1cycle

2.17 Phage induction with Mitomycin C or Norfloxacin

The method was modified from (Humphrey *et al*., 1995). Briefly, 50 ml of *B. burgdorferi* s.l. cultures after 7 days of incubation were split evenly into four parts, three aliquots were treated with Mitomycin C or Norfloxacin (Sigma-Aldrich, UK) at a final concentration of 10, 15, 30 µg/ml, respectively. One culture was used as control receiving no treatment. These cultures were incubated at 34°C for 48 h followed by centrifuging at 4500 × g for 20 mins. The resultant supernatants were filtered through a 0.22 µm filter. The filtrate was centrifuged down at 120 000 × g for 4 h at 4°C. The resulting pellet was re-suspend in SM buffer (10 mM NaCl, 8 mM MgSO4.7H2O, 50 mM Tris-Cl, pH 7.5) and was referred to as 'phage suspension'. Alternatively, the induced phages were precipitated using polyethylene glycol (PEG) (Eggers and Samuels, 1999, Eggers *et al*., 2000). Samples were analysed by TEM. All cultures were checked for contamination before and after induction by culturing them on Brain heart infusion agar plates.

2.18 Large scale of phage induction with Mitomycin C and purification with PEG

Once the optimal concentration of Mitomycin C or Norfloxacin in inducing phages was determined, a large-scale phage induction was carried out (Eggers and Samuels, 1999, Eggers *et al*., 2000). 500 ml of *B. burgdorferi* s.l. cultures were treated with Mitomycin C at a final concentration of 10 µg/ml for 48 h. Induced cultures were centrifuged down at 4,500 x g for 20 mins at room temperature. The supernatants were filtered through a 0.22 µm filter. NaCl was added to the filtrate at a final concentration of 1 M, and left on ice for 30 mins. 10% w/v of PEG 8000 (Fisher Scientific, New Jersey, USA) was slowly added followed by rotating for 1 h at 150 rpm. The mixture was centrifuged at 4500 x g for 30 mins at 8°C. The resultant pellet was re-suspended in 2 ml of SM buffer. An equal volume of chloroform was added followed by gentle mixing for 30 seconds. The organic and aqueous phases were separated by centrifugation at 4500 x g for 30 mins at 4°C. The resulting top aqueous phase containing phages was collected. The samples were stored at 4°C for further analysis.

2.19 Induction of *B. garinii* 190P9 strain using UV

14 ml of active *B. burgdorferi* s.l. liquid culture was poured in 90 mm petri dish plates and were exposed to UV light (stratalinker 2400, UK) at 3000 µJ for 0, 10, 20, and 30 seconds. The negative control was a culture of *B. burgdorferi* B31 without irradiation (0 second). The irradiated cultures were incubated at 35°C for 2h to increase the chance of a phage lytic cycle. The cultures were spun down at 4500 x g for 20 mins. The supernatants were filtered using a 0.22 µm filter. The filtrates were concentrated using high-speed centrifuge at 120, 000 x g for 3 h. The pellet was re-suspended in 2 ml SM buffer for further analysis.

2.20 Transmission electron microscopy (TEM)

About 4 µl of each induced prophage suspension was placed on carbon-coated copper grids and was allowed to stand for 4 mins for bacteriophage to bind. The grids were dried with Whatman filter paper, rinsed with 10 µl of double-distilled water, dried and stained with 10 µl of 1% w/v uranyl acetate for a few seconds. The grids were allowed to dry for approximately 5 mins and examined at 80 Kv with TEM-1230 transmission electron microscope (JEOL, Tokyo Japan) equipped with a Dual vision digital camera (Gatan, Pleasanton, CA) (Nale *et al*., 2012).

2.21 Phage enrichment

Enrichment for *B. burgdorferi* s.l. phages was carried out by inoculating 'promising' tick homogenates (those that produced terminase 676 PCR products) and *B. burgdorferi* s.l. cultures into c-BSKII medium. 'Promising' tick homogenates were filtrated through 0.22 µm. 200 µl of the tick homogenate filtrates mixed with 300 µl *B. burgdorferi* s.l. were inoculated into 14 ml of C-BSKII medium. Then, cultures were incubated at 34°C for one week. Cultures were centerfuged at 6000 x g for 10 mins. The supernatants were filtered using 0.22 µm Millipore filter. Phage DNA extraction was performed using the Phenol/Chloroform method (as stated below). The resulting DNA samples were subjected to PCR using Terminase 676 and specific *16SrRNA* primers. Terminase PCR positive and *16S rRNA* PCR negative samples were used for spots test and plaque assay against different *B. burgdorferi* s.l. species.

2.22 Phenol Chloroform Isoamyl alcohol DNA extraction

This protocol was adapted from a previous publication (Eggers and Samuels, 1999). The Phage DNA extraction was carried by adding 500 mM EDTA to 300 µl phage suspension. 5% SDS and 100 µg of proteinase K were added and incubated at 65 °C for 10 mins. DNase and RNase were added to the lysate and incubated at 37 °C overnight. An equal volume of phenol was added and left to stand for 2 mins. Centrifugation was carried out at 21000 x g for 10 mins. The aqueous layer was collected and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (V) was added mixed well and then left to stand for 2 mins before being centrifuged at 21000 x g for 10 mins. The aqueous layer was collected with an equal volume of chloroform: isoamyl alcohol (24:1, v/v) mixed well, left for 2 mins and centrifuged at 21000 x g for 10 mins. The resulting aqueous layer was mixed well with 0.4 volume of 7.5 M ammonium acetate and 2 volumes of isopropanol, and left on ice for 1 h before being centrifuged at 21000 x g for 20 mins at 4°C. After removing the supernatant and briefly air- drying, the resulting DNA pellet was washed once with 500 µl of 70% (v/v) ethanol followed by a final centrifugation of 21000 x g for 10 mins to recover the DNA pellet. The pellet was dissolved in 50 µl of Elution Buffer (EB, 10 mM Tris –Hcl, pH 8.5) a Nanodrop ND-1000 spectrophotometer was used to quantify the DNA samples.

2.23 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and visualise the PCR products; this was prepared by dissolving 1 gram of agarose (Bioline) in 100 ml of Tris base, acetic acid and EDTA (TAE) buffer, and microwave heating for 2 mins. 5 µl of Midori Green loading dye was added to the agarose and poured into a gel tray and allowed to set. PCR products were loaded alongside 1 kb or 100bp DNA ladder (Thermo Scientific ^{TM}, UK). The electrophoresis was performed at 100 volts for 90 mins. Gel electrophoresis was visualised using gel ChemiDOC^{TM} BIO-RAD, UK.

2.24 Live/dead assay using SYBR Green I / Propidium iodide (PI)

SYBR Green I and propidium iodide (PI)-based live/dead assay has been used for the viability assessment for *B. burgdorferi* s.l. (Feng *et al.*, 2014). Whereas SYBR Green I stains all cells, PI stains only cells with a compromised cell membrane. Thus, viable cells with intact membranes are stained green with SYBR Green I, while cells with a compromised membrane (dead) are stained orange-red by PI. The ratio of green-to-red fluorescence is proportional to the ratio of live/dead cells of *B. burgdorferi* s.l. cells. The higher ratio of live cells/dead cells means the higher number of *B. burgdorferi* s.l. present and indicates low level of killing. While low ratio of live cells/dead cells indicates a high level of killing. The test was used to determine killing activity of the induced *B. burgdorferi* s.l. phage lysates against different *B. burgdorferi* s.l. strains. Briefly, 200 µl of *B. burgdorferi* s.l was inoculated into 96-well plates (L form). Then, 20 µl of induced *B. burgdorferi* s.l phage suspension was added. The plate was incubated in 34ºC incubator for 5 days. Non-phage control wells were prepared by adding bacteria and 20 µl of complete BSKII medium. 20 µl tetracycline was added as a positive control. The plate was sealed with a thermal seal RTS^{Tm} film membrane to avoid evaporation. After 5 days of incubation, the 96-well plates were centrifuged at 1500 x g for 10 mins to pellet *B. burgdorferi* s.l. The resulting supernatants were removed, and the wells were rinsed with 200 µl fresh BSK-II medium and centrifuged at 1500 x g for 10 mins. 100 µl of fresh BSK-II medium was added to each well and mixed well. 10 µl of fluorescence dye mix (prepared by mixing 20µl of SYBR Green I 10,000 × stock, (Invitrogen) and 60 µl of 20 mM Propidium iodide (Sigma) into 2.0 ml of sterile dH_2O) was added to each well and mixed thoroughly. The plate was incubated

at room temperature in the dark for 15 mins. The fluorescence intensities at 535 nm (green emission) and 635 nm (red emission) were measured using Varioskan with excitation wavelength at 485 nm. The green/red fluorescence ratio was calculated for each well.

2.25 Development of a spot test on *B. burgdorferi* s.l. lawn

To measure 'anti- *B. burgdorferi* s.l.' activity using a spot test, the first thing is to grow *B. burgdorferi* s.l. into a lawn on a petri dish. This was achieved by mixing 300-500 µl of *B. burgdorferi* s.l. culture with 3 ml of p-BSKII and 0.4% of agar overlays and pouring onto p-BSKII agar plate. The plate was left to set for 30 mins. 10 µl of samples (filtrates from phage enrichment, or filtrates from phage induced *B. burgdorferi* s.l. cultures) were spotted on. Tetracycline was used as a positive control. Plates were sealed with parafilm. The plates were incubated at 35°C. The plates were then inspected for clear zones.

2.26 Plaque assay

Apart from spot test, lytic activity against *B. burgdorferi* s.l. can also be evaluated using classical plaque assay. 250 µl of phage suspension was mixed with 500 µl of *B. burgdorferi* s.l. culture with an OD_{600} of 0.2 in a 10ml falcon tube. The phage - bacteria mix was then mixed with 3 ml of soft P-BSKII 0.4% agar contain (1 M of MgCl, MgSO4, CaCl2, and NaCl) salt buffer followed by pouring plates (round petri dish). It will take 30 mins for soft agar to solidify. The plats were sealed with parafilm membrane and incubated at 35°C. The plates were then checked for complete lysis or plaques.

2.27 Preparation of *B. burgdorferi* s.l. DNA for Sanger sequencing

DNA was extracted from *B. burgdorferi* s.l. culture using E.N.Z.A.® genomic DNA isolation Kit (Omega Bio-Tek, USA). The quantity and quality of DNA were measured using Qubit for dsDNA high sensitivity kit before sending GATC company for sequencing.

2.28 Analysis of the PCR products

The chromatogram files (.abi format) were retrieved from the GATC website and analysed using APE 2.0.52. The good quality sequence should be seen by well-defined peak resolution, uniform peak spacing, and high signal-to noise ratios as suggested by https://www.seqme.eu/en/magazine/sanger-data-analysis. To remove the bad resolusion, all the sequences were trimmed by ~ 20 nucleotides downstream and upstream to delete any poor-quality sequence in the beginning and at the end of the sequence. The cleaned sequence was copied and blasted in nucleotide BLAST to confirm the identity. In order to confirm the result, the reverse complementary of the sequences can be blasted in NCBI and the gene identity can be identified.

2.29 Genome sequencing and *B. burgdorferi* s.l. prophage analysis

DNA isolated from 12 *B. burgdorferi* s.l. strains (six DNA samples isolated from *B. burgdorferi* s.l. strains that purified from ticks and six DNA samples were isolated from *B. burgdorferi* s.l. lab strains) were extracted and sent to Microbes NG for Whole-genome sequencing. The result analysis was received through (http://www.microbesng.uk), The *B. burgdorferi* s.l. genomes were downloaded and used for further analysis. *B. burgdorferi* s.l. genomes were analysed for prophage presence using PHASTER/PHAST analysis.

2.30 Prophage analysis using PHAST/PHASTER

PHAST and PHASTER tools were used to identify potential prophage regions and to access nucleotide and amino acid sequences for predicted genes. In this study, sequences of 12 *B. burgdorferi* s.l. strains were received as FASTA files from MicrobesNG, and were analysed for the prophage presence. The FASTA sequence was upload through PHAST at (http://phast.wishartlab.com) and confirmed by PHASTETR webserver at http://phaster.ca/. In addition one strain called *B. afzelii* ACA-1 was already sequenced and has accession number (GCA_000170935.2), the accession number was uploaded through PHAST/PHASTER webservers. PHAST/ PHASTER were produced extensive text summaries, downloadable figures, and circular and linear genome views as well as colorful, scalable, user-interactive graphics (Zhou *et al*., 2011, Arndt *et al*., 2016). The FASTA sequence of the prophage region was copied and saved in Notepad^{++} file.

CHAPTER THREE

Isolation and Characterisation of *B. burgdorferi* s.l. and phages from ticks

Chapter 3 Isolation and Characterisation of *B. burgdorferi* s.l. and phages from ticks

3.1 Introduction

Ticks are important vectors capable of transmitting a number of human and animal pathogens, including viruses, bacteria and protozoa. Ticks of the *Ixodes* spp. are the main vectors which carry *B. burgdorferi* s.l. strains, the causative agents of LB, among which *B. afzelii, B. garinii, B. spielmanii, B. burgdorferi* sensu stricto*, B. valaisiana*, and *B. lusitaniae* are thought to be pathogenic for humans and are therefore considered clinically important (Shapiro, 2014, Rudenko *et al.*, 2011). The diagnosis of LB relies on clinical practice. Having said that, there are microbiological and molecular tests available, such as indirect methods based on detecting the host immune response, direct culturing from clinical specimens and PCR against tissue and tissue fluids. However, all of these methods suffer from low sensitivity (Wilske *et al.*, 2007).

The current 'gold standard' method for detecting *B. burgdorferi* s.l. in clinical samples involves culturing the samples may contain *B. burgdorferi* s.l. Generally, the growing of cultures can take up to seven weeks before a sufficient amount of the bacteria is developed; however, such a test may not help differentiate between Lyme disease and other tick-borne bacterial infections (Marques, 2015). *B. burgdorferi* s.l. spp. are notoriously difficult to grow *in vitro* because they are well adapted for survival inside ticks and mammals. Therefore, a complex growth medium is needed to grow them *in vitro*. The media used for the culturing of *B. burgdorferi* includes various versions of the Barbour-Stoenner-Kelly (BSK) medium and the modified Kelly Pettenkofer (MKP) medium (Barbour, 1984, Zückert, 2007). The direct microscopic detection of *B. burgdorferi* sensu lato has limited utility in the laboratory due to the diversity of microorganisms in the tested samples (Aguero-Rosenfeld *et al*., 2005). Different techniques have been used to visualise *B. burgdorferi* s.l., such as dark-field microscopy or fluorescent microscopy after staining with acridine orange (Aberer and Duray, 1991, Wallach *et al*., 1993, Olsen *et al*., 1995) Phase-contrast microscopy is useful in examining the morphology of bacterial cells; phase-contrast illumination can be used to enhance the enormous contrast within the cells samples, without staining. Phase-contrast

microscopy has been used to identify *B. burgdorferi* s.l. from tick samples at 400X (Olsen *et al.*, 1995, Axline, 1981).

Different phenotypic and molecular typing methods have been developed to characterise *B. burgdorferi* s.l. (Wang *et al*., 2014b). Conventional bacterial phenotypic typing methods, such as those based on the evaluation of colony morphology, resistance to various chemicals (antibiotic susceptibility), as well as more bacteriophage typing, cannot be applied to the *B. burgdorferi* s.l. genus directly due to the extremely fastidious nature of *B. burgdorferi* s.l. when growing *in vitro* (Tang *et al*., 1997). A serotyping method based on two exterior surface proteins, the outer surface protein A (OspA) and the outer surface protein C (OspC), has been investigated on the basis of differential immune responses to different epitopes of OspA and/or OpsC (Marconi *et al*., 1999). Apart from classifying *B. burgdorferi* s.l. according to their antigenic structure or their ability to react with specific antibodies, other typing methods have been developed to harness *B. burgdorferi* s.l.'s genetic composition as ways of differentiation (Wang *et al.*, 1999, Wang *et al*., 2014b). These so-called 'molecular methods' include DNA-DNA homology analysis, as well as ribotyping, which is rRNA gene restriction analysis (van Dam *et al*., 1993), and PCR methods targeting conserved regions such as *16S rRNA* DNA sequences, flagellin (*fla*) and ospA. Other PCR-based methods such as pulsed-field gel electrophoresis (PFGE) (Belfaiza *et al*., 1993), random amplified polymorphic DNA (RAPD) fingerprinting (Wang *et al*., 1998), variable-number tandem repetition (VNTR) (Farlow *et al*., 2002) and whole generation sequencing (WGS) analysis have also been developed and used in the typing of *B. burgdorferi* s.l. (Vayssier-Taussat *et al*., 2013, Wang *et al*., 2014a).

3.1.1 *16S rRNA* gene

The *16S rRNA* gene was the first marker being explored as a PCR target in the detection of *B. burgdorferi* s.l. (Schwartz *et al*., 1992). *16S rRNA*-based primers were designed to amplify and differentiate spirochetes species associated with Lyme disease. The sensitivity and specificity of *16S rRNA* primers have been tested against more than 38 *B. burgdorferi* s.l. isolates; all were found to be positive (Marconi and Garon, 1992). Recently, the PCR amplification of a segment of the *16S rRNA* gene (referred to as "core genome" in the paper), coupled with sanger sequencing was proposed as a way to detect and distinguish *B. burgdorferi* s.l. strains (Lee *et al*., 2019). The diagnostic method involving the PCR detection of bacteria is often low

in sensitivity due to the fact that the concentration of the bacteria in clinical samples is extremely low. For example, previous findings have shown that only a third of clinical samples from patients in the USA with *B. burgdorferi* s.l. showed positive PCR results; when tested from cerebrospinal fluid samples, half of the clinical samples of patients in the early stages of LD showed negative PCR results (Stanek *et al.*, 2012).

3.1.2 Terminase gene

B. burgdorferi s.l.is a slow-growing organism and requires a complicated medium for *in vitro* culture, therefore, a culture-based method is not practical for the routine detection of *B. burgdorferi* s.l. To increase the sensitivity of *B. burgdorferi* s.l. identification, the previous study by (Clokie and Shan, 2019) discovered a phage terminase gene located in several plasmids, including the cp32 series within the *B. burgdorferi* s.l. genomes (Clokie and Shan, 2019). Terminase is an essential phage protein responsible for phage genome packaging (Zhang and Marconi, 2005, Brisson *et al*., 2012). Eight copies of phage genes coding for the terminase were identified on *B. burgdorferi* s.l. plasmids (Clokie and Shan, 2019). These terminase genes were highly similar to each other which allowed for the designing of primers and probes to target identical regions. Consequently, a phage terminase-based qPCR was developed and validated in the lab (Clokie and Shan, 2019). The performance of the phage-based qPCR also was shown to be almost three times more sensitive than the current most standard serological method when tested against Lyme patient samples (Clokie and Shan, 2019). Currently, no systematic experimental effort has been put into trying to understand and characterise *B. burgdorferi* s.l. phages. The only confirmed *B. burgdorferi* s.l. phage that can be induced out of *B. burgdorferi* s.l. is the circular plasmid (cp) 32 (cp32) carried by *B. burgdorferi* s.l. (Casjens, 2003).

3.1.3 PHAST and PHASTER

PHAST and PHASTER tools are online web servers designed for the screening of prophages or phage-like regions. PHAST was developed by (Zhou *et al*., 2011), and was designed to rapidly identify, annotate and graphically display prophage sequences within bacterial genomes or plasmids. PHASTER is the updated version of PHAST. It uses an up-to-date database, and is faster and more accurate in identifying prophages. PHASTER can analyse a raw sequence in three minutes and an annotated sequence in less than one and a half minute. The PHASTER web server can be accessed via www.phaster.ca (Arndt *et al*., 2016). Since its release in 2016 (Arndt *et al*., 2016), PHASTER has gained much popularity. Both PHAST and PHASTER identify and annotate the prophage using the GLIMMER gene prediction and local genome annotation tools. The prophage is annotated according to position, length, boundaries, number of the genes, attachments sites, tRNA, and the identified phage-like genes and attachment sites (*att*). The prophage region is identified as intact, questionable, or incomplete according to the 'completeness score'. A prophage region with a maximum score of 150 is an 'intact' phage (Zhou *et al*., 2011, Arndt *et al*., 2016). However, if the region contains more than 50% of the known phage-related genes, the score of the completeness is determined to be between 70-90, and the prophage is identified as questionable (Zhou *et al.*, 2011, Arndt *et al.*, 2016). If the region contains less than 50 % of the known phage-related genes, the prophage is identified in a score range of less than 70 and will be considered incomplete (Zhou *et al*., 2011, Arndt *et al*., 2016).

The first step in exploiting the therapeutic use of phages to combat *B. burgdorferi* s.l. infection is to isolate and characterise phages that are potentially lytic to their host, i.e. understanding the fundamental biology of *B. burgdorferi* s.l. phages. Until now, no *B. burgdorferi* s.l. lytic phages have been identified. This chapter represents the first attempt to isolate *B. burgdorferi* s.l. specific phages from ticks.

3.2 Methods

3.2.1 *16S rRNA* PCR for *B. burgdorferi* s.l. identification

A total of 187 tubes contain pooled ticks were delivered to the lab from different places. Ticks were homogenided and these homogenised ticks were inoculated in enrichment cultures. The presence of *B. burgdorferi* s.l. in tick homogenates and in tick enrichment cultures were determined using PCR targeting the conserved *16S rRNA* gene. This was performed by extracting DNA from both tick homogenates and tick enrichment samples. A total of 187 DNA samples isolated from tick homogenates, and 187 DNA samples isolated from their corresponding tick enrichment cultures were analysed. PCR was applied to test the DNA samples, the positive control was *B. burgdorferi* B31 DNA. The negative control was ultrapure water, The positive PCR samples have an expected amplicon size of 288 bp.

3.2.2 Terminase gene among *B. burgdorferi* s.l., multi-sequence alignment , and design of PCR primers (Ter676 primers).

In order to design a PCR which targets the *B. burgdorferi* s.l. terminase consensus region. A multi-sequence alignment of the full-length terminase gene of 1353 bp was performed using T. Coffee against the following *B. burgdorferi* s.l. strains: *B. afzelii* ACA-1, *B. bavariensis* PBi, *B. bissettii* DN127, *B. burgdorferi* B31, *B. garinii* PBr, *B. turicatae* BTE5EL, *B. valaisiana* VS116, *B. mayonii* MN14-1539 , *B. coriaceae* DOU, and *B. finlandensis* SV1. BoxShade server was used to present the alighnment.

Terminase 676PCR primers (Ter676 primers) targeting the conserved regions of the phage terminase gene were designed based on the terminase genes from *B. burgdorferi* s.l. genome using BLAST PCR primers (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Terminase gene sequence accession number (AAF07402.1) was determined previously by manually inspecting the *B. burgdorferi* s.l. genome sequences in NCBI. The full terminase sequence can be found in the Appendices sections (Appendices 4). The resulting PCR primers amplified a 676 bp region and were referred to as Ter676 primers, The primers consist of a forward primer (Fwd.Ter676): 5'-GGAGGCATAGCTAGTGGCAAA-3' and a reverse primer (Rs.Ter676): 5'-CCGCCAACACTAAATGCT-3'.

PCR primer status software (http://www.bioinformatics.org/sms2/pcr_primer_stats.html), was used to evaluate the quality of the primers. Parameters such as single base run, dinucleotide base runs, primer length, GC content, melting temperature, GC clamp, and self-annealing & hairpin formation were included. The optimal parameters for a good primer were set as following: length 18-22 base pair, melting temperature - 50-60ºC, GC content - 40-60%, GC clamp was avoided in the last 5 bases at the 3' end of the primer. No self-annealing, and hairpin formation were permitted. Before conducting 'wet PCR', *In silico* PCR (http://insilico.ehu.es/PCR/) was carried out to test the specificity of these primers against the sequence of *B. burgdorferi* s.l. spp. Terminase 676 PCR thermal cycle conditions are shown in Table 3-1.

Table 3-1 Thermal cycle conditions for Terminase 676 PCR

Primary DNA denaturation	94ºC	2 mins	1 cycle
DNA denaturation	94ºC	30 s	30 cycles
DNA annealing	55ºC	30 s	30 cycles
DNA extending	72ºC	40 s	30 cycles
Final extending step	72ºC	10 mins	1cycle

3.2.3 Terminsase 676 PCR for phage identification in ticks

Following on from the *in silico* PCR, the wet PCR was carried out. Terminase 676 PCR was applied to the same DNA samples extracted from tick homogenat and tick enrichments samples tested before for *16S rRNA.*

3.2.4 Single colony purification of *B. burgdorferi* s.l.

To develop a method to culture *B. burgdorferi* s.l. as a single colony from enriched tick cultures, a previously published method by (Zückert, 2007), was modified using p-BSKII media instead of the typical BSKII media for single colony purification. Agar was used for the plating of *B. burgdorferi* s.l. on the solid medium, with 1 % w/v agar for the bottom layer and 0.2 % w/v for the top layer.

3.2.5 Spot tests of tick homogenates filtrates on *B. burgdorferi* s.l. lawns

In this chapter, Spot test as in sections 2.25 was used to test 31 filtrates of tick homogenates that showed positive signals for the *B. burgdorferi* s.l. *16S rRNA* and terminase PCRs, against *B. burgdorferi* s.l. lawns alongside tetracycline (positive control). The presence of lytic zones from tested samples determine any killing activitiy.

3.2.6 Next-generation sequencing of *B. burgdorferi* s.l. isolates.

Twelve DNA libraries were whole-genome sequenced by Microbes NG using the Illumina platform, including six DNA samples derived from single colonies isolated from tick enrichment samples, these being 18, 19, 20, 21, 89 and 90 samples. In addition to this, six DNA samples extracted from the *B. burgdorferi* s.l. reference lab strains Bb VS185 P9, Bv NE218, BbUK filtered, Bg 190 P9, Bb China 23 and BbB31 were also sequenced. Row sequences data obtained from this sequencing in form FASTA format of FASTA Q files. The annotation was done with Prokka version 1.12 in genebank file (gbk) and general feature format (gff). The genome was analysed by microbes NG to see what the expected sample to be.

3.2.7 Identification of *B. burgdorferi* s.l. prophages using PHASTER/PHAST analysis and determine the similarties between them.

In order to understand prophage carriage, thirteen *B. burgdorferi* s.l. genomes, these being the 12 genomes obtained from Microbes NG from this study, and the reference lab strain *B. afzelii* ACA-1 genomes were analysed by PHASTER / PHAST. The 13 genomes are made up of the six new strains isolated from ticks, and the following seven lab strains: *B. burgdorferi* VS185 P9, *B. valaisiana* NE218, *B. afzelii* ACA-1, *B. burgdorferi* UK filtered, *B. garinii* 190 P9, *B. burgdorferi* China 23 and *B. burgdorferi* B31. The sequence similarity of the putative 'prophage-like' regions identified by PHASTER was annotated by sequences annotation using Rapid annotation subsystem technology (RAST) pipeline as described previously by (McNair *et al*., 2018) and sequences were presented using Easyfig version 2.2.3, a Python application used to create linear comparison figures of multiple genomic loci (Sullivan *et al*., 2011). BLAST comparisons between multiple genomic regions, ranging from single genes to whole prokaryote chromosomes, can be generated, visualized and interactively coloured, enabling a rapid transition between analysis and the preparation of publication-quality figures (Sullivan *et al.*, 2011).

3.3 Results

3.3.1 *16S rRNA* PCR for *B. burgdorferi* s.l. identification

PCR results are shown in the gel pictures below Figure 3-1. The data showed that on average, 3.2 % of pooled UK ticks were tested positive in *16S rRNA* PCR. Only 6 out of 187 tick homogenates were tested positive, and 181 out of 187 samples of tick homogenate were negative. *16S rRNA* PCR was also applied on DNA extracted from tick enrichment cultures and results as following: only 17% (31/187) of tick enrichment cultures were tested positive for *B. burgdorferi* s.l. *16S rRNA* PCR, and 156out of 187 samples of tick enrichment cultures were tested negative. *16S rRNA* PCR results are shown in Figure 3-1; the PCR results for the first batch of DNA extracted from tick homogenate numbered as 18, 19, 20, 21, 89, and 90 for ticks delivered from Lincoln, Bournemouth, Thetford Forest and Dorchester.

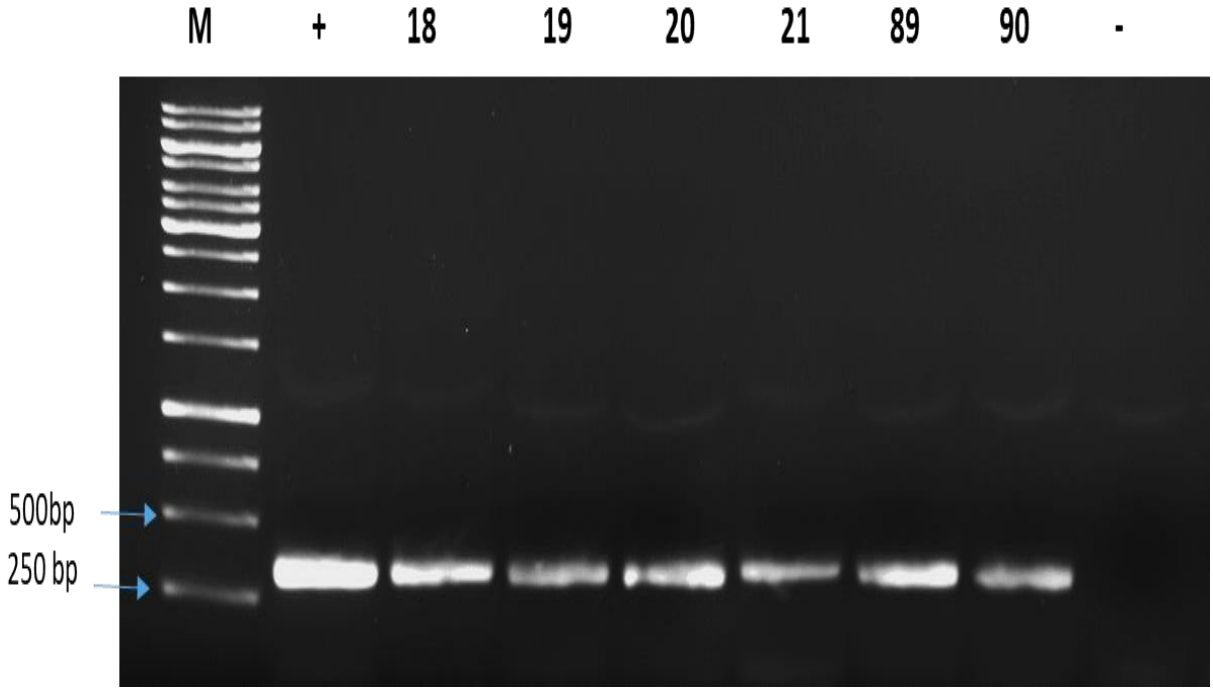


Figure 3-1 PCR targeting the *B. burgdorferi* s.l. *16S rRNA* gene in the tick homogenat DNA samples.

DNA was extracted from homogenate samples obtained from ticks delivered from Lincoln, Bournemouth, Thetford Forest and Dorchester, samples 18, 19, 20, 21, 89, and 90. The positive control used was *B. burgdorferi* B31 DNA. The negative control was ultrapure water. The expected 288 bp amplicon can be seen present for DNA samples 18, 19, 20, 21, 89, and 90, as well as in the B31 DNA. M is λ DNA marker.

Then PCR using DNA from the tick enrichment samples was done. Figure 3-2 and Figure 3-3 show the positive results of the PCR using DNA from tick enrichment samples.

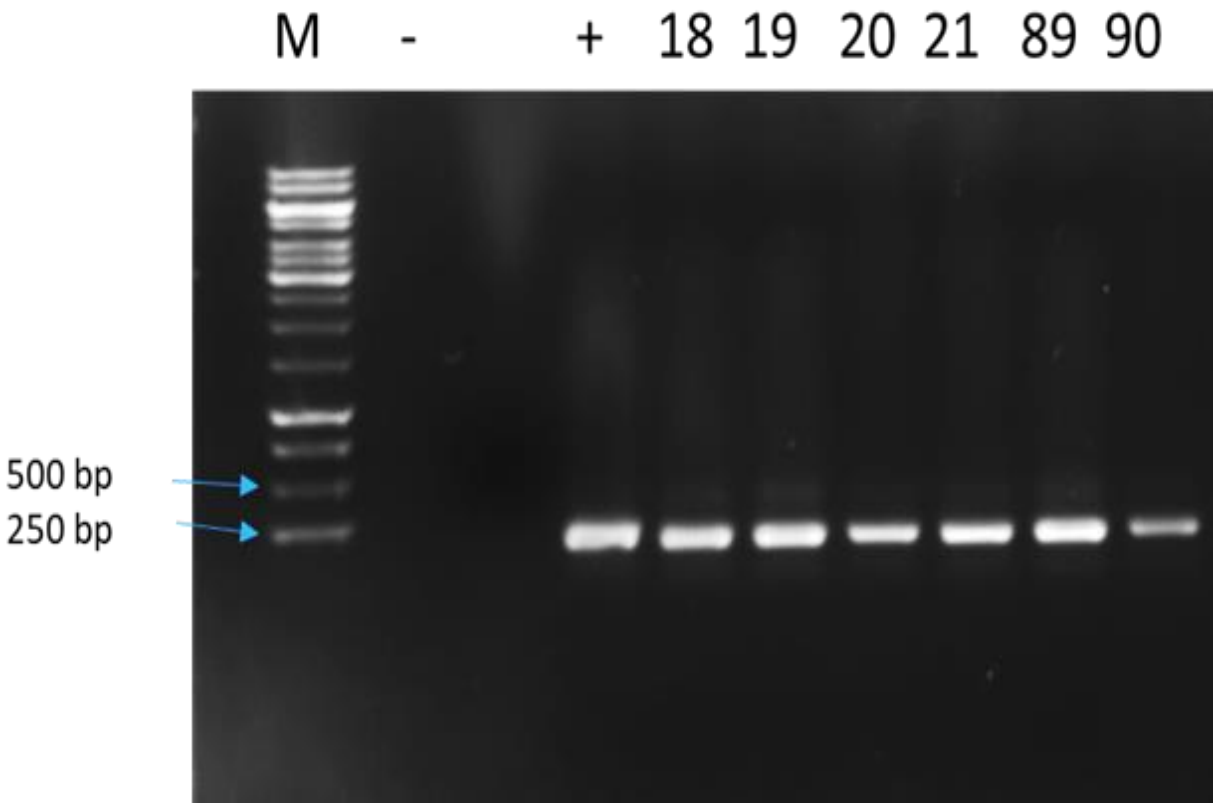


Figure 3-2 PCR targeting *B. burgdorferi* s.l. *16S rRNA* gene in tick enrichment DNA samples.

DNA was extracted from tick enrichment samples of the same ticks that delivered from Lincoln, Bournemouth, Thetford Forest and Dorchester (samples no. 18, 19, 20, 21, 89, and 90). The positive control was *B. burgdorferi* B31 DNA. The negative control was ultrapure water. The expected 288 bp amplicon was amplified in samples 18, 19, 20, 21, 89, and 90, as well as in the B31 DNA. M is λ DNA Marker

The rest of DNA sample extracted from tick enrichment samples from ticks delivered from Bournemouth, Thetford Forest, Woburn, Suffolk, Scottish Highlands, Ayrshire, Leicester, Southhampton Dorchester, Rusden and Attenborourgh, Exeter, Bristol, Dorset, and Essex as mentioned in Appendices 2. PCR products represent *B. burgdorferi* s.l. *16S rRNA* PCR amplicon with an expected size of 288 bp.

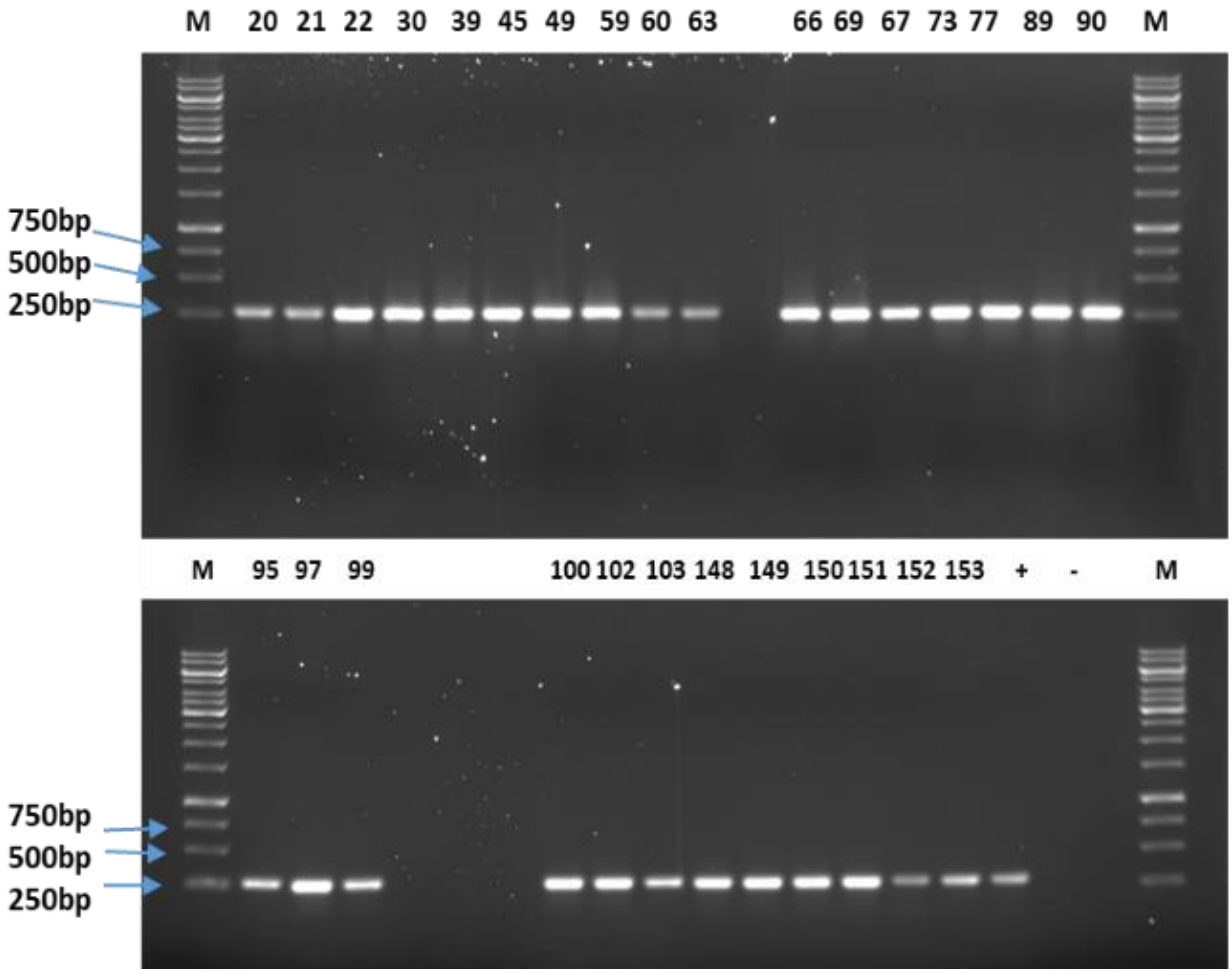
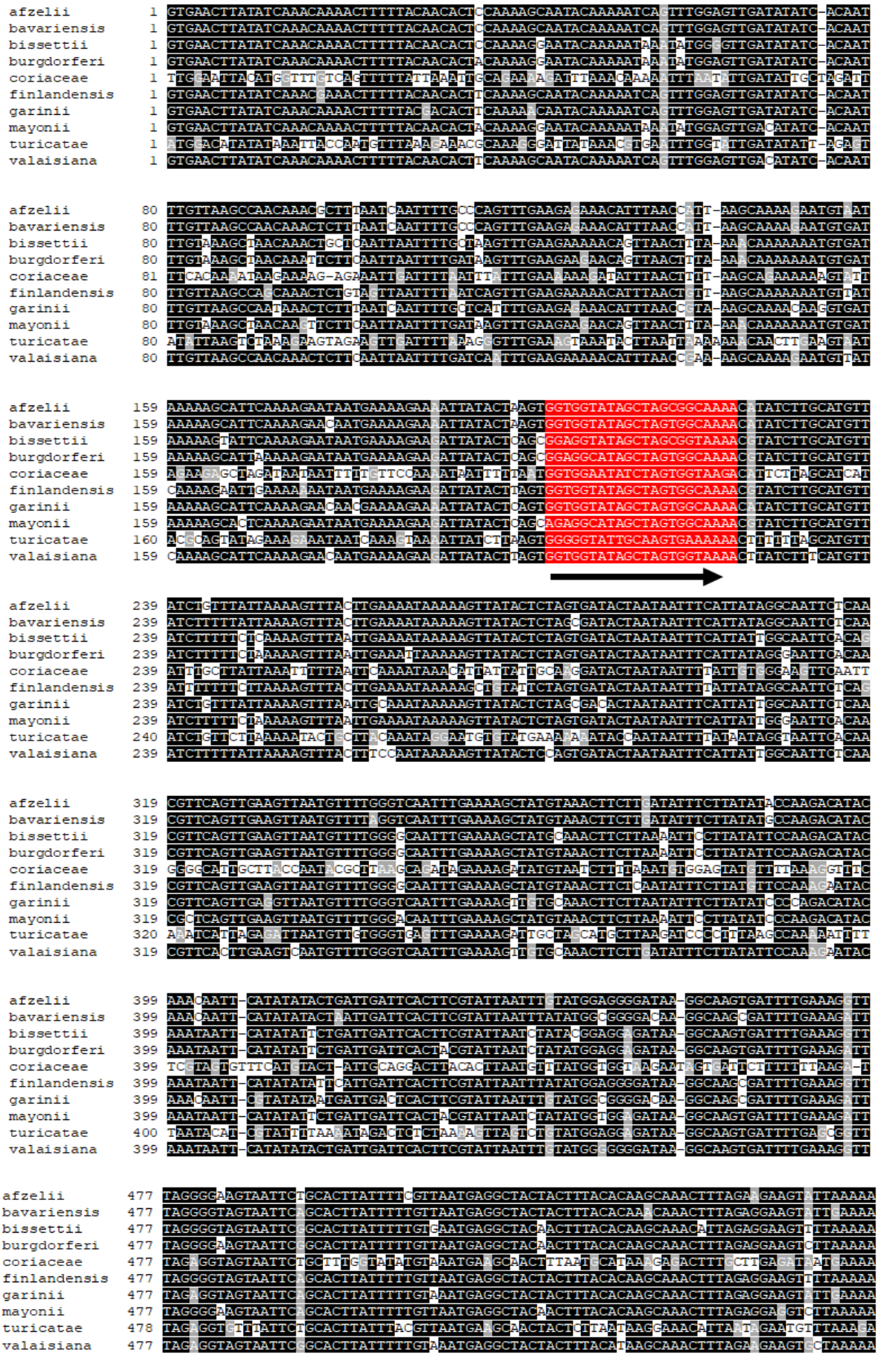


Figure 3-3 PCR targeting the *B. burgdorferi* s.l. *16SrRNA* gene in the rest of tick enrichment DNA samples.

An expected amplicon of 288 bp can be seen in the extra tick enrichment cultures of ticks delivered as mentioned above from Bournemouth, Thetford Forest, Woburn, Suffolk, Scottish Highlands, Ayrshire, Leicester, Southhampton Dorchester, Rushden and Attenborough, Exeter, Bristol, Dorset, and Essex. The positive control was *B. burgdorferi* B31 DNA, while the negative control was ultrapure water. M is λ DNA marker.

3.3.2 Multi-sequence alignment of terminase gene fragment among *B. burgdorferi* s.l. strains.

The wide prevalence of terminase genes was confirmed by blasting terminase genes against NCBI, which showed that terminase genes from different *B. burgdorferi* s.l. strains were similar to each other as shown in multi-sequence alignment in Figure 3-4. The alignment shows the variation and conservation between the terminase gene in *B. burgdorferi* s.l. strains: *B. afzelii* ACA-1, *B. bavariensis* PBi, *B. bissettii* DN127, *B. burgdorferi* B31, *B. garinii* PBr, *B. turicatae* BTE5EL, *B. valaisiana* VS116, *B. mayonii* MN14-1539 , *B. coriaceae* DOU, and *B. finlandensis* SV1. The consensus is highlighted in black, while the varied regions are highlighted in different colours. The conserved consensus can be seen mostly in strains such as *B*. *afzelii* ACA-1, *B. bavariensis* PBi, *B. bissettii* DN127, *B. burgdorferi* B31., *B. garinii* PBr, and *B*. *valaisiana* VS116, while the varied consensus can be seen mostly in *B. finlandensis* SV1, *B. mayonii* MN14-1539, and *B. turicatae* BTE5EL. The Ter676 forward primer (5`-GGAGGCATAGCTAGTGGCAAA-3`) and the reverse complement of the reverse ter676 primer (3`-AGCATTTAGTGTTGGCGG-5`) are highlighted in red. PCR direction is indicated with black arrows in the DNA alignment.


afzelii
bavariensis
bissettii
burgdorferi
coriaceae
finlandensis
garinii
mayonii
turicatae
valaisiana


afzelii 557 GACTTAGATGTGGTCAAG-AAACTATTATTTTTGATACCAATCCAGACCATCCAGAACATTATTTTAAAACCGATTATAT
bavariensis 557 GACTTAGTGCGCGCAAG-AAACTATTATTTTTGATACCAATCCAGACCATCCAGAACATTATTTCAAAACCGATTATAT
bissettii 557 GACTTAGTGTGGGCAAG-AAACTATTATTTTTGATACTAATCCCGATCATCCGGAACACTATTTTAAAACCGACTATAT
burgdorferi 557 GACTAAGATGCGGGCAAG-AAACTATTATTTTTGATACTAATCCTGATCATCCAGAACACTATTTTAAAACCGATTATAT
coriaceae 557 GCTTAGACAAAACCAG-CCATCATTATTTTTGATACTAATCCTGATCATCCTGCACATTATTTTAAGACTGATTATAT
finlandensis 557 GACTTAGTGTGGCCAAG-AAACTATTATTTTTGATACTAATCCGGATCATCCAGAACATTATTTCAAAACTGATTATAT
garinii 557 GACTTAGTGCGCGCAAG-AAACTATTATTTTTGATACCAATCCAGACCATCCAGAACATTATTTCAAAACCGATTATAT
mayonii 557 GACTAAGATGCGGGCAAG-AAACTATTATTTTTGATACTAATCCCGATCATCCGGAACACTATTTTAAAACCGATTATAT
turicatae 558 GACTTAGA-GTGGGTATGAAAACAATTATCTTTGATACCAATCCTGACACGCTTGTAGCATTTCTTTAAAACTGATTATAT
valaisiana 557 GACTTAGTGTGTGGTCAAG-AAACTATTATTTTTGACACCAATCCTGATAATCCAGAACATTATTTTAAAACTGATTATAT
afzelii 636 TGATAATATAGCGACTTTTAAGACATATAATTTTACAACTTATGATAATGTACTT-TTAAGTAAAGGATTTATTGAAACT
bavariensis 636 TGATAATGTTGCAACTTTTAAGACATATAACTTTACAACTTATGATAATATACTT-CTAAGTAAAGAATTTATCAAAACT
bissettii 636 TGATAATATAGCGACTTTTAAGACATATAATTTTACAACTTATGACAATGTTCTA-CTTAGCAAAGGATTTATAGAAACT
burgdorferi 636 TGATAATATAGCGACCTTTAAGACATATAACTTTACAACTTATGATAATGTGCTA-CTTAGTAAAGGATTTTTCGAAACA
coriaceae 636 TGATAGGCAGCAGCTTTATAGGACCTATAATTTTAGTATTATGATAATCCTTTAAATTCGCA-AAATTTTATTGAAACT
finlandensis 636 TGATAATACTGCGACTTTTAAGACATATAACTTTACAACTTATGATAATGTTCTT-CTTAGTAAAGGATTTATCGAAACG
garinii 636 TGATAATGTTGCAACTTTTAATACATATAACTTTACAACTTATGATAATGTACTT-CTAAGTAAAGAATTTATCAAAACT
mayonii 636 TGATAATATACCGACATTTAAGACATATAATTTTACAACTTATGATAATGTTCTA-CTTAGTAAAGGATTTATCGAAACA
turicatae 637 TGATAACACAAAAATTTACTCTACATATAACTTTACAACATATGATAATGGTTTA-ATTTCTAGCGATTTTATTAAAACC
valaisiana 636 TGACAATATACATACTTTCACTACGTATAATTTTACAACTTATGATAATGTACTT-CTTAGTAAAGGATTTATAGAAACT
afzelii 715 CAAGAGAAACTGTATAAAGATATACCAACATACAAGCAAGAGTTTTGTTAGGAGATGGATAGCAAGCACCGACTCAAT
bavariensis 715 CAAGAAAAACTCTATAAAGATATACCACCATATAAGCAAGAGTTTTGCTAGGAGATGGATAGCAAGCACCGACTCAAT
bissettii 715 CAAGAGAAACTCTATAAAGATATCCCATCATATAAGCAAGAGTTTTGCTAGGTGATGGATAGCAAGCACCGATTCAAT
burgdorferi 715 CAAGAAAAGCTATATAAAGATATACCATCATATAAGCAAGAGTTTTCTTAGGTGATGGATAGCAAGCACTGATTCAAT
coriaceae 715 CAAGAGCTAATTTACAAAGATTTGCATCCTTACAAGGCTAGCGTACTTCTTGGCGATGGACTGCTAGTGCTGATACGTG
finlandensis 715 CAAGAGAAACTCTATAAAGATATACCAACATATAAGCAAGAGTTTTGTTAGGAGATGGATAGCAAGCACTGATTCAAT
garinii 715 CAAGAAAAACTCTATAAAGATATACCAGCATATAAGCAAGAGTTTTGCTAGGAGATGGATAGCAAGCACCGACTCAAT
mayonii 715 CAAGAAAAACTCTATAAAGATATACCATCATATATAGCAAGAGTTTTGCTAGGTGATGGATAGCAAGCACTGATTCAAT
turicatae 716 CAAGAAGAGATTTACAGGGACATGCCAACATATAAAGCTAAAGTCCTTTAGGAGATGGCTTGCGCCATGTGATTCGAT
valaisiana 715 CAAGAGAAACTTTATAAAGATATACCAACATATAAGCAAGAGTTTTGCTAGGAGATGGATAGCAGCATTGATTCAAT
afzelii 795 TTTCGCACAAATTAATATTACTCAAGATTATGTGTTTACTAGCCCAATAGCATATTTAGACCCAGCGTTTAGTATTGGGG
bavariensis 795 TTTTACACAAATTAATATTACTCAAGATTATGTGTTTACTAGCCCAATAGCATATTTTGGATCCAGCATTTAGCATTGGGG
bissettii 795 TTTTACACAAATAAATATTACTGAGGATTATGTGTTTACTAGTCCAATAGCATATTTAGACCCAGCATTTAGCGTTGGAG
burgdorferi 795 TTTTACACAAATAAATATTACTGATGATTATGTATTTACTAGCCCGATAGCATATTTAGACCCAGCATTTAGTGTTGGCG
coriaceae 795 TTTTAATGAGTTTTTTAAATCAAGATTATGAATTTAAAGTCCAATCATGTATTGTTGATCCTGCATTTTCTGTTTGGTA
finlandensis 795 TTTTACACAAATTAATATTATTCAAGATTATGTGTTTACTAGTCCAATAGCATATTTAGATCCAGCATTTAGTATTGGGG
garinii 795 TTTTACACAAATTAATATTACTCAAGATTATGTGTTTACTAGCCCAATAGCATATTTAGATCCAGCATTTAGCATCGGGG
mayonii 795 TTTTACACAAATAAATATTACTGATGATTATGTATTTACTAGCCCGATAGCATATTTAGACCCAGCATTTAGTGTTGGAG
turicatae 796 ATTTACCAATATTAATATTACAACTGAACATGAATTTCTAAATCCAATAGCATATTTAGATCCTGCATACAGTATTGGAG
valaisiana 795 TTTTACACAAATTAATATTACTCAAGATTATGTGTTTAGCAGCCCTATAGCATATCTAGACCCAGCATTTAGTGTTGGTG
afzelii 875 GCGACAATACTGCTTTATGTGTTATGGAGAGAATTGATGATAAGTATTATGCTTTTGTATTTCAAGATCAACGACCAGCG
bavariensis 875 GCGAAATACTGCTTTATGTGTTATGGACGGGTTGATGAAAATATTATGCTTTTGTATTTCAAGACCAACGACCAGC
bissettii 875 GGGATAACACTGCATTATGTGTTATGGAGCGGGTTGATGATAAGTATTATGCTTTTGTATTTCAAGACCAACGACCAGCC
burgdorferi 875 GGGATAACACTGCATTATGTGTTATGGAGCGAGTTGATGATAAGTATTATGCTTTTGTATTTCAAGACCAAAGACCAGCT
coriaceae 875 TGGATAATACCGCCATTTGTGTCCTTGAGAGACTGGGTGATAAGTATTATTCTTTATTCTACCAAGATAGAAGACCTATA
finlandensis 875 GAGACAACACTGCTTTATGTGTTATGGAGAGAGTTGATGATAAGTATTATGCTTTTGTATTTCAAGATCAACGACCAGCT
garinii 875 GCGACAATACTGCTTTATGTGTTATGGATCGGTTGATGACAAAATATTATGCTTTTGTATTTCAAGACCAACGACCAGCC
mayonii 875 GAGATAACATTGCATTATGTGTTATGGAGTGAGTTGATGATAAGTATTATGCTTTTGTATTTCAAGACCAACGACCAGCC
turicatae 876 GAGATAATACAGCTATTTGTGTGTTAGAGCGAGTAGATCAAAGTTATTATGCGTTTTTATTTCAAGAAAGTTACCATCA
valaisiana 875 GGGATAATACTGCTTTATGTGTTATGGAGAGAGTTGATGATAAGTATTATGCTTTTGTATTTCAAGACCAAAGACCAGCG
afzelii 955 AATGACCCCTATATTATGAATATGGTCAAGACTGTTTTAGGAAATTTCAATGTACACACACTTTAGTTAGAGGATAGGGA
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bissettii 955 AATGATCCATATATTATGAATATGATCAAGACCGTTTTAGAGAATTTCAATGTACATACACTGTATTTAGAGGATAGAGA
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coriaceae 955 ATTCATGCATCTATGCTTAATTCAATCATTGTTCTTGCCGAGAACTTTAATATCAATACTCTTTATATTGAAGATCGGGA
finlandensis 955 AATGATCCTTATATTATGAATATGGTCAAAACTGTTTTAGAAAATTTCAATGTACATACACTTTATTTAGAAGATAGAGA
garinii 955 AATGATCCTTATATTATGAATATGGTCAAAACTGTATTAGAGAATTTCAATGTAAATACACTTTACTTAGAAGATAGAGA
mayonii 955 AATGATCCTTATATTATGAATATGGTAAAGACCGTTTTAGAGAATTTCAATGTGCATACACTGTATTTAGAGGATAGAGA
turicatae 956 GGTGATCCTAAGATGTTTAAATACAATTAAAACTATACTTACAAATCTTAATGTACATACACTATATGTTGAAGATAGAGA
valaisiana 955 AATGAGCCTTATATTATGAATATGGTCAAGACTGTATTAGAGAATTTCAATGTAAATACACTTTACTTAGAAGATAGAGA
afzelii 1035 TAATACTAAAGGTGCTGGTGCATTGACCCGTGAATAT-ATGACTCTTAGAAATAATATGAGCCACAATTTTAGAATTGCG
bavariensis 1035 TAATACTAAGGGTGCTGGTGCATTGACGCGTGAATAT-ATGGCTCTTAGAAACAACATGAGCCAATATTTTAGAATTGTG
bissettii 1035 CAATACAAAAGGTGCTGGCGGATTGACTCGTGAATAT-ATGTTGCTAAGAAATAATATGAGCCAATATTTTAGAATTGTT
burgdorferi 1035 TAATACAAAAGGTGCTGGTGGATTGACCCGTGAATAC-ATCTTGCTAAGAAGTAATATAAGCCAATATTTTAGAATTGTT
coriaceae 1035 TAATACAAATGGGTATGGATTTTTAACTAA-AACTATGATATTCTATTAGCAAAGTATAAATCATTATTTTAAGATATTA
finlandensis 1035 TAACACGAAAGGTGCTGGTGGATTGACTCGTGAATAT-ATAATGCTTAGGAGCAATATGAGCCAATATTTTAGAATTGCG
garinii 1035 TAATACTAAAGGTGCTGGTGCATTGACTCGTGAATAT-ATGACTCTTAGAAACAATATGGGTCATTATTTTAGAATTGTG
mayonii 1035 TAATACAAAAGGTGCTGGTGGATTGACCCGCGAATAC-ATCTTGCTAAGAAATAATATAAGCCAATATTTTAGAATTGTT
turicatae 1036 TAATATCTCATGACATGAAAATGTAACACAACTTTT-TTAAAACTTAGATCAAGTATGAATCATAAGTTTAGGATTGCA
valaisiana 1035 TAATACTAAAGGTGCTGGTGCGCTAACTCGTGAATAT-ATGACTCTTAGGAACAACATGAGTCACAATTTTAGAATTGTG

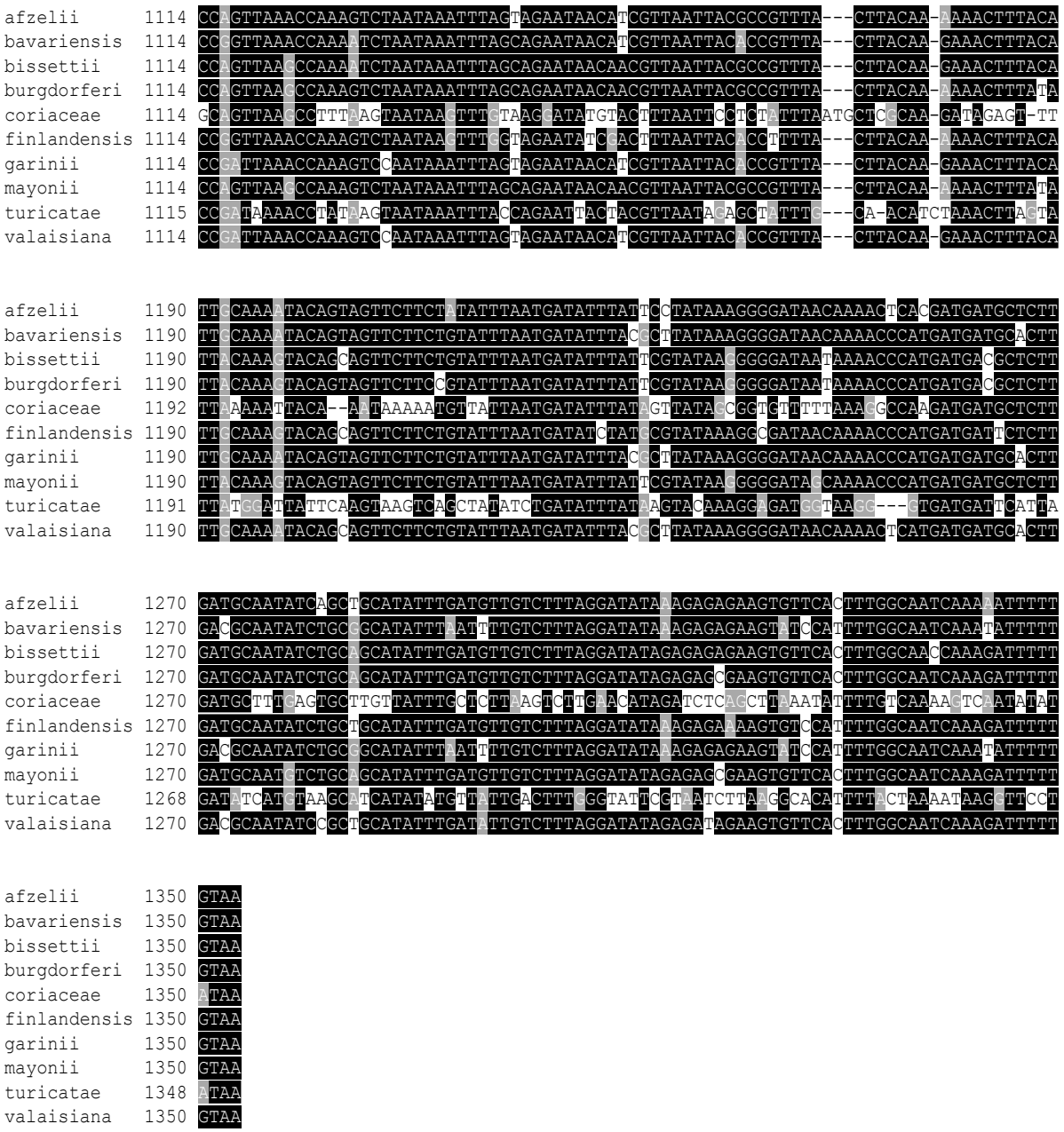


Figure 3-4 BoxShade Output for a T-Coffee multi-sequence alignment of *B. burgdorferi* s.l. strains.

The alignment shows the variation and conservation between the terminase gene in *B. burgdorferi* s.l. strains *B. afzelii* ACA-1, *B. bavariensis* PBi, *B. bissettii* DN127, *B. burgdorferi* B31, *B. garinii* PBr, *B. turicatae* BTE5EL, *B. valaisiana* VS116, *B. mayonii* MN14-1539 , *B. coriaceae* DOU, and *B. finlandensis* SV1. The consensus is shown in black, while the varied consensus are shown in various colours. The conserved consensus can be seen mostly in the strains *B. afzelii* ACA-1, *B. bavariensis* PBi, *B. bissettii* DN12*, B. burgdorferi* B31, *B. garinii* PBr*, and B. valaisiana* VS116*,* while the varied consensus can be seen mostly in *B. finlandensis* SV1, *B. mayonii* MN14-1539*, and B. turicatae* BTE5EL. The sections highlighted in red represent the Ter676 forward primer (5`- GGAGGCATAGCTAGTGGCAAA-3`) and the reverse complementary of the ter676 primer (3`-AGCATTTAGTGTTGGCGG-5`). The black arrows indicate the PCR direction.

3.3.3 *In silico* PCR Amplification

The *in silico* result of terminase 676 primers is shown in Figure 3-5. Out of 18 *B. burgdorferi* s.l. isolates listed in the NCBI database as shown in Table 3-2, Five bands corresponding to the correct amplicon were found in the tested *B. burgdorferi* s.l. strains: *B. bissettii* DN127, *B. burgdorferi* JD1, *B. burgdorferi* B31, *B. burgdorferi* N40, *B. burgdorferi* ZS7.

No PCR products were observed in other *B. burgdorferi* s.l strains such as *B. afzelii* HLJ01*, B. afzelii* PKo*,* and *B. garinii* NMJW1, *B. crocidurae* str. Achema, *B. duttonii* Ly, *B. hermsii* DAH, *B.miyamotoi* LB-2001, *B. recurrentis* A1, and *B. turicatae* 91E135 which did not show any amplification.

Table 3-2 *B. burgdorferi* s.l. isolates listed in the NCBI database were tested for *insilico* PCR

No.	Strain
1	*Borrelia afzelii* HLJ01
2	*Borrelia afzelii* PKo
3	*Borrelia afzelii* PKo
4	*Borrelia bissettii* DN127ß
5	*Borrelia burgdorferi B31*
6	*Borrelia burgdorferi* CA382
7	*Borrelia burgdorferi* JD1
8	*Borrelia burgdorferi* N40
9	*Borrelia burgdorferi* ZS7
10	*Borrelia crocidurae* str. Achema
11	*Borrelia duttonii* Ly
12	*Borrelia garinii* BgVir linear chromosome
13	*Borrelia garinii* NMJW1
14	*Borrelia garinii* PBi linear genome
15	*Borrelia hermsii* DAH
16	*Borrelia miyamotoi* LB-2001
17	*Borrelia recurrentis* A1
18	*Borrelia turicatae* 91E135

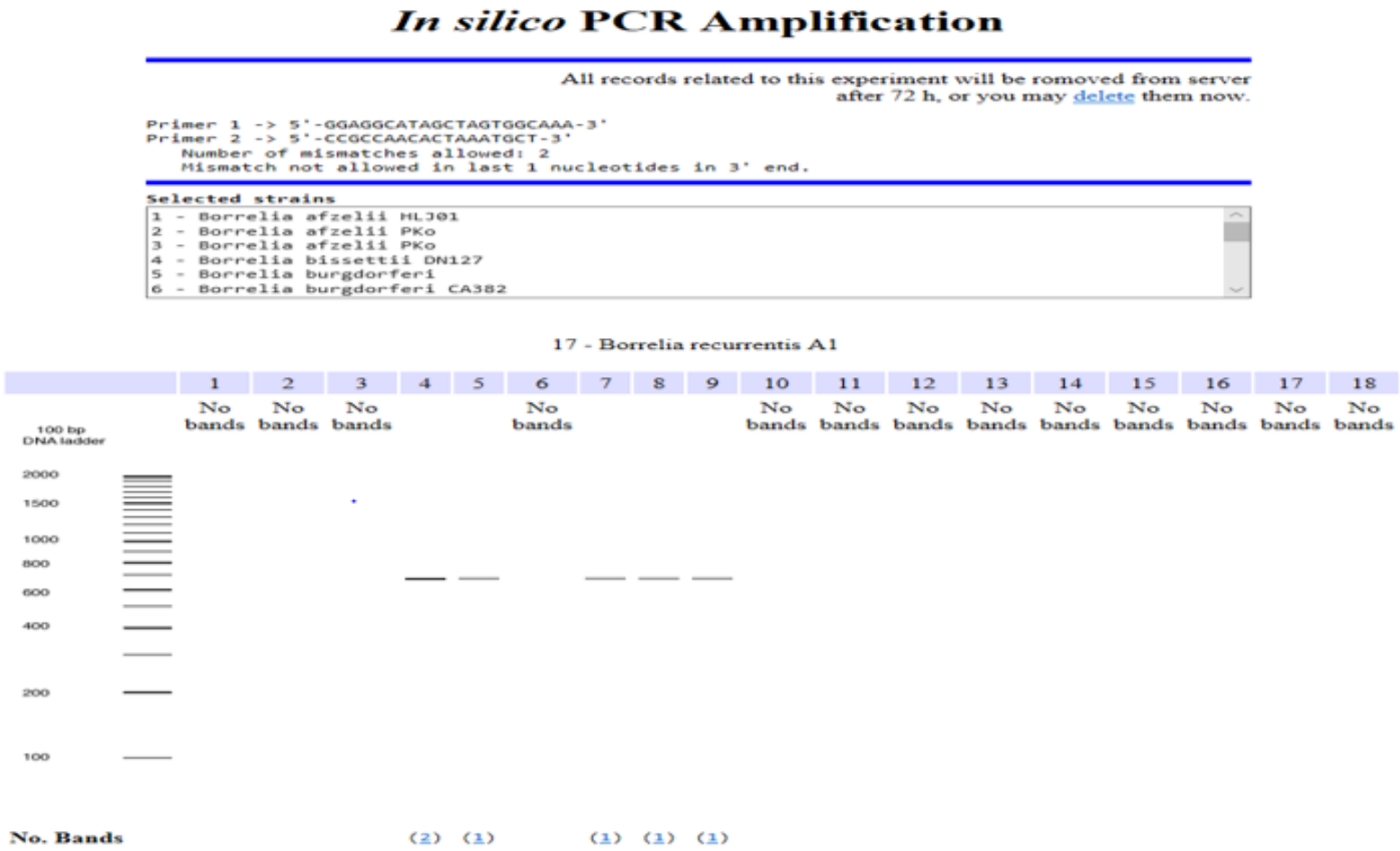


Figure 3-5 Results of *insilico* PCR amplification of the terminase gene tested with 18 *B. burgdorferi* s.l. strains.

A PCR product with the expected size of 676 bp was obtained with *Borrelia bissettii* DN127, *Borrelia burgdorferi B31i*, *Borrelia burgdorferi* JD1, *Borrelia burgdorferi* N40 and *Borrelia burgdorferi* ZS7. No bands were observed in other strains.

3.3.4 Terminsase 676 PCR for phage identification in ticks

The data showed 17 % of pooled UK ticks were positive for the presence of the phage terminase gene as following. Out of (31/187) DNA samples isolated from tick homogenates (previously tested for *16S rRNA* PCR) were tested positive for the presence of the terminase PCR, and out of (31/187) samples of the DNA samples extracted from tick enrichment cultures were tested positive to terminase PCR. The result of PCR is shown in Figure 3-6, PCR products of the expected size of 676 bp were observed from the DNA samples extracted from tick enrichment. The positive bands represents the terminase band.

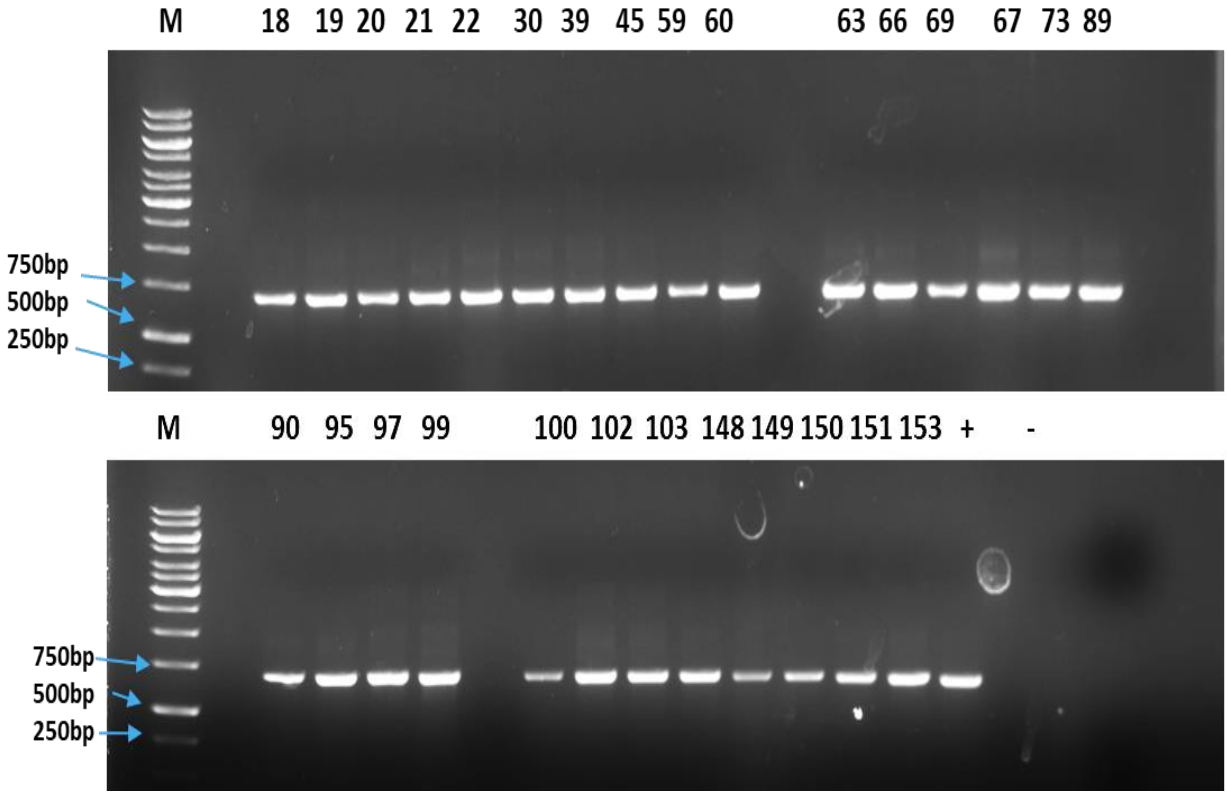


Figure 3-6 Gel photo showing PCR results based on the terminase 676 primers for tick enrichment DNA samples.

DNA was isolated from tick enrichment samples. The positive control was DNA isolated from *B. burgdorferi* B31. The negative control was ultrapure water. M is λ DNA Marker. PCR products with the expected size were observed from the following DNA samples isolated from tick enrichment samples The expected amplicon is 676 bp.

Figure 3-7 and Figure 3-8 show the results of terminase PCR for 31 tick homogenates. PCR amplicons of the expected size of 676 bp were observed for the DNA samples extracted from tick homogenates samples.

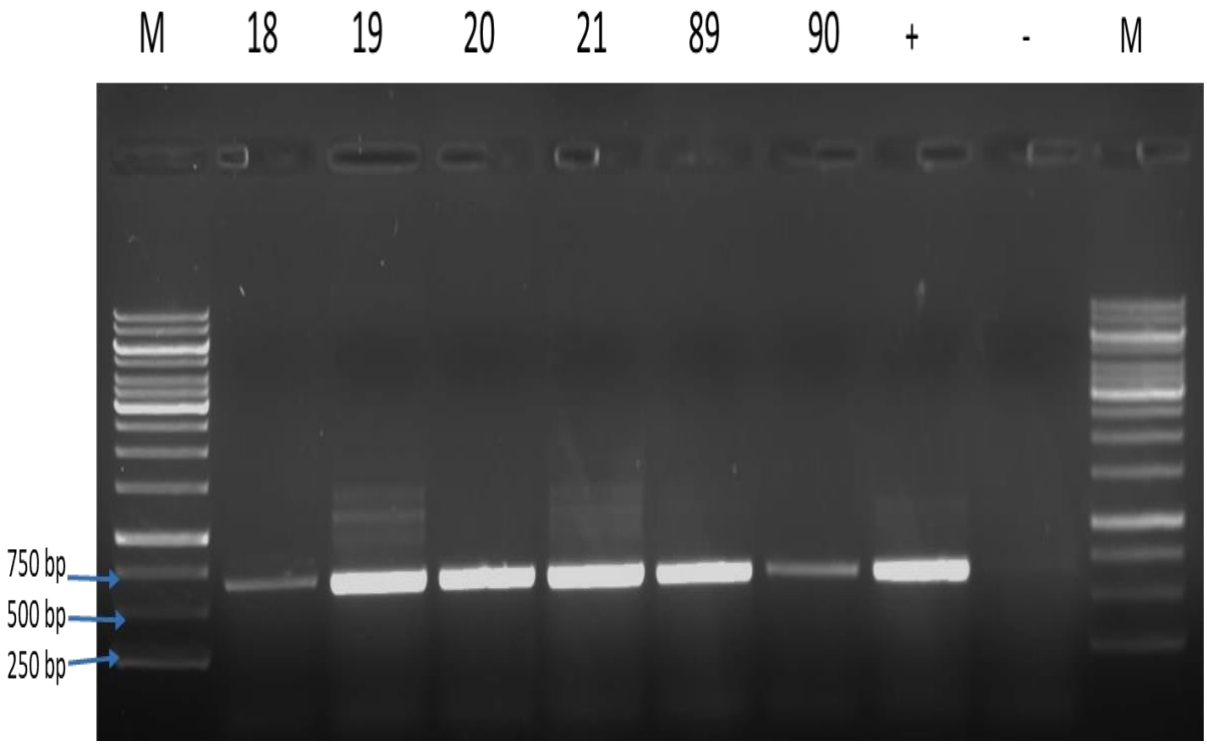


Figure 3-7 Gel photo showing PCR results based on the terminase 676 primers for 6 tick homogenates .

DNA was isolated from the tick homogenate samples 18, 19, 20, 21, 89 and 90 . The positive control DNA isolated from *B. burgdorferi* B31. The negative control was ultrapure water. PCR products with the expected size were observed from samples. M is λ DNA marker.

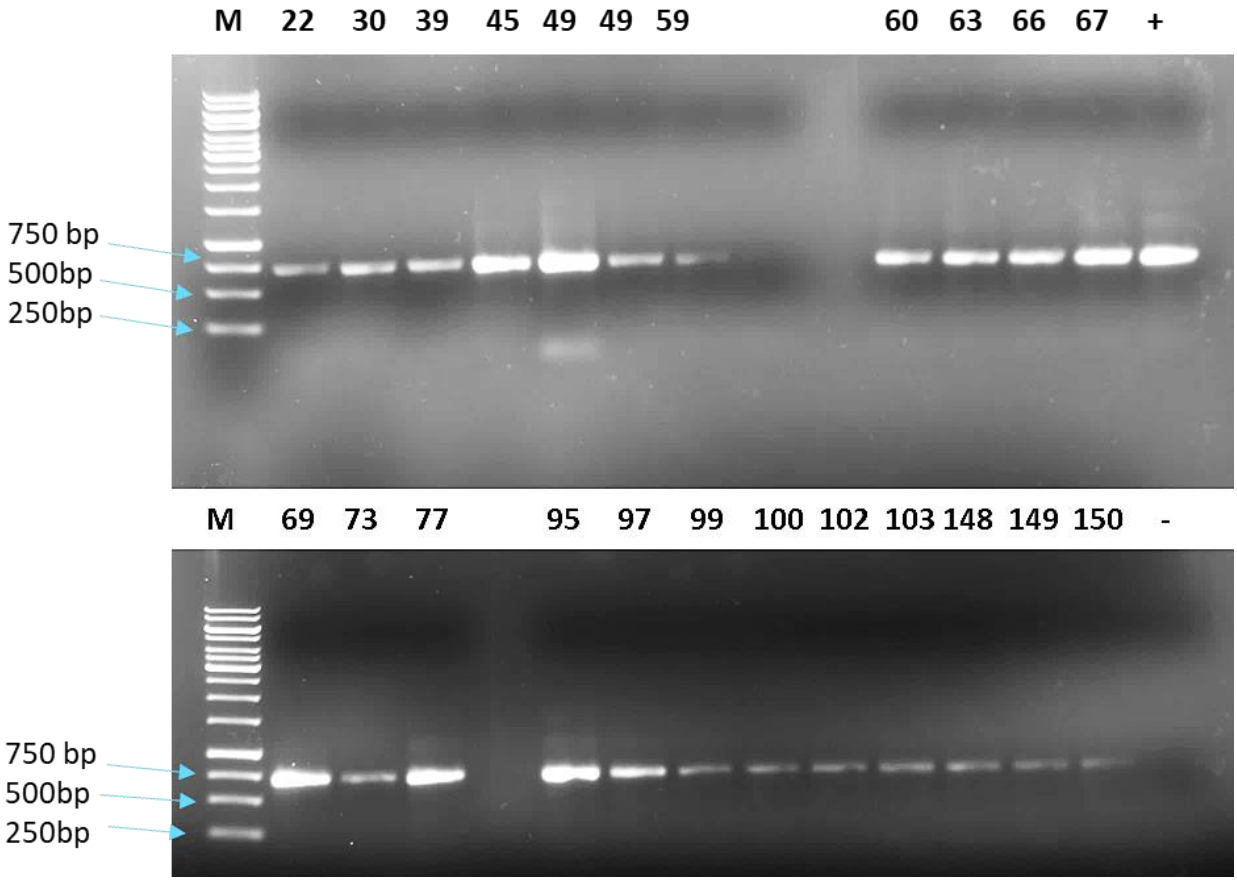


Figure 3-8. A gel photo showing results of PCR using the terminase 676 primers for the rest of tick homogenate samples.

PCR products with the expected bands of 676bp were amplified from these samples. The positive control was DNA isolated from *B. burgdorferi* B31. The negative control was ultrapure water. M is λ DNA marker.

The positive results from both PCR are summarised in Table 3-3. Finally, 156out of 187 DNA samples isolated from tick enrichments were found to be negative in terminase PCR; the negative samples are described in Appendix 3.

Table 3-3 Positive results of the *16S rRNA* and terminase PCR obtained for DNA extracted from tick homogenates and from tick enrichment cultures.

Sample	***16SrRNA* PCR against tick DNA (+/-)**	**Terminase PCR against tick DNA (+/-)**	***16SrRNA* PCR against tick enrichment DNA (+/-)**	**Terminase PCR against tick enrichment DNA (+/-)**
18	+	+	+	+
19	+	+	+	+
20	+	+	+	+
21	+	+	+	+
22	-	+	+	+
30	-	+	+	+
39	-	+	+	+
45	-	+	+	+
49	-	+	+	+
59	-	+	+	+
60	-	+	+	+
63	-	+	+	+
66	-	+	+	+
67	-	+	+	+
69	-	+	+	+
73	-	+	+	+
77	-	+	+	+
89	+	+	+	+
90	+	+	+	+
95	-	+	+	+
97	-	+	+	+
99	-	+	+	+
100	-	+	+	+
102	-	+	+	+
103	-	+	+	+
148	-	+	+	+
149	-	+	+	+
150	-	+	+	+
151	-	+	+	+
152	-	+	+	+
153	-	+	+	+

16S rRNA PCR products were sequenced, and the sequencing data were blasted against the NCBI database. As shown in Table 3-4, 26 % (8 out of 31 samples) were *B. garinii* isolates, 55 % (17 out of 31 samples) were *B. burgdorferi* s.s. isolates, and 19% (6 out of 31) samples were *B. afzelii* isolates. The accession number, Sequence similarity for the reference strain of hits are provided for all samples in Table 3-4.

Table 3-4 Blast results of *B. burgdorferi* s.l. *16S rRNA* PCR products

Sample	Species similarity	Base pair	Sequence Similarity %	Accession number
18	*Borrelia garinii* CIP	260	100%	NZ_CP018744.1
19	*Borrelia burgdorferi* B31	263	100%	CP019767.1
20	*Borrelia afzelii* Ip-21	261	100%	AY574639.1
21	*Borrelia burgdorferi* VS116	248	100%	X98233.1
22	*Borrelia garinii* vanino 2011	236	100%	KY346890.1
30	*Borrelia garinii* HLJ156	240	100%	MK121661.1
39	*Borrelia burgdorferi* DK29	233	100%	X85202.1
45	*Borrelia burgdorferi* B31	231	100%	HQ433658.1
49	*Borrelia garinii* 58dama	233	100%	KF422748.1
59	*Borrelia garinii* 58dama	242	100%	KF422748.1
60	*Borrelia garinii* 58dama	240	100%	KF422748.1
63	*Borrelia garinii* Ipv6262	248	100%	KU672546.1
66	*Borrelia burgdorferi* B31	253	100%	KY284013.1
67	*Borrelia afzelii* Tom3107	242	100%	CP009212.1
69	*Borrelia afzelii* Tom3107	244	100%	CP009212.1
73	*Borrelia afzelii* Tom3107	235	100%	CP009212.1
77	*Borrelia burgdorferi* B31	225	100%	CP019767.1
89	*Borrelia burgdorferi* B31.	244	100%	AE000783.1
90	*Borrelia garinii* SZ	242	100%	CP007564.1
95	*Borrelia burgdorferi* DK29	235	100%	X85202.1
97	*Borrelia burgdorferi* IIi1	241	100%	KY284013.1
99	*Borrelia. burgdorferi* B31	245	100%	CP009656.1
100	*Borrelia burgdorferi* DK29	250	100%	X85202.1
102	*Borrelia afzelii* HlJ01	239	100%	CP003882.1
103	*Borrelia afzelii* HlJ01	242	100%	CP003882.1
148	*Borrelia burgdorferi* IIi1	239	100%	KY284013.1
149	*Borrelia burgdorferi* IIi1	246	100%	KY284013.1
150	*Borrelia burgdorferi* IIi1	237	100%	KY284013.1
151	*Borrelia burgdorferi* B31	236	100%	CP009656.1
152	*Borrelia burgdorferi* B31	233	100%	CP009656.1
153	*Borrelia burgdorferi* B31	242	100%	CP009656.1

3.3.5 Observation of *B. burgdorferi* s.l. spirochetes using a phase-contrast microscope and their cultivation as single colonies.

31 out of 187 samples of tick enrichments cultures contining *B. burgdorferi* s.l. spirochetes were subcultured in cBSKII media supplemented with rifampicin and phosphomycin. *B. burgdorferi* s.l. enrichment cultures were stored at -80^{o}C. 6 out 31 *B. burgdorferi* s.l isolates were cultured as single colonies for next generation sequencing will described later. The remaining samples were not purified. During enrichment, observation of *B. burgdorferi* s.l. spirochetes was determined using Phase-contrast microscopy. Active-moving typical spirochetes were observed under the phase-contrast microscope. The presence of spirochetes (spiral shape) at 200X magnification in the enrichment culture is shown in Figure 3-9.

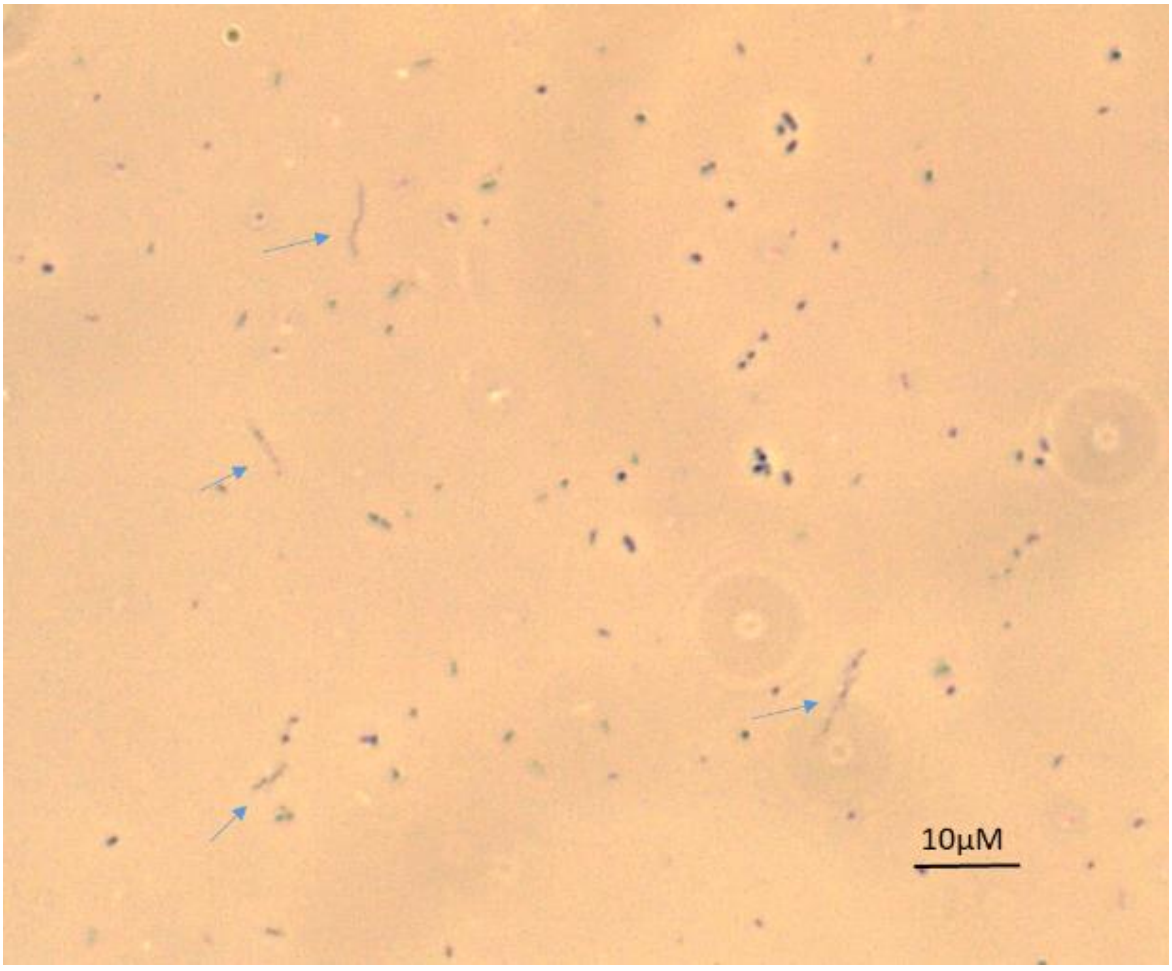


Figure 3-9 Spirochetes from an enrichment culture visualized via phase-contrast microscopy.

Cells were determined at 200 X magnification. Bar represents 10 µM. Blue arrows point to the spirochetes.

After enrichment, *B. burgdorferi* spirochetes were observed at 200X magnification in the culture, as shown in Figure 3-10. In this figure, healthy spirochetes of *B. burgdorferi* B31 were observed.

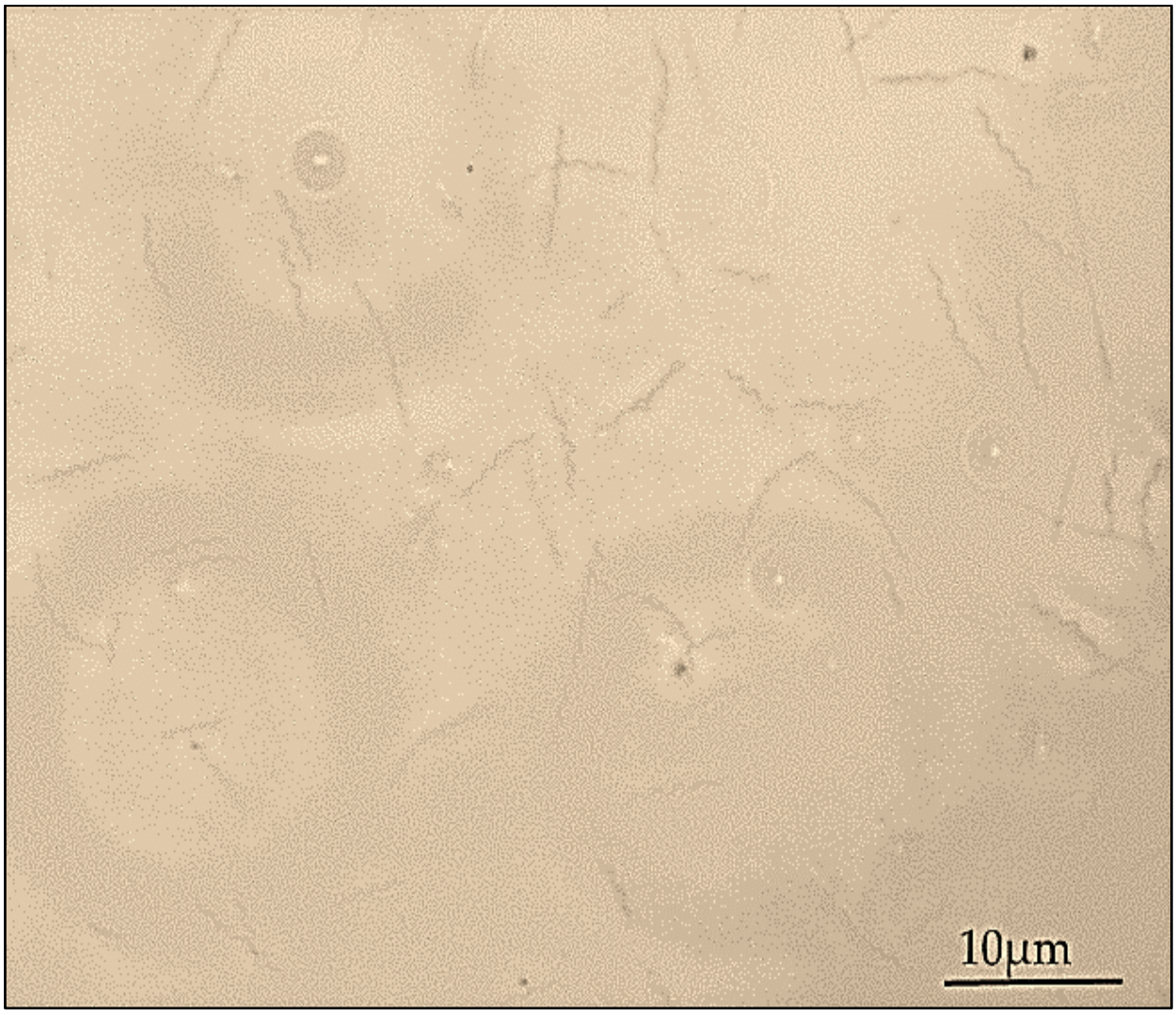


Figure 3-10 *B. burgdorferi* B31 cells visualized via phase-contrast microscopy**.**

Cells were determined at 200X magnification. Bar represents 10µM.

After numerous rounds of optimization, the working protocol was established as follows: plating on BSKII (P-BSKII) with 1% agar (VWRBDH chemical, UK), incubating at 34 ºC with 10 % rabbit serum for the bottom and top agar *B. burgdorferi* s.l. colonies were performed on modified p-BSKII media containing agar as shown in Figure 3-11 .*B. burgdorferi* s.l. produced white circular colonies after 7 days of incubation at 34°C.

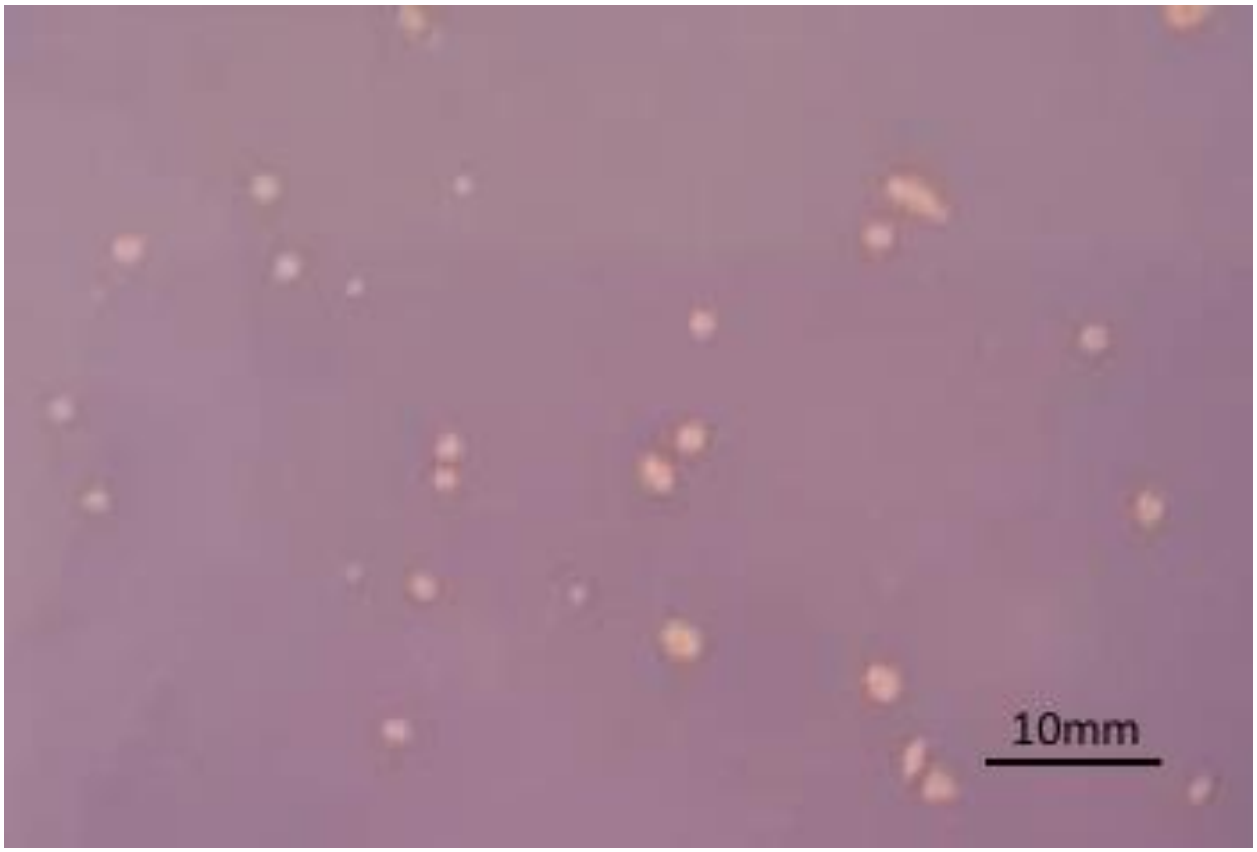


Figure 3-11 White single colonies appeared after 7 days of culturing *B. burgdorferi* s.l. on solid media. The culture was grown using the modified p-BSKII solid media. Scale bar is 10 mm.

3.3.6 Next-generation sequencing of *B. burgdorferi* s.l. isolates

The results of whole genome sequensing of *B. burgdorferi* s.l. strains are shown in Table 3-5. Among the strains, the genome of *B.valaisiana* NE218 has the largest contig (the length of the largest reads of nucleotides) is 923474 bp for *B.valaisiana* NE218, the contigs were sequenced with an average coverage (or depth) of more than 32 times and GC ratio of 27.54%. while, the genome of *B. afzelii* S20 has the largest contig of 221925 bp with an average coverage of 15 times and GC ratio of 27.70%. *B. afzelii* S20 consider the strain that has the smallest reads among *B. burgdorferi* s.l. strains analysed in this project. The six *B. burgdorferi* s.l. strains isolated from tick enrichment samples 18, 19, 20, 21, 89 and 90 were identified as *B. garinii* S18*, B. burgdorferi* S19*, B. afzelii* S20, *B. burgdorferi* S21, *B. burgdorferi* S89 and *B. garinii* S90, respectively. The identity of the six lab strains were confirmed to be *B. burgdorferi* VS185 P9, *B. valaisiana* NE218, *B. afzelii* Ukfiltered, *B. burgdorferi* China 23, *B. garinii* 190 P9, and *B. burgdorferi* B31.

Table 3-5 *B. burgdorferi* s.l. strains identified according to whole genom sequencing

Sample	**Species**	**annotation system**	**No. of contig**	**largest contige**	**GC %**
lab strain	*B. burgdorferi* VS185 P9	Prokka	32	905652	27.96
lab strain	*B. valaisiana* NE218	Prokka	32	923474	27.54
lab strain	*B. afzelii* Ukfiltered	Prokka	26	913014	58.27
lab strain	*B. garinii* 190 P9	Prokka	31	692746	27.81
Labstrain	*B. burgdorferi* China 23	Prokka	30	692746	27.95
Labstrain	*B. burgdorferi* B31	Prokka	32	859920	28.06
new strain	*B. garinii* S18	Prokka	31	906065	27.82
new strain	*B. burgdorferi B31*	Prokka	26	481779	28.07
new strain	*B. afzelii* S20	Prokka	15	221925	27.70
new strain	*B. burgdorferi* S21	Prokka	25	590138	28.08
new strain	*B. burgdorferi* S89	Prokka	18	468150	29.72
new strain	*B. garinii* S90	Prokka	32	906204	27.82

Note**:** Table information was provided by Microbes NG at http://www.microbesng.uk.

3.3.7 Spot tests of tick homogenates filtrates on *B. burgdorferi* s.l. lawns

In this study, Spot test was developed to screen for *B. burgdorferi* s.l. phages. The addition of tetracycline (positive control) produced a clear lytic killing area, showing that the spot test was working. However, no tick enrichment samples showed virulent activity against the lawn of *B. burgdorferi* s.l.

3.3.8 Identification of *B. burgdorferi* s.l. prophages using PHASTER/PHAST analysis

Putative prophage regions(Regions) in thirteen *B. burgdorferi* s.l. strains were analysed in this study by PHASTER/PHAST. The best alignment between the query sequence (the potential prophage region) and the sequences found from the NCBI database (hit) is shown in Table 3-6. Eight strains, Bb VS185 P9, Bv NE218, Ba Uk filtered, Bg 190 P9, Bb China 23, BbB31, BbS21, and BgS90, showed the presence of one incomplete prophage region called region1 (the number assigned to the region). Two strains, Ba ACA-1 and BgS18, showed two incomplete prophages regions named region 1 and region 2. However, three strains - Bb S19, BaS20, and BbS89 - were not analysed due to the size of the contigs, which was smaller than 2000 bp, the minimum size for analysis using PHASTER. The strains are not included in the table below. The score of the most prophage regions (Score) was 10-20 in the analysed strains based on specific criteria in PHASTER web. The completeness (the predictions of whether the region contain an intact, questionable, and incomplete prophages) was described. In this study most of the *B. burgdorferi* s.l. prophages were incomplete prophages. The length of the prophage region in kilo base pair (KB) for each region was described, for most of the *B. burgdorferi* s.l prophages to be 6.8KB region length. The number of the coding sequeces (#CDS) was described for each region, for the most of the *B. burgdorferi* s.l strains to be 6. The prophage GC ratio (percentage of GC nucleotides of the region) varied from 26.81 % to 31.49 %. Finally, the best alignment is the phage(s) with the highest number of the proteins most similar to those in region. In this study, the most common phages with the highest similarty to *B. burgdorferi* s.l. phages were phages of Bacilli AR9-NC-031039 and Bacilli BM5-NC029069.

Table 3-6 Putative prophage regions in thirteen *B. burgdorferi* s.l. strains analysed in this study using PHASTER/PHAST

Strain	Region	Completeness	Score	#CDS	Region Position	Region length KB	GC %	The best alignment	Remark
Bb VS185 P9	1	Incomplete	10	6	451829-460459	6.8	28.73	PHAGE_Bacill_AR9_NC_031039(3)	Sequenced in this project
Bv NE218	1	Incomplete	10	7	454028-462664	6.8	28.17	PHAGE_Strept_9872_NC_031094(3)	Sequenced in this project
Ba ACA-1*	2	Incomplete	10	6	13515-22163	6.8	28.34	PHAGE_Bacill_AR9_NC_031039	GCA_000170935.2*
			20	9	390983-402418	11.4	31.49	PHAGE_Bacill_AR9_NC_ 031039 (2).	
Ba ukfiltered	1	Incomplete	10	7	449884-458517	6. 8	28.24%	PHAGE_Bacill_AR9_NC_031039(3)	Sequenced in this project
Bg 190 P9	1	Incomplete	20	6	238887-247518	6.8	28.54%	PHAGE_BacillAR9_NC_031039(3	Sequenced in this project
Bb China 23	1	Incomplete	10	6	238900-247530	6.8	28.73%	PHAGE_Bacill_AR9_NC_031039(3	Sequenced in this project
Bb B31	1	Incomplete	10	6	402212-410829	6.8	28.53%	PHAGE_Strept 9872_NC_031094(4)	Sequenced in this project
Bg S18**	2	Incomplete	20	7	263205-272920	9.7	26.81%	PHAGE_Bacill_BM5_NC_029069	Sequenced in this project
			20	6	451972-460603	6.8	28.54%	PHAGE_Bacill_PfEFR_5_NC_031055.	
Bb S21	1	Incomplete	20	6	449184-457801	6.8	28.53	PHAGE_Bacill_AR9_NC_031039(2)	Sequenced in this project
Bg S90	1	Incomplete	10	6	452143-460774	6.8	28.54	PHAGE_Bacill_AR9_NC_031039(2)	Sequenced in this project

*-*Borrelia afzelii* ACA-1, the assembly was retrieved from ENA (European Nucleotide Archive). **Bg S18 contains two prophage regions.

- Region: number of prophage region within genome, Score: the score of the region analysed by PHASTER, #CDS: number of coding sequences.GC%: GC nucleotide percentage.

B. burgdorferi s.l. prophage-like regions (PO) identified in this project were aligned together to determine the similarity between them Figure 3-12. The prophage-like regions are named as PO, followed by the name of the *B. burgdorferi* s.l. strains and the number of identified prophage regions. The figure shows the alignment of all region positions with a minimum of 80% similarity. The studied *B. burgdorferi* s.l. prophage like regions for the strains Bb Vs185, Bv NE218, Ba ACA-1, Bb UK filtered, Bg 190p9, Bb China 32, Bb B31, Bg S21 and Bg S90 were highly similar in the structural proteins that they formed such as hypothetical proteins, phage like proteins and phage coat proteins. This was shown in the variation of the shades of grey between them. The dark grey shade was significant to the high similarity at percentage of 100%. However, two prophage-like elements such as (PO Ba ACA-1 [2], and PO Bgs18[1]) showed a similarity of less than 80% against the other prophage-like regions. No similarity is shown for anything less than 80%.

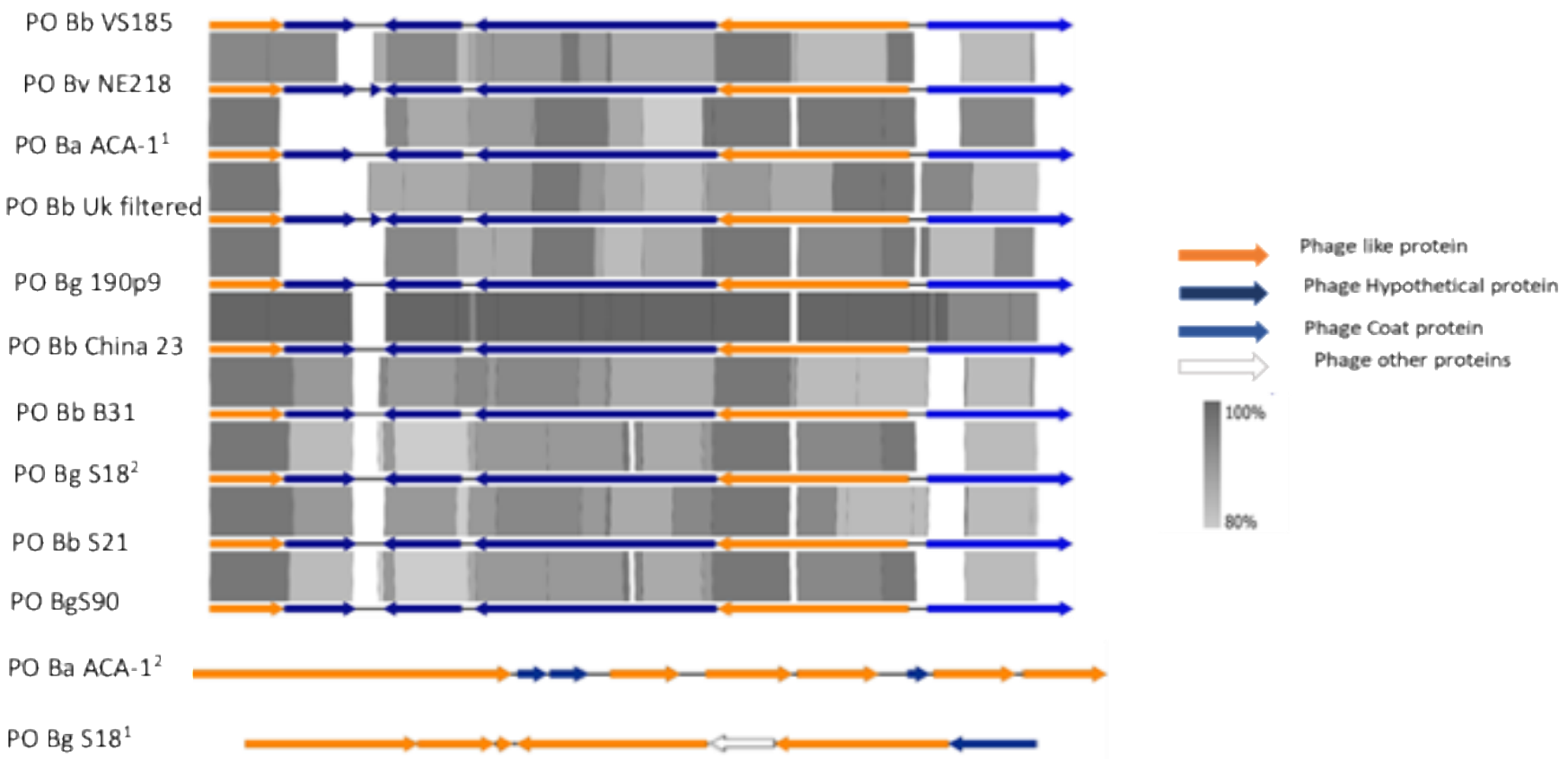


Figure 3-12 Sequence similarity of prophage-like regions identified by PHASTER, annotated by RAST and presented by Easyfig.

The prophage regions identified from PHASTER/ PHAST, annotated and aligned by RAST and presented through Easyfig version 2.2.3. The (PO) positions of *B. burgdorferi* s.l. prophage regions, followed by the name *B. burgdorferi* s.l. isolates used in this study. Some strains followed by the number of the region.The phage proteins are represented with orange arrows for phage-like proteins. The dark blue arrows represent phage hypothetical proteins. The light blue arrows represent phage coat proteins, and the white arrows represent the other phage-like proteins. The similarity between them is shown using varying shades of grey; dark grey signifies high similarity at a percentage of 100 %, while light grey signifies a percentage of 80 %. No similarity is shown for anything less than 80 %.

3.4 Discussion

3.4.1 *B. burgdorferi* s.l. carriage rate in ticks

Hard-bodied ticks, scientifically known as *Ixodes ricinus*, are the main vectors that transmit LB in the UK. The tick lifecycle involves four stages: eggs, larvae, nymphs, and adults (male and female). The ticks are infected by *B. burgdorferi* s.l. when feeding on a range of small mammals (such as squirrels and mice) and birds. While deer are the principal host for adult ticks, they do not infect ticks with *B. burgdorferi* s.l. These animals have been closely linked with the overall high number of ticks. Carriage of *B. burgdorferi* s.l. in ticks is typically determined using PCR assays; the percentage of *B. burgdorferi* s.l. carrying ticks varies drastically globally, ranging from 3.3 % in the UK to more than 40 % in Ontario, Canada (Ogden *et al*., 2008). In Europe, the *B. burgdorferi* s.l. carriage rate in adult ticks is 18.6 %, and in nymph ticks is 10.1 % (Rauter and Hartung, 2005). In this study, pooled ticks collected throughout the UK were analysed for their *B. burgdorferi* s.l. carriage using two PCR assays, one targeting the *B. burgdorferi* s.l. *16S rRNA* gene, and another targeting the phage terminase genes. The data showed that the terminase-targeting PCR is over five times more sensitive than the *16S rRNA*-based PCR. Phylogenetic analysis has confirmed a strong correlation between the terminase gene and the *B. burgdorferi* s.l. strain identity (Clokie and Shan, 2019). In other words, the presence of terminase genes points to the existence of *B. burgdorferi* s.l. Therefore, a terminase-based PCR can be highly sensitive, and with further development, could potentially detect and distinguish different *B. burgdorferi* s.l. strains.

The carriage rate of *B. burgdorferi* s.l. in ticks can vary drastically between regions and can be influenced by environmental factors that affect reservoir hosts and tick populations (James *et al*., 2013). The sensitivity of different assays can also vary, with PCR-based assays being the most sensitive in comparison to antibody-based techniques. Previous reports using PCR systems have shown carriage rates typically in the range of 3 % to 10 % in the UK (Bettridge *et al.*, 2013, James *et al*., 2013) and over 40 % in endemic regions such as Ontario, Canada (James, 2017, Kaffenberger *et al*., 2017, Clow *et al*., 2018). In this study, *B. burgdorferi* s.l. carriage rate in UK ticks was found to be higher in ratio of 17% in some Uk areas rather than others. The *16S rRNA* negative PCR results of samples collected may indicate that those ticks were either free from *B. burgdorferi* s.l. or under the detection limit of PCR. Therefore, the

16S rRNA-specific PCR screening may not have provided true positive results, the PCRs results were confirmed to be higher by developing terminase gene PCR. This was agreed with the previous study that have been confirmed that phage terminase gene was a good marker in detecting *B. burgdorferi* s.l. from human samples (Clokie and Shan, 2019). The newly designed terminase 676 PCR primers were sensitive and specific in generating positive PCR results compared to other PCR assays, such as the *16S rRNA* PCR. This was confirmed during PCR experiments, where 3.2 % of pooled UK ticks were *16S rRNA* PCR positive, while 17 % of pooled UK ticks were positive for the presence of the phage terminase gene.

3.4.2 *B. burgdorferi* s.l. culturing optimisation

Apart from the PCR-based method, a culture-based method was also used in this study in an attempt to isolate *B. burgdorferi* s.l. from ticks. Ticks can carry numerous microorganisms, Therefore, the *in vitro* cultivation of *B. burgdorferi* s.l. from ticks can be challenging. There was a study determined that ticks can carry numerous microorganisms such as *Chlamydophila psittaci*, *Anaplasma phagocytophilum*, *B. burgdorferi* s.l., *Campylobacter jejuni*, *Salmonella enterica*, *Pasteurella multocida* and *Mycobacterium avium* (Hubálek, 2004). To have the possibility of culturing *B. burgdorferi* s.l., the growth of other microorganisms must be prohibited. To do that, the typical complete BSKII (c-BSKII) medium was supplemented with Rifampicin at a final concentration of 1 µg/ml. Rifampicin at this concentration has been confirmed to inhibit the growth of other organisms without harming *B. burgdorferi* s.l. as suggested by (Sapi *et al.*, 2011). In this study, tick homogenates were inoculated into c-BSKII with Rifampicin, i.e. enrichment for *B. burgdorferi* s.l. growth. Tick enrichment culture was carried out in a narrow 15 ml Falcon tube with a cultivation volume of 14.7 ml to provide a microaerobic environment suitable for the growth of *B. burgdorferi* s.l. The presence of spirochete-like microorganisms in cultures was confirmed by phase-contrast microscopy under 200X magnification. Subcultures were conducted every three weeks with selective the c-BSKII medium (Sapi *et al*., 2013).

3.4.3 Cultivation of *B. burgdorferi* s.l. as single colonies

For any enrichment cultures that showed the presence of spirochetes under the microscope, single colony purification was performed to isolate a pure culture. Developing the method to culture *B. burgdorferi* s.l. as single colonies presented a great challenge. This was agreed by (Kurtti *et al*., 1987, Barbour and Hayes, 1986). In this study, the optimization the working protocol was done. Agar has been previously used in culturing *B. burgdorferi* s.l. spirochetes (Kurtti *et al*., 1987, Barbour and Hayes, 1986). Agar was more suitable than agarose to solidify the media and form single colonies. This was agreed with the previous study that used agar in plating relapsing fever spirochetes such as *B. hermisii* and *B. turicatae* (Raffel *et al*., 2018).. *B. burgdorferi* s.l. were recognized as single, mini opaque colony after 7 days of incubation in microaerobic conditions. This is because *B. burgdorferi* s.l. moves more freely through agar, which promotes the formation of a single colony. In addition, avoiding bubbles during the pouring of agars helped promote colony formation. Perhaps the oxygen-containing bubbles suppress the growth of *B. burgdorferi* s.l., given that it is a microaerophilic bacteria and as such, prefers a lack of oxygen. Success in obtaining single *B. burgdorferi* s.l. colonies were positive for further genotypic and phenotypic studies.

3.4.4 Sequence analysis of *16S rRNA* PCR product

In this study, Sequence analysis of the *16S rRNA* amplicon detected that the main common genotypes that have been identified are *B. garinii, B. afzelii,* and *B. burgdorferi* sensu stricto. This was agreed to the previous studies that have been confirmed the main genotypes in Europe are *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto (Mannelli *et al*., 2012, Casjens *et al.*, 2011), And further UK studies that have been previously reported that *B. burgdorferi* s.l. genospecies such as *B. afzelii*, *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana* were found in UK ticks (Bettridge *et al*., 2013, Hansford *et al*., 2017, Layzell *et al*., 2018, Abdullah *et al*., 2018), and can cause further health issues for the general public. Within the UK, Public Health England is currently studying the epidemiology of ticks, but not the disease causing bacteria that they carry. In Scotland, the European Space Agency is helping with similar research to map areas of highest risk for ticks. Further effective tests are needed in the UK to detect

infectious agents carried by ticks, due to the different genospecies and strains of *B. burgdorferi* s.l.

3.4.5 Identification of *B. burgdorferi* s.l. prophages using PHASTER/PHAST

Previous work has shown that it is difficult to obtain complete genomes (including proper assembly of all plasmids) for *B. burgdorferi* s.l. species using short-read sequencing technologies, such as Illumina (Margos *et al*., 2017). This study aimed to obtain more comprehensive data on the prophage regions within the *B. burgdorferi* s.l. genome. To achieve this, bacterial genomes of 12 isolates of different *B. burgdorferi* s.l. strains were sequenced using Illumina technology. Different assemblers were used. However, the assemblies failed to give consistent results for prophage numbers and structure. PHASTER/PHAST analysis was performed using the *B. burgdorferi* s.l. genome to identify the prophage regions, considering the fact that most of the *B. burgdorferi* s.l. temperate phages are present in *B. burgdorferi* s.l. genomes as plasmids called cp32 plasmids (Brisson et al., 2012, Brisson et al., 2013). Further confirmed that *B. burgdorferi* s.l. carry multiple and diverse cp32 plasmid types (Casjens *et al.*, 2018). The presence of cp32 prophage in *B. burgdorferi* s.l. genome is a promising indicator to initiate the study of *B. burgdorferi* s.l. prophages using bioinformatic analysis such as PHASTER/PHAST. The analysis confirmed the presence of one incomplete prophage inside the *B. burgdorferi* s.l. genome. Most of the analysed strains have one prophage region as an incomplete prophage, while two strains – *B. afzelii ACA-1* and *B. garinii* S18 – were found to have two prophage regions. One previous study stated that to obtain complete genomes of *B. burgdorferi* s.l. species, long and short-read data must be combined (Kingry *et al*., 2016, Margos *et al*., 2017). This might explain why only an incomplete image of the '*B. burgdorferi* s.l. complete phage hits' within the genome was obtained in this study. Intact (complete) or questionable prophages within the *B. burgdorferi* s.l. genome were not identified. This is because the percentage of the known phage gene was less than 50%. Thus, the prophage genome is considered to be incomplete. No previous studies have conducted *B. burgdorferi* s.l. prophage analysis using PHASTER or PHAST. The prophages were anaotated by RAST and easyfig was used after the alignment of all the annotated *B. burgdorferi* s.l. prophage positions in order to study the similarity between them. The data showed that they were highly similar. The current study is the first,

and aimed to draw a clear image of the *B. burgdorferi* s.l. temperate phages. Further analysis of the *B. burgdorferi* s.l. extrachromosomal DNA is recommended to gain a deeper understanding of temperate *B. burgdorferi* s.l. prophages.

CHAPTER FOUR

Induction of temperate phages from *B. burgdorferi* s.l.

Chapter 4 Induction of temperate phages from *B. burgdorferi* s.l.

4. 1 Introduction

A temperate phage is a phage that display the lysogenic life cycle in contrast to the virulent phage that could not able to be lysogenic. The template phage genome is either integrated into a bacterial genome or present as an extrachromosomal plasmid. These 'latent' forms of phages are also termed 'prophages', and can sometimes be excised from the bacterial genome and enter the lytic life cycle. Prophage induction can occur spontaneously or under stressed conditions; such as antibiotic treatment or other DNA-damaging chemicals or stimuli or following exposure to UV light (Aertsen and Michiels, 2005). A common trigger for prophage induction is DNA damage, which leads to an irreversible conversion of lysogeny to lysis (Campbell, 2003). Specifically in *E.coli,* the activation of the SOS response occurs as a result of cell stress or non-lethal DNA damage, which can be caused by ultraviolet or ionizing radiation (D'Ari, 1985). The SOS response involves two regulatory proteins, the LexA repressor and RecA inducer. The RecA protein cleaves the LexA repressor to activate the DNA repair pathways. The RecA protein can also cleave the cI repressor. The phage Lambda cI repressor is part of a phage molecular switch that converts the phage life cycle from being lysogenic to lytic. The Lambda cI mimics the *E. coli* LexA, and senses the physiological condition of the host cell; the cI undergoes self-cleavage under SOS activation and is catalysed by the active RecA nucleoprotein filament. The cI protein contains two domains that are connected to the cleavage site by a flexible "hinge" region. The self-cleavage of the repressor enables the prophage to shift efficiently from lysogeny when the majority of the phage genes are presented for induction and lytic growth (Galkin *et al.*, 2009).

Commonly used prophage-inducing agents include Mitomycin C and Norfloxacin (Ramirez et al., 1999). Mitomycin C is a DNA cross-linking activation agent and is used broadly in chemotherapy (Paz *et al.*, 1999). The mechanism of Mitomycin C in the induction of prophages is linked to the activation of the SOS response (Galkin *et al*., 2009). Mitomycin C has been previously used to induce prophages of spirochetes such as *Brachyspira hyodysenteriae*, and revealed a bacteriophage-like particle named VSH-1 (Humphrey *et al*., 1995). VSH-1 has a polyhedral head approximately 45 nm in diameter with a short, non-

contractile 64 nm-tail. The initial attempt to use Mitomycin C at 10 µg/ml to induce *B. burgdorferi* temperate phages was unsuccessful, and the prophage BB-1 was induced from *B. burgdorferi* CA-11.2A using 1 Methyl-3-nitro- 1-nitrosoguanidine (MNNG) (Eggers and Samuels, 1999, Eggers *et al*., 2000). A further report showed that Mitomycin C at a final concentration of 20 µg/ml was able to induce phage BB-1 out of its *B. burgdorferi* s.l. host strain (Eggers *et al.*, 2000). The induced culture supernatants were PEG-precipitated and analysed for phage DNA on a 0.5 % agarose gel, followed by ethidium bromide staining. The phage DNA was observed as a faint band on the gel (Eggers *et al*., 2000).

At a concentration of 1 mg/ml, Norfloxacin has been used successfully to induce two prophages out of their *E. coli* host (Matsushiro *et al*., 1999), and a range of prophages out of *Clostridium difficile* (Meessen-Pinard *et al*., 2012, Nale *et al*., 2012, Shan *et al.*, 2012). Norfloxacin is a gyrase inhibitor which leads to DNA damage, and further studies have shown that the molecular mechanism of prophage induction is closely linked to the RecA system (Matsushiro *et al.*, 1999). Norfloxacin has previously been used at a final concentration of 3 µg/ml to induce different bacteriophages from *Clostridium difficile* ribotype 027 (Nale *et al*., 2012). Recently, Norfloxacin has been used at 6 µg/ml to induce phage tail-like particles (PTLPs) from *C. difficle.* PTLPs were similar to phages but lacked a viral capsid and genome; some PTLPs were capable of killing target bacteria (Hegarty *et al.*, 2016b). In addition to chemical induction, environmental factors such as UV radiation can also cause DNA damage and prompt prophage release as a result of the hosts' SOS response activation (Lamont *et al*., 1989, Nanda *et al*., 2015). Spontaneous prophage induction (SPI) can also occur as a result of an accumulation of DNA damage and the activation of the host's SOS response (Cortes *et al*., 2019). SPI has been observed in some bacterial cultures, such as *Pseudomonas aeruginosa* (Fothergill *et al.*, 2011), and plays a role in the population of temperate phages in a mixture of susceptible cells and lysogens (Cortes *et al.*, 2019).

Currently knowledge of *B. burgdorferi* s.l. phages biological properties is limited. Bioinformatic data has shown that quite a few of the plasmids carried within *B. burgdorferi* s.l. probably originated from phages. Phage-Like plasmids associated with *B. burgdorferi* s.l. were first studied with the *B. burgdorferi* cp32 plasmids. These plasmids have been experimentally shown to be prophages (Radolf and Samuels, 2010, Casjens *et al*., 2017). A bacteriophage of *B. burgdorferi*, designated as ϕBB-1, has been confirmed to be the 32-kb

circular plasmid that is part of the cp32 family of *B. burgdorferi* plasmids (Zhang and Marconi, 2005). This cp32 prophage can be induced with chemicals such as Mitomycin C or 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG) from *B. burgdorferi* strain CA-11.2A (Eggers *et al*., 2000). Two phages have also been induced from *B. burgdorferi* s.l. using ciprofloxacin, one of these phages having an isometric head of 30 nm in diameter, a neck, a baseplate and a contractile tail of 50–64 nm in length, falling within the criteria for morphotype A1 (icosahedral capsid) within the *Myoviridae* family (Neubert *et al*., 1993). The other phage belonged to the *Siphoviridae* family and to the subgroup B1 (icosahedral capsid), with a 30 nm isometric head and a long non-contractile straight tail 115–130 nm in length (Neubert *et al*., 1993). This chapter describes attempts to induce, purify and characterize temperate phages from different *B. burgdorferi* s.l. strains in order to increase the understanding of *B. burgdorferi* s.l. prophage carriage and their biological properties.

4.2 Methods

4.2.1 Measuring *B. burgodorferi* s.l. growth with a Haemocytometer

The growth of *B. burgdorferi* s.l. strains were monitored by using a haemocytometer (counting chamber) under a phase-contrast microscope. *B. burgdorferi* s.l. counting started from the first day of the inoculation, was performed on a daily basis and lasted for 15 days. The morphological status of *B. burgdorferi* s.l. was also recorded and monitored. For example, the early log phase of the *B. burgdorferi* B31 culture was dominated by active-moving free spirochetes with a concentration of approximately (5.02×10^6 cells/mL). Conversely, in the late loge phase (2.00×10^8 cells/mL), *B. burgdorferi* s.l. began forming aggregates. The proportion of *B. burgdorferi* s.l. aggregates increased with time and by the end of day 15, the culture was more populated with aggregates; this was when the cell population increased to ~ 7×10^8 cells/mL. The growth parameters were determined according to the following formula (Heroldova *et al.*, 1998):

R (h^{-1}) = 1/log2 [(logN2 - logN1/t)].

R (h^{-1})= Growth rate per hour, which is the rate of change in the number of the cells in a culture per unit time, this required estimation of the cell dencity at series of time points (Hall *et al*., 2013).

N1= Bacteria present at an early stage in the exponential phase.

N2 = Bacteria present at some time of the exponential phase.

t = time interval between N1 and N2.

G (h)=generation time= 1/R.

μ (h^{-1})= Specific growth rate = 0.693/G.

Specific growth rate (period) was defined as the rate of increse of biomass of cell population per unit of biomass concentration (Kosseva and Kent, 2013).

4.2.2 Induction of laboratory *B. burgdorferi* s.l. strains via Mitomycin C and Norfloxacin treatment

Different *B. burgdorferi* s.l. strains, (*B. burgdorferi* VS185 P9*, B. valaisiana* NE218*, B. afzelii* ACA-1*, B. burgdorferi* UK filtered*, B. garinii* 190 P9, *B. burgdorferi* China 23 *and B. burgdorferi* B31) were used in prophage induction with Mitomycin C and Norfloxacin as in section 2.17. 0TEM analysis was performed for the induced lysates, DNA extracted from the induced lysates were tested using the terminase 676 PCR.

4.2.3 Measuring the killing activity of induced phage lysates using SYBR Green I/PI assay.

In this chapter, SYBR Green I and propidium iodide (PI)-based live/dead assay was used to determine killing activity of the induced phage lysates filtrates against *B. burgdorferi* s.l. strains such as *B. burgdorferi* VS185 P9, *B. valaisiana* NE218, *B. afzelii* ACA-1, *B. burgdorferi* UK filtered, *B. garinii* 190 P9 and *B. burgdorferi* B3. The killing ratio was measured according to the ratio of green-to-red fluorescence which is proportional to the ratio of live/dead cells of *B. burgdorferi* s.l.present, i.e. The high ratio of green to red fluorescence of the SYBR Green I/PI represents a high amount of live bacterial cells, a bigger ratio represents a high number of undamaged bacterial cells and indicates a low killing efficiency of the agent used. A lower ratio of the green to red fluorescence of the SYBR Green I/PI assay represents a lower number of intact cells and higher number of damaged and killed bacterial cells, and indicates a high killing efficiency of the agent used. The tested strains compared with Tetracycline as positive control and both SM buffer and No phage control (bacterial culture with 20μl of complete BSKII medium) as negative controls. The Green to red fluorescence ratios were measured for three replicates. The analysis was done using Two-way ANOVA (multiple comparisons).

4.2.4 Spot test and plaque assays for induced phage lysates.

To determine any potential virulent activity of the induced lysates against *B. burgdorferi* s.l., the classical spot test and plaque assay described in sections 2.25 , and 2.26 were performed. Induced lysates that filtered through 0.22 µm filters were spotted against different *B. burgdorferi* s.l. lawns.

4.2.5 Induction of *B. garinii* 190 P9 with UV

B. garinii 190 P9 was tested for prophage induction using UV irradiation as in section 2.19. To further understand these potential phage fractions from the UV-induced *B. burgdorferi* s.l. filtered lysates, DNA from the UV-induced phage fraction was extracted according to the classical phage DNA isolation protocol of Sambrook, which incorporates extra steps to remove free DNA and RNA before releasing phage DNA. The resulting putative phage DNA was examined using a 0.7% w/v gel electrophoresis (Sambrook and Russell, 2006b, Sambrook and Russell, 2006a). The killing of UV-induced lysate filtrates was also tested against *B. burgdorferi* B31 using the live/dead assay.

4.2.6 Induction of *B. burgdorferi* s.l. strains isolated from ticks

B. burgdorferi s.l. strains isolated from ticks were subjected to Mitomycin C induction only. Induced lysates samples (18M, 19M, 20M, 21M, 89M, and 90M) were subjected to TEM analysis. Killing test was also performed using SYBR Green I and propidium iodide (PI)-based live/dead assay. PCR targeting terminase gene, spot test and plaque assay were also performed.

4.3 Results

4.3.1 Measuring *B. burgdorferi* s.l. growth strains with a Haemocytometer

B. burgdorferi s.l. growth curves for different *B. burgdorferi* s.l. strains are shown in Figure 4-1, and the growth parameters are presented in Table 4-1. Results analysis showed that among these four strains, the shortest generation time (about 8.77 hours) was found for *B. burgdorferi* B31, whereas the longest generation time was found for *B. afzelii* ACA-1 (about 13.50 hours). Highest growth rate R ($0.11h^{-1}$) was observed in *B. burgdorferi* B31, while the lowest growth rate R ($0.05h^{-1}$) was observed in *B. afzelii* ACA-1. The maximum cell density was measured in *B. Burgdorfer*i B31 was $8.2x\ 10^8$ after 7 days of incubation compared with the other strains, while the maximum cell density for *B. afzelii* ACA-1 was measured at $1.2x\ 10^8$ after 9 days of incubation.

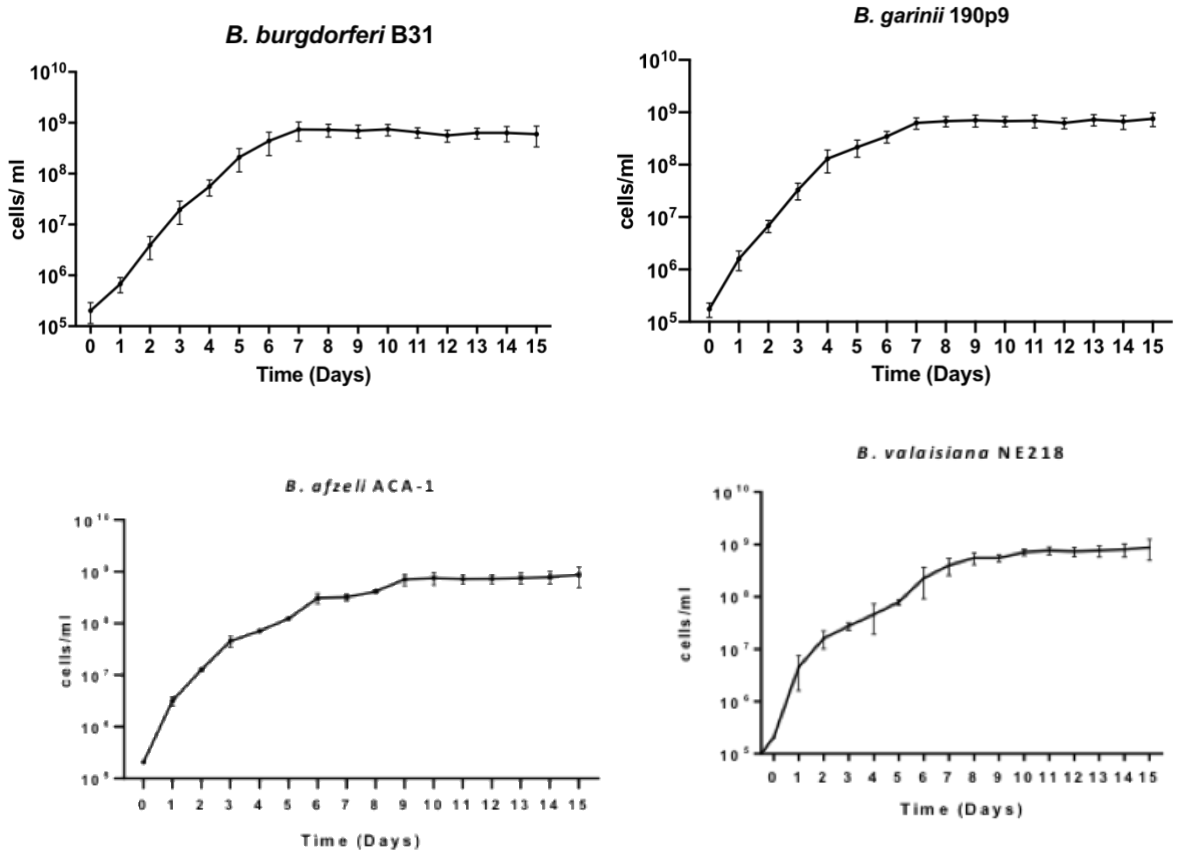


Figure 4-1 Growth curves of *B. burgdorferi* B31, *B. afzelii* ACA-1, *B. garinii* 190 P9, and *B. valaisiana* NE218 at 34 °C. Values are the average of three replicates (three readings for the same culture) determined using a DHC-N01-Chip haemocytometer.

Table 4-1 Growth parameters for *B. burgdorferi* B31, *B. afzelii* ACA-1, *B. garinii* 190 P9, and *B. valaisiana* NE218.

Growth parameters	***B. burgdorferi* B31**	***B. garinii* 190P9**	***B. afzelii* ACA-1**	***B.valaisiana* NE218**
G(h)*	8.77	12.05	13.50	9.09
$R(h^-)$**	0.114	0.083	0.074	0.110
$\mu(h^-)$***	0.079	0.057	0.050	0.076

G(h)* generation times $R(h^-)$** growth rate per hour $\mu(h^-)$*** specific growth rate.

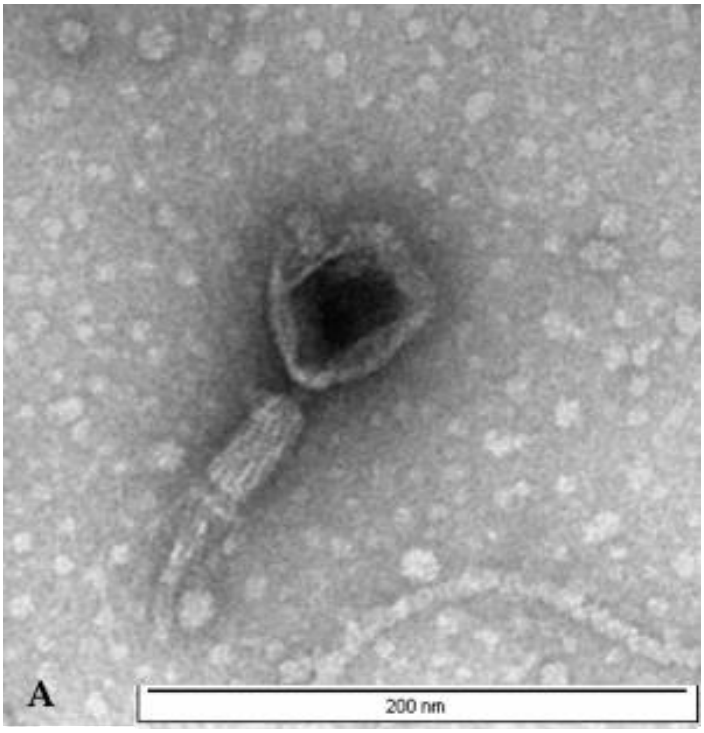
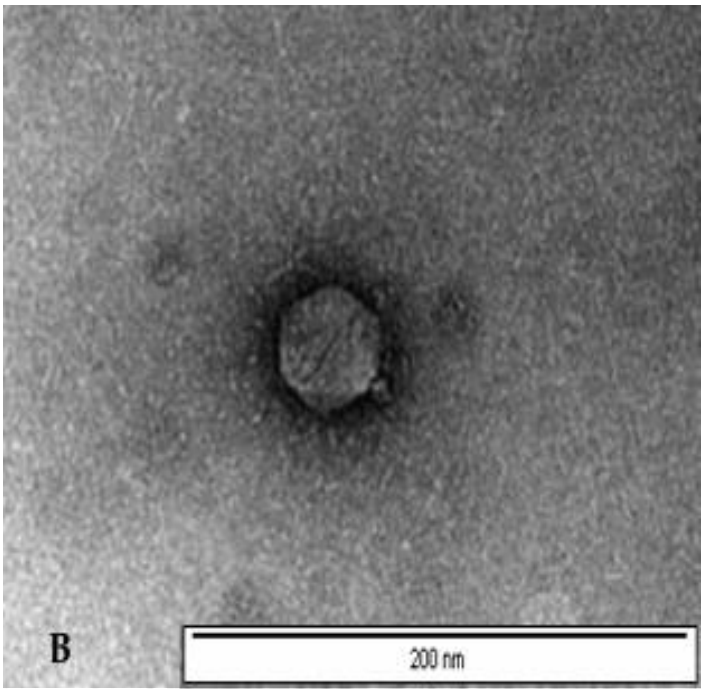
4.3.2 Induction of laboratory *B. burgdorferi* s.l. strains via Mitomycin C and Norfloxacin treatment

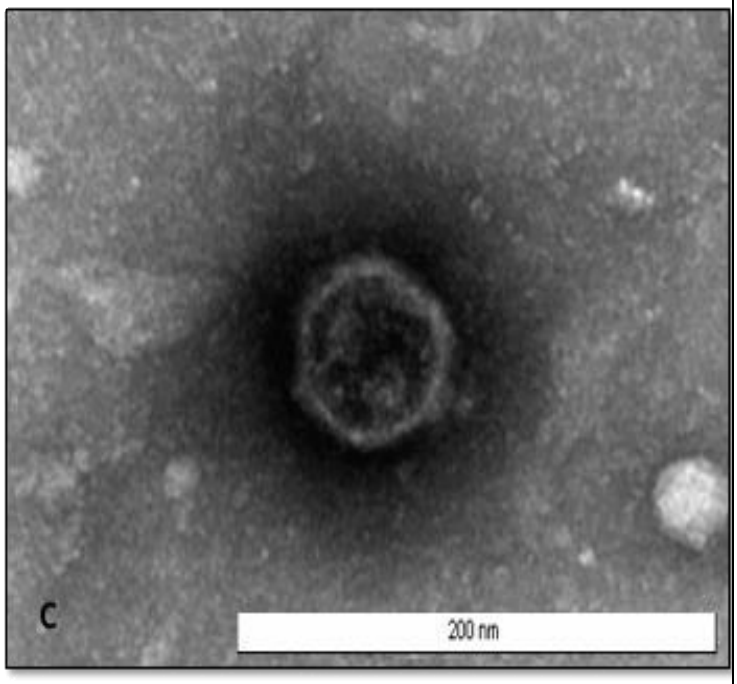
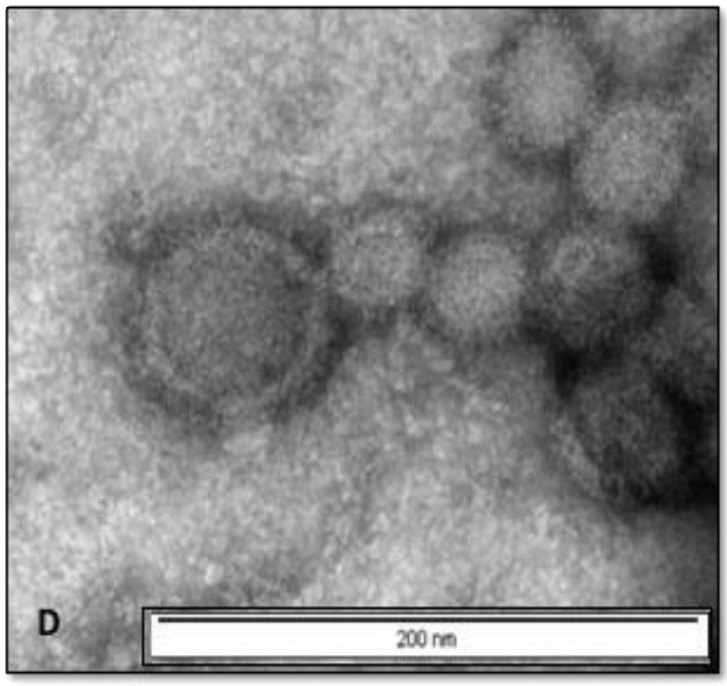
The results of the induction of *B. burgdorferi* s.l. strains were analysed using TEM for phage presence in Table 4-2. The TEM analysis of the Mitomycin C induced *B. afzelii* ACA-1 cultures revealed two phage particles named pL5-1 (myovirus A), which has a hexagonal head that is 70 nm in diameter and a 100 nm long tail, and pL5-2 (podovirus B), which has an isometric head of 50 nm in diameter and a 10 nm tail. No recognisable phage particles were observed from the other Mitomycin C-induced strains.

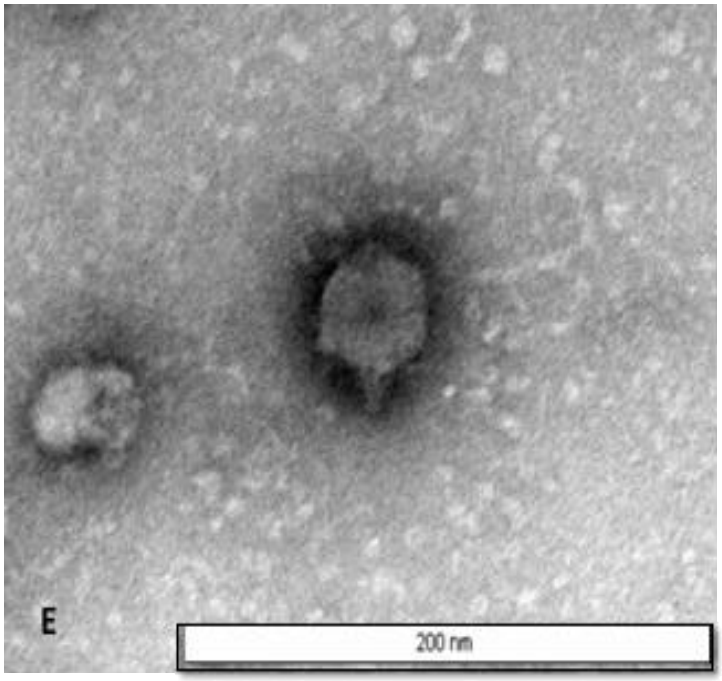
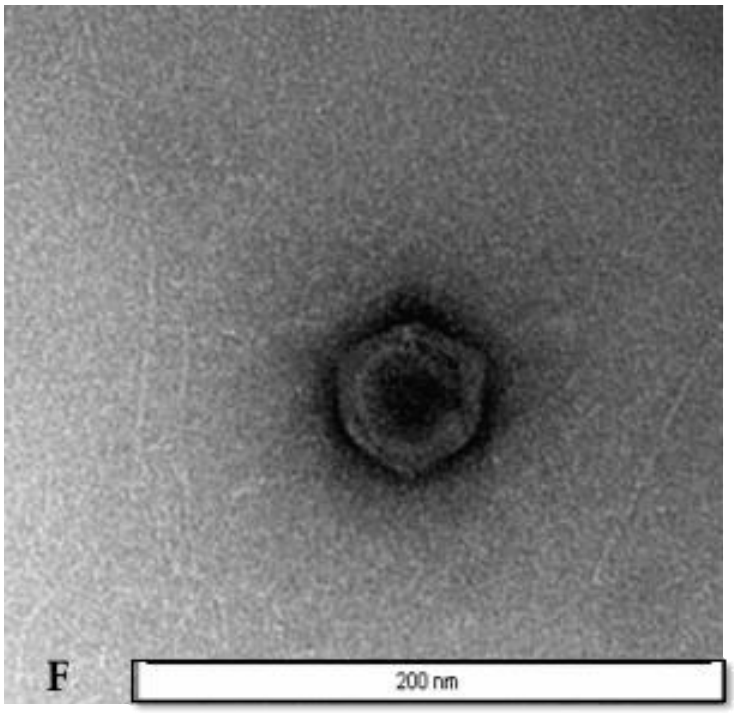
TEM analysis of the Norfloxacin-induced *B. garinii* 190P9 culture revealed one podovirus named pL7 (podovirus C), which has a head that is 64 nm in diameter, and tailless. In the case of inducing *B. valaisiana* NE218, TEM analysis revealed one podovirus named pL4 (podovirus D), which has a head that is 75 nm in diameter and a 10 nm tail.

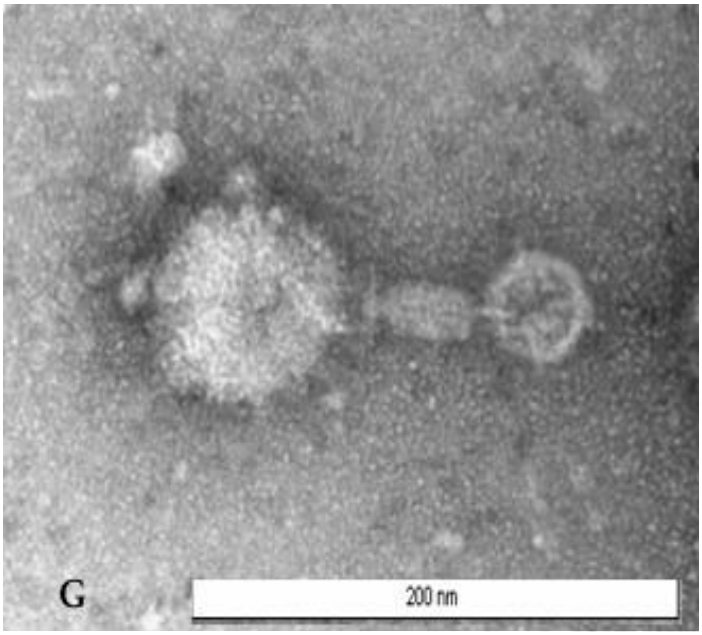
Norfloxacin also induced one podovirus named pL6 (podovirus E) from *B. burgdorferi* UK filtered, with a head that is 45 nm in diameter, and that is tailless. Moreover, a podovirus was also induced from *B. burgdorferi* vs185p9, named pL3 (podovirus F), with a head that is 55 nm in diameter and that is tailless. Finally, TEM revealed one myovirus named pLB31 (myovirus G) in the culture filtrate of *B. burgdorferi* B31, which had not been subjected to any treatment. This demonstrated the occurrence of spontaneous prophage induction (SPI) in *B. burgdorferi* B31. This phage-like particle has an isometric head of 50 nm in diameter and a 70 nm tail.

Table 4-2 Induced phages from lab *B. burgdorferi* s.l. strains

Sample	Strain	Inducer	Induced phage morphology
pL5-1	*Ba*ACA1	Mitomycin C	
pL5-2	*Ba*ACA1	Mitomycin C	

Sample	Strain	Inducer	Induced phage morphology
pL7	*Bg* 190P9	Norfloxacin	
pL4	*Bv* NE218	Norfloxacin	

Sample	Strain	Inducer	Induced phage morphology
pL6	*Bb* UK filtered	Norfloxacin	
pL3	*Bb*VS185 p9	Norfloxacin	

Sample	Strain	Inducer	Induced phage morphology
pLB31	*Bb* B31	Spontaneous Induction	

In summary of this experiments, seven phages like particles were induced from *B. burgdorferi* s.l. strains. Mitomycin C was able *B. burgdorferi* s.l. strains. However, TEM confirmed the presence of only two phages like particles from *B. afzelii* ACA-1. One was podovirus and the second one was myovirus. TEM did not revealed the presence of phage like particles in other strains such as *B. valaisiana* NE218, *B.burgdorferi* Uk filtered, *B. burgdorferi* VS185p9 and *B. garinii* 190p9.

Norfloxacin was also able to induce *B. burgdorferi* s.l. strains. Norfloxacin induced four podovirus. TEM confirmed the presence of podovirus in each of *B. valaisiana* NE218, *B. burgdorferi* Uk filtered, *B. burgdorferi* VS185p9 and *B.garinii* 190p9. TEM did not confirm the presence of any phage like particles in *B. afzelii* ACA-1. In addition to one spontaneous induced phage from *B. burgdorferi* B31. The results confirmed that inducing agent have different efficiency in *B. burgdorferi* s.l. prophage induction due to the number of phages and morphotypes of them. All the myovirus types have different morphotypes. Some of them have a hexagonal capsid. Others have isometric head in range of (55-65)nm head and 100 nm tail. All the podoviruses induced from those strains have (50-60)nm an isometric head and most of them are tailless.

4.3.3 Measuring the killing activity of Mitomycin C induced lysates

In this study, MitomycinC induced phage lysate filtrates were tested for killing activity against *B. burgdorferi* s.l. strains. The killing efficiency was measured according to the ratio of green-to-red fluorescence (G/R) which is proportional to the ratio of live/dead cells of *B. burgdorferi* s.l. present. Results are shown in Figure 4-2. Multiple Bar chart shows Green/Red fluorescence ratio of the SYBR Green I/PI for Mitomycin C induced phage lysates. First comparison between no phage control treatment and TC treatment to show the viability of *B. burgdorferi* s.l. strains, when TC was added the G/R ratio (live cells) sharply dropped with *P* value of < 0.0001. The second comparison between no phage control treatment and SM treatment to show the impact of adding SM buffer on the viability of the bacteria, which was significantly less between SM and no phage control with *P* value < 0.0001. From the above comparisons, the main control for the tested groups will be SM treatment because all induced phages were resuspended in SM buffer.

All the induced phage lysates such as 3M, 4M, 5M, B31M, and 7M were significantly less than SM with *p* value of < 0.0001. There was significant reduction in the G/R of all induced phage lysates against the tested *B. burgdorferi* s.l. strains.

Within the groups, firstly, MitomycinC-induced phage lysate from *B. burgdorferi* VS185P9 (3M) showed significant difference between the strains with *P*value < 0.0001. G/R was significantly different. The highest G/R was observed against BbB31 in ratio of 3.77. The lowest G/R was observed against Bv NE218 in ratio of 1.50. This means the highest killing of 3M was determined against Bv NE218 with low G/R ratio of 1.50.

In addition to, MitomycinC-induced phage lysate from *B. valaisiana* NE218 (4M) showed significant difference between the strains with *P* value of <0.0001. 4M had a significant reduction in G/R. The highest G/R was observed against Bb B31 in ratio of 3.80, the lowest G/R was against Bv NE218 in a ratio of 1.75. This means that the highest killing was determined against Bv NE218 with low G/R ratio of 1.75. Moreover, Mitomycin C-induced phage lysate from *B. afzelii* (5M) showed significant difference between the strains with *P* value of <0.0001. 5M has highest G/R was observed against Bb B31 in ratio of 3.66. The lowest G/R was observed against Bg 190p9 in ratio of 1.43. This means the highest killing of 5M was determined against Bg 190p9 with low G/R ratio of 1.43. Furthermore, Mitomycin C-induced phage lysate obtained from *B. burgdorferi* B31 (B31M) showed significant difference between

the strains with *P* value of <0.0001. The highest G/R was observed against BbB31 in ratio of 3. 53 against BbB31. The lowest G/R was observed against Bv NE218 in ratio of 1.65. This means that the highest Killing was determined against Bv NE218 with low G/R ratio of 1.65. Finally, Sample MitomycinC- induced phage lysate of *B. garinii* 190 P9 (7M) showed significant difference between the strains with *P* value of <0.0001. The highest G/R of 3.50 was observed in Bb B31; the lowest G/R ratio of 1.45was observed in Bv NE218 with mean killing G/R ratio of 1.45.

In summary, MitomycinC-Induced phage lysates had significant killing effects against *B. burgdorferi* s.l. The highest killing activity for all MitomycinC-induced phage lysates was determined against *B. valaisiana* NE218. 5M was the highest killing activity against Bv NE218 with the lowest ratio of G/R in 1.43 compared with the other induced phage lysates. This strain appeared to be more susceptible to the induced phage lysates compared to the other strains. In contrast, *B. burgdorferi* B31 was most resistant to all induced phage lysates used in this experiment.

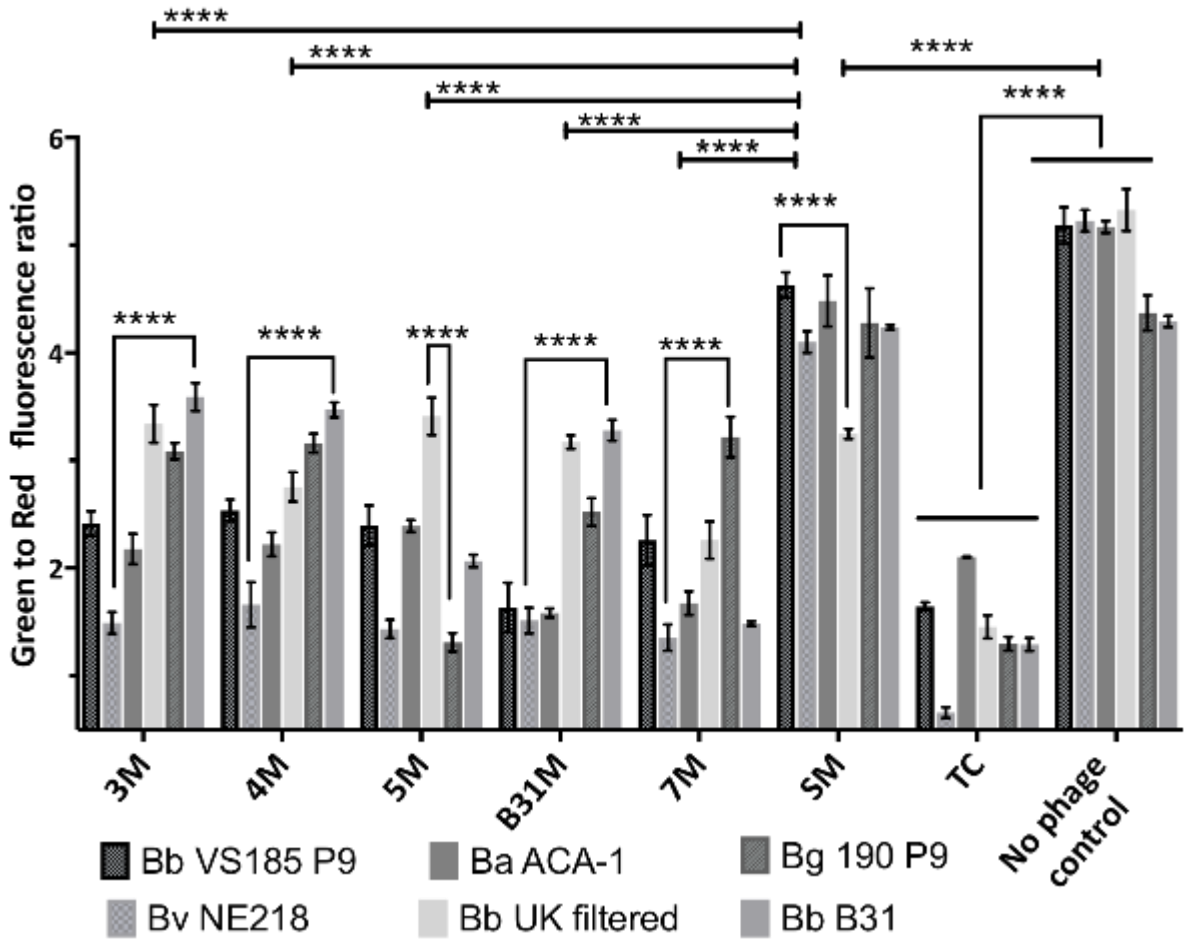


Figure 4-2 Green/Red fluorescence ratio (G/R) of the SYBR Green I/PI assay of Mitomycin C induced lysates.

(Y axis) is the green to red of fluorescence ratio. (X axis) MitomycinC- Induced phage lysates. killing efficiency was measured according to the ratio of green-to-red fluorescence, Sample 3M is the Mitomycin-C induced phage lysate from *B. burgdorferi* VS185P9, sample 4M is the Mitomycin C-induced phage lysate from *B. valaisiana* NE 218, and 5M is the Mitomycin C-induced phage lysate from *B. afzelii*. B31M is the Mitomycin C-induced phage lysate induced from *B. burgdorferi* B31, and 7M is the Mitomycin C-induced phage lysate from *B. garinii* 190 P9. TC is Tetracycline as a positive control. SM is SM buffer-treated *B. burgdorferi* s.l. strains. No phage control was the culture of *B. burgdorferi* s.l. strains treated with cBSKII media only as a negative control. The data is recorded as the average of three measurements. Legend represents *B. burgdorferi* s.l. strains: Bb VS185P9, Bv NE218, BaACA-1,Bb uk filtered,Bg 190 P9, BbB31:*B. burgdorferi* B31. ****P values<0.0001. Bars were analysed according to Two- way ANOVA (multiple comparisons).

4.3.4 Measuring the killing activity of Norfloxacin induced lysates

Norfloxacin induced phage lysate samples were also tested for killing activity against *B. burgdorferi* s.l. strains. The killing activity was measured as mentioned above. The results are shown in Figure 4-3. Multiple Bar chart shows the Green /Red fluorescence (G/R) ratio for Norfloxacin induced phage lysates against *B. burgdorferi* s.l. As the previous analysis the comparison was performed between TC and No phage control to measure the viability of the bacterial strains tested in this experiment, which was significantly dropped by addition of TC with P value of < 0.0001. SM and No phage control were also compared between each other, and they less significant with P value < 0.0001.

As suggested from the previous graph, SM was confirmed to be the main control. The analysis showed that the reduction of G/R of fluorescence ratio in all treatment of 3N, 4N, 5N,7 N, and B31N compared with SM treatments.

Within the groups, the reduction of killing was significant with P value of < 0.0001. Sample 3N has highest G/R ratio against Ba ACA-1 in ratio of 4.30. The lowest ratio was observed against Bv NE218 at ratio of 2.25. this means the highest killing activity was against Bv NE218 with low ratio of 2.25.

In the same way, sample 4N has the highest G/R ratio against Bb VS185P9 in ratio of 4.37. The lowest G/R ratio was against BbUk filtered in ratio of 2.50. the highest killing activity was against BbUk filtered with low ratio of 2.50. Sample 5N has the highest G/R ratio against BbB31 in ratio of 5.80, the lowest ratio was against Bv NE218 in ratio of 3.10

Further samples such as 7N and B31N, 7N has the highest G/R ratio against Bb VS185P9 with ratio of 3.00. the lowest ratio was against Bv NE218 in ratio of 2.70. the highest killing was determined with the low G/R ratio to be 2.70. Finally, sample B31N has killing ratio against Bv ACA-1, the lowest G/R was in ratio of 2.30. The highest ratio of G/R was 4.70 against BbB31, the same strain that the phages were induced.

In summary Norfloxacin induced phage lysates showed killing activity against all the tested *B. burgdorferi* s.l. strains. The killing was significant between them. Interestingly sample 3N was the most active due to the lowest G/R ratio of 2.25 against *B. valaisiana* NE 218 among all the induced phage lysates. In addition to 4N and B31N showed the highest killing ratio when G/R was low as 2.50 and 3.10 respectively, both were more active in killing compared with 7N and 5N.

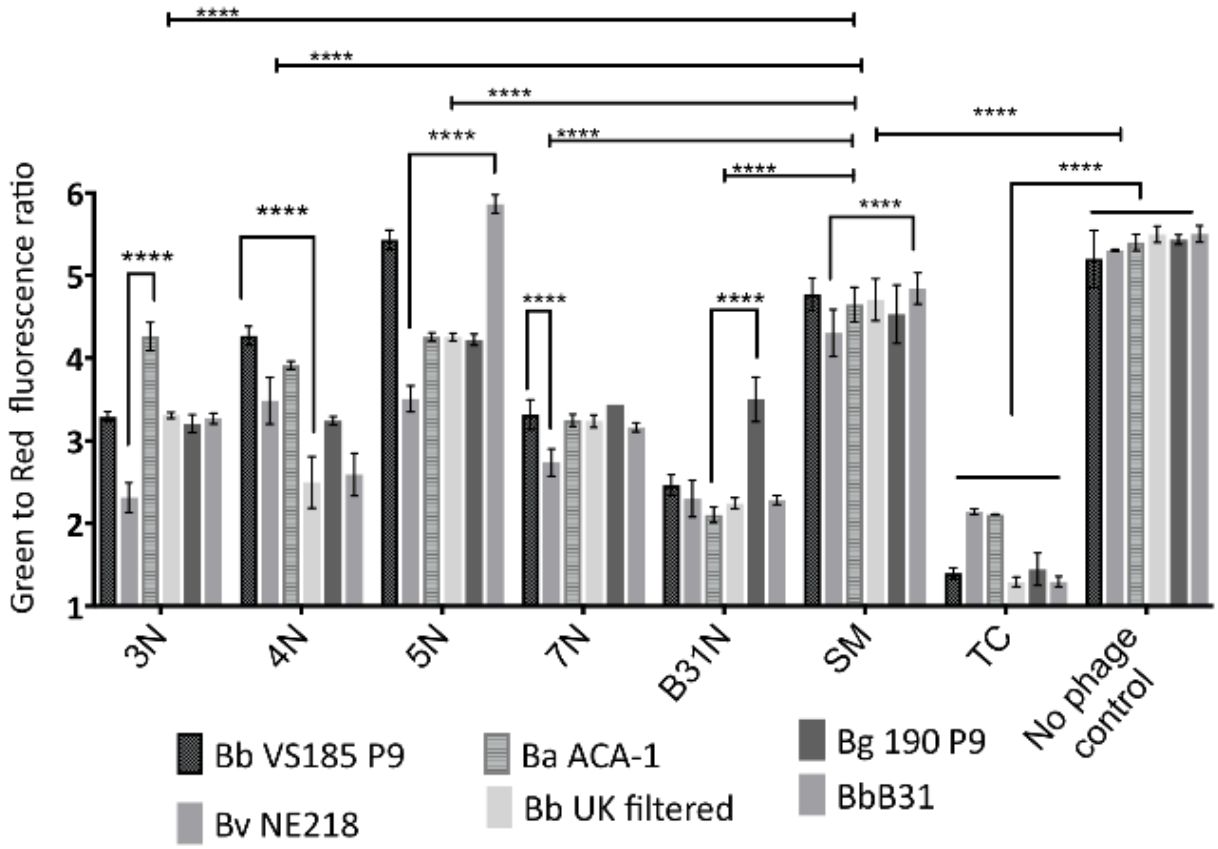


Figure 4-3 Green/Red fluorescence ratio (G/R) of the SYBR Green I/PI assay of Norfloxacin induced lysates.

(Y axis) is the green to red of fluorescence ratio. (X axis) Norfloxacin- Induced phage lysates . killing ratio was measured according to the ratio of green-to-red fluorescence. Sample 3N is the Norfloxacin-induced phage lysate from *B. burgdorferi* VS185P9. Sample 4N is the Norfloxacin-induced phage lysate from *B. valaisiana* NE 218. 5N is the Norfloxacin-induced phage lysate from *B. afzelii* ACA-1. 7N is the Norfloxacin-induced phage lysate from *B. garinii* 190 P9. B31N is the Norfloxacin-induced phage lysate from *B. burgdorferi* B31. TC is the Tetracycline used as a positive control. SM refers to the *B. burgdorferi* s.l. strains treated with SM buffer, no phage control was the culture of *B. burgdorferi* s.l. strains treated with cBSKII media only as a negative control. The data is recorded as the average of three measurements. Legend represents *B. burgdorferi* s.l. strains: Bb VS185P9, Bv NE218, BaACA-1,Bb UK filtered, Bg 190 P9, BbB31: *B. burgdorferi* B31. P values<0.0001. Bars analysed as Two- way ANOVA (multiple comparison).

4.3.5 PCR test, Spot test and plaque assays for induced phage lysates.

The result of the terminase 676 PCR against the induced lysate filtrates is presented in Figure 4-4. A distinct PCR band with the expected size of 676 bp was observed in all lysates, indicating the presence of *B. burgdorferi* s.l. prophages or might be DNA fragments contain terminase gene. For example, a sharp PCR band was observed from the Mitomycin C-induced lysate from *B. afzelii* ACA-1, as in lane 1, and Norfloxacin-induced lysate from *B. afzelii* ACA-1, as in lane 7. Moreover, a clear PCR band was seen for the Norfloxacin-induced lysate for *B. valaisiana* NE218, as in lane 2, and Mitomycin C-induced lysate for *B. valaisiana* NE218, as in lane 8. Furthermore, there is a clear band in the Norfloxacin-induced lysate of *B. burgdorferi* UK filtered, as in lane 4. The terminase PCR band was also present in the spontaneous prophage induction (SPI) of *B. burgdorferi* B31, as in lane 6. The positive control (DNA extracted from *B. burgdorferi* B31) also generated a clean PCR product of the correct size. No PCR product was observed from the PCR negative controls, which confirmed that no contamination occurred during PCR.

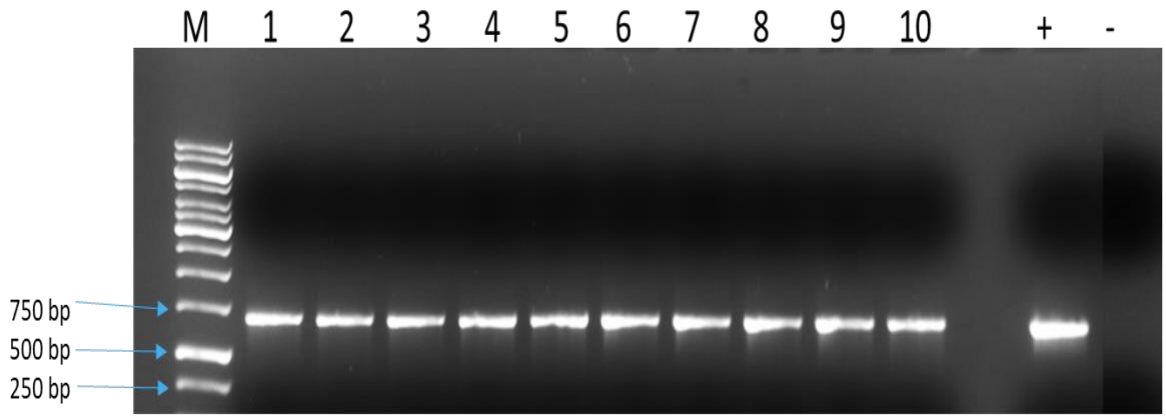


Figure 4-4 Terminase 676 PCR targeting the terminase gene in DNA samples isolated from induced lysates with Mitomycin C and Norfloxacin.

PCR was applied to DNA extracted from Mitomycin C and Norfloxacin-induced lysates. Lane (1) Mitomycin C-induced lysate from *B. afzelii* ACA-1. Lane (2) Norfloxacin-induced lysate of *B. valaisiana* NE218. Lane (3) Norfloxacin-induced lysate from *B. garinii* 190P9. Lane (4) Norfloxacin-induced lysate of *B. burgdorferi* UK filtered. Lane (5) Norfloxacin-induced lysate of *B.burgdorferi* VS185p9. Lane (6) lysate of *B. burgdorferi* B31 without treatment with Norfloxacin or Mitomycin C. Lane (7) Norfloxacin-induced lysate of *B. afzelii* ACA-1. Lane (8) Mitomycin C-induced lysate of *B. valaisiana* NE218. Lane (9) Mitomycin C-induced lysate of *B. garinii* 190P9. Lane (10) Mitomycin C-induced lysate of *B. burgdorferi* VS185p9. A positive control was DNA extracted from *B. burgdorferi* strain B31. Negative control was a PCR mixture with ultrapure water as a template. M represents a 1 KB DNA marker. Amplicons of the expected size of 676 bp were detected.

Induced lysates of *B. burgdorferi* s.l. were spotted against other *B. burgdorferi* s.l. lawns. Figure 4-5 illustrates example of the spot test of Mitomycin C induced lysates samples 3M, 4M, 5M, 7M and 8M on the lawn of *B. burgdorferi* s.l lawn, no clear zones were identified for the Mitomycin C-induced lysates. However, the positive controls (Tetracycline and Norfloxacin) shows positive lytic zones against the tested *B. burgdorferi* s.l. strain. In the same way, Norfloxacin-induced lysates were also spotted against *B. burgdorferi* s.l. lawns. However, the result was negative. A plaque assay was also performed in an attempt to identify any lytic activity against *B. burgdorferi* s.l. strains. However, no plaques were observed.

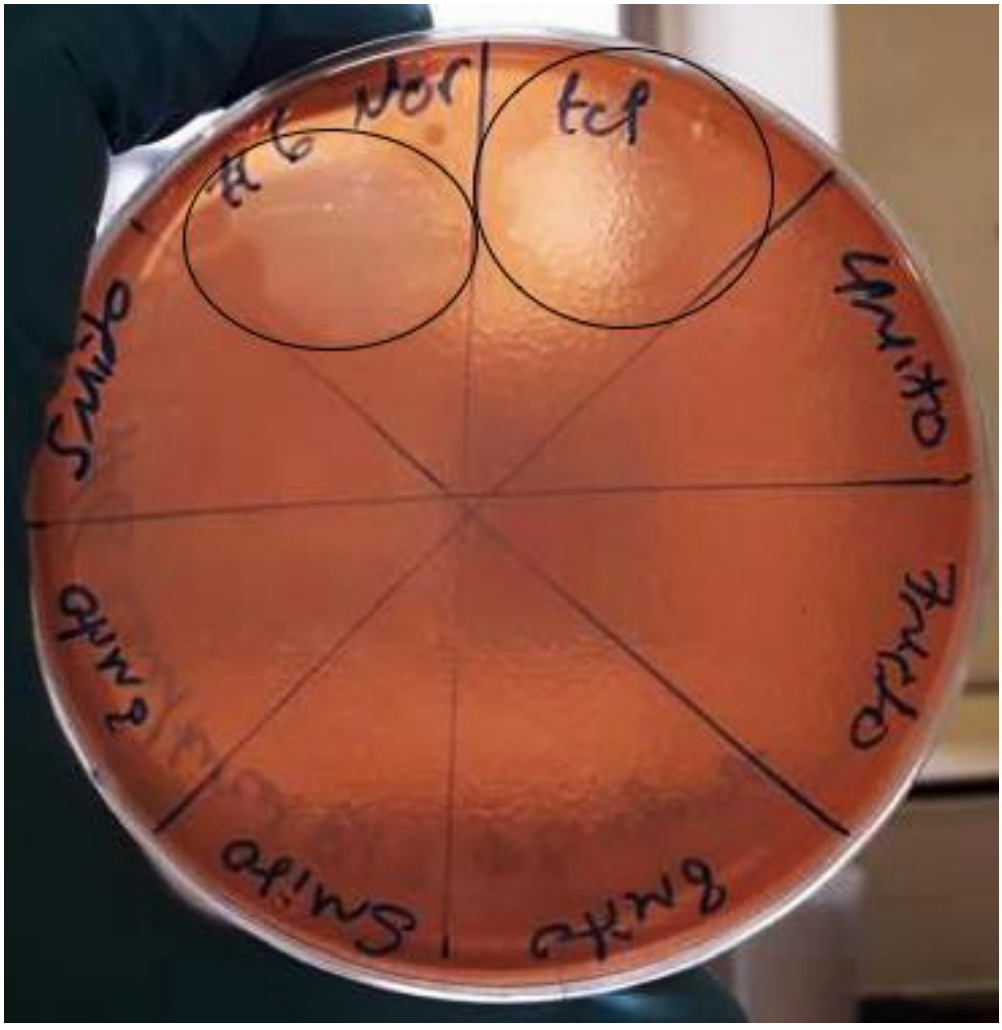


Figure 4-5 Spot test of MitomycinC induced lysates.

TC and Norfloxacin shows lytic lysis determined by black circles. Mitomycin C-induced lysates samples 3M, 4M, 5M, 7M, and 8M were spotted on the *B. burgdorferi* B31 lawn. All are negative.

4.3.6 Induction of *B. garinii* 190 P9 with UV

TEM analysis of the UV-induced culture revealed podovirus-like particles with a head that is 64nm in diameter and no visible tail as shown in Figure 4-6. Phage DNA is shown as single band with a molecular weight of ~30KB. UV-induced lysate filtrates of *B. garinii* 190p9 were tested for killing against *B. burgdorferi* B31. The analysis of green to red fluorescein ratio was performed using one-way ANOVA. As the previous graphs, the analysis was performed firstly between No phage control and TC to see the viability of *B. burgdorferi* B31. There was significant reduction in the G/R ratio of fluorescence, when TC was added the ratio was dropped significantly due to the high number of the damaged cells.

SM treatment was already confirmed to be the main control. The comparison was performed between the SM treatment and all UV induced phage lysates. Firstly, there was no significant reduction in the ratio of G/R between sample at 0 seconds which has ratio of 4.90 and SM. The second one was treatment with UV induced phages lysate (10 seconds), the ratio was less significant compared with the SM treatment with p value <0.05. The ratio of G/R was 3.70.

Same comparison was performed between treatment with UV induced lysates (20 seconds) and (30 seconds), and SM. The ratio of G/R was highly significant for the 20 seconds treatment, which has low G/R ratio of 2.54, compared with sample 30 seconds which has G/R ratio of 3.50. They were significantly less than SM treatment which has ratio of G/R at 4.90.

In conclusion, the highest ratio of killing was lower G/R to be 2.54, observed in sample 20 seconds (UV induced phage lysate treated at 20 seconds). The ratio of G/R was too low due to low number of live cells and this sample might contain *B. garinii* 190P9 phages.

Spot test and plaque assays were also performed for UV-induced lysates of *B. garinii* 190P9 against *B. burgdorferi* B31. The results were negative. No lytic zones for the spot test or plaques were identified in these samples.

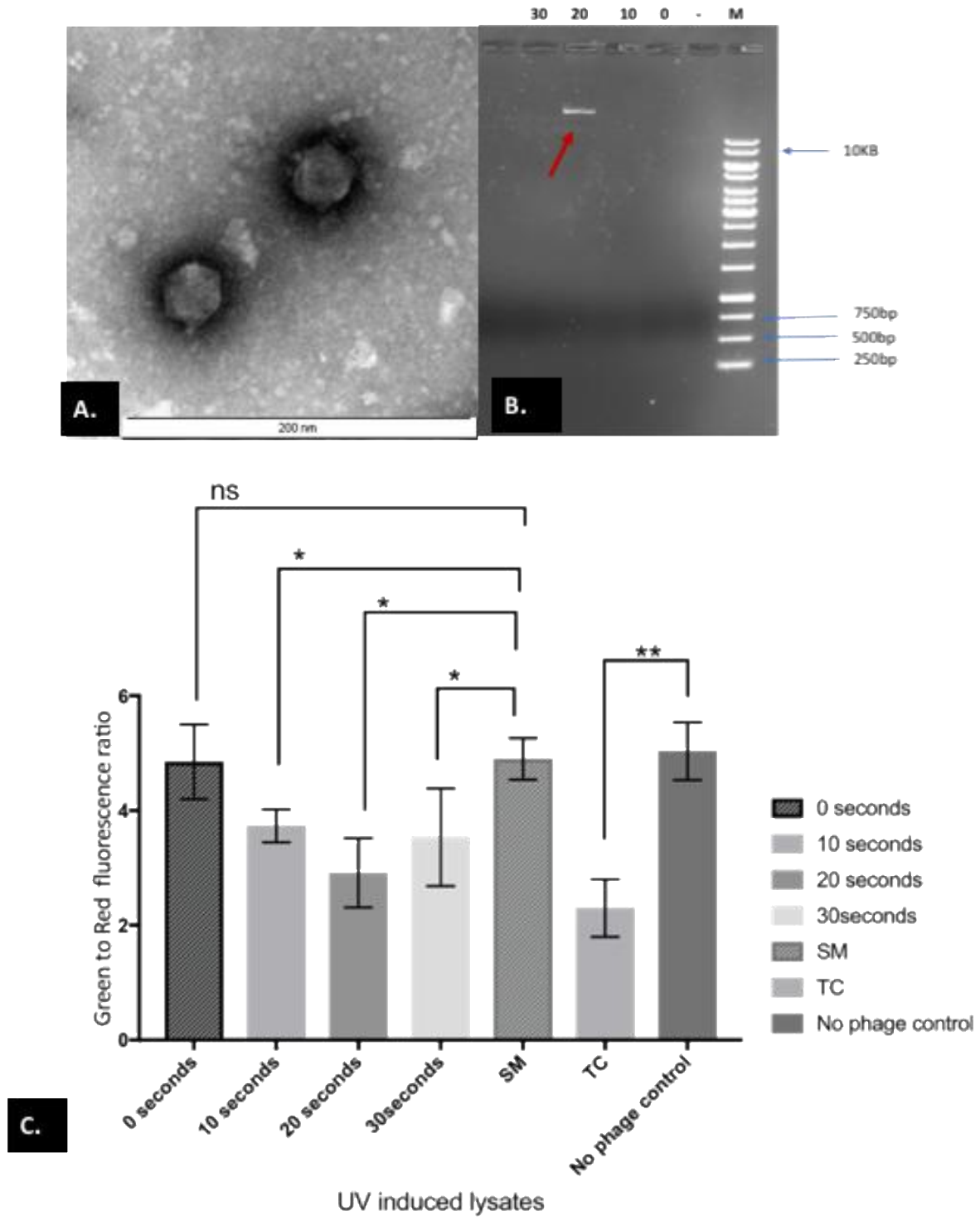


Figure 4-6 Characterisation of lysate filtrates derived from UV-induced *B. garinii* 190 P9.

(A) Induced phages under TEM. (B) Agarose gel for DNA isolated from induced lysate filtrates. (C) Killing test of UV-induced lysate filtrates for various lengths of time was measured. Values are means with a standard deviation of different independent cultures as three replicates of *B. burgdorferi* B31 treated with induced phage lysate filtrates at 0, 10, 20 and 30 seconds, and with TC as the positive control. SM buffer and no phage control (*B. burgdorferi* B31 treated with CBSKII only) as the negative controls. Analysis by one-way ANOVA.$^{*}P$-value < 0.05.

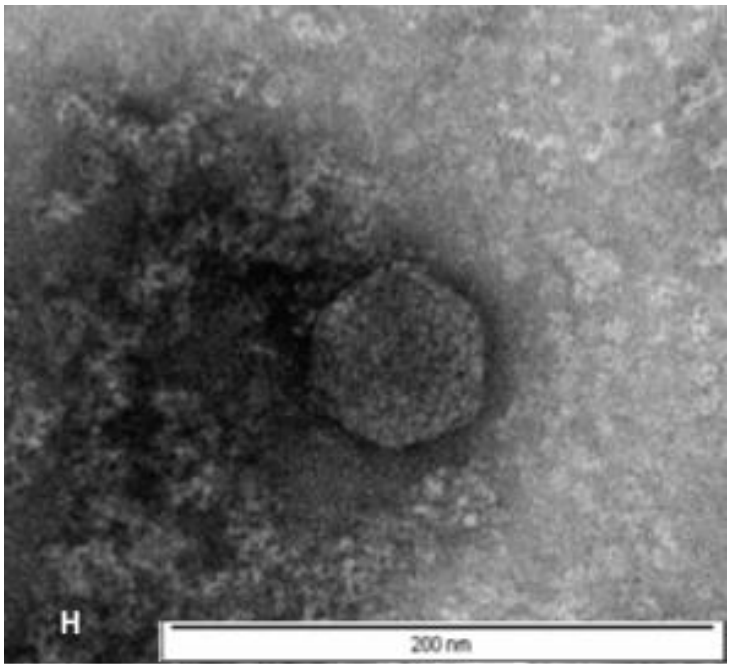
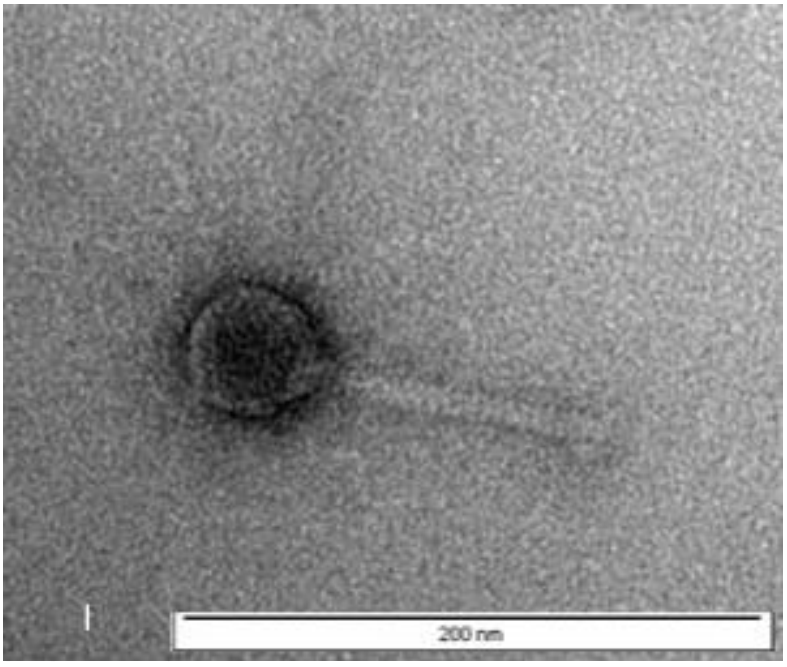
4.3.7 Induction of *B. burgdorferi* s.l. isolated from ticks

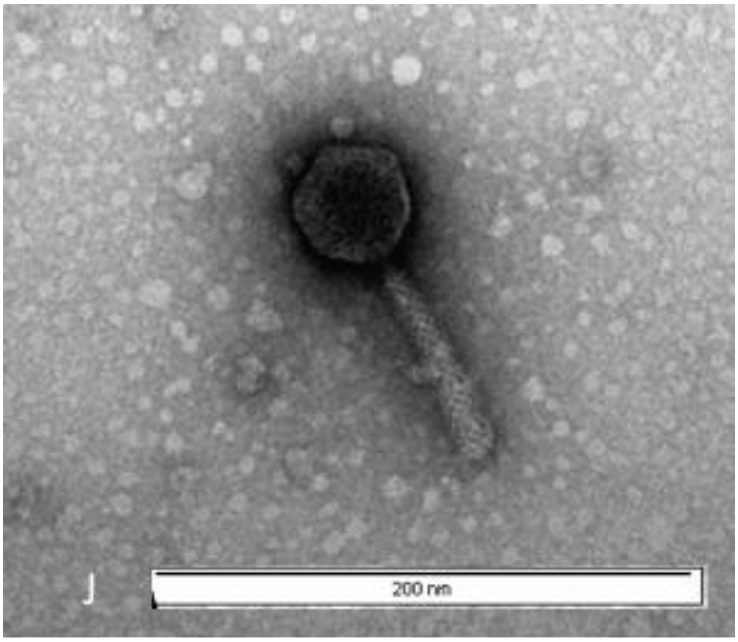
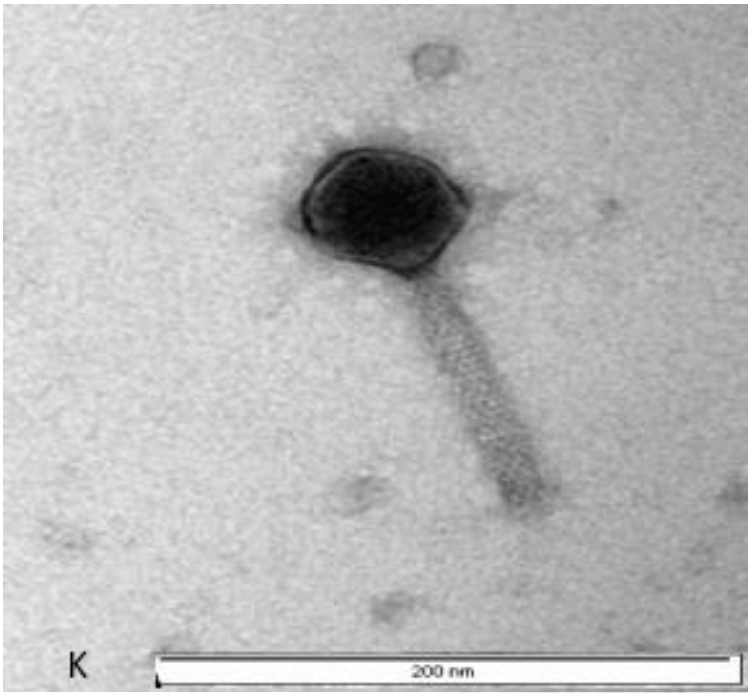
B. burgdorferi s.l. strains isolated from ticks were subjected to Mitomycin C induction only. Table 4-3 summarises information about the induced phages. TEM analysis of the induced *B. burgdorferi* s.l. filtrates revealed the presence of phages named as pL18-1, Pl18-2, pL19, pL20, pL21, pL89, and pL90. Specifically, strain BgS18 revealed two phage-like particles, the first one pL18-1(podovirus H) with an isometric head that is 56 nm in diameter and no visible tail, and the other one was pL18-2(myovirus I) with isometric head that is 60 nm in diameter and has a 130 nm tail.

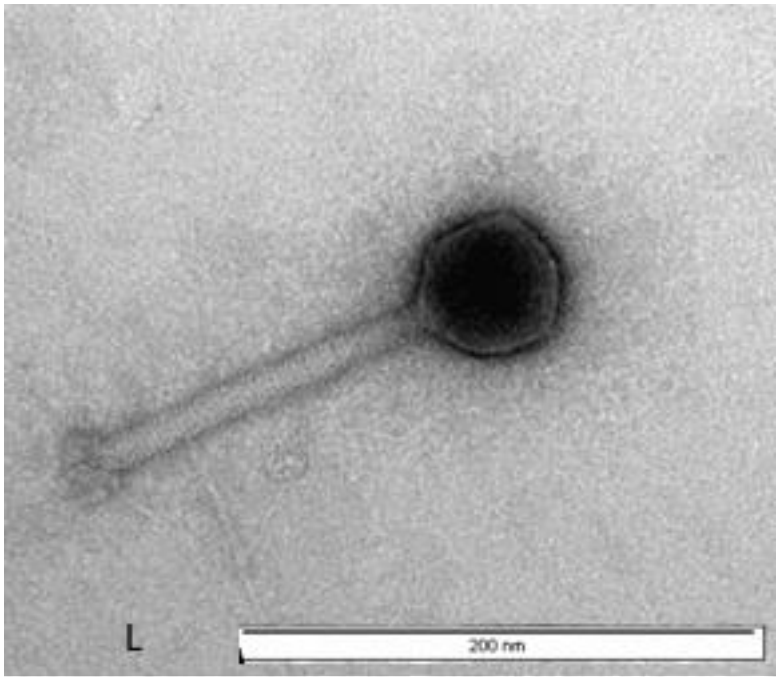
For the strains BbS19 and BbS21, TEM analysis revealed two phages named pL19 (myovirus J) and pL21 (myovirus K), respectively. Phage pL19 has a hexagonal head that is 65nm in diameter and a 100 nm tail. Phage pL21 has a head that is 57 nm in diameter and a 100 nm tail. Additionally, the strain BgS90 produced one phage named as pL90 (myovirus L) with an isometric head that is 60 nm in diameter and a 130 nm tail. Most *B. burgdorferi* s.l. phage particles were found to be associated with membrane-derived vesicles. Finally, *B. burgdorferi* s.l. strains such as BaS20 and BbS89 did not generate any visible phage-like particles under TEM.

In summary, Mitomycin C induced five phages like particles from *B. burgdorferi* s.l. strains that isolated from ticks. The phage like particles named as pL18-1, PL18-2, pL19, pL21, and pL90. PL18-1 was podovirus with 56nm head and tailless. PL18-2, pL19, pL21, and pL90 were myoviruses. All have head in size of (55-65) and (100-130) nm tail. The induced filtrates were tested for terminase PCR using terminase 676 primers. All the induced lysates were tested positive.

Table 4-3 Mitomycin C-induced phages from *B. burgdorferi* s.l. strains isolated from ticks

Sample	Strain	Inducer	Morphology
pL18-1	BgS18	Mitomycin C	
pL18-2	BgS18	Mitomycin C	

Sample	Strain	Inducer	Morphology
pL19	BbS19	Mitomycin C	
pL20	BaS20	Mitomycin C	No TEM image
pL21	BbS21	Mitomycin C	
PL89	BbS89	Mitomycin C	No TEM image

Sample	Strain	Inducer	Morphology
pL90	BgS90	Mitomycin C	

4.3.8 Measuring the killing activity of Mitomycin C induced lysates

Induced lysates samples (18M, 19M, 21M, and 90M) that have phage like particles were tested for killing against *B. burgdorferi* s.l. strains using SYBR Green I/PI assay in the same manner as stated above. The ratio of the green-to-red fluorescence (G/R) was tested as shown in Figure 4-7. Multiple Bar chart shows Green/Red fluorescence ratio of the SYBR Green I/PI assay of Mitomycin C induced lysates. As previous graphs, the comparison between TC and No phage control to test the viability was performed and it was high significant with $P<0.0001$.

SM buffer treatment was compared with all induced phage lysate to show the reduction in the viable cells. The result show there was significant reduction I G/R ratio for all samples with P value <0.0001. Within the groups, sample 18M was significant difference between the highest ratio of G/R against BbB31 at ratio of 5.30 and the lowest ratio of G/R against BvNE218 at G/R ratio of 2.15.

The second sample was 19M, there was significant difference between the highest ratio of G/R against Bb B31 at ratio 5.50 and the lowest ratio against Bb vs185p9 at ratio of 3.15.

The third sample was 21M has significant difference between the highest ratio of G/R against BbB31 at ratio of 4.00 and the lowest ratio against Bb vs185p9 at ratio of 2.00.

Finally, sample 90M has significant difference between the highest ratio of G/R against BbB31 and the lowest ratio against Bb vs185p9 at ratio of 3.20.

In summary, the induced phage lysates tested in this experiment, sample 18M, 19M, 21M, and 90M showed killing activity. Sample 21M showed the highest killing activity among the other lysates with the lowest viable cells at lower ratio of G/R of 2.00.

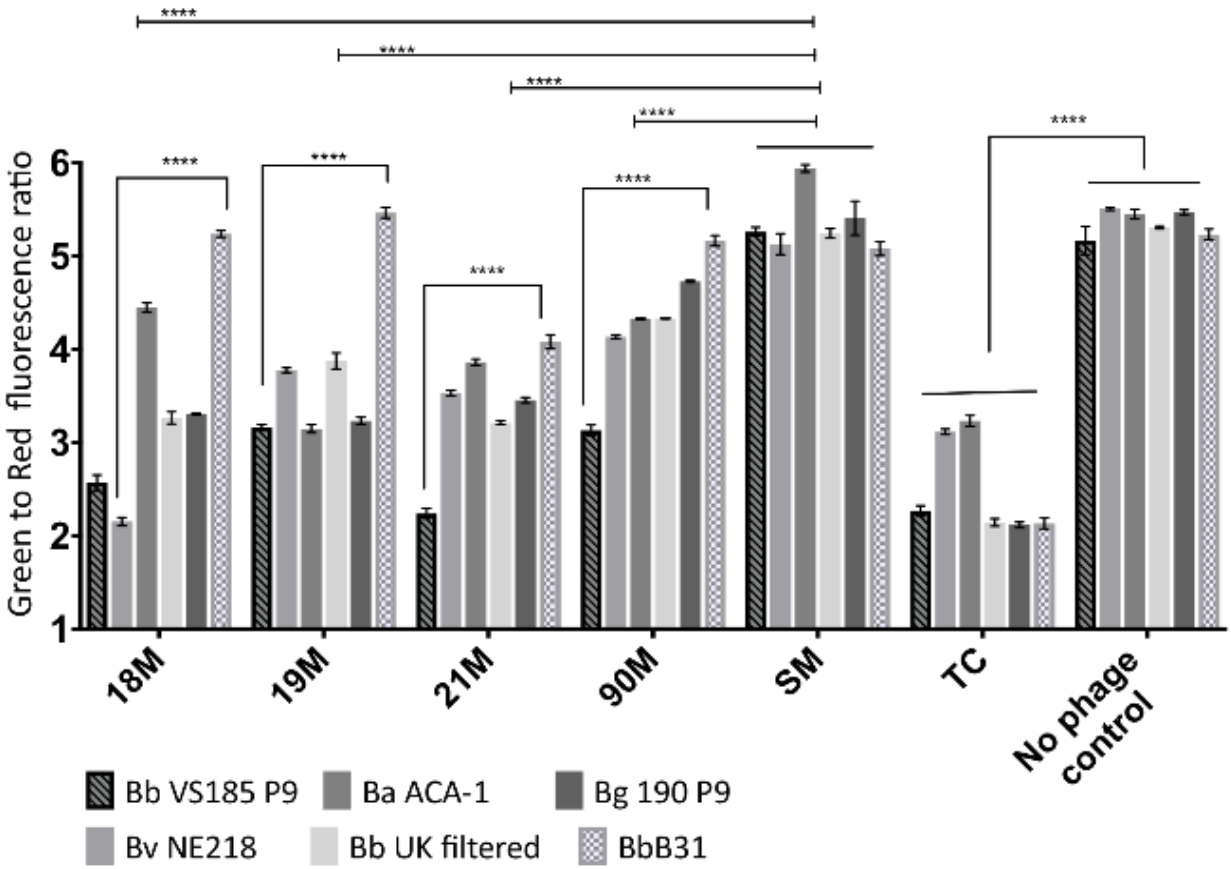


Figure 4-7 Green/Red fluorescence ratio of the SYBR Green I/PI assay of Mitomycin C induced lysates samples 18M, 19M, 21M and 90M.

Induced phage lysate samples 18M, 19M, 21M,and pL90. Legends from left to right represent *B. burgdorferi* s.l. strains such as Bb for *B. burgdorferi* VS185 P9, Bv for *B. valaisiana* NE218, Ba for *B. afzelii* ACA-1, Bb, *B. burgdorferi* UK filtered. Bg is for *B. garinii* 190 P9 and BbB31 for *B. burgdorferi* B31. TC is Tetracycline as a positive control. SM represents SM buffer-treated *B. burgdorferi* s.l. cultures. *B. burgdorferi* s.l. Free treatment refers to a culture of *B. burgdorferi* s.l. treated with only CBSKII media as a negative control. The green-to-red fluorescence ratio was measured as the average of three replicate readings. ****P values<0.0001. Bars were analysed according to Two- way ANOVA(Multiple comparison).

4.3.9 Spot test and plaque assay

Induced phage lysates samples 18M, 19M, 20M, 21M, 89M, and 90M were also used for spot tests and plaque assays against *B. burgdorferi* s.l. strains. No clear zones or plaques were observed from any samples, as described in Figure 4-8, which shows the spot test of the Mitomycin C-induced phage lysate filtrate samples 18M, 19M, 20M on the *B. burgdorferi* B31. In the same way, the spot test was performed for the rest of the Mitomycin C-induced lysates. the spot tests were negative. the plaque assay was negative.

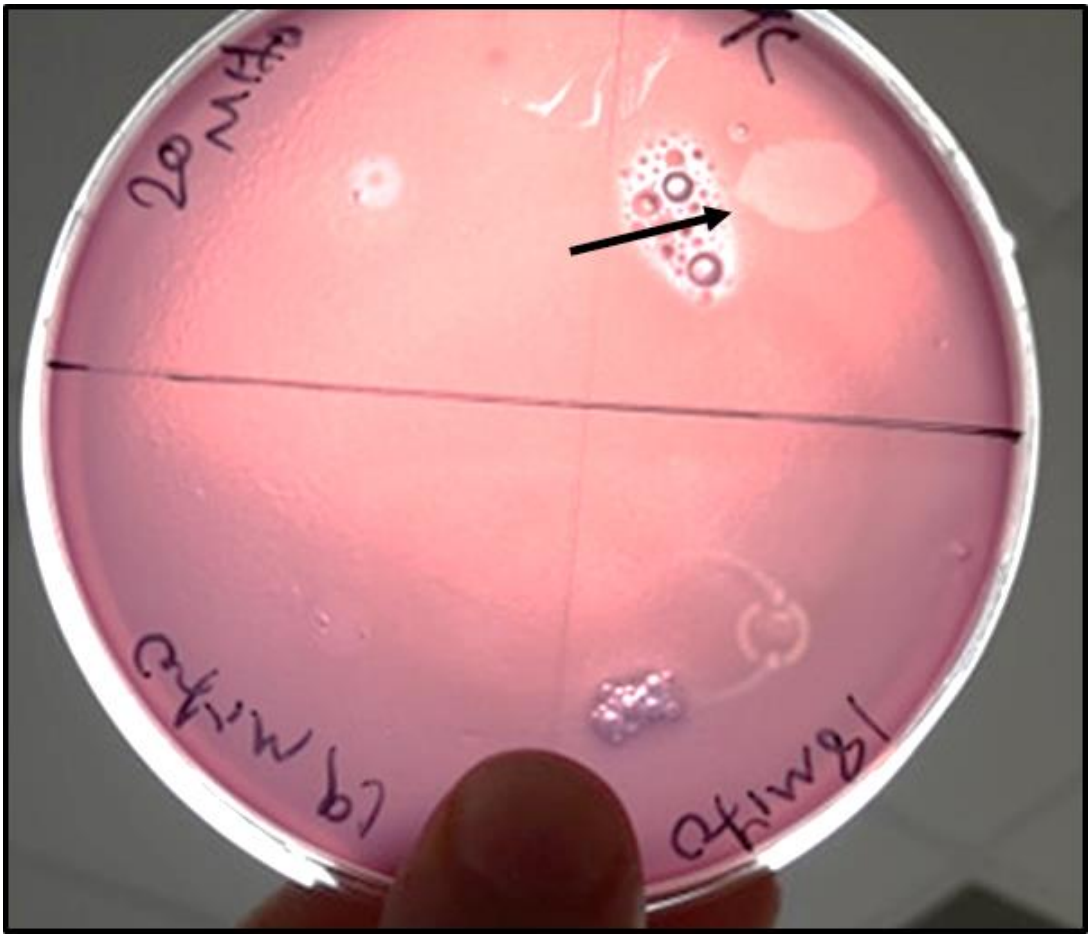


Figure 4-8 Spot test of the Mitomycin C-induced phage lysate filtrate samples 18 M, 19 M and 20M on the *B. burgdorferi* B31.

Samples were induced from strains BgS18, BbS19, and BaS20, The black arrow indicates the lytic zone due to the activity of the Tetracycline.

The summary of all the induced phages including PCR, spot tests, plaque assays and live/dead assays are listed in Table 4-4.

Table 4-4 Summary of characterisation of all induced phage lysates in this study.

Sample	Strain	Inducer	Phage	Morphology	Size	PCR	Spot test	Plaque Assay	Lowest ratio/strain
5M	*B. afzelii* ACA1	Mitomycin C	pL5-1	Myovirus	70 nm head, 100 nm tail	+ve	-ve	-ve	1.43/Bv NE218
5M	*B. afzelii* ACA1	Mitomycin C	pL5-2	Podovirus	50 nm head, 10 nm tail	+ve	-ve	-ve	1.43/Bv NE218
7N	*B. garinii* 190P9	Norfloxacin	pL7	Podovirus	64 nm head, tailless	+ve	-ve	-ve	2.45/Bv NE218
4N	*B. valaisiana* NE218	Norfloxacin	pL4	Podovirus	75 nm head, 10 nm tail	+ve	-ve	-ve	2.50/Bb uk filtered
6N	*B. burgdorferi* UK filtered	Norfloxacin	pL6	Podovirus	45 nm head, tailless	+ve	-ve	-ve	Not analysed
3N	*B. burgdorferi* VS185p9	Norfloxacin	pL3	Podovirus	55 nm head, tailless	+ve	-ve	-ve	2.25/Bv NE218
8N	*B.burgdorferi* China 23	Norfloxacin	No	No phage	No phage	+ve	-ve	-ve	Not analysed
B31SPI	*B. burgdorferi* B31	Spontaneous Induced	pLB31	Myovirus	50 nm head, 70 nm tail	+ve	-ve	-ve	1.50/Bv NE218
18M	*B. garinii* S18	Mitomycin C	pL18-1	Podovirus	56 nm head, tailless	+ve	-ve	-ve	2.15/ Bv NE218
18M	*B. garinii* S18	Mitomycin C	pL18-2	Myovirus	60 nm head, 130 nm tail	+ve	-ve	-ve	2.15/ Bv NE218
19M	*B. burgdorferi* S19	Mitomycin C	pL19	Myovirus	65 nm head, 100 nm tail	+ve	-ve	-ve	3.15/Bb VS185p9
20M	*B. afzelii* S20	Mitomycin C	No	No phage	No size	+ve	-ve	-ve	Not analysed
21M	*B.burgdorferi* S21	Mitomycin C	pL21	Myovirus	57 nm head, 100 nm tail	+ve	-ve	-ve	2.00/Bb VS185p9
89M	*B.burgdorferi* S89	Mitomycin C	No	No phage	No phage	+ve	-ve	-ve	Not analysed
90M	*B. garinii* S90	Mitomycin C	pL90	Myovirus	60 nm head 130 nm tail	+ve	-ve	-ve	3.20/Bb VS185p9
20s	*B. garinii* 190P9	UV	pL7	Podovirus	64 nm head, tailless	+ve	-ve	-ve	2.54/BbB31

4.4 Discussion

4.4.1 Measuring *B. burgdorferi* s.l. growth with a Haemocytometer

Working with *B. burgdorferi* s.l presents many challenges: firstly, *B. burgdorferi* s.l is a fastidious bacterium to culture *in vitro*; it needs a super-rich complex medium, and is therefore susceptible to contamination. Secondly, it takes time and effort to master the right skills to be able to recognise and count *B. burgdorferi* s.l. with a microscope via a haemocytometer. The difficulty of culturing and identifying *B. burgdorferi* s.l. is partially due to its pleomorphic forms, which include spirochetal, L-forms, round bodies, aggregates and biofilm-like structures. All of these forms were observed when working with the Leicester lab *B. burgdorferi* s.l cultures. These different forms have contributed to certain complications regarding the counting of *B. burgdorferi* s.l. and consideration. In addition to using of different strains have different growth parameters. In this study, *B. burgdorferi* s.l. cells were counted with a haemocytometer under phase-contrast microscopy. The generation time for *B. burgdorferi* s.l. varied between strains. The data showed that *B. burgdorferi* B31 have shortest generation time to be 8.77 hours in, and longest one was found in *B. afzelii* ACA-1 to reach 13.50 per hours. This was agreed with previous studies suggested that generation time of *B. burgdorferi* s.s. has a range between 7 and 20 hours, depending on the culture conditions and temperature (Mursic *et al.*, 1996, Heroldova *et al*., 1998, Aslam *et al*., 2013). In spite of the difficulties regarding *B. burgdorferi* s.l. counting using haemocytometer. It was attempt to optimise the right way to measure *B. burgdorferi* s.l. growth due to the limitation of adequate light absorption for direct optical density measurement.

4.4.2 Induction of laboratory *B. burgdorferi* s.l. strains via Mitomycin C and Norfloxacin treatment

It was published that *B. burgdorferi* s.l. prophage can induced by antibiotics such as MNNG and Ciprofloxacin (Neubert *et al*., 1993, Eggers and Samuels, 1999, Eggers *et al*., 2000). In this study, prophages from *B. burgdorferi* s.l. were induced using different agents such as Mitomycin C and Norfloxacin, in order to differentiate between them and identify which inducing agent is more efficient. Several methods were used to confirm the presence of phage particles. Previously MitomycinC was used to induce *B. burgdorferi* CA-11.2A (Eggers and Samuels, 1999, Eggers *et*

al., 2000). However, it was unsuccessful. This might relate to the cell's status or culture growth condition. In this study, the prophages were induced from other *B. burgdorferi* s.l. strains. The induction was successful. The optimization was done by using three different concentration of Mitomycin C such as 30, 20, and 10 µg/ml. The data showed that using Mitomycin C at final concentration of 10 µg/ml was optimal for induction. This was not the same with previous study by (Eggers and Samuels, 1999, Eggers *et al.*, 2000). They did not gain any phage like particles. In order to search about the Mitomycin C ability in induction, different *B. burgdorferi* s.l. strains were used. The data showed that not all the strains prophages were induced with Mitomycin C. in other word, Mitomycin C was not always be the right inducer or efficient. For this reason, another inducing agent was used which is Norfloxacin.

It was previously reported that Norfloxacin was used to induced prophages from *E. coli* O157:H7 (Matsushiro *et al*., 1999, Pohlhaus and Kreuzer, 2005, Herold *et al*., 2005) and many prophages were induced from *Clostridium difficile* 027 (Meessen-Pinard *et al.*, 2012, Nale *et al*., 2012, Shan *et al*., 2012, Sangster *et al*., 2015, Hegarty *et al*., 2016a). Preliminary time course studies were do not use Norfloxacin in *B. burgdorferi* s.l. prophage induction. This is the first to do so; it seems that Norfloxacin is efficient and capable in inducing phages out of *B. burgdorferi* s.l. The protocol was used the same as Mitomycin C induction with final concentration of 10 µg/ml. Induction was successful and more efficient.

In the published literatures, Neubert and Schaller (1994) used ciprofloxacin which is one of the fluoroquinolone antibiotics, the same as Norfloxacin. Ciprofloxacin and Norfloxacin are gyrase inhibitor lead to DNA damage. They have the molecular mechanisms which make them suitable to induce prophages (Matsushiro *et al*., 1999, Schaller and Neubert, 1994, Nale *et al*., 2012, Zhang et al., 2000) . They were used before in induction of higher level of phages from *Pseudomonas aeruginosa* (Fothergill *et al*., 2011, James *et al*., 2012, Davies, 2015). In this study, Induction of *B. burgdorferi* s.l. prophages were performed and TEM analysis of *B. burgdorferi* s.l. cultures treated with Mitomycin C, Norfloxacin revealed the presence of putative phages particles in twelve samples. In details, five myoviruses were induced from five strains of *B. afzelii* ACA-1, *B. garinii* S18, *B. burgdorferi* S19, *B. burgdorferi* S21, and *B. garinii* S90. Six podoviruses were induced from strains of *B. afzelii* ACA-1, *B. garinii* 190P91, *B. valaisiana* NE218, *B. burgdorferi* UK, *B. burgdorferi* vs185p9, and *B. garinii* S18 respectively.

Most *B. burgdorferi* s.l. phage particles were found to be associated with membrane-derived vesicles. In fact, it is not clear whether these vesicles are lytic artefacts or whether they are produced by bacterial cells to act as decoys, as previously reported for *E. coli* 19 (Manning and Kuehn, 2011). This phenomenon has previously been described in *B. burgdorferi* invasiveness and immune evasion (Toledo *et al*., 2012).

It was shown that phages-like particles (myovirus form) induced in this study were similar to phage BB-1 in morphology (Eggers and Samuels, 1999, Eggers *et al*., 2000). Most of them have head in rang of 55-65nm and 100-130nm tail. However, they slightly differ in size to the known LE1, LE3, and LE4 phages which were observed after induction with Mitomycin C from *L. biflexa* (Schiettekatte *et al.*, 2018a). It is unclear why they are different in size. The reason may relate to the size of their genomes.

From reviewing the published literature, there were no podovirus morphotype induced from spirochetes while in this study podovirus morphotype phages were induced. Norfloxacin was able to induce most of them, only one induced with Mitomycin C from *B. afzelii* ACA-1, they have head in range of (50-60)nm and tailless. This is the first time that podoviruses were detected after induction of *B. burgdorferi* s.l. I have not found any separated phage tail-like particles (PTLPs) in the TEM analysis similar to that induced from *C. difficle* 078 (Sangster et al., 2015).

It has been reported that the induction of prophages from *B. burgdorferi* B31 can occur, albeit rarely, as a spontaneous release from this strain (Eggers and Samuels, 1999, Eggers et al., 2000). This was observed in this study, as myovirus induced from *B. burgdorferi* B31, as the strain was used as a control in both inductions with MitomycinC or induction with Norfloxacin. This phage could be the cp32 prophage. There was an attempt to induce this phage with both Mitomycin C and Norfloxacin. However, no TEM image was obtained, possibly due to the TEM sample preparation being incorrect or the induction conditions not being good.

4.4.3 Induction of *B. burgdorferi* s.l. with UV

UV light was also successfully used to induce phage-like particles from *B. garinii* 190P9. The phage, named pL7, is a podovirus which has a 64 nm head, and is tailless. This phage pL7 was undistinguishable from the phage that was induced by Norfloxacin from the same strain, so it was probably the same phage. However phage pL7 could not be induced by Mitomcycin C.

Previously, UV light has been reported to induce phages from *E. coli* K12 (George *et al*., 1975, Wood, 1985), and phage from *Haemophilus influenza* (Barnhart *et al.*, 1976). The idea is that a very low dose of UV can activate the SOS response which activates the induction of prophages (Witkin and Wermundsen, 1977). In this project, UV irradiation was performed using UV light against *B. garinii* 190P9 at the energy of 3000 micro Jules/ second (µj/s), which is equivalent to 0.3 J/s for different lengths of time such as 0, 10, 20 and 30 seconds. The data showed the first successful attempt of inducing phages out of *B. garinii* 190P9 with UV. The rationale for choosing this strain to carry out UV induction work was because phage-like particles were induced from it using Norfloxacin. The reason for using 3000 µj/s was because this setting was the only one available in the lab stratalinker 2400 machine. Previously, UV light has been tested before and reported in the literature in the induction of Lambda prophage using different ranges of energy between 0.2 to 6.4 Jules per second (J/s)to irradiate *E.coli* B/r (Witkin and Wermundsen, 1977). There were no phages from filtrates irradiated for 0 (negative control), 10, and 30 seconds. The induction at 0.3 J/s for 10 seconds was probably not enough to activate the SOS response, and irradiation for 30 seconds may have limited the viability of *B. burgdorferi* s.l. This indicating that the intensity of UV light plays a vital role in inducing *B. burgdorferi* s.l. phages.

To further investigate phage-like particles induced by UV light, the total DNA was extracted from the supernatant filtrate of the culture irradiated for 20 seconds. The DNA was then analysed by gel electrophoresis according to the classical protocol using 0.7 % agarose gel (Sambrook and Russell, 2006). A distinctive clear band ~30KB in size was observed on the gel, which might correspond to the putative phage genome. If I had more bench time during my study, I would have purified and sequenced this putative phage DNA. The previous investigation of the *B. burgdorferi* s.l. phage DNA using a gel method was performed by using agarose gel at 0.5%; the size of the phage BB-1 was 32KB (Eggers and Samuels, 1999, Eggers *et al.*, 2000), which is similar to that observed in this study. UV induction was not performed with other *B. burgdorferi* s.l. strains used in this study due to time limitations.

4.4.4 The killing activity of induced phage lysates

To investigate whether the induced phages have any killing effect against *B. burgdorferi* s.l. in a liquid culture, a live and dead assay using SYBR Green/PI was adopted. This assay has been used successfully before in identifying the best antibiotics in eliminating *B. burgdorferi* s.l. *in vitro* (Feng *et al.*, 2016, Feng *et al*., 2014). The protocol used was adopted from a protocol that has been used previously to screen the viability of *B. burgdorferi* when the Food and Drug Administration tested drugs against induced *B. burgdorferi* s.l. round bodies, and stationary phase *B. burgdorferi* s.l. persisters cells (Feng *et al.*, 2016). In the current study, the killing effects of induced phage lysates were measured. The killing mediated by the MitomycinC-induced phages lysates filtrates was highly significant when compared with killing of tetracycline (TC) used as a positive control and with SM buffer and *B. burgdorferi* s.l. cells treated with CBSKII broth (No phage control) as negative controls.

The highest killing of *B. burgdorferi* s.l. was mediated by the Mitomycin C induced phage filtrates from *B. afzelii* ACA-1 (5M) against *B. valaisiana* NE218 (with a ratio of 1.43). This compared with the positive control (tetracycline treatment) . When *B. burgdorferi* s.l. were treated with SM buffer, the undamaged to damaged cells were at a ratio of 5.20. From the TEM analysis, it was known that this sample contains two phages morphologies, one myovirus and one podovirus, called pL5-1 and pL5-2. The result indicated that there is a good inhibition in the growth of *B. burgdorferi* s.l. by induced phage lysates comparing with the others. This may relate to many reasons. One of the most likely reasons is that at the used culture status, there was insufficient number of live *B. burgdorferi* s.l. cells. Another reason may relate to the incubation period for the culture with the phage sample was too long which lead to increase in the number of dead cells.

In order to compare the killing results with other inducing lysates, the strains were treated with different inducing phage lysates such as Norfloxacin induced lysates. The data of this assessment showed that different induced lysates have different ratios of killing. Specifically, the lowest ratio of fluorescence was observed when the Norfloxacin induced lysate of *B. burgdorferi* VS185P9(3N) against *B. valaisiana* NE218 (ratio of 2.25), compared with the tetracycline treated sample (ratio of 2.00), and SM buffer treated *B. valaisiana* NE218 and no phage control (ratios of 4.7 and 5.00, respectively).

Furthermore, a preliminary investigation was performed by using UV induced lysates, the highest killing ratio was seen in the induced phage filtrate of *B. garinii* 190 P9 irradiated at 0.3 J/ S for 20 seconds was tested against *B. burgdorferi* B31, the ratio of fluorescence was 2.54 compared to the positive control, tetracycline treated *B. burgdorferi* B31, with a ratio of 2.40, and the negative control of 5.76, which was No phage control (the *B. burgdorferi* B31 treated with CBSKII broth only). A low ratio in the sample treated with the induced lysate indicates that *B. burgdorferi* s.l. cells were greatly damaged. It was previously reported that *B. burgdorferi* s.l. was determined at a ratio of fluorescence of 4.35 and 35 % of live cells when treated with Doxycycline and 5.76 and 66% of live cells when treated with Amoxicillin (Feng *et al.*, 2014). If we can compare the current findings with Feng *et al*., 2014 's results and consider them as standered. The conculsion will be induced phage lysates were able to supress the growth of *B. burgdorferi* s.l. with high ratios of killing. The suggestion will be that induced phage lysats contain high amount of the induced phages that killed the cells, or *B. burgdorferi* s.l. growth was affected by the concentration of the inducer present in the lysates.

Since findings of the live to dead test that was used in this project yielded interesting results, indicating that using of SYBR Green/PI was most advantageous to detect the killing activity of the induced phage lysates. However, it required significant time and skills. In addition to, using of different strains against the same sample was necessary, and a long incubation period of the PCR 96 plate was needed (5 days), this led some times to the evaporation of some samples. To overcome this problem, a sealing film membrane was used. Finally, this protocol required suggestive time starting from culturing *B. burgdorferi* s.l*.,* which is a slow-growing bacterium, and time for killing, which is 5 days. So, it took in general 10-14 days from the start of the experiment to the start of the analysis.

4.4.5 Spot test on *B. burgdorferi* s.l. lawn

To physically isolate potential phages, induced phage samples were spotted on *B. burgdorferi* s.l. lawns. It was noticed that spotting of induced lysates on *B. burgdorferi* s.l. lawn was feasible. This contradict the result of (Eggers and Samuels, 1999, Eggers *et al.*, 2000), they reported that growing of *B. burgdorferi* s.l. on a lawn was infeasible. The lawns were prepared using pBSKII media with optimised agar concentrations in the lower layer to be 1% agar and 0.4% of agar in the sloppy (upper layer). However, this methodology needs more optimisation. No lysis zones

were identified , while there was a clear zone of clearance from the spotting of tetracycline as the positive control. The failure in identifying any virulent phages might be related to many factors. It is possible that the host strain used was unsuitable for the lytic *B. burgdorferi* s.l. phages, or the *B. burgdorferi* s.l. phages titer for propagation were not sufficient to kill the bacteria. Lastly reason might be that the induced phages were not functional and may have required some helper phages, similarly to the lysogenic phage P1 and P2 of *E. coli* that replicate via the lytic cycle (Sternberg and Coulby, 1987). The induced phages may also be able to reintegrate again within the genome and not entering a lytic life cycle. This is most likely temperate prophage state.

CHAPTER FIVE

Conclusions and general discussion

Chapter 5 Conclusions and general discussion

Lyme disease is the most prevalent vector-borne disease in the northern hemisphere, it is caused by a group of bacteria *B. burgdorferi* s.l. The disease spreads to humans via bites from infected ticks. There is no vaccine available for Lyme disease. The current treatments are based on the use of antibiotics. However, a failure to treat patients rapidly and adequately enables symptom progression. At later stages, the disease can manifest with increasingly debilitating complications (Johnson *et al.*, 2011). Lyme disease prevention is, therefore, critical to risk management. Strategies to combat this growing threat include robust surveillance measures to detect the carriage of pathogens in ticks and identification of geographic areas of concern. As bacteria's natural enemy, phages have regained attention from scientists around the world due to their potential in combating bacterial infections. No systematic research has been carried out to explore phages of spirochetes causing Lyme disease. The aim of the study was to understand the basic biology of *B. burgdorferi* s.l. phages with an overall objective to isolate and characterise phages that could infect pathogenic *B. burgdorferi* strains. The investigation was directed by the following working hypotheses: prophages residing inside *B. burgdorferi* s.l. strains can be induced out with chemicals; lytic phages specific for *B. burgdorferi* strains can be isolated from environmental samples, such as ticks. In this chapter, the following conclusions and discussion of the main points will be present :

- **The phages induced from *B. burgdorferi* s.l. strains showed different morphotypes, which are different from the *B. burgdorferi* s.l. phages currently known.**

Previously, only limited examples of data have shown that prophages could be induced from *B. burgdorferi* s.l. cultures using 1-methyl-3-nitroso-nitroguanidine (MNNG), and phage BB-1 was the phage that was induced from *B. burgdorferi* CA-11-2A. It has an isometric head of 50 nm in diameter, a contractile tail 90 nm in length and a genome size of 32KB (Eggers and Samuels, 1999, Eggers *et al*., 2000). In this project, several induced *B. burgdorferi* s.l. phages showed morphotypes which were different from previously described phages. Three chemicals were used such as Mitomycin C, Norfloxacin and UV light to induce phages from thirteen *B. burgdorferi* s.l. strains, including seven lab *B. burgdorferi* s.l. strains named as *B. burgdorferi* VS185 P9*, B. valaisiana* NE218*, B. afzelii* ACA-1*, B. burgdorferi* UK*, B. garinii* 190 P9, *B. burgdorferi* China 23, and *B. burgdorferi* B31. In addition to the lab strains, six strains isolated

in this project from ticks named as *B. burgdorferi* S19, *B. afzelii* S20*, B. burgdorferi* S21*, B. burgdorferi* S89*,* and *B. garinii* S90 were also used. The study findings revealed that phage-like particles with distinctive morphological types were observed in the cultures. Firstly, Mitomycin C induced seven phages like particles from *B. burgdorferi* s.l. strains. Particularly, two podovirus and five myovirus. The podoviruses were induced from *B. afzelii* ACA-1 and *B. garinii* S18, both have 50-60 nm head and small tail with 10nm. The myovirus were induced from *B. burgdorferi* strains: *B. afzelii* ACA-1, *B. garinii* S18, *B. burgdorferi* S19, *B. buegdorferi* S21, and *B. garinii* S90. The induced phages were observed with head in range of size between 55-65nm and 100-130 nm tail. Some strains such as *B. garinii* 190p9, *B. valaisiana* NE218, *B. burgdorferi* UK filtered, and *B. burgdorferi* VS185p9 did not induced any phage with MitomycinC. The limitation in the number of prophages from those strains might be related to the low titer of phages present in the induced lysate sample, or that Mitomycin C might be not the right inducer for releasing phages out of these strains. Secondly, Norfloxacin induced four podovirus from *B. garinii* 190p9, *B. valaisiana* NE218, *B. burgdorferi* UK filtered, and *B. burgdorferi* VS185p9. All phages induced with Norfloxacin have head in range of 50-60, and most of them were tailless. Finally, the phage induced from *B. garinii* 190p9 by UV light was the same one that induced with Norfloxacin. No more strains were induced with UV.

It was noticed that all phages induced with Mitomycin C and Norfloxacin were different from the putative cubic phages that were induced previously with ciprofloxacin from *B. burgdorferi* CA-11.2A (Neubert *et al*., 1993). And different from phage BB-1(Eggers and Samuels, 1999, Eggers *et al*., 2000). The difference can recognize from the size of head and tail. Some of them have hexagonal capsid. Other have isometric one. Some of them were tailless, other have long tail with 130nm. The variation in there morphology even if the original strains are closely related might relate to divers in the host life style and genome, And the result obtained in this project confirm the variation in the *B. burgdorferi* s.l. phage morphotypes.

- **Different inducing agents seem to induce different types of phages from some *B. burgdorferi* s.l. strains, which indicates that *B. burgdorferi* s.l. strains may be carrying multiple prophages.**

It was previously reported that *B. burgdorferi* s.l. cp32s were present in the genome of *B. burgdorferi* s.l. as circular episomes were further confirmed as prophages (Brisson *et al*., 2013).

One objective of this study is to induce these prophages. Different inducing agents were used in this project such as Mitomycin C, Norfloxacin and UV light. The outcomes of the induction revealed that Mitomycin C induced two phages from *B. afzelii* ACA-1, one as podovirus. the other one as myovirus. However, Norfloxacin could not induced phages from the same strain, as determined by TEM. In addition, Mitomycin C was able to induce two phages from *B. garinii* S18. However, Norfloxacin was not. In the same time Norfloxacin was able to induce one phage from other *B. burgdorferi* s.l. strains such as *B. valaisiana* NE218, *B. burgdorferi* UK filtered, *B. burgdorferi* VS185p9 and *B. garinii* 190p9. However, MitomycinC was not able to induce these strains.

It was found that all induced phages by Mitomycin C and Norfloxacin were different to the previously *B. burgdorferi* s.l. phage induced by MNNG. One most likely reason is due to the different inducing mechanisms associated with different antibiotics (Eggers and Samuels, 1999, Eggers *et al*., 2000). Mitomycin C is a DNA cross-linking activation agent. It causes DNA bases to be mispaired, DNA strand breakage and the supplementary strand cross-linking (preventing DNA synthesis) in bacteria (Iyer and Szybalski, 1964, Hopkins *et al*., 1991, Paz *et al*., 1999) Norfloxacin is a DNA gyrase inhibitor, it also causes DNA damage, and published studies have shown that the molecular mechanism of prophage induction by this antibiotic is closely related to the activation of RecA system (Matsushiro *et al.*, 1999, Zhang *et al*., 2000). Taken together, these findings indicate that *B. burgdorferi* s.l. strains may be carrying multiple prophages and can induce by different inducing agents.

- **PCR primer targeting the phage terminase gene showed increased sensitivity in detecting phage from induced *B. burgdorferi* s.l. cultures and tick enrichment samples.**

In order to detect the presence of phages using PCR, essential phage genes are normally targeted. For *B. burgdorferi* s.l. phages, the putative terminase-encoding gene was identified on several plasmids, including cp32 series in all genomes of the *B. burgdorferi* s.l. Targeting this gene has been proposed and developed into a PCR assay to detect *B. burgdorferi* s.l. (Clokie and Shan, 2019). There was no previous studies targeting *B. burgdorferi* s.l. terminase in tick samples. This is the first one, PCR with primers targeting the *B. burgdorferi* s.l. phage terminase was used in an attempt to determine the presence or absence of *B. burgdorferi* s.l. phages in induced *B. burgdorferi* s.l. cultures and in tick enrichment cultures. In this study, new PCR

primers targeting the terminase gene fragment, different from those described by (Clokie and Shan, 2019) were designed and named as 'terminase 676 PCR primers' as they amplified a PCR product of 676 bp. It was suggested that PCR-based assays are more sensitive than culture-based and antibody-based techniques in term of identifying *B. burgdorferi* s.l. (Engleberg, 1994, Moniuszko *et al*., 2015, Nunes, 2016). In this study, the two PCR assays using *16SrRNA* and the *terminase* genes showed different sensitivity when analysed tick samples. *B. burgdorferi* s.l. carriage rate according to the *16SrRNA* PCR was found to be 3.2% in ticks. When terminase 676 PCR was applied to the same tick samples that were analysed by the *16SrRNA* PCR, a higher *B. burgdorferi* s.l. carriage rate was found. The results revealed that the apparent *B. burgdorferi* s.l. carriage rate was 17%, was substantially higher than that measured by the *16S rRNA* PCR. This may be because there are multiple copies of the terminase gene carried on the cp32-related plasmids of *B. burgdorferi* s.l. (Clokie and Shan, 2019), while only one copy of the *16SrRNA* gene is present in the *B. burgdorferi* s.l. genome. Using PCR primers targeting the phage terminase gene was also applied to analyse the induced phage filtrates. The lysates were filtered through 0.22µm filters to remove any intact bacteria. DNase and RNase were then added to the filtered lysate to destroy any contaminating bacterial DNA; this treatment does not destroy DNA within intact phages. The samples were then used as templates for PCR. Analysis of PCR-amplified fragments revealed the presence of the phage terminase-encoding gene in all samples that originated from lysates with the presence of phages under TEM. The output confirmed that the terminase was one of the phage-encoded genes, and that functional and inducible prophages are present in at least some of the tested *B. burgdorferi* s.l. strains. Thus, the terminase 676 primers targeting *B. burgdorferi* s.l. designed in this study allowed for a fast and sensitive way to determine the presence of *B. burgdorferi* s.l. phages in samples with even a small amount of induced phage DNA present in the sample.

- ***B. burgdorferi* s.l. strains can be isolated from tick enrichment samples by plating to obtain single colonies. Whole-genome sequencing and *16S rRNA* PCR analysis of isolated strains indicated that the dominant *B. burgdorferi* s.l. strains in UK ticks are *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*.**

There are little studies on the platting of *B. burgdorferi* s.l. as single colonies (Kurtti *et al.*, 1987, Rosa and Hogan, 1992, Samuels, 1995), and further one publish on the plating of relapsing fever (Raffel *et al.*, 2018). To date culturing of *B. burgdorferi* s.l. was problematical due to the *B.*

burgdorferi s.l. culture demands that need high skills and knowledge in microbiology of this bacteria. In this study, several *B. burgdorferi* s.l. strains were successfully isolated and purified from tick enrichment samples as single colonies. Plating was performed using pBSKII media, and solidified by agar as 1% for the bottom layer and 0.2 % for the top layer instead of agarose. It was noticed that pBSKII media was efficient in culturing *B. burgdorferi* s.l. After inoculation, the plates were incubated at 34°C, and in order to provide microaerophilic conditions, the plate was sealed with parafilm. Putative *B. burgdorferi* s.l. colonies appeared as small round opaque, single colonies after 7 days of incubation. The success in isolating single colonies from several tick enrichment samples provided the first indication that many UK ticks collected for this study did carry *B. burgdorferi* s.l.

It was advocated that whole genome sequencing of bacterial pathogens have been successfully organised in public health circumstance for high detection of bacterial genotype, phylogeography, detecting high-risk clone and source attribution (Kurtenbach *et al*., 2006, Gilmour *et al*., 2013). Whole genome sequencing of *B. burgdorferi* s.l. isolated from ticks can identify the source of the pathogenic invasion and can develop the ecology of *B. burgdorferi* s.l. In this study, *B. burgdorferi* s.l. single colonies were analysed by PCR and whole-genome sequencing to confirm their identity. Specifically, total DNA isolated from single colonies, derived from six tick enrichment samples, was extracted and submitted for sequencing to Microbes NG. The analysis of this sequence data showed that the *B. burgdorferi* s.l. strains isolated from UK ticks in this study were *B. garinii*, *B. afzelii,* and *B. burgdorferi* sensu stricto.

Sequencing of *B. burgdorferi* s.l. strains that isolated in this project will help the further researches and shade the light on evolutionary history of *B. burgdorferi* s.l. in UK.

- **Downstream phage purification from tick enrichment samples or induced *B. burgdorferi* s.l. cultures is the bottleneck in *B. burgdorferi* s.l. phage research; more optimisation is needed for *B. burgdorferi* s.l. phage purification.**

Although studies on *B. burgdorferi* s.l. phage purification are limited. One of the objectives of this study was to isolate and characterise *B. burgdorferi* s.l. phages from wild ticks and from induced *B. burgdorferi* s.l. cultures. In this study 31 samples of tick enrichment cultures and all induced lysates prepared in this project were analysed for phage terminase PCR. The finding data confirmed the presence of terminase in tick enrichment and induced phage lysates. Since

spot test consider the golden method in the purification of *B. burgdorferi* s.l. phages. No lysis zones were identified. The restriction in identifying any lytic phages from filtrates of *B. burgdorferi* s.l. enrichment cultures and from induced phage lysate might be related to many factors. It is possible that the host strain used was unsuitable for the lytic *B. burgdorferi* s.l. phages, or there were no free lytic phages in the tick enrichments. The negative outcomes in the isolation of *B. burgdorferi* s.l. phages deliberate the main problem in developing researches in *B. burgdorferi* s.l., this study considers the first one in *B. burgdorferi* s.l. phage research and attempted to overcome on this problem. More optimisation in *B. burgdorferi* s.l. phage purification is needed.

- **Spot test or plaque assay for detecting *B. burgdorferi* s.l. phages are feasible but needs more optimisation and validation. While live/dead assay might be effective for screening for antibiotics, but the adaptation of the current live/dead assay protocol for *B. burgdorferi* s.l. phage research needs further time and effort.**

In literatures, It was reported that spot test of *B. burgdorferi* s.l. phages is infeasible (Eggers and Samuels, 1999, Eggers *et al*., 2000). In this study, spot test and plaque assay were developed. This start from developing the protocol that used to prepare *B. burgdorferi* s.l. lawn. As mentioned in this project, the lawn was prepared firstly by using pBSKII instead of BSKII media prepared by (Zückert, 2007). pBSKII media was used and it was more enriched of the ingredients than BSKII. Agar was used instead of agarose. Spot test was performed to screen for the lytic phages from ticks and from induced phage lysates. Unfortunately, there was no lytic zone was identified to confirm the killing of lytic phages. Plaque assay was also un promising due to the negative results. The revers outcomes of both spot test and plaque assay might related to many reasons. The most likely one is due to the phage titer concentration that spotted from induced phage lysates was not enough to form lytic zone . The second one was the lawn was prepared with un suitable host bacterium. More optimisation and validation are needed.

In this study live/dead assay was used to determine the killing activity of the induced phage lysates. It was reported that live/dead assay was effective in measuring the antibiotic activity against *B. burgdorferi* s.l. (Feng *et al*., 2014, Feng *et al*., 2016). This project adopted the protocol to screen for the *B. burgdorferi* s.l. phages from induced phage lysate filtrates. It was

noticed that all induced phage-filtrates that have a potential ratio of fluorescence in live and dead assay. It was promising that there was a way to determine that the induced phage lysates from *B. burgdorferi* s.l. have a killing activity. However, these findings did not support by the spot test and plaque assay. Therefore, using of live/dead assay was useful in *B. burgdorferi* s.l. phage research. However, it needs more time and efforts to consider it as standard for the future *B. burgdorferi* s.l. phage researches.

As a final statement, it can be said that this study provided information about possible approaches to identify and study *B. burgdorferi* s.l. phages. It will undoubtedly help the efforts to find and isolate active phages which can be used as an alternative therapeutic treatment of Lyme disease.

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APPENDICES

APPENDICES

Appendices 1 Media, Buffers and Solutions

Media

Bacteriological agar (VWRBDH chemical, UK)

Agar……..2 g

Distilled water ……..200 ml

Weigh 2g of agar in 200ml of distilled water. Sterilised by autoclave at 121ºC for 15 mins.

Brain heart Infusion agar (Oxoid, UK)

Brain heart infusion agar……3.7 g

Bacteriological agar ……1 g

Distilled water……100 ml

Weigh 3.7 g of brain heart infusion agar, 1g of agar in 100ml distilled water, Sterilised by autoclave at 121ºC for 15 mins

Nutrient Agar (VWR chemical, UK)

Nutrient agar…… 1.5 g

Distilled water……100 ml

Weigh 1.5 g of nutrient agar in 100ml of distilled water. Sterilised by autoclave at 121ºC for 15 mins

Buffers

SM buffer (10 mM NaCl, 8 mM $MgSO_4.7H_2O$ and 50 mM Tris-Cl) in 1L

NaCl …….. 5.8 g

$MgSO_4.7H_2O$……..2 g

1M Tris-Cl……50 ml

Distilled water……to make up 1000 ml

Weigh 5.8 g of NaCl, 2g of $MgSO_4.7H_2O$ in 50 ml of distilled water, Once they dissolved, complete up to 1000ml of distilled water. Autoclave and store at room temperature.

Salt solution for agar sloppy in 100 ml (1 M of MgCl, $MgSO_4$, $CaCl_2$ and NaCl)

$MgCl_2$…….. 20.33 g

$CaCl_2$……..14.70 g

$MgSO_4$……..24.65 g

NaCl……..5.84 g

Distilled water ……to make up 100 ml

Weigh 20.33 g of $MgCl_2$, 14.70 g of $CaCl_2$, 24.65g of $MgSO_4$, and 5.84 g of NaCl in 100 ml distilled water. Autoclave and store at room temperature.

Phosphate buffer saline (PBS)

Phosphate buffer saline tablet.....1 tablet

Distilled water……100 ml

Dissolve one tablet in 100 ml of distilled water using a magnetic stirrer.

10 x TE buffer (100 mM Tris. Cl, 10 mM EDTA) (pH 8.0) in 100 ml

1M Tris.Cl ……10 ml

0.5 M EDTA……2 ml

Distilled water…….. up to 100 ml

Mix 10ml of 1M Tris.Cl buffer with 2ml of 0.5 M EDTA buffer. Complete the size of the buffer up to 100ml by distilled water. Autoclave and store at room temperature

1 X TE in 100 ml

10 X TE……10 ml

H_2O……90 ml

Mix 10 ml of 10X TE buffer and 90 ml of ultra-pure H_2O, store at room temperature.

50 x TAE buffer (2 M Tris-acetic acid and 0.05 EDTA) buffer

Tris Base……242 g

H_2O……..600 ml

Allow to dissolve and then add 57.1 ml Glacial Acetic Acid and 100 mL 0.5 M EDTA, mix and bring to a final volume of 1 L with ultra-pure H_2O. Store at room temperature.

Antibiotics and enzymes were filter sterilised.

Rifampicin stock solution 50 mg/ml

Rifampicin……..0.5 gm

DMSO……10 ml

Weigh 0.5 gm of Rifampicin, dissolve it in 10 ml of DMSO (Sigma- Alderich), then store at -20°C

Phosphomycin stock solution 100mg/ml

Phosphomycin …… 1 g

Distilled water……..10 ml

Weigh 1 g of Phosphomycin, dissolve it in 9 ml of distilled water. Once Phosphomycin has dissolved completely, bring the volume up to 10 ml. Dissolve completely and filter using 0.22µM syringe filter, Store at -20°C

Tetracycline 12 mg/ml stock solution (10 ml)

Tetracycline……120 mg

70%Ethanol……9 ml

Weigh 120 mg of Tetracycline, dissolve it in 9 ml of 70% ethanol. Once tetracycline has dissolved completely, bring the volume up to 10 ml.

Mitomycin C (Fisher Scientific, Loughborough, UK) 300 µg/ml w/v

Dissolve content of one 2 mg bottle of Mitomycin C (2 mg of Mitomycin C and 48 mg of NaCl) in 6.67 ml of UP water. Protect from light by wrapping tube with aluminum foil. Store at 4 ^{o}C.

Norfloxacin (Sigma-Aldrich, Dorset, UK) 1000 µg/ml w/v

Norfloxacin powder……100 mg

Absolute alcohol ……10 ml

Weigh 100 mg of Norfloxacin powder, dissolve it in 10 ml of absolute alcohol. Cover tube with aluminum foil. Store at 4 ^{o} C.

5 % SDS (Sodium dodecyl sulfate) in 10 ml

SDS……..5 g

H_2O……..10 ml

Weigh 5g of SDS in 10ml of distlled water, Store at room temperature.

50 mg/ml Proteinase K

Proteinase K powder……..50 mg

Ultra-pure H_2O…….. 1 ml

Weigh 50mg of Proteinase K in 1ml of ultra-pure water, Store at -20 ^{o}C

Ultra-pure (UP) water

Add certain amount of Nano pure water in duran bottle. Autoclave.

Rabbit serum

Rabbit serum were prepared as aliquots of 12ml and stored at -20 ^{o}C.

Solutions

2.5 mM dNTPs stock solution 25 mg/ml

100mM dATP……20 ml

100mM dCTP……20 ml

100mM dTTP ……20 ml

100mM dGTP ……..20 ml

0.25 mM dNTPs working solution

25 mg/ml of dNTPS……100 µl

Ultrapure distilled water ……900 µl

Use 1:10 in PCR to give 0.25 mM final concentration.

Phenol / tris

Phenol (Sigma- alderich, UK) should prepared for working solution in 100ml in Duran bottle.

The bottle should wrap with aluminium foil.

Phenol / Chloroform

Phenol……50 ml

Chloroform (Sigma- Aldrich, UK)……..50 ml

Mix 50ml of Phenol and 50ml of Chloroform gently in 250 ml bottle. Store at 4 °C

Phenol/Chloroform / Isoamyl alcohol (25:24:1) 50ml

Phenol……25 ml

Chloroform……24 ml

Iso amyl alcohol……1 ml

Store at 4 °C

Cold Isopropanol alcohol

Isopropanol alcohol……100 ml

Store isopropanol in 250 ml bottle at 4 °C

70% Ethanol

Absolute Ethanol……70 ml

Distilled water……30 ml

Mix 70ml of absolute ethanol with 30ml of distilled water in 250ml bottle, store at 4 °C

50% Glycerol

Glycerol……50 ml

Distilled water ……50 ml

Mix 50ml of glycerol and 50ml of distilled water. Sterilise by autoclaving at 121°C for 15 mins. Store at room temperature.

5 % Chelex 100 in 5 ml water

Distilled water …… 5 ml

Chelex resin ……0.25 g

Dissolve 0.25 g of Chelex resin in 5ml of distilled water. Use immediately or store at 4°C.

Appendices 2 Ticks' collections, location, and positive PCR results of *16SrRNA* and terminase gene against DNA samples extracted from tick homogenate and tick enrichment culture.

Sample	NO. of ticks	Larva	nymph	adult	Location	Host	*16SrRNA* PCR against tick homogenate DNA (+/-)	terminase PCR against tick homogenate DNA (+/-)	*16SrRNA* PCR against tick enrichment DNA(+/-)	terminase PCR against tick enrichment DNA (+/-)
18	1	X	X	1	Lincoln	NA	+	+	+	+
19	1	X	X	1	Bournemouth	NA	+	+	+	+
20	1	X	X	1	Bournemouth	NA	+	+	+	+
21	7	X	5	2	Thetford Forest	Forest	+	+	+	+
22	1	X	X	1	Yorkshire	Forest	-	+	+	+
30	1	X	X	1	Woburn	NA	-	+	+	+
39	1	X	X	1	Suffolk	dog	-	+	+	+
45	1	X	X	1	Suffolk	dog	-	+	+	+
49	8	X	7	1	Glasgow	NA	-	+	+	+
59	10	X	X	10	Dundee	hedgehog	-	+	+	+
60	10	X	X	10	Dundee	hedgehog	-	+	+	+
63	14	X	X	14	Scottish highland	deer	-	+	+	+
66	10	X	X	10	Ayrshire	deer	-	+	+	+
67	10	X	X	10	Ayrshire	deer	-	+	+	+
69	11	X	X	11	Leicester	NA	-	+	+	+
73	2	X	1	1	Stranraer	deer	-	+	+	+
77	1	X	X	1	Southampton	dog	-	+	+	+
89	4	X	3	1	Dorchester	dog	+	+	+	+
90	10	X	4	6	NA	dog	+	+	+	+
95	1	X	X	1	Rushden	dog	-	+	+	+
97	20	X	13	7	Argyll	dog	-	+	+	+
99	2	X	1	1	Attenborough	NA	-	+	+	+
100	3	X	X	3	Exeter	NA	-	+	+	+
102	9	X	2	7	Suffolk	Dog	-	+	+	+
103	4	X	2	2	Bristol	Dog	-	+	+	+
148	8	X	4	4	Bampton	forest	-	+	+	+
149	7	2	5	x	Cornforth	forest	-	+	+	+

Sample	NO. of ticks	Larva	nymph	adult	Location	Host	*16SrRNA* PCR against tick homogenate DNA (+/-)	terminase PCR against tick homogenate DNA (+/-)	*16SrRNA* PCR against tick enrichment DNA(+/-)	terminase PCR against tick enrichment DNA (+/-)
150	2	X	X	2	Cornforth	Fox	-	+	+	+
151	4	X	X	4	Dorset	Fox	-	+	+	+
152	10	6	2	2	Essex	Deer	-	+	+	+
153	1	X	X	1	Essex	Deer	-	+	+	+

NA: No available information, x: No ticks.

Appendices 3 Tick samples collections, location, and the negative PCR results for of *16SrRNA* and terminase gene against DNA samples extracted from tick homogenate and tick enrichment culture.

Sample	NO. of ticks	larva	nymph	adult	Location	host	*16SrRNA* PCR against tick homogenate DNA (+/-)	terminase PCR against tick homogenate DNA (+/-)	*16SrRNA* PCR against tick enrichment DNA(+/-)	terminase PCR against tick enrichment DNA (+/-)
1	2	1	1	x	Devon	dog	-	-	-	-
2	1	x	1	x	Devon	dog	-	-	-	-
3	1	x	x	1	Devon	dog	-	-	-	-
4	1	x	x	1	Devon	dog	-	-	-	-
5	1	x	x	1	Hampshire	deer	-	-	-	-
6	1	x	1	x	Hampshire	dog	-	-	-	-
7	1	x	x	1	Hampshire	deer	-	-	-	-
8	1	x	x	1	Hampshire	deer	-	-	-	-
9	1	x	x	1	Hampshire	deer	-	-	-	-
10	1	x	x	1	Hampshire	rat	-	-	-	-
11	1	x	x	1	Hampshire	rat	-	-	-	-
12	1	x	x	1	Hampshire	fox	-	-	-	-
13	1	x	1	x	NA	NA	-	-	-	-
14	1	x	x	1	Wareham	dog	-	-	-	-
15	1	x	x	1	Wareham	dog	-	-	-	-
16	1	x	x	1	Wareham	dog	-	-	-	-
17	1	x	1	x	Wareham	dog	-	-	-	-
23	1	x	x	1	Hampshire	deer	-	-	-	-
24	1	x	x	1	Hampshire	deer	-	-	-	-
25	1	x	x	1	Hampshire	deer	-	-	-	-
26	1	x	x	1	Hampshire	deer	-	-	-	-
27	23	x	x	23	Dorset	deer	-	-	-	-

28	8	x	x	8	Nottingham	dog	-	-	-	-
29	4	x	x	4	Bedfordshire	dog	-	-	-	-
31	1	x	x	1	Cambria	dog	-	-	-	-
32	1	x	x	1	NA	NA	-	-	-	-
33	1	x	x	1	NA	NA	-	-	-	-
34	1	x	x	1	Wareham	dog	-	-	-	-
35	1	x	1	x	Wareham	dog	-	-	-	-
36	1	x	x	1	Wareham	dog	-	-	-	-
37	13	x	6	7	Wareham	dog	-	-	-	-
38	1	x	x	1	Suffolk	dog	-	-	-	-
40	1	x	x	1	Suffolk	dog	-	-	-	-
41	2	x	x	2	Suffolk	dog	-	-	-	-
42	2	x	x	2	Suffolk	dog	-	-	-	-
43	2	x	x	2	Suffolk	dog	-	-	-	-
44	1	x	x	1	Suffolk	dog	-	-	-	-
46	1	x	x	1	Suffolk	dog	-	-	-	-
47	1	x	1	x	Suffolk	dog	-	-	-	-
48	8	2	6	x	Suffolk	dog	-	-	-	-
50	10	2	7	1	Dorset	dog	-	-	-	-
51	14	4	6	4	Wareham	NA	-	-	-	-
52	14	5	6	3	Wareham	NA	-	-	-	-
53	10	x	x	10	Dundee	hedgehog	-	-	-	-
54	10	x	x	10	Dundee	hedgehog	-	-	-	-
55	10	x	x	10	Dundee	hedgehog	-	-	-	-
56	10	x	x	10	Dundee	hedgehog	-	-	-	-
57	10	x	x	10	Dundee	hedgehog	-	-	-	-
58	10	x	x	10	Dundee	hedgehog	-	-	-	-
61	10	x	x	10	Dundee	hedgehog	-	-	-	-
62	10	x	x	10	Dundee	hedgehog	-	-	-	-

64	9	x	x	9	Scottish Highlands	Grass	-	-	-	-
65	8	x	8	x	Scottish Highlands	Grass	-	-	-	-
68	27	x	x	27	NA	NA	-	-	-	-
70	6	x	x	6	Hartland	NA	-	-	-	-
71	4	x	x	4	Edinburgh	NA	-	-	-	-
72	6	x	x	6	Edinburgh	deer	-	-	-	-
74	5	x	x	5	NA	NA	-	-	-	-
75	3	x	2	1	Hampshire	dog	-	-	-	-
76	3	x	1	2	Devon	grass	-	-	-	-
78	2	x	x	2	Hampshire	dog	-	-	-	-
79	4	x	1	3	Hampshire	dog	-	-	-	-
80	6	x	2	4	Hampshire	NA	-	-	-	-
81	1	x	x	1	Hampshire	dog	-	-	-	-
82	3	x	x	3	NA	NA	-	-	-	-
83	7	x	1	6	NA	NA	-	-	-	-
84	11	x	5	6	Hampshire	deer	-	-	-	-
85	2	x	1	1	Hampshire	deer	-	-	-	-
86	13	x	x	13	Hampshire	dog	-	-	-	-
87	1	x	1	x	Orkney	dog	-	-	-	-
88	7	x	x	7	Orkney	dog	-	-	-	-
91	2	2	x	x	Orkney	dog	-	-	-	-
92	2	x	x	2	Bristol	dog	-	-	-	-
93	1	1	x	x	NA	NA	-	-	-	-
94	9	x	5	4	Scottish Highlands	dog	-	-	-	-
96	26	1	20	5	Exeter	dog	-	-	-	-
98	1	x	x	1	Exeter	dog	-	-	-	-
101	3	x	x	3	Exeter	dog	-	-	-	-

104	2	x	x	2	Bristol	dog	-	-	-	-
105	2	x	x	2	Bristol	dog	-	-	-	-
106	2	x	x	2	Perth	deer	-	-	-	-
107	1	x	x	1	NA	NA	-	-	-	-
108	1	x	x	1	Rushden	dog	-	-	-	-
109	4	x	4	x	NA	NA	-	-	-	-
110	9	x	2	7	Perth	deer	-	-	-	-
111	10	x	9	1	Scottish Highlands	Grass	-	-	-	-
112	3	2	1	x	Ipswich	dog	-	-	-	-
113	5	3	2	x	Northland	deer	-	-	-	-
114	3	x	2	1	NA	NA	-	-	-	-
115	9	x	x	9	NA	NA	-	-	-	-
116	1	x	x	1	Cornwall	dog	-	-	-	-
117	1	x	x	1	Cornwell	dog	-	-	-	-
118	12	x	x	12	York	Pig	-	-	-	-
119	1	x	x	1	Probus	dog	-	-	-	-
120	1	x	x	1	Probus	dog	-	-	-	-
121	2	x	x	2	Probus	dog	-	-	-	-
122	6	2	4	x	Southampton	deer	-	-	-	-
123	1	1	x	x	Southampton	deer	-	-	-	-
124	3	x	x	3	Dorchester	cow	-	-	-	-
125	1	x	x	1	Dorchester	cow	-	-	-	-
126	1	x	x	1	Dorchester	Grass	-	-	-	-
127	1	x	x	1	Dorchester	Grass	-	-	-	-
128	1	x	x	1	Arnside	dog	-	-	-	-
129	1	x	x	1	Dorset	deer	-	-	-	-
130	1	x	x	1	Dorchester	deer	-	-	-	-
131	1	x	x	1	Dorset	deer	-	-	-	-

132	1	x	x	1	Sussex	deer	-	-	-	-
133	1	x	x	1	Knighton	dog	-	-	-	-
134	2	x	1	1	York	dog	-	-	-	-
135	1	x	x	1	Suffolk	dog	-	-	-	-
136	10	x	x	10	Arnside	dog	-	-	-	-
137	10	x	x	10	Scotland	deer	-	-	-	-
138	10	x	x	10	Knighton	Pig	-	-	-	-
139	3	x	x	3	Knighton	Pig	-	-	-	-
140	1	x	x	1	Scotland	dog	-	-	-	-
141	1	x	x	1	probus	dog	-	-	-	-
142	7	x	x	7	Scotland	deer	-	-	-	-
143	7	x	x	7	Essex	Fox	-	-	-	-
144	1	x	x	1	Essex	Fox	-	-	-	-
145	8	x	2	6	Brampton	NA	-	-	-	-
146	11	x	x	11	Brampton	NA	-	-	-	-
147	7	x	x	7	Brampton	NA	-	-	-	-
154	4	x	x	4	Essex	NA	-	-	-	-
155	1	x	x	1	Essex	deer	-	-	-	-
156	1	x	x	1	Essex	deer	-	-	-	-
157	3	x	x	3	Dorset	Grass	-	-	-	-
158	9	x	4	5	Lancaster	Grass	-	-	-	-
159	1	x	x	1	Lancaster	Grass	-	-	-	-
160	1	x	x	1	NA	NA	-	-	-	-
161	3	x	x	3	NA	deer	-	-	-	-
162	4	x	x	4	Moray	cat	-	-	-	-
163	1	x	x	1	Angus	deer	-	-	-	-
164	4	x	4	x	Angus	deer	-	-	-	-
165	4	x	4	x	Moray	cat	-	-	-	-
166	2	x	x	2	Essex	dog	-	-	-	-

167	1	x	x	1	Essex	dog	-	-	-	-
168	2	x	x	2	Essex	Fox	-	-	-	-
169	7	x	x	7	Essex	fox	-	-	-	-
170	1	x	x	1	Devon	dog	-	-	-	-
171	3	x	x	3	Devon	dog	-	-	-	-
172	6	3	2	1	Devon	dog	-	-	-	-
173	1	x	1	x	Devon	dog	-	-	-	-
174	1	x	x	1	Suffolk	dog	-	-	-	-
175	1	x	x	1	NA	NA	-	-	-	-
176	4	x	x	4	NA	NA	-	-	-	-
177	8	2	6	x	Lancaster	dog	-	-	-	-
178	4	1	3	x	Lancaster	dog	-	-	-	-
179	2	x	2	x	Essex	dog	-	-	-	-
180	2	1	1	x	Essex	dog	-	-	-	-
181	3	x	1	2	Essex	deer	-	-	-	-
182	1	x	x	1	Suffolk	deer	-	-	-	-
183	1	x	x	1	Scotland	deer	-	-	-	-
184	4	x	4	x	Essex	fox	-	-	-	-
185	1	x	x	1	Essex	fox	-	-	-	-
186	1	x	x	1	Essex	fox	-	-	-	-
187	5	5	x	x	Essex	fox	-	-	-	-

NA: No available information, x: No ticks.

Appendices 4 Terminase gene and terminase 676 PCR primers sequences

Terminase gene of *B. burgdorferi* B31 plasmid cp32-1. Sequence ID: AE001575.1

>GTGAACTTATATCAAACAAAACTTTTTACAACACTACAAAAGGAATACAAAAATAAATATGGAGTTGATATATCACAATT
TGTAAAGCTAACAAATTCTTCAATTAATTTTGATAAGTTTGAAGAAGAACAGTTAACTTTAAAACAAAAAAATGTGATAAA
AAGCATTAAAAAGAATAATGAAAAGAAGATTATACTCAGCGGAGGCATAGCTAGTGGCAAAACGTATCTTGCATGTTATC
TTTTTCTAAAAAGTTTAATTGAAATTAAAAAGTTATACTCTAGTGATACTAATAATTTCATTATAGGGAATTCACAACGTTC
AGTTGAAGTTAATGTTTTGGGGCAATTTGAAAAGCTATGTAAACTTCTTAAAATTCCTTATATTCCAAGACATACAAATAAT
TCATATATTCTGATTGATTCACTACGTATTAATCTATATGGAGGAGATAAGGCAAGTGATTTTGAAAGATTTAGGGGAAGT
AATTCGGCACTTATTTTTGTTAATGAGGCTACAACTTTACACAAGCAAACTTTAGAGGAAGTCTTAAAAAGACTAAGATGC
GGGCAAGAAACTATTATTTTTGATACTAATCCTGATCATCCAGAACACTATTTTAAAACCGATTATATTGATAATATAGCGA
CCTTTAAGACATATAAGTTTACAACTTATGATAATGTGCTACTTAGTAAAGGATTTGTCGAAACACAAGAAAAGCTATATA
AAGATATACCATCATATAAAGCAAGAGTTTTGTTAGGTGAGTGGATAGCAAGCACTGATTCAATTTTTACACAAATAAATA
TTACTGATGATTATGTATTTACTAGCCCGATAGCATATTTAGACCCAGCATTTAGTGTTGGCGGGGATAACACTGCATTAT
GTGTTATGGAGCGAGTTGATGATAAGTATTATGCTTTTGTATTTCAAGACCAAAGACCAGCTAATGATCCTTATATTATGA
ATATGGTAAAGACTGTTATAGAAAATTTCAATGTGCATACACTGTATTTAGAGGATAGAGATAATACAAAAGGTGCTGGT
GGATTGACCCGTGAATACATCTTGCTAAGAAGTAATATAAGCCAATATTTTAGAATTGTTCCAGTTAAGCCAAAGTCTAAT
AAATTTAGCAGAATAACAACGTTAATTACGCCGTTTACTTACAAAAAACTTTATATTACAAAGTACAGTAGTTCTTCCGTAT
TTAATGATATTTATTCGTATAAGGGGGATAATAAAACCCATGATGACGCTCTTGATGCAATATCTGCAGCATATTTGATGT
TGTCTTTAGGATATAGAGAGCGAAGTGTTCACTTTGGCAATCAAAGATTTTTGTAA

Terminase 676 primers regions were highlighted in blue highlight.

Fwd Ter676: GGAGGCATAGCTAGTGGCAAA — Forward

Rs Ter 676: CCCCGCCAACACTAAATGCT — Reverse complementary

Product size: 676 bp. TM from calculator for one tag: 55°C.

Appendices 5 Sanger sequences of *16SrRNA* products of the DNA samples extracted from positive *B. burgdorferi* s.l. cultures

>18 length 260

TCAGAAAGAATACCGGAGGCGAAGGCGAACTTCTGGGTCAAGACTGACGCTGAGTCACGAAAGCG

TAGGGAGCAAACAGGATTAGATACCCTGGTAGTCTACGCTGTAAACGATGCACACTTGGTGTTAACT

AAAAGTTAGTACCGAAGCTAACGTGTTAAGTGTGCCGCCTGGGGAGTATGCTCGCAAGAGTGAAAC

TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGC

>19 length 263

TGAGATGGGGATAACTACTAGAAATAATGGCTAATACCGAATAAGGTCAATTAATTTGTTAATTGAT

GAAAGGAAGCCTTTAAAGCTTCGCTTGTAGATGAGTCTGCGTCTTATTAGTTAGTTGGTGGGGTAAA

TGCCTACCAAGGCGATGATAAGTAACCGGCCTGAGAGGGTGAACGGTCACACTGGAACTGAGATAC

GGTCCACACTCCTACCGGAGGCAGCAGCTAACAATCTTCCGCAATGGGCGAAGGCCTGACGGA

>20 length 261

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>21 length 248

TACCAGGGCTTGACATATATAGGATATAGTTAGAGATAATTACTCCCCGTTTGGGGTCTATATACAG

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>22 length 236

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>30 length 240

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GCTTGTAGATGAGTCTGCGTCTTATTAGCTAGTTGGTAGGGTAAATGCCTACCAAGGCGATGATAAG

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>39 length 233

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GCTTGTAGATGAGTCTGCGTCTTATTAGCTAGTTGGTAGGGTAAATGCCTACCAAGGCGATGATAAG

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>45 length 231

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GATACGGTCCAGACTCCTACGGGAGGCAGCA

>49 length 233

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>59 length 242

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> 60 length 240

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>63 length 248

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>66 length 253

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>67 length 242

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> 69 length 244

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>77 length 225

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>89 length 244

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>90 length 242

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>95 Length 235

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GCTTGTAGATGAGTCTGCGTCTTATTAGCTAGTTGGTAGGGTAAATGCCTACCAAGGCGATGATAAG

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TGGGGTCTATATACAGGTGCTGCATGGTTGTCGTCAGCTCGT

>99 length 245

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>100 length 250

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>103 length 242

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>151 length 236

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>152 length 233

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