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Susceptibility of *Borrelia burgdorferi* Morphological Forms to Chemical Antimicrobials

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Susceptibility of *Borrelia burgdorferi* Morphological Forms to Chemical Antimicrobials

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Master of Science

*Borrelia burgdorferi* is the etiological agent of Lyme disease. Not much is known about the susceptibility of this organism to chemical disinfection. Current antimicrobial susceptibility test methods, such as those published by the American Society for Testing and Materials (ASTM), usually require assessment of the number of colony forming units (cfu) of growing organisms on plates following exposure to an agent. For fast-growing organisms, plates are ready for counting 1-2 days post plating, while several weeks may be needed for slower growing organisms. Spirochetes, like *B. burgdorferi* are difficult to grow on solid media and typically require long incubation periods, sometimes up to several weeks, to generate visible colonies. These issues make *B. burgdorferi* cfu assessment by plate counting difficult and unreliable. Furthermore, *Borrelia* have a demonstrated capacity for pleomorphic forms, and can exist in spirochete, round body, or biofilm forms, depending on culture conditions. Plate counts, by nature, do not allow for assessment of morphological form changes. Additionally, the susceptibility of *B. burgdorferi* pleomorphic forms to chemical disinfectants has not been tested. In this study, we used the SYBR GREEN I/Propidium Iodide (SG I/PI) viability assay to rapidly estimate the percent kill of *B. burgdorferi* pleomorphic forms to chemical disinfection. Planktonic spirochete populations in 30-second treated samples showed viability percent values of: >95% for Hanks balanced salt solution (HBSS), ~60% for distilled deionized H$_2$O (dd H$_2$O), <5% for ACS 200, and 1% for 1% glutaraldehyde (GTA). Solutions containing 70% ethanol (ETH) and 1% hypochlorite (HC) showed no viable spirochetes following treatment. The percent of live round body cells following different treatments were: >99% for HBSS and <25% for dd H$_2$O. ACS 200, 1% GTA, and 70% ETH treatments resulted in <1% live round body forms, whereas HC showed no live round cell forms. The susceptibility of *B. burgdorferi* biofilms to various treatments was also assayed using a SG I/PI viability stain after 30-minute contact times. The percent of viable organisms (green) in the treated biofilms was estimated by microscopic observations. HBSS controls showed >98% of bacteria in the biofilm were alive, while treated biofilms showed the following percent viabilities: ACS 200 - ~2%, 1% HC - <1%, 5% HC - <1%, 1% GTA - ~10%, 70% ETH - ~ 2%, and dd H$_2$O ~40%. These techniques merged standardized assessment of antimicrobial activity in liquid culture using an ASTM-type kill-time procedure with viability techniques used in antibiotic susceptibility testing to rapidly evaluate the percent kill of *B. burgdorferi* pleomorphic forms in vitro following disinfectant exposure. These results showed that *B. burgdorferi* biofilm forms are orders of magnitude more resistant to chemical disinfection than other morphological forms of this organism.

Keywords: *Borrelia burgdorferi*, biofilm, round body, spirochete, disinfectant, antimicrobial
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INTRODUCTION

1.1 Disinfection of Lyme Spirochete

Literature suggests that *Borrelia burgdorferi sensu lato* can be inactivated with heat, UV light, 1% HC and 70% ETH [37]. Heat-killed *Borrelia* or isopropyl/ethyl-alcohol killed *Borrelia* are often used in producing “dead” spirochetes for various assays [73-74] [89]. However, to our knowledge, no studies involving kill-time disinfectant assays have been recorded in the literature. Additionally, a rapid contact disinfection assay has not been established to assess the susceptibility of the major morphological forms of *Borrelia*; single spirochetes, round body, and biofilm-like colonies. The purpose of these studies is to establish an assay that can be used to determine the antimicrobial susceptibility of the different morphological forms of *B. burgdorferi* to chemical disinfection.

1.2 Difficulty in Classical Disinfection testing with *B. burgdorferi*

*Borrelia* are slow growing with a doubling time between 12 and 24 hours and require special growth media supplements [39-42]. This makes typical contact disinfection analyses difficult, as standardized testing usually requires plate counts of colony forming units. The plates used in standard *Borrelia* growth are bi-layered with a thicker bottom layer and a less dense top agar overlay. This method of plating is difficult to replicate, and exceedingly labor intensive when used in a standard disinfection assay. It has been reported that once prepared under these optimal conditions, *Borrelia* colonies can be seen with the unaided eye 3-4 weeks post inoculation [43]. Colonies that do form are also quite small and can be difficult to locate [44]. Barbour described *Borrelia burgdorferi* growth on plates as a “lawn”, prior to the generation of media that allowed for individual isolated colonies of the Lyme spirochete [40]. Moreover, potentially not all viable *Borrelia* on plates will form colonies, as *Borrelia* spirochetes are prone to forming aggregates, and can also change to round body forms, which may require further
extended incubation periods prior to reverting into spirochetes that can then form colonies. These issues complicate disinfection protocols and require several weeks of incubation time prior to data collection. In addition, plate counts limit the ability to assess quantitative and qualitative morphological changes of *Borrelia* spirochetes to round body forms.

1.3 Purpose of *B. burgdorferi* Disinfection Testing

To better understand the effect of chemical antimicrobials on *B. burgdorferi*, the standard chemical antimicrobial assay was modified for rapid assessment of susceptibility and morphological change from spirochete to round body. A better understanding of the effects of chemical antimicrobials on the major morphological forms of *B. burgdorferi* is critical to the proper handling and disinfection of this microbe.

In these studies, we developed an evaluation method for the analysis of chemical disinfectant efficacy against the different morphological forms of *Borrelia burgdorferi*, using viability dye assessment in combination with standardized ASTM-type kill-time suspension testing. Novel growth and viability assessment techniques formulated in the laboratory of Eva Sapi, that were originally designed for the analysis of antibiotic efficacy against three different forms of *Borrelia burgdorferi* (spirochetes, round bodies, and biofilm-like colonies) [2] [9], were utilized in combination with standardized kill-time suspension techniques, to test the effectiveness of chemical disinfectants that could be used to kill *Borrelia burgdorferi* in a lab or clinical setting. Our goal was to establish an *in vitro* system to analyze contact kill times for *Borrelia burgdorferi* morphological forms, using chemical antimicrobials, in order to better understand the susceptibility of the pleomorphic variations of the Lyme spirochete.
BACKGROUND

2.1 Discovery of Lyme Disease

Lyme disease is a tick-borne illness caused by the *Borrelia burgdorferi* spirochete. Over the last decade, Lyme disease has become more prominent and grown into a major public health problem. The disease has been noted in the United States, Central Europe, and parts of Asia. Lyme disease shows equal prevalence in men and women, and can be found among all age groups, including children [13-14]. First described and discovered in Old Lyme, Connecticut in 1975, adolescent arthritis became a major noted concern among residents, and this ultimately led to the discovery of *Borrelia burgdorferi* as the causative agent of Lyme disease [10-11]. Dr. Alan Steere initially described Lyme disease as a clinical manifestation of an infectious illness and suggested the transmission of the agent through arthropod vectors. Thereafter, Dr. Willy Burgdorfer and coworkers isolated spirochetes from the midguts of ticks. They found that when these spirochetes were injected into rabbits, it resulted in the production of a rash similar to the erythema migrans seen in some Lyme patients [38]. Subsequently, the bacterial species was named *Borrelia burgdorferi*.

2.2 Introduction to *Borrelia Burgdorferi*

In Europe and Asia, *Borrelia Burgdorferi senu strictu (s.s)*, *B. garinii*, and *B. afzelii*, which are collectively known as *B. burgdorferi sensu lato (s.l)*, are the three major genotypes of *Borrelia* responsible for most human cases, although additional genotypes are being identified. Lyme disease in the US is generally thought to be caused by *Borrelia burgdorferi* alone [65]. The United States Center for Disease Control and Prevention (CDC) estimates about 300,000 [79] new infections annually in the United States alone [15] [67-68]. Increased exposure to tick habitats or *Ixodes* tick hosts, increases the risk for contracting Lyme disease. Current research has been focused mainly on antibiotic efficacy against *Borrelia*, the different pleomorphic forms
of the organism, protein regulation necessary for survival in multiple hosts types, and persistent infections [9]. However, very little research has been done on this organism’s susceptibility to disinfection.

2.3 Transmission and Life Cycle

Lyme Disease is known to be transmitted by tick vectors, namely of the genus *Ixodes* in the United States. These tick vectors find hosts among a wide range of vertebrate animals including small and large mammals, birds, and lizards [38] [78]. Though less studied and less understood, clinical signs attributed to Lyme disease have been reported in dogs, cattle and horses [16] [28-29]. Worldwide geographic distribution of Lyme disease in Humans tracks with several species of ticks that serve as vectors, and varying genospecies of *Borrelia*. In the western United States, *I. pacificus* (the western black-legged tick) and in the northeastern and midwestern U.S., *I. scapularis* (the black-legged tick), are the primary tick species that cause human disease. In Europe, *I. ruminus* (the European sheep tick) is the main agent of Lyme dissemination, and in Asia, *I. persulactus* (the taiga tick) is the main vector [80-81]. However, newly discovered tick vectors and *Borrelia spp.* variants are becoming apparent [82]. Larval, nymphs, and adult ticks acquire spirochetes when feeding on infected animals. Frequently, larva feed on small animals such as infected rodents, while nymphs and molted adult ticks often feed on larger animals [38]. Once they become carriers, ticks host the spirochetes often for life. Infected ticks feed on a large range of animals, which then become new reservoirs to continue the cycle. When an infected tick feeds upon a human, the spirochete can permeate the skin and begin dissemination through the body. Nymphs are small and often difficult to detect, which make them more likely to attach long enough to transmit the spirochete before being removed.
Ticks and mammalian hosts are exceedingly different environments for bacterial growth. Tick ambient temperature will fluctuate, depending on its surroundings and host attachment, while mammalian hosts regulate their body temperature between 37°-39°C. Additionally, mammalian host blood and tissue pH are more neutral, compared to the more basic environment found in the tick midgut. *Borrelia* adapt to these environmental changes with variable gene expression, which alters protein components and stimulates regulatory migration based on external cues during a tick blood meal [83].

Though controversial, there is some evidence to support the idea that *Borrelia* can be transmitted by sexual contact. *Borrelia* spirochetes have been cultured from and identified in human vaginal and seminal secretions [79]. Serological and genetic identification of *Borrelia* in genital secretions suggests at least the possibility that sexual transmission of the spirochete and initiation of disease may occur.

2.4 Symptomology of Lyme Borreliosis

Lyme disease is a multisystem disorder that results in flu-like symptoms, which if left untreated, can develop into more severe complications [84]. Lyme patients often exhibit nonspecific symptoms such as headache, myalgia, arthralgia, shaking chills, and abdominal complaints [76]. More severe symptoms might include extreme fatigue, musculoskeletal symptoms, neurological problems, and/or cardiac abnormalities, which may present weeks to months after initial infection [10-11]. The most easily recognizable and diagnosable presentation sign of Lyme disease is a bulls-eye skin rash called erythema migrans, which usually appears at the bite site [14]. The rash exhibits, as the name suggests, a rash that looks like a bull’s eye target with rash rings centered around a focal point. This focal point is thought to be the where the tick initiated a blood meal, with subsequent dissemination of Lyme spirochetes throughout the skin.
Though the well-known bull’s eye rash is easily identified and recognized, only a fraction of Lyme patients exhibit this presentation. In mouse models, *Borrelia burgdorferi* has also been implicated in bone building inhibition, resulting in trabecular bone loss and demineralization [86].

2.5 Treatment of Lyme Borreliosis

The increasing prevalence of Lyme disease has been attributed to poor preventative measures, suboptimal treatments, and lack of information regarding *Borrelia* infection. Antibiotics administered as a frontline treatment for Lyme disease include doxycycline, minocycline, amoxicillin, cefuroxime, and ceftriaxone [9]. Although antibiotic treatment for 2-4 weeks is typically successful for early stages of Lyme disease, studies have shown that not all patients are cleared of infection. These patients then continue to have Lyme disease symptoms that persist. Morphological forms of *Borrelia burgdorferi* may contribute to treatment failures [87-88].

2.6 Morphological Forms of *Borrelia burgdorferi*

*Borrelia burgdorferi* is known to be capable of existing in several alternate morphologies when exposed to harsh or changing environmental conditions [1-6]. In addition to its typical spirochete form, the Lyme bacteria can transform from this corkscrew shape into round body cyst-like forms. Unfavorable condition such as high pH, adverse temperatures, nutrient starvation, and altered osmotic pressure can induce formation of the round body form from spirochetes [7-8]. These round bodies also have the capacity to revert back into vegetative motile spirochetes *in vitro* [1] and *in vivo* [4]. In addition to these single cell forms, *Borrelia* can aggregate into functional biofilms which can protect internal resident spirochetes from unfavorable external conditions [2] [9]. These alternate morphologies may be a critical part of *Borrelia burgdorferi*’s ability to survive
hostile environments. It has been shown that these morphological forms (single spirochetes, round body forms, and biofilm-like-colonies), can protect the bacteria in different instances, such as when they are exposed to antibiotics in vitro [9].

2.6.1 *Borrelia burgdorferi* Spirochete

*Borrelia burgdorferi* is well known as the Lyme Disease spirochete. Characterized as a flat-wave spirochete, it is known that the flagella component of *Borrelia* is critical in determining its shape [65-66]. The cell envelope of *Borrelia burgdorferi* includes a protoplasmic cylinder which is covered by two lipid membranes [55]. The periplasmic space contains the peptidoglycan layer and flagellar filaments, located between the outer and inner membrane [57]. *Borrelia burgdorferi* have multiple periplasmic flagella bundled together in structures called axial filaments that attach at each end of cell and overlap in the middle. This constrains the bacterium into an elongated spirochete shape. The two flagellar bundles located at each end of the cell provide motility by propelling the spirochete in a corkscrew like motion, which allows for easier movement through viscous media [60-63]. The intersection of the two axial filaments in the middle of the cell is also the site of a flexing motion, often seen while spirochetes are suspended in free motion [64]. Characteristically, *Borrelia* are approximately 0.2 µm in diameter and 10-30 µm in length, though length can vary significantly depending on growth conditions [66]. Unlike classic gram-negative bacteria, *Borrelia burgdorferi* lack lipopolysaccharides (LPS), which are normal outer membrane components, and have immunoreactive glycolipids in their place [58-59].

2.6.2 *Borrelia burgdorferi* Round Body

Current literature has reported that *Borrelia* spirochetes can transform into round body forms, which are also called L-forms, cystic forms, and cell wall deficient forms [30-31]. These
round body forms have been observed as a small fraction of a typical *Borrelia* culture and can be induced with antimicrobials and human spinal fluid when cultured *in vitro* [1]. Induction of round body *Borrelia* can be accomplished by stressing the cells through osmotic stress, starvation, antibiotic treatment and pH extremes outside of the typical *Borrelia* growth range [5-6]. Round body forms have been reported to be a functional way of potentially protecting the spirochete for a time until more favorable conditions arise, at which time spirochetes can reemerge and continue metabolic activity. As the spirochete changes to a round form, the outer membrane make up also changes in a way to protect the spirochete from unfriendly environments, such as antibiotic treatment [33-35]. Rapid induction of the round body form can be accomplished by subjecting spirochetes to distilled deionized (dd) H₂O. On contact with this hypotonic environment, spirochetes will shift to round body forms, with longer exposures resulting in increased round body counts, and increased spirochete reversion time when they are returned to optimal growth conditions [34]. To date, round body susceptibility to antibiotics have been shown *in vitro* [2], but no similar assessments of round bodies to chemical antimicrobials have been tested.

2.6.3 *Borrelia burgdorferi* Biofilm

Among the *Spirochaetales*, *Leptospira spp.*, and *Treponema denticola* have been reported to contribute to biofilms [20-22]. Similarly, biofilm formation has been reported in *Borrelia burgdorferi*, and has been associated with protection of the organism from antibiotic treatment *in vitro* [9] [18] [25-28]. These biofilms are a complex conglomeration of *Borrelia burgdorferi* spirochetes that serve to protect associated spirochetes from hostile or unfavorable environments. *Borrelia* have been shown to display several known hallmarks of biofilms *in vitro* [9] [18]. For instance, structural rearrangements of the aggregates, variable stages of
development on varying substrate matrices, and secretion of a protective extracellular polymer substance (EPS) matrix have been observed. Sapi et al. showed multi-level rearrangements take place at different stages of the aggregate biofilm colony development. Atomic force microscopy, which allows live imaging of cells without fixation, showed the arranging formation of Borrelia spirochetes in a biofilm to be progressive over time [2]. The Sapi lab also showed that individual spirochetes were observed on agarose layers, but often were observed in pairs, which, within a few days would begin to acquire more spirochetes and generate complex structure formations, until large aggregates and biofilm were generated. These aggregates then produced a complex and continuously changing structure, thought to reintroduce fresh resources and exchange waste to each segment for the biofilm. This same group also showed that these developing biofilms form on several abiotic and biotic substrates [2]. In addition, Borrelia burgdorferi spirochetes are also capable of forming floating aggregate biofilms in liquid media. Analysis of the extracellular substances of these aggregates showed them to be exopolysaccharides containing both sulfonated and non-sulfonated/carboxylated substrates, which are common in various bacterial biofilms. Literature also has shown that the exopolysaccharides are predominately composed of an alginate in combination with calcium and extracellular DNA. These components present in Borrelia burgdorferi biofilms are known components of biofilms produced by other medically relevant bacteria [2][9] [19] [23]. The rigidity of the biofilm structure can be felt by rubbing the coverslip over the wet mount of a biofilm sample and an audible crunching sound is produced (A. Reid, unpublished observations). Borrelia biofilm formation has been shown to protect the organism from antibiotic penetration and allow organism persistence through treatment in vitro [9].
2.7 Growth Conditions and Division

Originally, *Borrelia* was cultured in specialized liquid Barbour-Stonner-Kelly (BSK) medium. Currently, there are variations of BSK medium, such as BSK-H, BSK-S, BSK-II and others, which have been altered with variable serum concentrations needed to optimally culture *Borrelia in vitro* [40-41] [44]. BSK medium is highly enriched with bovine serum albumin and heat-inactivated rabbit serum to promote growth. Cultures are typically incubated in a microaerophilic environment of 5% CO$_2$ at the optimal temperature of 33°C [9] [53]. *Borrelia burgdorferi* is a human pathogen, so even though the optimal growth temperature is about 33°C, it can manage at 37°C. Generally, spirochetes grow slowly and *Borrelia burgdorferi* doubles about every 12 hours. In a fashion unique to the genus *Borrelia*, these spirochetes elongate from specific zones of peptidoglycan accumulation which are regulated spatially and temporally. A new cell is born with a central region that produces larger quantities of peptidoglycan relative to the rest of the cell, while the poles of the spirochete remain inert. As the cell cycle progresses, the spirochetes produce additional regions of increased peptidoglycan production at about the $\frac{1}{4}$ and $\frac{3}{4}$ regions on the spirochete. These areas elongate the cell in both directions, followed by DNA segregation, and septate formation. As the mid-cell region completes septation, the region becomes an inert region relatively lacking peptidoglycan on either end of the new daughter cells. The daughter cells mid-cell origins are then established where the mother cells $\frac{1}{4}$ and $\frac{3}{4}$ peptidoglycan dense regions formed. [75-76]

2.8 Live/Dead Differentiation of Lyme Spirochetes

To differentiate live and dead *Borrelia* forms: spirochetes, round body, and biofilm, darkfield microscopy was used to visualize the planktonic or aggregated cells. Darkfield microscopy allows for easy viewing of an otherwise thin and difficult to view bacterium. As the
organisms appear as bright objects surrounded by a dark background, the contrast allows for simple spirochete identification [69]. Often, live spirochetes are characterized by active motility while impaired or no motility can indicate cell death [33] [69-72]. These motile cells can be counted to estimate the number of viable spirochetes in a population [62-63]. In addition to darkfield microscopy, fluorescent viability dyes can be used to stain the organisms and achieve percent live/dead measurements [2] [9]. These dyes offer a more accurate measure of a cell’s viability status through selective membrane permeability of the dyes; where live cells are impermeable to selective dyes while dead cells with damaged membranes allow such dyes to pass into the cell. [53] [73].

**MATERIALS AND METHODS**

3.1 Strain, Media and Culture

*B. burgdorferi* B31 strain was obtained from the American Type Tissue Collection (ATCC 35210). *B. burgdorferi* was cultured in BSK-H medium containing 6% rabbit serum. BSK base was prepared in 250 ml batches. Each batch was made by mixing part A components in 200ml of dd H2O using magnetic stirring for 1 hour at room temperature, after which part B components were added and the medium was further mixed for another hour. Dd H2O was used to bring the total volume to 250 ml. It was then mixed an additional 30 minutes prior to filtration. All culture media were filter-sterilized through a 0.22 µm filter. Cultures were incubated in sterile 15 ml closed conical tubes at 33⁰C and 5% CO2, without antibiotics, and grown to mid-late log phase. Cultures were kept at low passages (3-10 passages). Homogenous cultures of spirochetes without biofilm formation were obtained by maintaining the cultures in a shaking incubator with tight lids at 33⁰C and 250 rpm [9].
The methods used to prepare *B. burgdorferi* biofilm cultures using 24 well tissue culture plates, were as previously described [9]. Briefly, for the generation of *Borrelia burgdorferi* biofilms, 100 µl of active culture (2 X 10⁶ spirochetes) were transferred into 24 well plates containing one ml fresh BSK-H media supplemented with 6% rabbit serum and incubated at 33⁰C and 5% CO₂ for 10 days without shaking. After 10 days of incubation, biofilm-like colonies were examined and confirmed by darkfield microscopy. Contents of the wells were transferred to 1.5 ml centrifuge tubes, centrifuged and washed with one ml of Hank’s Balanced Salt Solution (HBSS), then exposed to chemical antimicrobials prior to crystal violet assay or SG I/PI viability assay.

3.2 Microscopes

Specimens of *Borrelia burgdorferi* cultures or cells were examined using a Zeiss Imager A1 microscope equipped with a darkfield condenser and a darkfield oil immersion 63X objective lens. A separate Zeiss A1 imager equipped with fluorescence capabilities was used for fluorescent analysis. Both microscopes were equipped with Zeiss Axiocam 503 color cameras.

3.3 Darkfield Microscopy

Initially, darkfield microscopy was used to assess cell viability by observing motility following disinfectant exposure. Samples were analyzed through cell counts in a Petroff-Hauser counting chamber-3902, under darkfield microscopy post contact with disinfectants. *Borrelia burgdorferi* were grown to log phase, washed in Hanks Balanced Salt solution (HBSS), and concentrated to 5 X 10⁸ spirochetes/ml. Spirochetes were placed in 1.5 ml centrifuge tubes (~5 X 10⁸ spirochetes/ml) and centrifuged at 10,000 xg for 10 minutes, then washed with HBSS. Cell pellets were re-suspended in one ml of disinfectant and homogenized gently by pipetting. After specified contact times (30 seconds, 1 minute, 3 minute), 0.1 ml of cell suspension was placed in
0.9 ml of neutralizer (~ 5 X 10^7 spirochetes/ml). After mixing, the suspension was centrifuged and washed in HBSS. Cells were viewed and counted at 200X magnification in a Petroff-Hauser counting chamber-3902 under darkfield microscopy. Motile and non-motile spirochetes or round body forms were counted. Assays were performed in triplicate.

3.3.1 Step-wise *Borrelia* Motility Darkfield Microscopy Assay:

1. Transfer one ml of 1 X 10^9 spirochetes into 1.5 ml centrifuge tubes
2. Centrifuge at 10,000 xg for 5 minutes
3. Discard the supernatant
4. Wash with one ml of HBSS
5. Centrifuge at 10,000 xg for 5 minutes
6. Discard the supernatant
7. Add one ml of disinfectant to the bacterial pellet
8. Re-suspend the cells with gentle and swift pipette motions in disinfectant
9. At contact time 30 seconds, 1 minute, and 3 minutes transfer 100 ul of treated cells into 900 ul of neutralizer in a separate 1.5 ml centrifuge tube
10. Vortex
11. Centrifuge at 10,000 xg for 5 minutes
12. Discard the supernatant
13. Wash with one ml of HBSS
14. Centrifuge at 10,000 xg for 5 minutes
15. Discard the supernatant
16. Gently re-suspend the pellet in one ml HBSS
17. Count with Petroff-hauser counting chamber-3902 under darkfield at 200X magnification

18. Count cells for total concentration, round body presence, and motility

Statistical analysis run with Two-Way ANOVA with Dunnett’s multiple comparison test; **** where p<0.0001 and * where p<0.02 using Graph Prism software.

3.4 Heat Kill Protocol

An active culture of *Borrelia burgdorferi* was pelleted by centrifugation at 3,000 xg for 15 minutes at room temperature, and the pellet was washed once in HBSS. The pellet was re-suspended in HBSS (preheated to 55°C) and the suspension was maintained in a 55°C water bath for 20 minutes. Spirochete death was confirmed by loss of motility under darkfield and SG I/PI viability staining. Spirochete density was adjusted to 10⁹ spirochetes/ml stock solution as counted in a Petroff-Hauser counting chamber-3902 under darkfield microscopy.

3.5 SG I/PI 96-well Plate Viability Assay and Fluorescent Microscopy

For assaying the live/dead percentages, cells in 96-well plates were stained with SG I and PI nucleic acid stains. SG I (10,000 X stock, Invitrogen) (10 µl) and 30 µl of PI (20 mM, Sigma) were added to 1.0 ml of sterile dd H₂O and the tube was vortexed until completely mixed. The staining mixture (10 µl) was added to each well containing 100 µl of cell suspension and the contents were mixed thoroughly. The plates were incubated in the dark at room temperature for 15 minutes. The plates were assayed for fluorescence using a BioTek Synergy HT plate reader, with the excitation wave length set at 485 nm, and the fluorescence intensities monitored at 535 nm (green emission) and 635 nm (red emission).

The prepared suspensions of *B. burgdorferi* for the generation of standard curves used live and heat killed *Borrelia burgdorferi* in seven different proportions (100%, 87.5%, 75%,...
50%, 25%, 12.5%, 0% live cells). These were prepared in 96-well plates using the mixy-mix method. The SG I/PI dye mix was added to each well and the Green/red fluorescence ratios were measured at each of the proportions in multiple replicates, using the BioTek plate reader. Regression equations were generated using the least-square fitting analysis of the ratios of green:red (live: dead) *Borrelia burgdorferi*. The regression equation obtained was used to calculate the percentage of live *Borrelia burgdorferi* cells in each well.

*Borrelia burgdorferi* spirochete homogenous cultures were grown to mid-late log phase, washed twice in HBSS, and concentrated to 1 X 10^9 cells/ml. To generate mixy-mix proportions for the standard curve, a 1 X 10^8 cells/ml dilution was prepared and split in half. Half of the mixture was heat killed and the other half was kept in HBSS at room temperature to serve as live cells. The heat-killed suspension was placed on ice and warmed to room temperature before mixing with live cells when preparing the standard curve proportions.

Test samples were made by placing one ml (~1 X 10^9 cells/ml) of culture in 1.5 ml centrifuge tubes. Samples were centrifuged for 10 minutes at 10,000 xg at room temperature. Supernatant fluid was discarded, and pellets were washed with one ml HBSS and centrifuged again to pellet the sample. Pellets were re-suspended in one ml of disinfectant. After specified contact times (30 seconds, 1 minute, 3 minutes), 100 µl was added to 900 µl neutralizer in a 1.5 ml centrifuge tube and vortexed (~1 X 10^8 cells/ml). The tube was centrifuged for 10 minutes at 10,000 xg at room temperature and the cells were washed once with HBSS. The pellet was re-suspended in one ml HBSS and mixed gently. A 200 µl aliquot of the samples was transferred to a 96-well plate (~2 X 10^7 spirochetes per well). Twenty µl of SG I/PI dye was added to each well and the contents were mixed well. Plates were incubated in the dark for 15 minutes, then assayed for fluorescence on a BioTek Synergy HT plate reader with the excitation wave length set at 485
nm, and the fluorescence intensities at 535nm (green emission) and 635 nm (red emission).
Samples were also examined with a fluorescent microscope to confirm counts in comparison
with the darkfield counts and the percent live calculations from 96-well plate assays.
Additionally, fluorescent direct counts were used to estimate percent change of spirochete to
round body forms induced by exposure to the disinfectant. Microscopy was performed on a Zeiss
Axio A1 fluorescent imager. Images were taken at 400 X.

3.5.1 Step-wise *Borrelia* SG I/PI 96-well plate viability assay:

1. Transfer one ml of 1 X 10⁹ spirochetes into 1.5 ml centrifuge tubes
2. Centrifuge at 10,000 xg for 5 minutes
3. Discard the supernatant
4. Wash with one ml of HBSS
5. Centrifuge at 10,000 xg for 5 minutes
6. Discard the supernatant
7. Add one ml of disinfectant to the bacterial pellet
8. Re-suspend the cells with gentle and swift pipette motions in disinfectant
9. At contact times of 30 seconds, 1 minute, and 3 minutes, transfer 100 ul of treated cells
into 900ul of neutralizer in a separate 1.5ml centrifuge tube
10. Vortex
11. Centrifuge at 10,000 xg for 5 minutes
12. Discard the supernatant
13. Wash with one ml of HBSS
14. Centrifuge at 10,000 xg for 5 minutes
15. Discard the supernatant
16. Gently re-suspend the pellet in one ml HBSS
17. Transfer 200 ul of treated sample into 96-well plate
18. Add 20ul of SG I/PI dye stock mixture and mix into each well
19. Incubate plates in the dark for 15 minutes at room temperature
20. Assay for fluorescence with the excitation wave length set at 485nm, and the fluorescence intensities at 535nm (green emission) and 635nm (red emission)
21. Use 20 ul of dyed sample to observe under fluorescent microscopy, count round body to spirochete ratio in 5 fields of view

Statistical analysis run with One-Way ANOVA with Dunnett’s multiple comparison test; **** where p< 0.0001 using Graph Prism software.

3.6 Compete-Kill Growth Assay

To determine if all viable cells were killed by antimicrobial agents with strong percent kill values as assessed by the 96-well plate viability assay and visual observance of non-motile cells via darkfield microscopy, a complete-kill test was implemented. Viable spirochetes were treated with antimicrobial agents as previously described at 30 seconds, 1 minute, and 3 minute contact times for homogenous spirochete cultures and a 30 minute contact time for biofilm cultures.

After exposure to listed disinfectants, treated cells were washed and replaced with fresh BSK-H media to assess for growth (G), or no growth (NG) observing spirochete motility under darkfield microscopy. Spirochete motility was assessed at 3 weeks post inoculation.

3.6.1 Step-wise *Borrelia* Compete-Kill Growth Assay:

1. Follow 3.5.1 “Step-wise *Borrelia* SG I/PI 96-well plate viability assay” to step sixteen
2. Centrifuge at 10,000 xg for 5 minutes
3. Transfer 100 ul of treated cells into 900 μl of BSK-H media in 24 well plates
4. Wrap edges in parafilm to prevent drying of plates
5. Incubate at 33°C and 5% CO₂ for 3 weeks
6. Assay Growth (G) or no growth (NG) by darkfield microscopy at 630X magnification

3.7 Quantitative Crystal Violet Biofilm Assay

To quantify *Borrelia burgdorferi* biofilm-like colonies after treatment with various chemical antimicrobials, 24-well plate were inoculated with 100 µl containing $\sim$2 X $10^6$ spirochetes into plates containing one ml fresh BSK-H media supplemented with 6% rabbit serum. The plates were incubated at 33°C and 5% CO₂ for 10 days without shaking. Contents of the wells were transferred to 1.5 ml centrifuge tubes, washed, and exposed to chemical antimicrobials for specified contact times and neutralized, as described above. Control wells were treated with HBSS alone and run in triplicate.

This assay was used to determine the destruction of biofilm mass after a specified contact time of 30 minutes, a significant increase from the 30 second contact times used on planktonic cells, by the following tested chemical antimicrobials: 70% ETH, 1% GTA, 1% HC, 5% HC, dd H₂O, and ACS 200.

After 10 days incubation to generate a biofilm, the contents of the well were transferred into a 1.5 ml centrifuge tube and centrifuged at 10,000 xg for 5 minutes. After discarding the supernatant fluid, 0.5 ml of HBSS was added to all wells. Cell were re-suspended, washed once with HBSS, and centrifuged again. Pellets were re-suspended in one ml of chemical disinfectant for a 30-minute contact time, after which contents were placed into 9 ml of neutralizer, mixed, centrifuged, and washed once with HBSS. Cells were re-suspended in 0.01% crystal violet for 10 minutes, centrifuged, and washed once with HBSS. Cells were centrifuged again and re-
suspended in 200 µl cold 10% acetic acid for 15 minutes. Cells were centrifuged and supernatant fluid was collected and placed into a 96-well plate, which was assayed for absorbance at 590 nm.

3.7.1 Step-wise *Borrelia* Biofilm Assay Using Crystal Violet:

1. Transfer the contents of each well into individually labelled 1.5 ml centrifuge tubes
2. Centrifuge at 10,000 xg for 5 minutes
3. Discard the supernatant
4. Add 500 µl of PBS to each 96-well plate well and add contents to the pellets in the centrifuge tubes
5. Resuspend the cells by vortexing
6. Centrifuge at 10,000 xg for 5 minutes
7. Discard the supernatant
8. Add one ml HBSS
9. Resuspend the cells by vortexing
10. Discard supernatant
11. Add one ml chemical antimicrobial being tested
12. Resuspend by vortexing
13. Incubate at room temperature for 30 minutes
14. Take one ml of disinfectant/cell mixture from 1.5 ml centrifuge tube and transfer to 9 ml of neutralizer.
15. Vortex
16. Centrifuge at 10,000 xg for 5 minutes
17. Discard supernatant
18. Wash with one ml HBSS and mix by vortexing, then transfer back to new individually labelled 1.5 ml centrifuge tube.

19. Add 100 µl of 0.01% crystal violet to the pellet.

20. Re-suspend the cells by vortexing.

21. Incubate at room temperature for 10 minutes.

22. Centrifuge at 10,000 xg for 5 minutes.

23. Discard the supernatant.

24. Add 0.5 ml HBSS to the pellet to wash the biofilm.

25. Re-suspend the cells by vortexing.

26. Centrifuge at 10,000 xg for 5 minutes.

27. Discard the supernatant.

28. Add 200 µl of 10% acetic acid to the pellet.

29. Re-suspend the cells by vortexing.

30. Incubate at room temperature for 15 minutes.

31. Centrifuge at 10,000 xg for 5 minutes.

32. The clear solution is transferred to a 96-well plate and OD is measured at 590 nm.

Statistical analysis run with One-Way ANOVA with Dunnett’s multiple comparison test; **** where p<0.0001 and * where p<0.05 using Graph Prism software.

3.8 Qualitative Assessment of *Borrelia burgdorferi* Biofilm Using SG I/PI

To determine a qualitative value for the effect of chemical bactericidal agents on biofilm-like colonies of *Borrelia burgdorferi*, 2 X 10^6 cells from a homogenous culture of spirochetes generated by shaking incubation at 250 rpm were inoculated into one ml of BSK-H in 24 well plates and incubated at 33°C and 5% CO2 for 10 days. After 10 days incubation without shaking,
biofilm-like colonies were observed in the wells. The contents of the well were removed and placed into 1.5 ml centrifuge tubes and centrifuged at 10,000 xg for 5 minutes and washed once in HBSS. One ml of disinfectant was added to the tube, which was then vortexed. After a 30 min contact time, the contents were added to 9 ml of neutralizer in a 15 ml conical tube. After mixing, the tube was centrifuged at 10,000 xg for 5 minutes and the pellet was re-suspended in one ml HBSS and transferred back into a 1.5 ml centrifuge tube. Cells were then washed once with HBSS and re-suspended in one ml HBSS. Ten µl SG I/PI dye mixture were added for each 100 ul of sample. The tube sat in the dark at room temperature for 15 minutes, and cells were then viewed with a fluorescence microscope. Control wells were treated with HBSS alone and all tests were repeated in triplicate. Images were taken at 400X magnification.

3.8.1 Step-wise SG I/PI Qualitative Assay Protocol for *Borrelia* Biofilm 30-minute Contact Time:

1. Transfer the contents of the well into individually labelled 1.5ml centrifuge tubes
2. Centrifuge at 10,000 xg for 5 minutes
3. Discard the supernatant
4. Add 500 µl of HBSS to all wells. The contents of each well is transferred to centrifuge tubes
5. Centrifuge at 10,000 xg for 5 minutes
6. Resuspend the cells in one ml HBSS by vortexing
7. Centrifuge at 10,000 xg for 5 minutes
8. Discard the supernatant
9. Add one ml HBSS
10. Resuspend the cells by vortexing
11. Discard supernatant
12. Add one ml chemical antimicrobial being tested
13. Resuspend by vortexing
14. Let sit for contact time of 30 minutes
15. Take one ml of disinfectant-organism from 1.5ml centrifuge tube and transfer to 9 ml neutralizer in 15 ml conical tube
16. Vortex
17. Centrifuge at 10,000 xg for 5 minutes
18. Discard supernatant
19. Wash with one ml HBSS and mix by vortexing
20. Transfer back to new labelled 1.5 ml centrifuge tube
21. Re-suspend in one ml HBSS by vortexing
22. Add 100 µl SG I/PI dye mixture
23. Vortex
24. Incubate at room temperature in the dark for 15 minutes.
25. View under fluorescent microscopy on a microscope slide
26. Count 5 fields of view

Statistical analysis was run with One-Way ANOVA with Dunnett’s multiple comparison test; **** where p<0.0001 using Graph Prism software.

RESULTS AND DISCUSSION

4.1 Specified Modifications

The liquid suspension test methods utilized in this study were based on the ASTM E2315-16 standard test method suitable for assessing bactericidal activity in liquid suspension
Modifications were made to scale down the volumes to one-ml test sample sizes. In addition, live/dead viability assessment of the samples via fluorescent viability dyes SG I and PI replaced standard colony forming unit counts. All disinfection efficacy tests were conducted in triplicate.

4.2 Motility Viability Assay

To evaluate *in vitro* chemical antimicrobial sensitivity of *B. burgdorferi* spirochete and round body morphological forms, *B. burgdorferi* (B31-35210) was subjected to timed contact with different disinfectants. Disinfectant sensitivity of spirochetes was evaluated using direct cell counting and motility assessment using darkfield microscopy (Figure 1). Treatment with HBSS showed that >99% of counted spirochetes were motile, while treatments with 70% ETH, 1% GTA, ACS 200, and dd H2O showed <1% of organisms present were motile. In addition, 1% HC showed no countable cells, presumably by inducing cell lysis.

![Figure 1: B. burgdorferi Spirochete Darkfield Motility Counts.](image)

Using this method, we also calculated the change in round body presence when cells were subjected to chemical antimicrobials (Figure 2). *B. burgdorferi* cultures naturally contain a
small population of round body forms. However, the predominant form is the spirochete. Our results showed that when in HBSS, the cultures contained ~5% round body forms. However, ACS 200 and dd H2O increased round body presence to ~80% of the total countable population, which is almost 13 times that of the control group. Interestingly, 1% GTA and 70% ETH showed ~ 5% round body forms (Figure 2A). No significant differences in round body induction were observed between 30-sec and 10-min contacts (Figure 2A). Using this method, we found that 30 sec appeared to be a suitable contact time for the single cell disinfection assay. From this, we concluded that though useful in viability detection in antibiotic assays, motility was not sensitive enough to be useful in disinfection assays. Therefore, we used fluorescent live/dead viability staining and direct counts for subsequent disinfection experiments.
FIGURE 2: *B. burgdorferi* Round Body Darkfield Counts. (A) *B. burgdorferi* percent round body estimation by darkfield microscopy after various contact times with different solutions. (B) *B. burgdorferi* exposed to HBSS viewed with dark-field microscopy in a Petroff-Hauser bacterial counting chamber; image taken at 400X magnification. (C) Darkfield image of *B. burgdorferi* round body forms induced by exposure to dd H2O; image taken at 630X magnification. Statistical analysis run with Two-Way ANOVA with Dunnett’s multiple comparison test; **** where p<0.0001 and * where p<0.02 using Graph Prism software.

4.3 SYBR GREEN I/PI Planktonic Cell Direct Counts

To examine *in vitro* viability of planktonic cells beyond motility, we used SG I/PI viability stains to assess membrane damage to the spirochetes and round body forms present in disinfectant-treated samples. SG I is a common dye used to stain purified nucleic acids in various molecular biology techniques. The SG I/PI assay has been used for viability assessment of *B. burgdorferi* after antibiotic exposure, and on other bacteria [73-74]. Like the SYTO 9/PI (BacLight) assay, Sybr Green I (SG I) is a membrane permeable fluorescent dye that will stain all cells, whereas Propidium Iodide (PI) is a red membrane impermeable fluorescent dye that will only stain dead cells or those with significant membrane damage. Thus, live cells fluoresce green and dead cells fluoresce red due to PI permeability. Previous studies with *B. burgdorferi* determined the optimal cell staining conditions and dye concentrations used in this study [74].

Percent live spirochetes were calculated based on direct cell counts using fluorescence microscopy, where numbers of live (green) and dead (red) cells were counted in 5 fields at 400X magnification. Live spirochete populations in treated samples showed >95% for HBSS, ~60%
for dd H$_2$O, <5% for ACS 200, ~1% for GTA, ~1% for ETH, and <1% for HC (Figure 3A). Live round body cells in the population were also assessed. Greater than 99% of HBSS treated round body forms were live (Figure 4 B). Distilled deionized H$_2$O showed <25% live round body types present in the population, while ACS 200, 1% GTA, and 70% ETH showed <1% live round body forms. 1% HC showed no live cell forms. Round body induction, as assessed by both darkfield and fluorescent microscopy cell counts, showed comparable readings (Figure 4 A) for ACS 200 and dd H$_2$O, resulting in ~13-fold increase of round body forms compared to the HBSS control.
FIGURE 3: B. burgdorferi Spirochete Live/Dead Viability Counts. (A) B. burgdorferi live spirochete percentage estimated by live/dead staining with SG I and PI after a 30 second contact time. (B) Representative image of SG I/PI staining of untreated B. burgdorferi (Darkfield, 1000X magnification). Statistical analysis run with One-Way ANOVA with Dunnett’s multiple comparison test; **** where p<0.0001 using Graph Prism software.
FIGURE 4: *B. burgdorferi* Round Body Live/Dead Viability Counts. (A) Percent *B. burgdorferi* round body form induction following a 30 sec treatment as estimated by live/dead staining with SG I and PI. (B) Percent of live Round Body forms following a 30 sec treatment as detected by fluorescent live/dead staining. (C) Representative image of live/dead staining of *B. burgdorferi* Round Body forms post 30 second treatment with dd H₂O (Fluorescent microscopy, 1000X magnification).

Similarly, 1% GTA and 70% ETH showed similar results as the HBSS control group, in round body form induction on contact. HC resulted in complete destruction of cells, as previously seen. We determined that, as shown in previous studies [1] [4-6], the round body form can be induced through hypotonic stress. Distilled deionized H₂O and ACS 200 showed immediate induction of round body forms on contact, with a >10-fold increase of round body forms over the control. Similarly, ACS 200, which is colloidal silver in dd H₂O, also resulted in >10-fold increase in round body form induction from the control. In contrast, ACS 200 showed no viable (green) round body forms, whereas water did. Although 1% GTA and 70% ETH were also prepared with dd H₂O, these preparations did not significantly increase round body formation compared with the HBSS control. In addition, 1% HC resulted in almost immediate lysis of planktonic cells. It was interesting to note the fragility of *B. burgdorferi* spirochetes and round body forms to various disinfectants, even with a short contact time of 30-seconds.
4.4 SYBR GREEN I/PI Planktonic 96-Well Plate Assay

Direct microscopic fluorescent cell counting can be time consuming and inaccurate. For these reasons, we explored a fluorescent plate reader assay as a rapid means of evaluating samples. First, we confirmed that the use of the fluorescent dyes SG I and PI could be used in generating a linear model for counting cells in a 96-well plate. Live \textit{B. burgdorferi} cells were diluted from $10^4$-$10^8$ cells/well. The resulting linear fit revealed an R$^2$ value of 0.997 between total fluorescent signal and the number of live \textit{Borrelia burgdorferi} cells in solution (Figure 5A). Separated R$^2$ values for each log section revealed that the limit of resolution for cell detection was near $5 \times 10^5$ cells, which correlates with previous studies [73].
FIGURE 5: Linear Relationship between Total fluorescent signal of live B. burgdorferi and the Total Available Number of Cells in Solution. (A) Linear relationship of total fluorescent signal of $10^5$-$10^8$ B. burgdorferi cells in test wells. (B) Separated from 5A, $10^7$-$10^8$ B. burgdorferi cells. (C) Separated from 5A, $10^6$-$10^7$ B. burgdorferi cells. (D) Separated from 5A, $10^5$-$10^6$ B. burgdorferi cells. (E) Separated from 5A, $10^4$-$10^5$ B. burgdorferi cells.

From the linear representation of the data between $5 \times 10^6$ to $1 \times 10^8$ cells we determined that a final working concentration of near $10^7$ cells per well would be ideal for further experiments. For our purposes, we became interested in using the ratio of live to dead cells in solution using the SG I (live) and PI (dead) viability dyes. Although a SG I/PI assay has been used for viability assessment for other bacteria and B. burgdorferi in antibiotic susceptibility [73-74], it has not been used to evaluate B. burgdorferi susceptibility to chemical antimicrobials. B. burgdorferi culture wells containing $1 \times 10^6$, $2 \times 10^6$, $5 \times 10^6$, $8 \times 10^6$, and $1 \times 10^7$ cells were made and stained with SG I/PI mixture, and then read at an excitation wavelength of 485nm and emission wavelengths of 535nm (green) and 635 nm (red). This was
done to test the linear relationship of serially diluted *B. burgdorferi* cells to the green/red ratio of the viability dyes. The linear relationship between green/red fluorescence ratios of *B. burgdorferi* cells showed an $R^2=0.974$ (Figure 6A) representing the variation of the green/red ratios as explained by the number of known *B. burgdorferi* cells present in the well. This data is comparable with previously described SG I/PI assays performed for antibiotic susceptibility testing [73-74]. Having established that the green/red ratios correlate with known total *B. burgdorferi* cells, we measured the green/red ratios of known proportions of percent live *B. burgdorferi* cells at our working concentration of $10^7$ cells. This would provide a means of percent live cell estimation from the solution. Heat-killed *B. burgdorferi* were made in HBSS, which was heated for 20 minutes at 55°C and cooled to room temperature on ice before combining with live *B. burgdorferi* at 5 different proportions, 0, 20, 50, 80, and 100% live cells prepared by mixy-mix method (Figure 6B). The resulting curve is consistent with similar curves developed for *B. burgdorferi* antibiotic susceptibility testing [73-74]. In addition, we tested this method with a total concentration of $1 \times 10^7$ and $2 \times 10^7$ *B. burgdorferi* live cells at 7 different proportions, 0, 12.5, 25, 50, 75, 87.5, and 100% to assess if there was a major difference in the green:red ratio linear fit to a doubling of fluorescent signal. A doubling of fluorescent signal revealed similar green:red ratio results to our original test (Figure 6C). Furthermore, fluorescence microscopy imaging was consistent with live/dead ratio values (Figure 6D). Fluorescence imaging produced comparable values with predetermined proportions of live/dead *B. burgdorferi* cells.
FIGURE 6: Linear Relationship between B. burgdorferi Viability and the Green/Red Fluorescence Ratios. (A) The linear relationship between the green/red fluorescence ratios and number of live B. burgdorferi spirochetes in solution. (B) The linear relationship between the green/red fluorescence ratios and percent live B. burgdorferi spirochetes at concentrations 10⁴-10⁷. Percent live (vs heat-killed) spirochetes used were: 100, 80, 50, 20, and 0. The line is a least-squares fit of the relationship between percentage of live bacteria and the green/red fluorescence ratio. (C) The linear relationship between the green/red fluorescence ratios and percent live B. burgdorferi spirochetes at a concentration of 1 X 10⁷ and 2 X 10⁷ using live proportions of 100, 87.5, 75, 50, 25, 12, and 0. (D) Representative fluorescence microscopy images showing live/dead staining of known proportions (0, 25, 50, 75, 100%) of live B. burgdorferi spirochetes (i-v, respectively). 100, 87.5, 75, 50, 25, 12, and 0
Having confirmed that the SG I/PI assay could be effectively utilized in the rapid assessment of viable *B. burgdorferi*, we then proceeded to apply the assay to disinfectant-treated samples. HBSS treatment showed >99% of cells were alive (Figure 7). Distilled deionized H₂O treatment showed that ~10% of cells present were living, while ACS 200, 1% GTA, 70% ETH and 1% HC treatments resulted in <4% cells surviving. Ratios of green/red were comparable to previously published live/dead ratios obtained when the background interference of the carrier medium was taken into account [73-74].

![Figure 7: The Effects of Different Antimicrobials on the Viability of B. Burgdorferi. Percent live B. burgdorferi after 30 (A) and 60 (B) second contact times with various disinfectants as measured by green/red ratios using SG I/PI staining. Statistical analysis run with One-Way ANOVA with Dunnett’s multiple comparison test; **** where p < 0.0001 using Graph Prism software.](image)

We determined that the Lower Limit of Quantitation (LLOQ), where various proportions of live *B. burgdorferi* were tested in 18 wells each, was ~4%. Results were similar to direct counts, with regards to all tested chemical antimicrobials. The variation of 3-4% was similar to that for the limit of quantification of 10⁵ cells established in the literature with green/red ratios using SG I/PI [74]. As some of these solutions are potent disinfectants which may cause lysis or extreme fragility of treated cells, which can then be lysed upon pipetting, it is reasonable to
assume that as cells in the wells fell below $10^5$ cells per well, there would be errors in the
green/red ratios determined by the assay. To confirm complete kill by HC treatment, samples
were inoculated into fresh BSK-H medium in 24-well plates and incubated for 3 weeks. At
various time points, samples were assessed for motile spirochetes with darkfield microscopy up
to 3 weeks (Table 3). Both HC solutions and ACS 200 treatments showed no recovery of motile
spirochetes after 3 weeks. Exposure to dd H$_2$O, however, showed recovery of viable motile
spirochetes within 1 week. This is consistent with round body reversion from dd H$_2$O and spinal
fluid noted in the literature [1, 6], and the doubling time of viable spirochetes. All neutralizer-
disinfectant controls showed motile spirochetes similar to the HBSS controls.

Having shown that the SG I/PI assay was an effective tool in measuring disinfectant
efficacy using $B. burgdorferi$, we next evaluated the effectiveness of ACS 200 in a silver-binding
environment. ACS 200 was diluted 1/2, 1/4, and 1/8 in dd H$_2$O and HBSS. $B. burgdorferi$
suspensions were exposed to these dilutions for 30 seconds, followed by neutralization and assay
for green/red ratios using SG I/PI viability staining in 96-well plates. When diluted in dd H$_2$O,
ACS 200 maintained a consistent kill of >95% through all dilutions (Figure 8A). As previously
shown, even small quantities of silver ion in dd H$_2$O have potent antimicrobial activity [91].
However, when diluted in HBSS, ACS 200 was significantly less effective at killing $B.$
burgdorferi. After exposure to ACS 200 diluted 1:2 in HBSS, about 60% of the cells were still
viable; with ACS 200 diluted 1:4, ~70% of the cells were viable, and with 1:8, ~80% of the cells
were viable. Fluorescent microscopy results were comparable to those from the plate reader, for
all ACS dilutions (Figure 8B i-viii).
FIGURE 8: The Antimicrobial Effects of ACS 200 Diluted in dd H₂O and HBSS on B. burgdorferi. (A) Percent live B. burgdorferi after a 30 second contact as measured by green/red ratios following SG I/PI staining. (B) Fluorescence microscopy images of ACS 200 dilutions in dd H₂O and HBSS; HBSS (i), ACS 200 (ii), 1:2,4,8 dd H₂O dilutions (iii-v respectively), 1:2,4,8 HBSS dilutions (vi-viii respectively). Statistical analysis run with One-Way ANOVA with Dunnett’s multiple comparison test; **** where p<0.0001 using Graph Prism software.

4.5 Crystal Violet Biofilm Assay

The crystal violet biofilm assay was used to quantify the amount of biofilm remaining following a 30-minute treatment with various disinfectants. Though typically used to assess firmly attached biofilms, the crystal violet assay can also be applied to suspended biofilm-like colonies [9]. First, we confirmed a linear relationship between crystal violet absorbance and concentration, using both 1:10 and 1:2 dilutions (Figure 9A and 9B). The least-squares fit line showed a R²=1 for 10-fold serial dilutions of 0.01% crystal violet and a R²=0.999 for 2-fold serial dilutions of the same solution. B. burgdorferi biofilms were generated through static culture in 24-well plates for 10 days, followed by treatment with various antimicrobials. Treatments were compared to the HBSS control to obtain percent of control values of the remaining B. burgdorferi biofilm.
The remaining biofilm after each treatment was as follows (Figure 9C): ACS 200, ~45%, 1% HC, ~15%, 5% HC, ~1%, 1% GTA, ~20%, 70% ETH, ~20%, and dd H₂O, ~95%. As expected, HC was best at biofilm degradation. However, both concentrations of HC resulted in no viable cells of any kind when examined microscopically, yet a small amount of crystal violet signal remained. We concluded that a small amount of biofilm matrix and cellular debris were detected by the crystal violet assay.

4.6 SYBR GREEN I/PI Biofilm Viability Assay

To qualitatively assess the effects of different disinfectants on *B. burgdorferi* biofilms in wells, disinfectants were applied for 30 minutes, wells were neutralized and then stained with SG I/PI. The percent estimates of green (live) cells in the biofilm were determined by visual
estimation of green present in the biofilm. HBSS controls showed >98% of cells in the biofilm to be live, while these same values for the other treatments were: ACS 200, ~2; 1% HC, <1%; 5% HC, <1%; 1% GTA, ~10%; 70% ETH, ~2%; and dd H2O, ~40% (Figure 10). Both ACS 200 and dd H2O induced heavy increases in round body forms within the biofilm. We believe this is due to the low osmotic strength of these solutions. ACS 200-treated biofilms, unlike the dd H2O, however, showed nearly all-round body and spirochete forms to be nonviable (red). Fluorescence microscopy analysis using viability stains of biofilms, in tandem with quantification of biofilms using the crystal violet assay, revealed the nature of antimicrobial-treated biofilms. The crystal violet assay showed that following dd H2O treatment, ~95% of the biofilm remained. However, when viewed with fluorescent microscopy, it became clear that most of the organisms in the dd H2O-treated biofilm had undergone a shift to primarily round body forms, and that about ~40% of the biofilm stained viable (green). The two HC solutions showed nearly identical results with remaining biofilm debris fragments, if any, staining red. GTA and ETH treatments showed that only a small fraction of the biofilms were viable. It was notable that 70% ETH occasionally produced excessive clumping of treated biofilm formations. Biofilm viability estimations by SG I/PI staining are shown in Table 1. The spirochetes and round body forms of *B. burgdorferi* appeared to be particularly susceptible to most of the antimicrobials tested here. However, the biofilm-like colonies showed surprising resilience to a 30-minute contact with these disinfectants.
FIGURE 10: Qualitative Assessment of Viable B. burgdorferi Biofilm-Like Colonies. Representative fluorescence microscopy images of 30-minute disinfectant-treated biofilm-like colonies stained with SG I/PI. (A) HBSS, (B) 1% GTA, (C) 5% HC, (D) dd H₂O, (E) ACS 200, (F), 70% ETH, and (G) 1% HC. Numbers following letters indicate replicate number (20X Magnification).

TABLE 1: Qualitative Assessment of Viable B. Burgdorferi in Biofilms Stained with SG I/PI.

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
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<tbody>
<tr>
<td>HBSS</td>
<td>100%</td>
<td>100%</td>
<td>98%</td>
</tr>
<tr>
<td>1% GTA</td>
<td>10%</td>
<td>10%</td>
<td>5%</td>
</tr>
<tr>
<td>5% HC</td>
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<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>dd H₂O</td>
<td>40%</td>
<td>40%</td>
<td>35%</td>
</tr>
<tr>
<td>ACS 200</td>
<td>1%</td>
<td>2%</td>
<td>1%</td>
</tr>
<tr>
<td>70% ETH</td>
<td>0%</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>1% HC</td>
<td>0%</td>
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</table>

Assessment summary table of Figure 10 images with estimations in percent of live (green) staining by SG I within the tested biofilms. Five fields were viewed for each tested well, and all tests were done in triplicate.
4.7 Complete-Kill Assay

Due to the limits of testing the entire volume of a test sample with microscopy and the 4% potential error in the 96-well plate assay, we were interested to know if any of the cells in wells with signals below 4% were alive and could be resuscitated. Samples were run as previously tested, washed, placed into fresh BSK-H medium, and incubated for 3 weeks. Assessment for viability was performed with direct visualization of motile spirochetes in solution using darkfield microscopy after 3 weeks of incubation. Table 2 shows the growth results of the
spirochete cultures post contact with chemical disinfectants for 30 seconds, 1 minute, and 3 minutes. While HBSS and dd H₂O showed growth recovery, GTA and HC solutions remained sterile. ACS 200 silver solution showed 1 of 3 wells resulting in growth recovery. Biofilm recovery was also tested using a 30-minute contact time (Table 3). All samples tested resulting in a recovery of growth except for the HC solutions.

**TABLE 2: Three Week Complete Kill Assessment Single Spirochetes**

<table>
<thead>
<tr>
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<th>30sec</th>
<th>1 minute</th>
<th>3 minutes</th>
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<tbody>
<tr>
<td>1% GTA</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
</tr>
<tr>
<td>5% HC</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
</tr>
<tr>
<td>ACS 200</td>
<td>G, NG, NG</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
</tr>
<tr>
<td>70% ETH</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
</tr>
<tr>
<td>1% HC</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
<td>NG, NG, NG</td>
</tr>
</tbody>
</table>

Complete kill assessment of single spirochete wells post treatment with antimicrobials at 30 seconds, 1 minute, and 3 minutes contact time. Wells were assessed for cell growth with darkfield microscopy through motility observation; growth (G), no growth (NG) repeated in triplicate. Spirochete motility was assessed at 3 weeks post inoculation.

**TABLE 3: Three Week Complete Kill Assessment of B. burgdorferi Biofilms**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>HBSS</td>
<td>G, G, G</td>
</tr>
<tr>
<td>1% GTA</td>
<td>G, G, G</td>
</tr>
<tr>
<td>5% HC</td>
<td>NG, NG, NG</td>
</tr>
<tr>
<td>dd H₂O</td>
<td>G, G, G</td>
</tr>
<tr>
<td>ACS 200</td>
<td>G, G, G</td>
</tr>
<tr>
<td>70% ETH</td>
<td>G, G, G</td>
</tr>
<tr>
<td>1% HC</td>
<td>NG, NG, NG</td>
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</table>

Complete kill assessment of B. burgdorferi biofilm wells post treatment with antimicrobials at a 30-minute contact time. Wells were assessed for cell growth with darkfield microscopy through motility observation; growth (G), no growth (NG) repeated in triplicate. Spirochete motility was assessed at 3 weeks post inoculation.
4.8 Summary

In summary, the goal of these studies was to identify a method for the rapid evaluation of disinfectants on the various morphological forms of *B. burgdorferi*. We showed SG I/PI staining to be a novel and rapid method for disinfection assessment on the various morphological forms of *B. burgdorferi*. In addition, SG I/PI staining provided valuable information as to the differential susceptibility of the various morphological forms: spirochetes, round body forms, and biofilm-like colonies. A major finding of this work is that biofilms of *B. burgdorferi* are orders of magnitude more resistant to chemical disinfection than the spirochete or round body forms. Although our study focused specifically on *B. burgdorferi*, this methodology could be adapted to assess disinfectant efficacy for other medically relevant, difficult to cultivate spirochetes. In addition, our study provides evidence that the common published information for *B. burgdorferi* spill cleaning methods may need to be revised [37].

FUTURE WORKS

Now that this protocol has been shown useful for assaying disinfectant efficacy for spirochetes and other morphological forms of *B. burgdorferi*, the next steps would be further accuracy enhancements. The current protocol is based on manual visual counts and live: dead ratio determinations using a fluorescence plate reader. A more ideal method would be a multi-staining technique processed with flow cytometry that would discriminate between morphological states and live/dead viability. In addition, more research exploring a broader range of disinfectants and antimicrobials on *B. burgdorferi* is needed. Although this method is relatively rapid and can be completed within two days (and is much quicker than waiting for organisms to grow 3 weeks post contact), it is demanding, and would benefit from further optimization of work flow and simplification where possible. Currently the protocol requires,
from start to finish, 18 hours of continuous work. All solutions were immediately before each test to ensure optimal antimicrobial activity. Currently, the protocol also demands the complete monopolization of a lab centrifuge to wash cells between contacts. Further optimization of work flow and simplification of the protocol would be beneficial. For instance, whether fewer wash cycles would give the same results, or result in less physical damage of the cells is unknown. Furthermore, if the 96-well protocol could be run with fewer transitions prior to reading, this may also provide more assurance that damaged cells are the result of the antimicrobial compounds and not manipulation-induced damage. Each wash cycle consists of 10 minutes in the centrifuge to spin down cells. With many replicates and test agents, this amounts to a significant centrifuge wait time. If possible, it would be ideal to reduce the amount of time used to pellet cells, while still maintaining confidence in complete cell collection. Ideally, the test would greatly benefit from being able to be run through the 96-well plate assay with the viability dyes in the neutralizer. This would reduce pipetting time, the physical damage to the cells, and the total number of wash cycles in which cells might be lost.
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