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Galleria Mellonella as an Alternate Infection Model for *Burkholderia* Species and a Comparison of Suspension and Surface Test Methods for Evaluating Sporicidal Efficacy

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A thesis submitted to the faculty of
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Master of Science

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ABSTRACT

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Melioidosis is a neglected tropical disease that continues unabated in many countries, particularly in Southeast Asia. There is no vaccine and antimicrobial treatment is expensive and complicated. Virulence models are important tools used to investigate genes involved in pathogenesis. *Galleria mellonella* is the larvae of the wax worm moth that has been used to model various infections. Based on previous studies, we attempted to establish an infection model using *Burkholderia pseudomallei* and *Burkholderia thailandensis*, a related species which is avirulent in humans. Injections of various forms of these species (fresh and frozen) were used to develop Kaplan-Meier plots. We also tested *Burkholderia cepacia, Burkholderia vietnamiensis, Burkholeria ambifaria,* and *Burkholderia multivorans* to understand how they affect the larvae. We found that larvae injected with *B. pseudomallei* and *B. thailandensis* did not accurately model the respective infections these species cause in humans, while the other non-virulent species did not produce disease, as expected. We conclude that *G. mellonella* is not an appropriate infection model for *B. pseudomallei* and *B. thailandensis*.

Healthcare-associated infections (HAI) are on the rise, and place a heavy burden on our healthcare system each year. Disinfectants used in healthcare settings can reduce HAIs, but first must be evaluated for proper efficacy. To date there are few statistical models that are useful in comparing disinfectant test methods. We conducted a head-to-head comparison of two common test methods, suspension and surface, using *Clostridium difficile* spores as the test organism. A novel statistical method was developed to evaluate which test method better predicted disinfectant performance. An activated disinfectant that gradually lost activity over time was used in these evaluations. Results showed that the suspension test method was less variable, and was a better predictor of disinfectant efficacy over time.

Keywords: infection model, disinfectant test, *Galleria mellonella*, *Burkholderia pseudomallei,* suspension test, surface test

ACKNOWLEDGEMENTS

Scientific advance is only made possible when people collaborate, give freely of their time, knowledge and expertise, and when one stands on the shoulders of the hundreds who have gone before them. This is no more apparent than when a young budding scientist begins their journey during graduate school. My experience has reinforced this norm, with many supportive mentors, colleagues, friends and family at my back, encouraging me throughout the process. While I have gained much in scientific knowledge and practical skills, I value even more the life lessons taught to me through the rigors and repetition of failure and perseverance. I am incredibility grateful to those that have voluntarily assisted me at any point in the process of my education and learning.

Particular thanks goes to my primary investigator, Dr. Richard Robison, along with my committee comprised of Dr. Kim O'Neill and Dr. Brad Berges. Their guidance and advice have been monumental in steering me during this rocky journey. I am also indebted to my wife, Megan, for her continued encouragement during countless nights at which I recounted the days failed experiments. Her confidence in me has been invaluable. I am grateful to my fellow graduate students who have troubleshot my issues, helped me with their ideas, and lifted me with their comradery. Finally, I am thankful to this University for the privilege of obtaining an education and conducting research.

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Introduction

Infection models

When researching an infectious bacterium or virus, infection models are needed to study the infection, resultant signs, and factors involved in virulence. Accurate infection models can help deduce the mechanisms of infection and thereby help identify treatments and other methods that might be used to combat disease. It is important that the infection model be technically and financially practical, and mimic human infection characteristics as closely as possible. The first chapter of this thesis endeavors to explore a novel infection model for various *Burkholderia* species.

Burkholderia

Burkholderia species are saprophytic soil dwelling bacteria endemic to South East Asia, including the countries Thailand, Vietnam, Myanmar, and also Northern Australia. (34) Naturally competent, they take on helpful gene clusters and pathogenic islands acquired from their soil habitat, resulting in robust organisms that are intrinsically resistant to a wide spectrum of antibiotics. They also possess an impressive array of pathogenic mechanisms that allow them to invade, hide, and survive in their natural hosts. As a result of their varied methods of pathogenicity, this genus commonly infects both plant and animal species (36). In the *Burkholderia* genus, there are two main species that readily infect and cause disease in humans and animals. *Burkholderia pseudomallei* infects many species, causing the disease melioidosis in humans, while *Burkholderia mallei* primarily infects equestrian species, causing

the disease glanders. However, there are cases of disease in other species as well. The *Burkholderia cepacia* complex is a set of up to 20 *Burkholderia* species. They are also known to infect humans, but as they are opportunistic organisms, these cases are observed less frequently. Infected patients usually have pre-existing lung conditions, such as cystic fibrosis or chronic granulomatous disease, or are immunocompromised. (20) The species in this *Burkholderia cepacia* complex also infect plants, and come with a variety of useful survival traits, such as nitrogen fixation or the ability to use alternate carbon sources. Some of the better studied species include *B. multivorans, B. vietnamensis,* and *B. cepacia.* As a genus, there is much research to be done to understand this complex and fascinating group of species.

Burkholderia pseudomallei

B. pseudomallei infects humans, and causes the disease melioidosis. (29) Due to its wide range of symptoms, specific antimicrobial resistance, elusive behavior relative to the immune system, and location of natural habitat, the disease is very difficult to diagnosis and treat. *B. pseudomallei* is a gram negative bacterium, with a genome split between two chromosomes. Since it is readily found in specific soils, areas where there is increased human-soil contact provide the bacterium opportunity to invade and establish an infection. This is often the case in countries where daily activities take people into rural areas, such as rice paddy fields. *B. pseudomallei* has been suggested to have evolved from an earlier species, *Burkholderia thailandensis*, by sluffing off unnecessary genes and acquiring those needed to make humans suitable hosts (7, 19). *B. pseudomallei* is transmitted often through direct contact with, or inhalation or ingestion of contaminated water or soil. This can be through cuts and abrasions on the skin surface that allow the bacteria access. The infectious dose for this organism is very

small, requiring as little as 10 CFU to establish an infection. Zoonotic transmission, sexual transmission, and vertical transmission are all believed to be uncommon for this bacterium (43).

B. pseudomallei uses a variety of virulence factors to infect and persist in a host. It is also hardy in challenging environmental conditions, including a broad pH range, antibiotic presence, nutrient deficiency, antiseptic and disinfectant treatments, and extreme temperatures (29). In addition, it is able to overcome a variety of host innate natural defenses. Examples are lysosomal defensins and cationic peptidases (29). While there are some differences in virulence factors corresponding to severity and type of infection, the following few factors are common and of particular note. *B. pseudomallei's* capsule provides an important defense against a range of phagocytic cells. The capsule is comprised of a group of 3 capsular polysaccharides. It interferes with the complement factor C3b and its ability to opsonize the surface of the bacterium, leading to reduced levels of phagocytosis (45).
The capsule also aids in epithelial cell attachment, and has other hypothesized functions (37).
Secretion systems play on important role in many of the bacterium's survival functions. The Type 3 secretion system (T3SS) is particularly important, as it produces and exports effector products into the host cell that help the bacterium to invade and establish an infection. Examples are BopE and Bsa, which, when knocked out, leave the bacterium unable to hijack the host cell's actin (45). These are important virulent factors that allow the bacterium to take control of the immune cell, and like *Listeria*, hide from the immune system while moving from cell to cell without exiting the cell. This creates problems for the immune system in reaching the bacteria directly. Type IV pili are known to be important factors for many gram-negative

bacteria. In *B. pseudomallei,* the Type IV Pilin, PilA, has been shown to aid the bacterium's adherence to epithelial cells (31).

Burkholderia thailandensis

B. thailandensis is typically avirulent towards humans. There are very few exceptions to this. The following are recorded cases of *B. thailandensis* infection. In 1999, a 16-year old male was treated after a motorcycle accident in Thailand. He was treated with an antibiotic regime against *B. pseudomallei* and survived (23). In 2006, a 2-year old child was hospitalized in Texas, USA after near drowning and developed a *B. thailandensis* infection. He survived (28). In 2013, a 67-year-old man in China was found with a chest infection after two weeks of fever. After days of diagnostics, he was discharged at the request of family, and died 2 days later. (32). In all three cases, the patients experienced a traumatic event, which is believed to have assisted *B. thailandensis* in establishing an infection. As a result of its general avirulence in humans, *B. thailandensis* is classified as a Biosafety Level 1 agent. It is also a gram negative rod found in certain soils, but with minor and important differences from *B. pseudomallei*.

B. thailandensis vs *B. pseudomallei*

In many ways *B. thailandensis* and *B. pseudomallei* are quite similar. In culture, both organisms produce a variety of colonial morphologies. The reason for these different forms is yet unknown. Both species possess an innate resistance to multiple antibiotic families. They share the same natural environment, and possess similar growth and survival characteristics. Studies have been done to show that when cloned into *B. thailandensis*, the Burkholderia type 3 secretion apparatus (Bsa) causes *B. thailandensis* to acquire virulence similar to *B. pseudomallei* (26). Due to its low health risks, lack of government regulation, lower cost to

research, genomic similarity to *B. pseudomallei,* and ease in distinguishing the difference, *B. thailandensis* makes an ideal candidate to study in tandem with, and in some cases, in place of *B. pseudomallei* (26). While these two species are almost genomically homologous, there are some important differences. The most important is the lack of the *ara* operon in *B. pseudomallei*. This operon codes for the proteins needed to assimilate arabinose, and its presence or absence has been the main distinguishing phenotypic factor between the two species for years. Another important difference is the lack of a capsule in *B. thailandensis* (13). This may be one of the main differences in virulence known so far. Beyond this, there are other gene clusters that have been lost or gained in the *B. pseudomallei* genome, thought to be associated with evolving virulence in humans. Much research is currently being dedicated to the understanding of additional virulence factors of *B. pseudomallei*, and how they contribute to the overall virulence of this formidable pathogen.

Type 3 Secretion System

Secretion systems are protein structures embedded in the membrane of prokaryotic cells that allow passage of proteins and other substances into the host cell to aid the pathogen's survival. The Type Three Secretion System (T3SS) is one of six known secretion systems that the *Burkholderia* genus utilizes for pathogenicity and survival (Fig 1). In general, it consists of three main components: the base complex, the needle, and the translocon. The base complex forms across the inner and outer membrane, and is made up of multiple ring structures surrounding a central rod (4). The next structure, the needle, is connected to and protrudes from the rod. This needle is hollow, allowing secreted proteins, or effectors, passage to the target organism. The third structural component is the translocon, or the tip of the

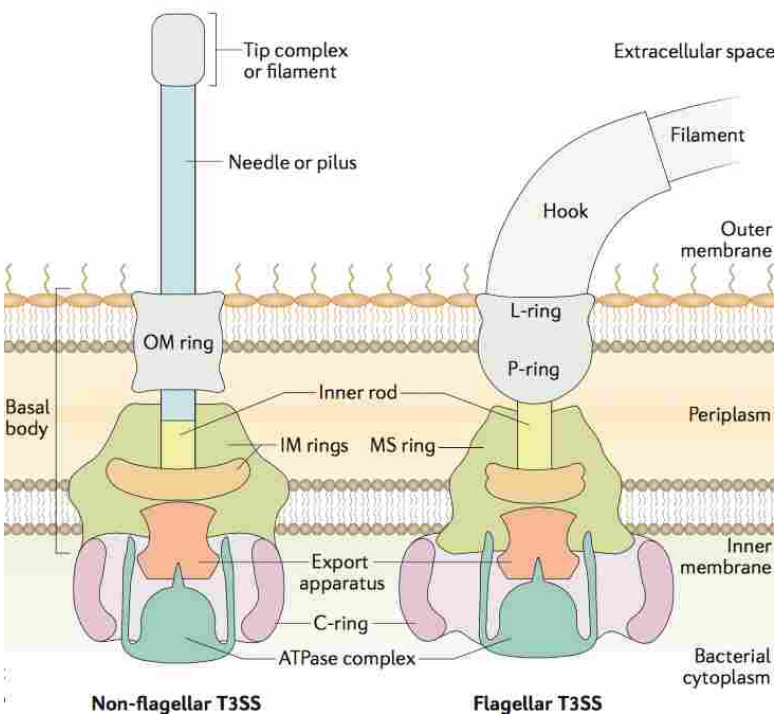


Figure 1. General overview of the structure of the Type Three Secretion System. From Finlay et al. (81)

needle. This structure senses contact with the host cell and regulates the secretion of the effectors (4). When properly attached to the host, a pore is formed through which effectors pass, allowing the bacteria to disrupt, change and establish new functions in the host cell, the goal of which is to allow persistence of the pathogen (4).

Burkholderia pseudomallei is known to have three T3SSs. T3SS-1 and T3SS-2 are not well characterized, but are known plant secretion systems homologous to those in *Ralstonia* and *Xanthomonas*. The T3SS-3 structural proteins are better understood, and are conserved across other bacterial families (27).

Due to the similarity in which *B. pseudomallei* invades hemocytes and macrophages, we believe that the various *Burkholderia* species use the T3SS-3 to invade and establish an infection in the hemocytes of *Galleria mellonella* larvae (3, 30).

Melioidosis

Melioidosis, or Whitmore's disease, is a systemic disease with a wide spectrum of symptoms. This makes diagnosis difficult, and misdiagnoses are not uncommon (43). This increases the number of deaths attributed to the disease (22). There are four major types of infection: pulmonary, septicemic, localized, and disseminated (43). Each of these types of infections carry distinctive symptoms. The most common disease is a pulmonary infection in which diagnosis is problematic, as symptoms can range anywhere from those of a mild bronchitis to those associated with a severe pneumonia. Additional symptoms include a high fever, headache, chest pain, and can sometimes also produce muscle soreness and anorexia (43). Localized infections are the easiest to diagnosis, as they usually manifest with swelling and a noticeable nodule, ulcer or skin abscess. These can be accompanied by fever or mild muscle aches (43). Disseminated infections commonly produce abscesses in the liver, spleen, lungs and prostate (43). Finally, during a bloodstream infection, individuals may have abscesses throughout the body, including the liver, spleen or prostate. The symptoms progress rapidly, and can include disorientation, muscle soreness, joint pain and abdominal pain (43). Those with diabetes or renal diseases are most affected by this type of infection, and it often results in septic shock. The incubation time can range from a few days to years, adding another difficult variable towards correct diagnosis. Currently there are no approved vaccines, so the public health concern remains high (14). Previous vaccine candidates have been evaluated, and none are currently ready for a clinical or commercial setting (14). Endemic regions of *Burkholderia* include South-East Asian countries and Northern Australia, and the countries with the highest number of reported cases of the disease are Thailand and Australia. However, this does not

take into account the constant problem of melioidosis underreporting, and therefore, there may be other countries with significant mortality and infection rates (22).

Worldwide there are an estimated 165,000 cases annually, with 89,000 deaths (Fig 2, 34, 22). Mortality remains at a surprising 40%, regardless of treatment. It is believed that the disease is underreported in as many as 79 countries. Roughly 75% of reported cases occur during the rainy season (21). This wide reaching disease needs further research, and developing cheap alternative virulence models is paramount to its study. Those populations most at risk include: diabetics, people with a pre-existing lung disease or complication, people with renal disease or complications, alcoholics, and immunocompromised patients.

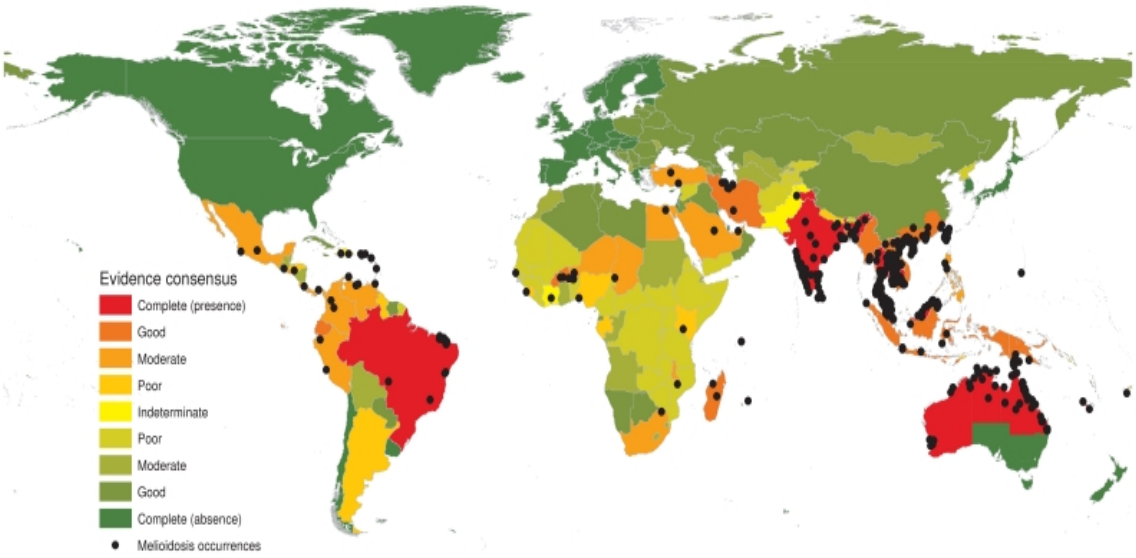


Figure 2. Global evidence consensus and geographic locations of melioidosis occurrence from 1910 to 2014. Country coloring is based on evidence-based consensus with green representing a complete consensus on absence of *B. pseudomallei* and red a complete consensus on presence of *B. pseudomallei*. Black dots represent geo-located records of melioidosis cases or presence of *B. pseudomallei*. From Limmathurotsakul et. al. (21).

Galleria mellonella

G. mellonella larvae can be found worldwide (46). In the past decade, increasingly more research has found this organism to be a useful alternative virulence model for bacterial, viral and fungal invasion, as well as for bacteriophage, toxin and other therapeutic treatments (25,6, 39, 40). It possesses a number of advantages over traditional mammalian virulence models. The financial input to set up and maintain a constant *G. mellonella* stock is far less than a rodent model, and there are no regulatory bodies that limit experimentation (47). They do not require feeding or other routine care, and can be housed for weeks until needed (25, 35). The cheap aspect of their breeding and maintenance allows high-throughput testing, providing the option for higher statistical weight. The larvae can be kept at 37°C, which provides an environment conducive to the human body and the optimal growth temperature for many pathogens. Due to their size, a precise inoculum of bacteria can be injected without difficulty or concerns of variability. The larvae possess a basic but similar innate immune system to humans, allowing limited but easy comparisons for early studies of virulence factors and treatments. Their immune system is comprised of an innate response, providing an assortment of phagocytic cells, nodulization and large scale encapsulation, melanization, haemolymph clotting, and anti-microbial peptide production (41).

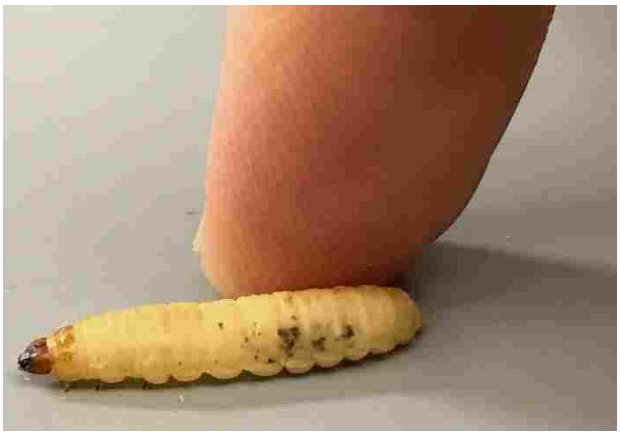


Figure 3. A larvae of the Greater Wax Moth, *Galleria mellonella.* Nodulation has begun to occur, as seen from the black splotches on the lower half of the larvae.

The primary immune cell of the larvae, hemocytes, are surprisingly similar in cellular structure and function to mammalian phagocytes. There are at least 8 types of hemocytes known, each with specific function and purpose (41). Some of the similarities are as follows; during a cellular immune response, hemocytes are able to differentiate between self and non-self. The phagocytic cell's activity is activated by the process of opsonization, utilizing complement-like proteins. Insect cells also produce a superoxide after a pathogen is engulfed due to a membrane-bound enzyme system being activated. Hemocytes possess surface receptors, allowing pathogen recognition. Nodulation, the binding of hemocytes to bacteria to form clusters, is the primary defense mechanism (Fig 3). Once complete, melanization of the nodules will occur (Fig 4). While this is not the same process as in humans, the complement pathway used is similar (41). Table 1 shows the similarities between hemocytes and neutrophils relative to the innate immune response.

Table 1. Summary of similarities between insect hemocytes and human neutrophils. From Browne et. al (41)

	Hemocytes	**Neutrophils**
Phagocytosis	Lectin-mediated	Lectin-mediated
ROS	O_2^-, H_2O_2, NO^-	O_2^-, H_2O_2, NO^-
Degranulation	Yes	Yes
AMPs	Peroxynectin, transferrin, lysozyme, defensins	MPO, transferrin, lysozyme, defensins
Receptors	TLRs, B-1,3-glucan, IL-IR	TLRs, B-1,3-glucan, IL-IR
Transcription factors	NFκB, IκB	NFκB, IκB
Cascades	IMD, JNK, JAK-STAT	IMD, JNK, JAK-STAT
Kinases	p38 MAPK, ERK, PKC, PKA	p38 MAPK, ERK, PKC, PKA
Neutrophil extracellular traps (NET)	NET-like structures present	NETs present

Previous *G. mellonella* model use with *Burkholderia*

Table 2 shows studies in which *G. mellonella* has been used to model infections with *B. thailandensis* and *B. pseudomallei*. In the few studies that have been performed, there is considerable variation in the survival percentages, revealing the need for further studies to better establish this model.

Table 2. Studies that used *G. mellonella* as a model for *B. thailandensis* and *B. pseudomallei* infections in the last 10 years.

Species and Strain	Concentration CFU/ml	Survival (%)	Time post injection(hrs)	Source
B. thailandensis				
E264	10	100	24	This study
E264	10^2	90	24	This study
E264	10^2	50	24	3
E264	10^3	3	24	This study
CDC2721121	10^2	0	24	3
CDC2721121	10^4	0	24	6
B. pseudomallei				
K96243	10	<20	48	10
K96243	10	100	24	This study
K96243	10^2	0	24	3
K96243	10^2	77	24	This study
K96243	10^3	50	24	5
K96243	10^3	0	24	This study
K96243	10^4	30	24	16
K96243	10^4	0	30	16

G. mellonella is a cheap and efficient infection model for *B. pseudomallei* and *B. thailandensis* that gives an accurate representation of human infection.

Materials and Methods

Bacterial strains and culture growth

All bacterial strains were grown in/on LB nutrient broth and agar unless specified otherwise. All strains were obtained from the American Type Culture Collection (ATCC), Manassas, VA, excluding *B. pseudomallei* K96243, which was obtained from The National Collection of Type Cultures (NCTC), London, UK. The fresh injection inoculum for the various strains was prepared by inoculating 5ml LB broth with an isolated colony, and incubated at 37°C with shaking at 200 rpm. After ~18 hours, 100µl was added to a fresh 5ml tube of LB broth,

which was incubated at 37°C with shaking at 200 rpm until an OD_{600nm} of 0.1 was reached. Serial dilutions were performed in PBS to reach the desired CFU/ml. Spread plates from these dilutions were performed in three independent tests to obtain viable counts at 0.1 OD_{600nm}. For frozen inoculation suspensions, a 5ml LB broth was inoculated with an isolated colony and incubated at 37°C and shaken at 200 rpm for ~18hrs. From this culture, 100 μl was used to inoculate a 5ml LB broth subculture. This was grown into log phase and serial dilutions were performed in 15% glycerol and PBS. Stocks of 100 μl aliquots were created and stored at -80°C. After one week, three stocks were thawed and viable concentrations were assayed by further dilution and spread plating, followed by incubation at 37°C for 48hrs, and colony counting.

Galleria mellonella care and injection

Galleria mellonella larvae were purchased from Best Bet Waxworm, MN, and maintained at 15°C in the dark until injection. Larvae were used within 2-3 weeks after purchase. Larvae were injected in the left upper proleg, using a 50μl Hamilton gas syringe with 10 μl of inoculum. Death was scored by observed darkening due to melanization and/or no movement after gentle manipulation. Controls for needle trauma, carrier used, and injection location on larvae were performed. Briefly, 10 μl PBS was injected into 10 larvae and death over time was recorded. Larvae were also injected in their lowest left proleg. If any of the controls died, the results from this test were discarded.

Statistical Analysis

The statistical software GraphPad was used to create the Kaplan-Meier graphs, bar graphs, to perform the t-tests, and the Wilcoxon signed-rank tests.

Results

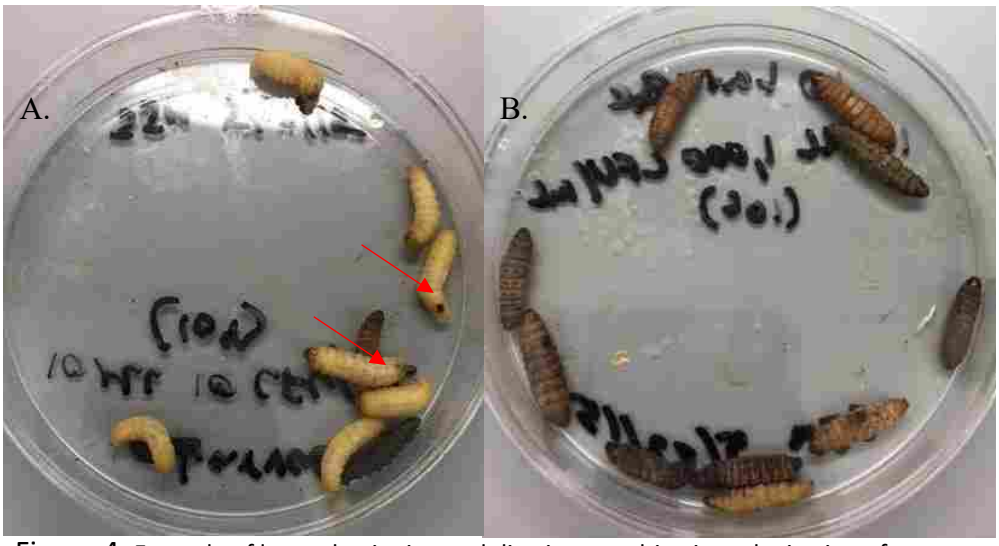


Figure 4. Example of larvae beginning nodulization, resulting in melanization of individual segments. A) Larvae mostly still healthy, with some nodulization (red arrows). B) Complete melanization of larvae, resulting in death.

Infection of *G. mellonella* with *B. pseudomallei*

Two types of *B. pseudomallei* were prepared for injection, fresh and frozen stocks. The frozen suspensions produced a uniform increase in time for an LD_{50} as the concentration of inoculum decreased, roughly two more hours for every ½ log decrease in inoculum (Figure 5). These were 1,000 CFU at 24hrs, 500 CFU at 26hrs, 100 CFU at 28hrs, 50 CFU at 30hrs, and 10 CFU at 36hrs. Likewise, with the fresh *B. pseudomallei* suspension, the survival curves followed a similar pattern, but did not result in the same LD_{50} values as the frozen stocks. They reached the LD_{50} in less time. Values were 1,000 CFU at 20hrs, 100 CFU at 26hrs, and 10 CFU at 30hrs. Figure 5C shows a comparison of the two suspension types at 1000 CFU. Using the Wilcoxon signed-rank test, we find that there is a highly significant difference in LD_{50} between the suspension types ($p = 0.0005$).

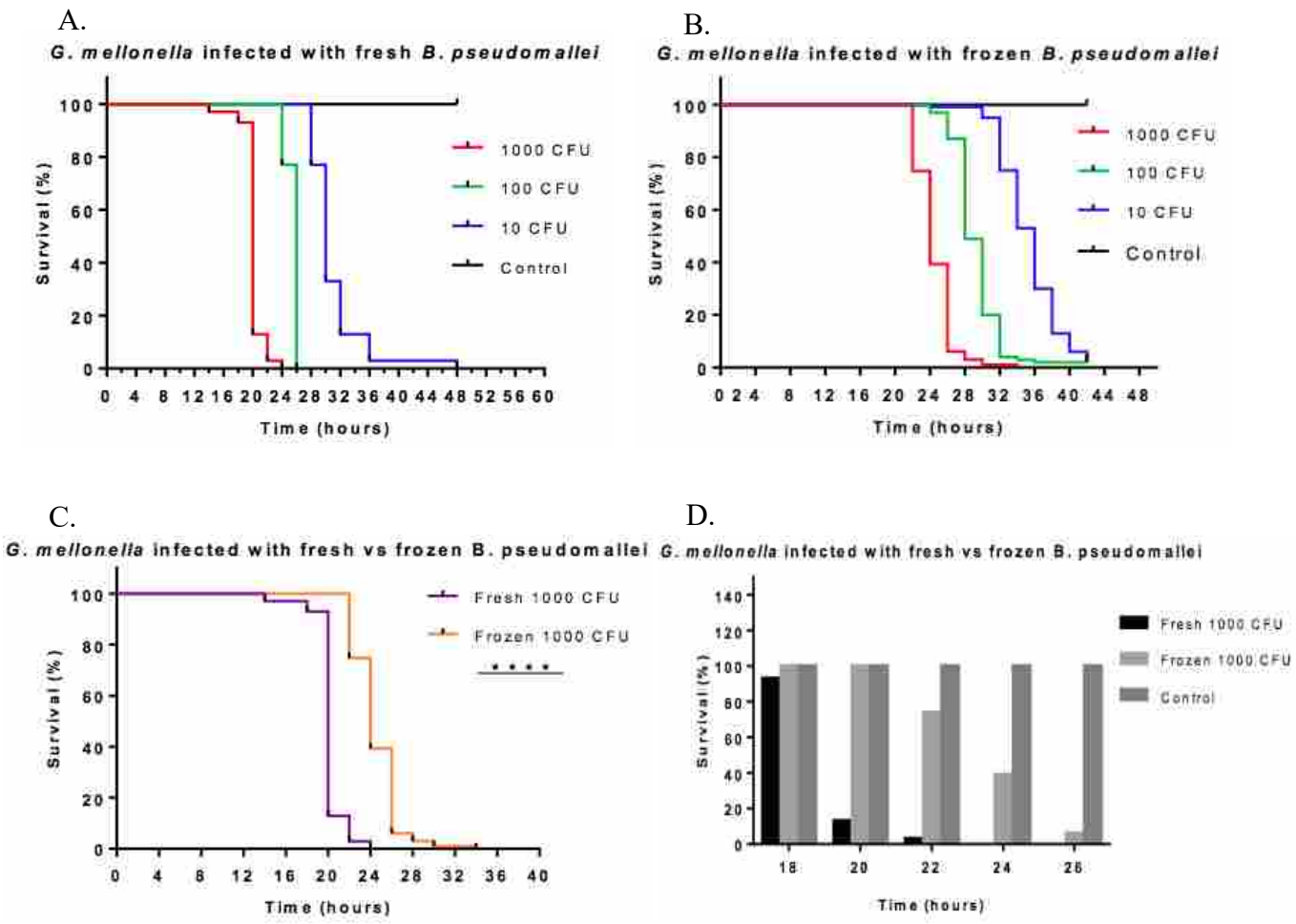


Figure 5. Survival of *G. mellonella* larva injected with various concentrations of *B. pseudomallei*. Two suspensions of *B. pseudomallei* were prepared, fresh and frozen. At least 90 separate larvae were infected at each CFU concentration and their death rates were averaged to calculate the survival curves. A) *G. mellonella* infected with fresh inoculum of *B. pseudomallei.* B) *G. mellonella* infected with frozen inoculum of *B. pseudomallei.* C) Kaplan-Meier survival curve comparison of fresh and frozen suspensions of *B. pseudomallei* at 1,000 CFU. D) Bar chart comparison of % survival using the two suspensions of *B. pseudomallei* at 1,000 CFU. **** denotes P value < 0.0001 using the Wilcoxon signed-rank test.

Infection of *Galleria mellonella* with *B. thailandensis*

Fresh and frozen suspensions of *B. thailandensis* were prepared and injected into *G. mellonella*. Similar to the *B. pseudomallei* results*, B. thailandensis* caused the larvae to die in the same pattern. In Figure 6A we see after inoculation with the fresh stocks, the larvae reach an LD_{50} 4-5hrs later as the CFU decreases by a log. In Figure 6B we find the frozen stocks of *B. thailandensis* follow the same trend. Beginning with 1,000 CFU at the LD_{50}, there is a consistent

delay of 6hrs added as the CFU dropped one log. Interestingly, there was only a 2hr increase in time to reach the LD_{50} when the CFU dropped half of a log.

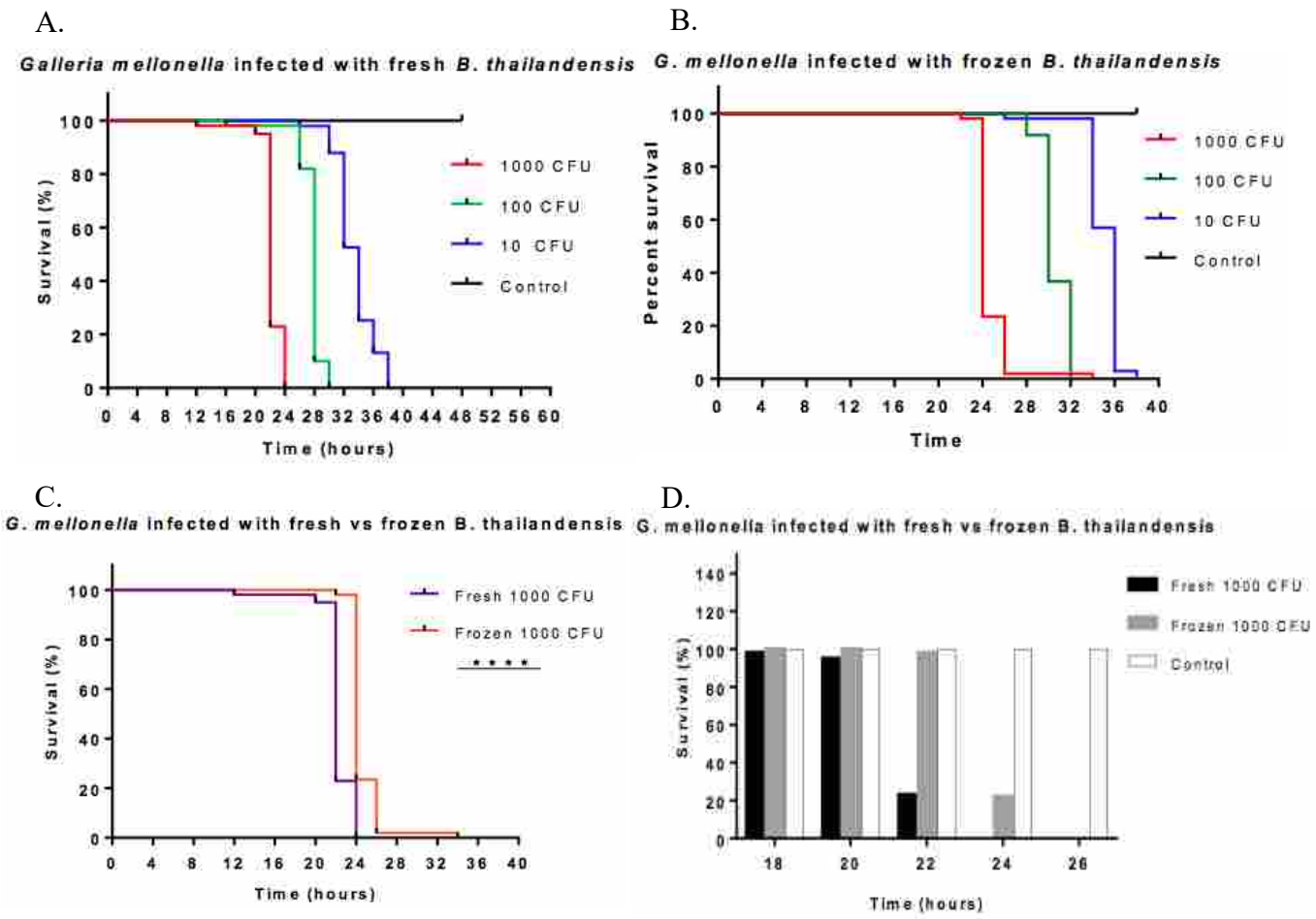


Figure 6. Survival of *G. mellonella* larva injected with various concentrations of *B. thailandensis*. Two suspensions of *thailandensis* were prepared, fresh and frozen. ≥90 separate larvae were infected at each CFU concentration and their death rates were averaged to calculate the survival curves. A) *G. mellonella* infected with fresh inoculum of *B. thailandensis.* B) *G. mellonella* infected with frozen inoculum of *B. thailandensis.* C) Kaplan-Meier survival curve comparison of the fresh and frozen suspensions of *B. thailandensis* at 1,000 CFU. D) Bar chart comparison of the two suspensions of *B. thailandensisi* infected with 1,000 CFU. **** denotes P value < 0.0001 using the Wilcoxon signed-rank test.

Comparison of *G. mellonella* survival between species

When injected with the fresh inoculums of both species there was a statistically significant difference in survival of larvae (Figure 7A, B). Interestingly, unlike the fresh stocks, the frozen stocks did not show any statistical difference at the LD_{50} between *B. pseudomallei*

and *B. thailandensis* (Figure 7C, D).

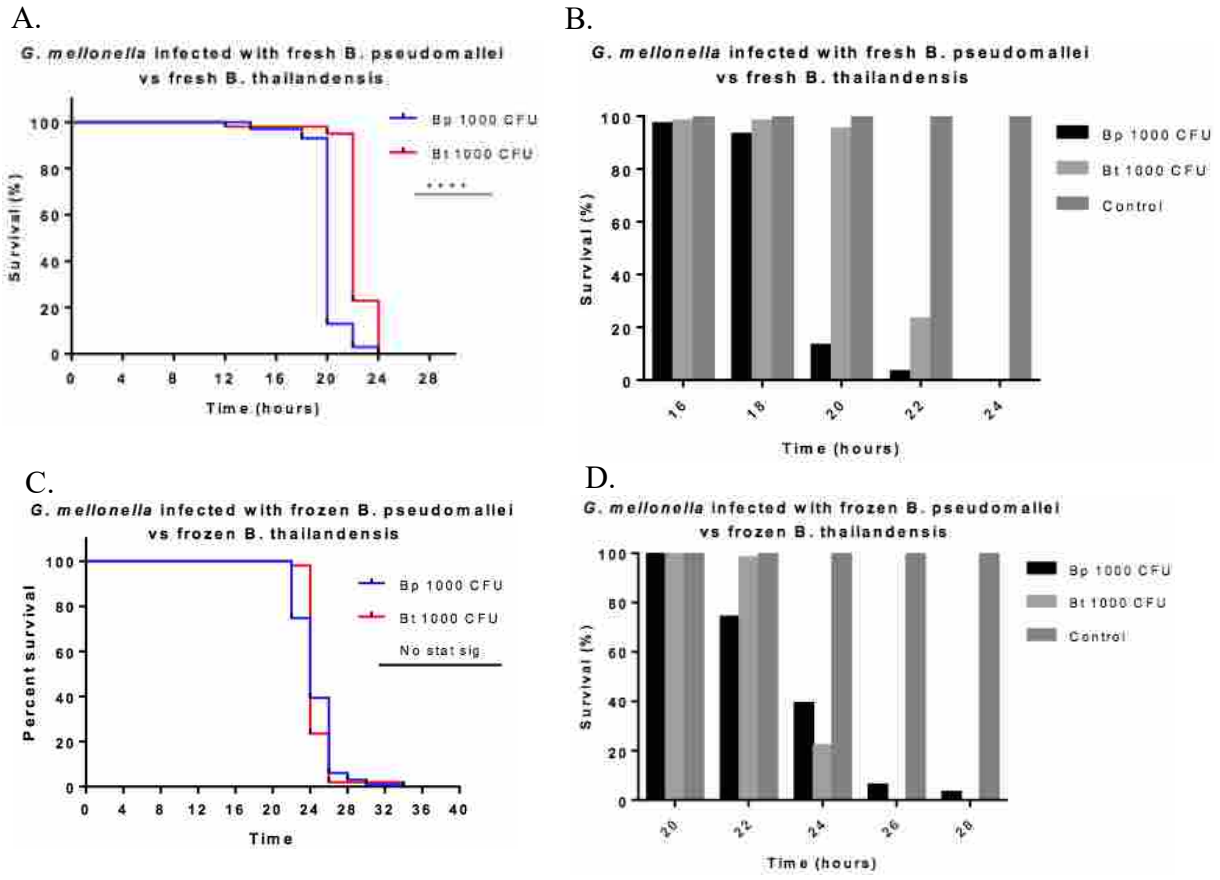


Figure 7. Kaplan-Meier survival curves and bar charts of *G. mellonella* infected with *Burkholderia*. A) Comparison of *G. mellonella* death after injection of *B. pseudomallei* and *B. thailandensis* fresh suspension. B) Bar chart comparison *G. mellonella* death after injection of *B. pseudomallei* and *B. thailandensis* fresh suspension. C) Comparison of *G. mellonella* death after injection of *B. pseudomallei* and *B. thailandensis* frozen suspensions. D) Bar chart comparison *G. mellonella* death after injection of *B. pseudomallei* and *B. thailandensis* frozen suspension. **** denotes P value <0.0001 using the Wilcoxon signed-rank test.

Species from the *B. cepacia* complex

Further injections of *G. mellonella* were performed using species from the *B. cepacia* complex, to evaluate their effects with this model. None of these species produced an LD_{50} within a reasonable length of time. These results were expected and are consistent with their reported virulence in other animals and humans (Figure 8).

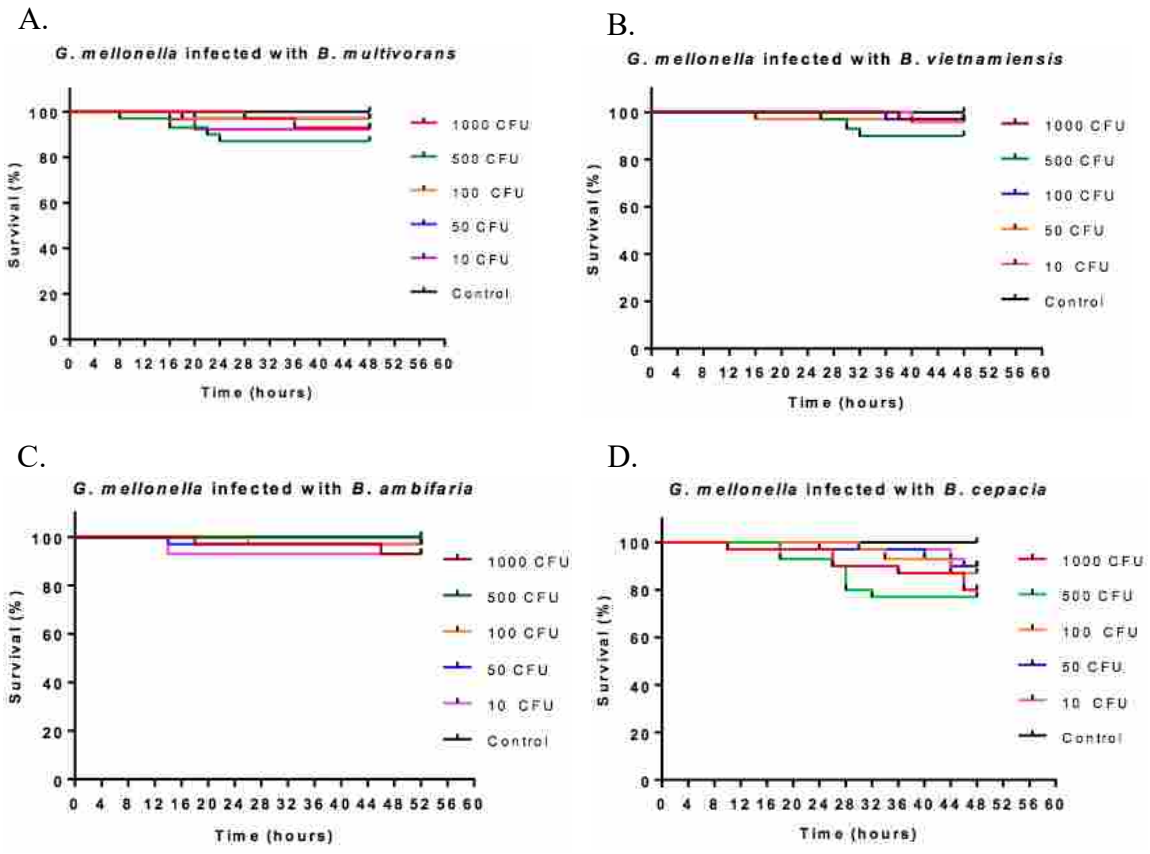


Figure 8- Kaplan-Meier survival curves of *Galleria mellonella* injected with various *Burkholderia cepacia* complex species. A) Survival of *G. mellonella* injected with *B. multivorans.* B) Survival of *G. mellonella* injected with *B. vietnamiensis. C)* Survival of *G. mellonella* injected with *B. ambifaria. D)* Survival of *G. mellonella* injected with *B. cepacia.*

Discussion

A cheap alternative pathogenesis model for the study of *Burkholderia* would enhance the research efforts of scientists attempting to develop vaccines and treatments for those in endemic areas. We have expanded the efforts of other labs in attempting to use *G. mellonella* as a viable model to evaluate virulence for the *Burkholderia* species. Figure 5 showed that the difference in suspension preparation can cause a statistically significant difference in survival time of the larvae. We observed that larvae infected with the fresh suspension died 2-4 hours faster than those infected with the frozen suspension, at all CFU levels. This may be due to the

stress-induced effects of freezing on the bacteria, which could have affected their virulence capabilities. While not investigated at the molecular level, this concern was considered by Chantratita et al. when characterizing morphology types of *B. pseudomallei*, and the effect freezing had on their morphological changes (38). Figure 5 also showed that by 24 hrs post-injection (pi), no *G. mellonella* infected with 1,000 CFU of *B. pseudomallei* survived. A study in 2011 by Vanaporn et al. showed that 50% of their larvae remained alive using the same parameters (10). Further conflicting data from Muller et al. showed that 30% of larvae remained alive after 24 hrs pi with a log higher infection inoculum of 10^4 (16). In Figure 5, 77% of larvae infected with 100 CFU *B. pseudomallei* remained alive at 24hr pi, whereas a study in 2011 by Wand et al. found none survived using these same parameters (3). The causes for these differences are hard to identify, since much effort was made in this work to follow the methods of previous studies in order to maintain the consistency of the model.

B. thailandensis mirrored the same trend as observed for *B. pseudomallei*. Figure 6 shows results for the fresh and frozen prepared suspensions. There was a statistically significant difference between the Kaplan-Meier curves of the frozen and fresh 1,000 CFU *B. thailandensis* infections. While there are fewer studies with *B. thailandensis* in the literature, we saw the same variability. We show that at 24 hrs pi with 100 CFU, there was a 90% survival rate (Figure 6A), while Wand et al. showed a 50% survival using the same parameters (3). Again, there are conflicting finding from the current studies available.

We showed that with fresh suspensions at 1,000 CFU, there was a statistically significant difference between *B. thailandensis* and *B. pseudomallei* at 24 hrs pi (Figure 7A). While the difference in survival rates of larvae injected with fresh *B. pseudomallei* and *B. thailandensis*

(Figure 7A) was significant, it carries with it serious practical issues. The window of time between both populations succumbing to infection and death is only about 2 hrs. This leaves a small margin with which to experiment with virulence mutants, and may restrict the impact of otherwise promising findings. Surprisingly, with frozen prepared suspensions at the same parameters, there was no statistical difference (Figure 7C). Again, we hypothesize that the damage caused by freeze/thaw may have affected the bacterium's virulence capabilities, and delayed the death of the larvae infected.

Infections of *G. mellonella* with species from the *B. cepacia* complex did not yield noticeable kill within the tested time frame (Figure 8A-D). This is consistent with other studies using the same infection model (8, 9, 18). These species are not able to establish an infection in healthy humans easily, and most clinical cases are in cystic fibrosis or otherwise immunocompromised patients (9).

Previous studies have been performed to test the usefulness of *G. mellonella* as a virulence model for the *Burkholderia* species. The literature on this topic is limited in a number of ways. These limitations restrict the implications of their conclusions, making them less robust. These factors include: amount of data reported, length of survival experiment (many were terminated prematurely), lack of challenge variability, lack of *Burkholderia* species tested, and small population size tested. We have taken these factors into account, as well as others to improve upon and add to this virulence model. Additional variables that we included in our testing beyond those stated were age of larvae at time of injection, incubation temperature of larvae post injection, temperature of inoculation preparation, frozen vs. fresh inocula, sterility of larvae pre-injection, and standardization of CFU in the inocula. We found no difference in

survival rates between disinfected vs. non-disinfected larvae pre-injection (data not shown). The temperature of incubation post injection had a large effect on survival of larvae (data not shown). This is assumed to be due to the bacteria not being at their optimal growth temperature, and thus not replicating or producing the proteins needed for establishing an infection in the larvae.

It was interesting to note the patterns of death produced by different species of *Burkholderia*. Both *B. thailandensis* and *B. pseudomallei* exhibit kill in which one to three larvae die due to infection after at least 10 hours. This is followed by the majority of larvae suddenly dying, with up to 80% of larvae deceased within a 2-hour period. Finally, any remaining larvae that were holding out succumb to the infection and die within a few hours more. This pattern was very consistent and reproducible. While more research must be done, we can hypothesize about what is causing the sudden death of the larvae. One train of thought follows the notion that there is an established point at which a certain level of melanization causes the larvae to simply shut done, no longer able to maintain organ functionality. This is confirmed visually as the larvae increase in nodulization and black pigmentation up to the point that they die. Another contrasting view is that at a certain time point, the bacteria are able to freely roam through the larvae, causing a systemic infection. This hypothesis would help explain the larvae which do not follow the traditional pattern of increasing in discoloration up to death. We found that there were occasional larvae that not only showed a lack of melanization at death, but were still 'healthy' looking according to the color. (These larvae were confirmed dead after gentle manipulation) Whatever the cause, there was a clear pattern of infection and reactionary behavior from the larvae.

As a model organism, *G. mellonella* is becoming more frequently used for the benefits stated previously. The greatest problem with this model is the lack of standardization of important factors involved in rearing, care, and injection of the larva. In their review, Cook et al. discussed the ramifications of a lack of genetic data, stating the negative outcome of, "genetic variability or epigenetic difference between populations on experimental outcomes." (35) Since there are a variety of locations from which these larvae may be acquired, and labs can rear them in disparate ways, differences in larvae will continue to be a significant variable (35). Tinsley et al. showed that genetic variations in populations of *D. melanogaster* caused varying susceptibility against some microbial pathogens (42). These factors could be a reason for the conflicting results many of the studies have produced. For now, it would be hard to establish one genetically stable population as this model is used internationally and one source of the larvae would negate the advantage of being able to obtain them cheaply and quickly.

Conclusion

We have found that *G. mellonella* was not an accurate representative of human infection for *B. pseudomallei* and *B. thailandensis*. These findings come contrary to previously published studies, which state that this model follows the natural *human* infection response against members of the *Burkholderia* genus.

Author Contributions

Experiments were performed by Joseph Thiriot and Taalin Rassmussen. Thesis was written by Joseph Thiriot. Experimental design, experiment performance and editing of thesis was overseen by Richard Robison.

CHAPTER 2- A comparison of Suspension and Surface Test methods for evaluating sporicidal efficacy

Introduction

Nosocomial Infections

Each year a discouraging number of patients acquire a healthcare-associated (HAI) or nosocomial infection while visiting the hospital. Roughly 1.7 million HAIs are reported in hospitals around the US each year. These infections cause 99,000 deaths, and ~$20 billion additional healthcare related expenses (68). This heavy burden on our healthcare system, and staggering number of preventable deaths, is a grave concern for all involved in the healthcare industry. The number of deaths here is truly worrying, as it towers over other disease-related mortality rates each year, such as pneumonia deaths at 51,811, HIV at 6,465, Hepatitis A-C at 7461, and flu deaths of >80,000 during the past 2017-18 season (69,70). The government has made noticeable efforts to reduce these numbers, and in 2009 initiated the 'National Action Plan to Prevent Health Care-Associated Infections: Road Map to Elimination' (79). This plan outlines five year goals for various parties to work towards in a collaborative effort. Despite these efforts, and the positive changes we have seen so far, further efforts are needed to prevent these infections. One approach is the improvement of disinfectants used in the hospital environs and on equipment that comes in contact with patients.

Disinfectant Testing

Before a product can be used in a Health Care setting it must first undergo rigorous testing to ensure its efficacy, and to fully understand any safety issues it presents to the environment and personnel. As such there is a need to have tests that correctly and accurately evaluate a given disinfectant. To date there is no international governing body that controls the

regulation of such products, and there is no set standard testing used throughout the world. As a result, there are many different methods used to test the efficacy of disinfectants. However, some nations have methods approved and enforced, such as the USA, which are enforced by the Environmental Protection Agency (EPA) and Food and Drug Administration (FDA). The wide range of test methods can be separated broadly into three categories: Suspension, Carrier, and Surface tests (64). There are different tests within these three categories, but our primary focus will be on the broad categories. As will be discussed, each carries benefits and weaknesses. Each method provides a different look into the usefulness of a given disinfectant against the chosen organism under various conditions.

Suspension Method

Suspension tests are the most basic of the three, and provide the best full contact between the disinfectant and the challenge organism. Briefly, an aliquot of a defined bacterial suspension is added to a known volume of liquid disinfectant, which is immediately mixed. At this point there is also the option of adding a soil load (55). After specified contact times, an aliquot of the mixture is added to a tube of neutralizer, thereby stopping disinfectant action. The suspension is assayed for viable organisms via serial dilution and various plating methods, including membrane filtration. Colony forming units (CFUs) are counted and a reduction factor is calculated. A current Suspension Method example is BS EN 13704 (75). The advantage of this method is the unimpeded and intimate contact between the disinfectant and the organism, allowing for maximum disinfectant activity. Because of this, suspension tests tend to be much more reproducible, and provide a truer picture of the disinfectant kill kinetics (55). It lacks, however, simulation to a plausible real-life situation in which the conditions would be similar.

Carrier Method

The Carrier Method involves small carriers (penicylinders) of various material (glass, metal, porcelain) that are suspended in a solution containing the challenge organism for a set time period. These now contaminated carriers are allowed to dry, then submerged in disinfectant for a specified time. Once removed from the disinfectant solution, they are placed in nutrient broth and incubated to verify kill of all challenge organisms (55). For kill to count, no organism can be found in the tube, which will be manifest by clear broth after incubation (76). This method is currently used frequently in the US, named the AOAC Use-Dilution Test, MB-05-14 (76). Due to the short contact time and the need to penetrate a dried biofilm, the antimicrobial solution needs significant biocidal activity, ensuring a high quality disinfectant. One can test many penicylinders in the same test. This method also has weaknesses. There is some statistical variability due to the test not requiring all carriers to pass for it to be counted as a pass test. Any physical variation in the carriers that give the challenge organism an edge to survive may be the cause of a failed tube. The test is hard to perform, and unskilled workers may produce false positives if not trained well or have achieved sufficient skill and experience (55).

Surface Method

In general, this method consists of taking a known volume of challenge organism and drying it on a surface, such as a small disk. Surface disks have long been used by the EPA and federal institutions of other countries in a standardized test method to measure the efficacy of disinfectants. These disks can be made of different materials, including plastic, metal and porcelain (55). The test procedure is as follows: an aliquot of an organism suspension with a

pre-determined concentration is placed on the disk and allowed to dry. The test disinfectant treatment is applied directly to the dried organisms on the disk, which is allowed to sit for the test contact time. Once completed, neutralizer is added to stop all disinfectant activity. This solution is assayed for viable organisms via serial dilution and a suitable assay for viable organisms like membrane filtration. This method has advantages over others such as its closer simulation of actual disinfection applications, and as a result, has gained acceptance over the other two test methods. Due to the process of drying organisms such as spores on the disk before treatment, this method represents a typical disinfectant to spore encounter on an environmental surface. In this regard the surface test gives us the best representation of how a disinfectant will perform under actual use conditions. However, there are limitations. The surface disks, while very similar, are never exactly the same. This is particularly true with stainless steel, where there is a brushed finish to the surface. Due to the variability of the brush pattern on each carrier and the unique way in which the spores dry on the carrier, there will likely always be slight differences in the nature of the dried spore films (64). Because of this, the reproducibility of this test suffers, and a standard is hard to establish. Surface-based methods are also inherently more variable because they involve at least two kinetic events: penetration of the dried biofilm by the disinfectant followed by its killing actions.

Spores

Endospores, or spores, are a non-reproductive, dormant stage that some bacterial genera produce to ensure their survival during harsh conditions (63). They are induced to form by external and environmental pressures, such as a lack of nutrients and water (62). Spore formation is most often a trait of some gram-positive rods. Spores can survive in their dormant

state for thousands of years. Due to their hardy nature, they become much more of an issue when found in any healthcare setting, as they require harsher disinfectants to affect the necessary kill.

Spore Attributes and Structure

Spores are very resistant to environmental stresses and chemical control measures, including extremes in temperature and pH, salinity, UV radiation, desiccation, and chemical disinfectants (63). This is due in large part to their structure. Spores are generally comprised of the following layers: exosporium, spore coat, outer membrane, cortex, germ cell wall, inner membrane, and core. However, there are exceptions to this order.

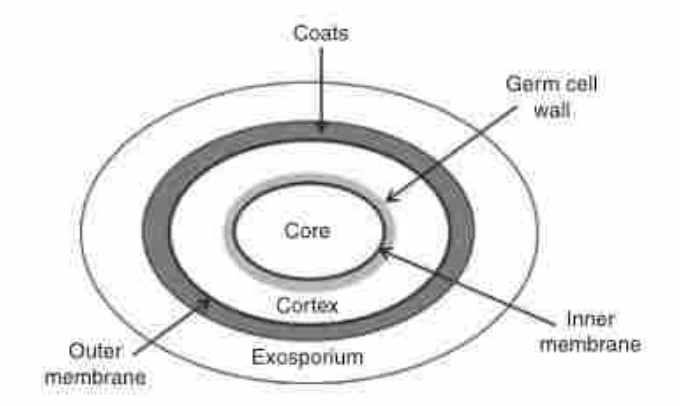


Figure 9. Spore structure. A representation of a 'typical' bacterial spore (structures not drawn to scale). Modified from Setlow (2006). -From Leggett et. al. (49)

Exosporium

The exosporium is the outer layer of *Clostridium difficile* spores. It is composed of protein (43-52% dry weight), carbohydrates (20-22% dry weight), and lipids (15-18% dry weight), with small amounts (~4%) of calcium and magnesium (49). In the context of clostridia, much remains to be studied to fully understand their spore structure. This is the layer that any

disinfectant will encounter once it gets past the biofilm and extracellular matrix build up. Up to now, it does not appear that the exosporium aids greatly to its chemical resistance (49).

Spore Formation

The formation of the endospore from a vegetative state is a survival tactic, and can occur for a variety of reasons. Once formed, the spore has the ability to remain inactive, or to reactivate into its vegetative state (80). Again, this is the result of external factors, like the sudden availability of nutrients. Figure 10 depicts the different phases of an endospore formation. While this is the norm, there are exceptions to this order of events among different bacteria (80).

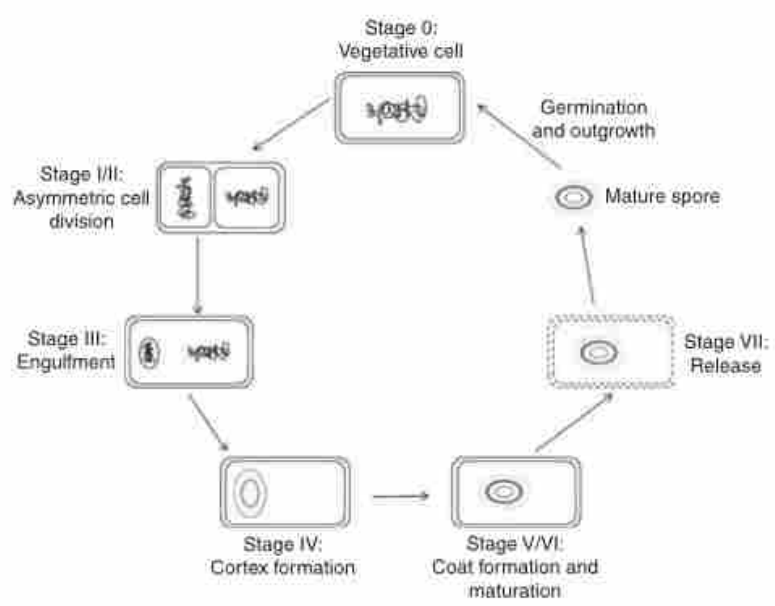


Figure 10. Key morphological changes that take place during sporulation. Modified from McDonnel (2007). From Leggett et. *al*. (49)

Current Statistical Models

To date there is a lack of statistical modelling for disinfectant testing in the literature. Some studies have tried to approach their analysis through tests of reproducibility for an

individual method. Others, to provide a statistical method to determine the pass-error and fail-error rates of the standardized Use Dilution Method (71). Another approach has been to develop a statistical model to establish the error rates of studies involving multiple laboratories and/or organisms (72, 74). These studies are important in providing statistical transparency and credibility to methods that are currently used, or that are being proposed for future use. They help in the essential role of reproducibility among different laboratories. While these are important studies, very little has been done in comparing different methods, and creating a statistical model to calculate their ability to assess a product successfully. In light of this absence, this study explores a novel statistical approach to compare the two methods of suspension and surface testing.

Materials and Methods

Spore Preparation

A test suspension containing endospores from *C. difficile* (ATCC #43598) was prepared following the US EPA procedure MB-28-01 (30). Briefly, an isolated *C. difficile* colony was used to inoculate 10 mL of pre-reduced Reinforced Clostridium Medium (RCM) broth, which was incubated anaerobically for 24 hr at 37 °C. From this culture, lawns were created on CDC Anaerobic Blood Agar (CABA) plates and grown anaerobically for ten days at 37 °C. The spores were harvested from each CABA plate by scraping them into suspension. The resultant suspension was centrifuged to pellet the spores. Spores were re-suspended in sterile PBS-Tween 80 solution. This centrifugation/resuspension process was repeated a total of three times. The spore suspension was then placed at 65 °C for 10 min to kill vegetative organisms, and the spores were purified using a HistoDenz (Sigma Aldrich, St. Louis) gradient. The final

viable *C. difficile* spore suspension was ascertained to be 1.11 x 10^8 spores/mL by serial dilution and colony growth on CABA plates. It was greater than 95% spore purity as determined by phase-contrast microscopy. The final spore suspension was stored at 4 °C until used.

Disinfectant Solution

A peracetic acid-based disinfectant was activated on Day 0 of the testing following the manufacturer's instructions. Briefly, the inner cap was compressed, releasing part B into part A. The 2 solutions were mixed thoroughly by repeatedly inverting the bottle. This same bottle of disinfectant was used throughout these studies, and evaluated as it decayed with age.

Neutralization Solution

The following neutralizer formulation was made fresh each day and used for both the surface and suspension tests. The Neutralizer solution consisted of 12.7% Tween 80, 6.0% Tamol SN, 1.7% lecithin, 1% Peptone, 1.0% Cysteine and 500 mM Tris (pH 7.0). The neutralizer was sterilized by autoclaving. For the carrier test, 10 ml of neutralizer was used per carrier, while 9 mL of neutralizer was used per tube for the suspension test.

Suspension Test

A 9.9 ml aliquot of the disinfectant was added to a sterile 50 ml polypropylene centrifuge tube. These tubes were equilibrated in a 20 °C water bath. Then, 0.1 ml of the *C. difficile* spore suspension was added at time zero. After the specified contact times (1, 2, and 5 min), 1 ml of this mixture was added to 9 ml of neutralizer. The tube was mixed thoroughly. After two min, the neutralized suspension was serially diluted in sterile 9-ml physiological saline solution (PSS) blanks. The number of viable organisms in selected dilution tubes was assayed by membrane filtration. One ml aliquots were plated in triplicate. The membranes were washed with about

100 ml of sterile PSS and removed to FA-HT plates (Fructose agar with horse blood and taurocholate). The plates were incubated at 37 °C for 72 hours under anaerobic conditions (ANOXOMAT system). The number of colonies on each filter was counted and log reduction and percent kill values were computed.

Surface Test

The ASTM E2197-11 protocol was followed (78). Briefly, the surface test consisted of the following steps. Ten µl of the spore suspension (containing approximately 10^6 spores) was deposited onto each carrier and the carriers were dried under vacuum in a desiccant chamber. At time zero, 50µl of the disinfectant was added to an inoculated carrier in a flat-bottom vial. After the specified contact time (5 min), 10 mL of neutralizer was added to the vial, and the vial was vortexed for 45-60 sec. The number of viable organisms released from the carrier were assayed by membrane filtration by pouring the entire contents of the vial into the funnel, and washing the vial with 10 mL of PSS three times. The membranes were placed on FA-HT plates. The process of preparing a disk and testing it thus far was repeated an additional nine times for a total of ten replicates. The plates were incubated at 37°C for 72 hours under anaerobic conditions (ANOXOMAT system). The number of colonies on each filter was counted and log reduction and percent kill values were computed.

Controls

Surface Test

An estimate of the number of viable spores present on a disk was computed by performing membrane filtration assays on selected 1:10 dilutions in PSS, on a vial containing 1 inoculated dried disk treated with 50µl PSS and 10 ml of neutralizer, and vortexed in an identical manner

to the disinfectant-treated disks. These controls were plated in triplicate.

A disk sterility control was completed by performing membrane filtration assays of a vial containing 50 ml of disinfectant applied to a sterile disk, and 10 ml of neutralizer. The entire contents of the vial were poured into a funnel with a membrane. The vial was rinsed with 10 mL of PSS three times.

Suspension Test

An estimate of the number of viable spores used in the suspension test was computed by performing membrane filtration assays on selected 1:10 dilutions in PSS of the spore suspension.

A neutralizer control for the test disinfectant was performed by inoculating a mixture of 9.0 ml of neutralizer and 1.0 ml of test disinfectant with 0.1 ml of the $1{:}1x10^5$ (from day 0 to 3) or $1{:}1x10^4$ (from day 4 to 25) dilution of the test suspension, which was then allowed to stand for 20 minutes prior to dilution and assay by membrane filtration using triplicate 1 ml samples.

Results

Log reductions of the suspension method at various contact times

Within the first 2 days of the testing, the disinfectant experienced a significant drop in sporicidal activity (Figure 11). This was expected, as the disinfectant efficacy begins gradual degradation. After this time point, the log reduction values exhibited decay at a significantly slower rate. At day 10, a noticeable decline in activity with time was seen with the 1 and 2-

minute contact times. The log reductions effected by a 5-minute contact time were consistent until day 15, after which a steep decline was noted.

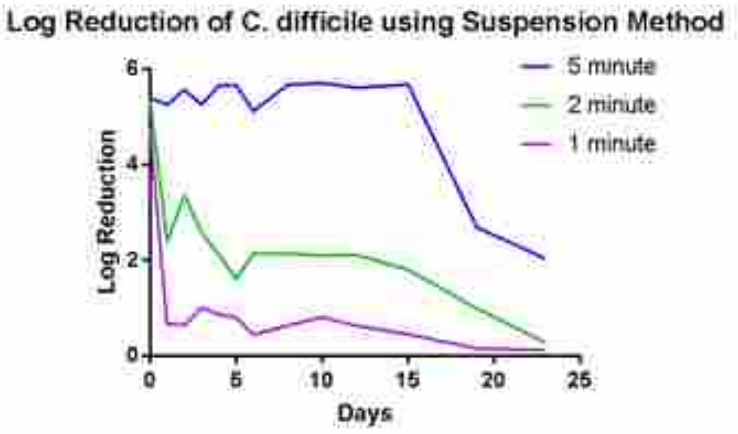


Figure 11. Log Reduction of *C. difficile* spores after disinfectant treatment using a suspension method. Three contact times were performed over 23 days. The same disinfectant solution was used.

A comparison of log reduction values obtained by suspension vs. surface methods

The log reduction values obtained from the surface method increased during the first 3 days (Fig 12). This was not expected. These values then decreased steadily until day 10, at which point the values increased slightly until day 15. After day 15, there was a steady decrease in activity with time until the termination of the experiment.

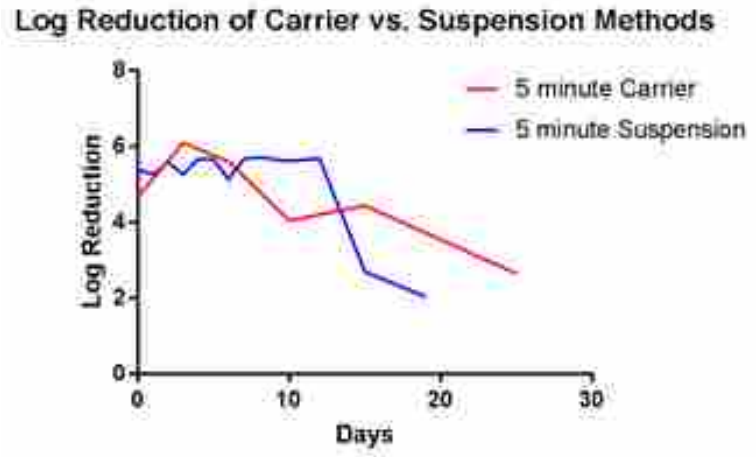


Figure 12. Log Reduction of *C. difficile* spores after disinfectant treatment. Surface and suspension methods are compared using a 5-minute contact time. The same disinfectant and spore solutions were used in both methods throughout the experiment.

The log reduction values obtained from the suspension method remained fairly steady with only slight fluctuations (<0.5 log reduction) up until day 12. After this time point, there was a steady decline in activity with time.

Statistical Analysis

A log linear model with Poisson distribution was attempted to model the colony counts from the two test methods. In particular, we modeled the log reduction of the colony counts, $-log\frac{N}{N_0}$, using the number of days passed after the disinfectant was activated for the carrier test. We modeled the same type of log reduction using the number of days passed after the disinfectant was activated and contact time of the disinfectant with the spores. Please note that N represents the observed count of the colonies under the experimental conditions, such as on which day and with what contact time, and N_0 represents the count of the colonies which we obtained through the corresponding control tests. The following two paragraphs describe the models that are obtained by SAS PROC GENMOD.

For the surface test with 5-minutes contact time, we obtain that

$$-log\frac{N}{N_0} = 3.8275 - 0.0531 * Days$$

where $Days$ are the number of days passed after the disinfectant was activated. It suggests that with every one day passed, the log reduction was reduced by 0.0531, and the log reduction of the disinfectant on day 0, the estimated log reduction of the number of colonies is estimated to be 3.8275.

For the suspension test with 1-minute contact time, we obtain that

$$-log\frac{N}{N_0} = 0.8388 - 0.0331 * Days$$

This suggests that with every one day passed, the log reduction was reduced by 0.0331, and the initial log reduction of the disinfectant on day 0 is estimated to be 0.8388.

For the suspension test with 2-minute contact time, we obtain that

$$-log\frac{N}{N_0} = 2.8181 - 0.1074 * Days$$

This suggests that with every one day passed, the log reduction was reduced by 0.1074, and the initial log reduction of the disinfectant on day 0 is estimated to be 2.8181.

For the suspension test with 5-minute contact time, we obtain that

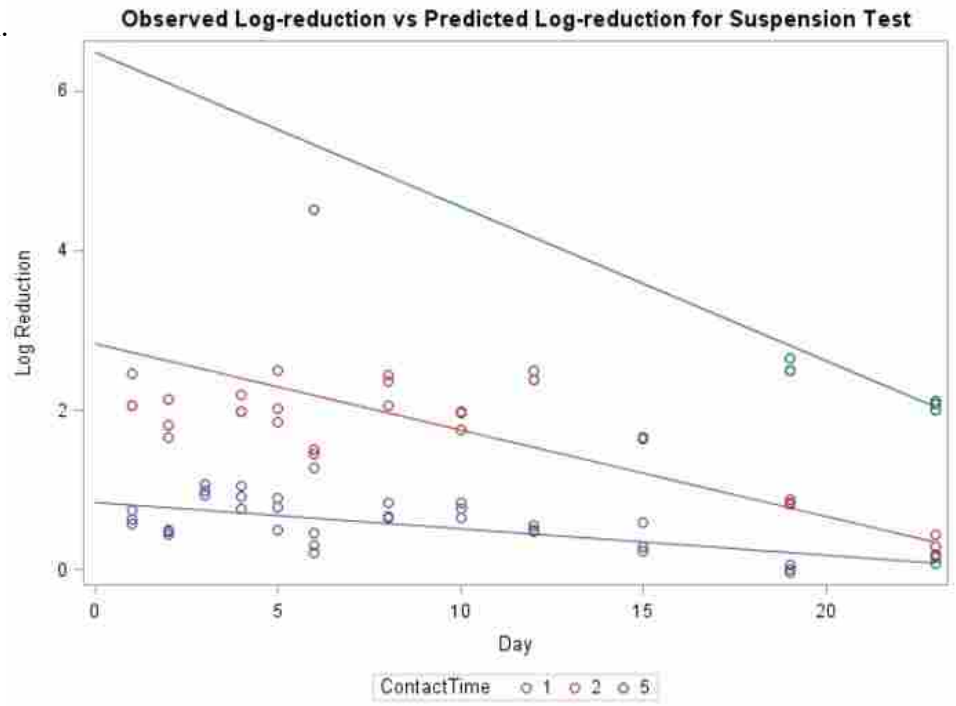
$$-log\frac{N}{N_0} = 6.1891 - 0.1750 * Days$$

This suggests that with every one day passed, the log reduction will reduce 0.1750, and the initial log reduction of the disinfectant on day 0 is estimated to be 6.1891.

Comparison of predicted log reduction values versus actual log reduction values

Using the formulae obtained from the statistical analysis, the predicted log reduction values over time were plotted against the observed log reduction values. In both suspension and surface methods we see that the predictive model strongly followed the observed values (Fig 13A, B). However, the surface method values had much greater variability compared to the predicted values than did the suspension values (Fig 13B).

A.



B.

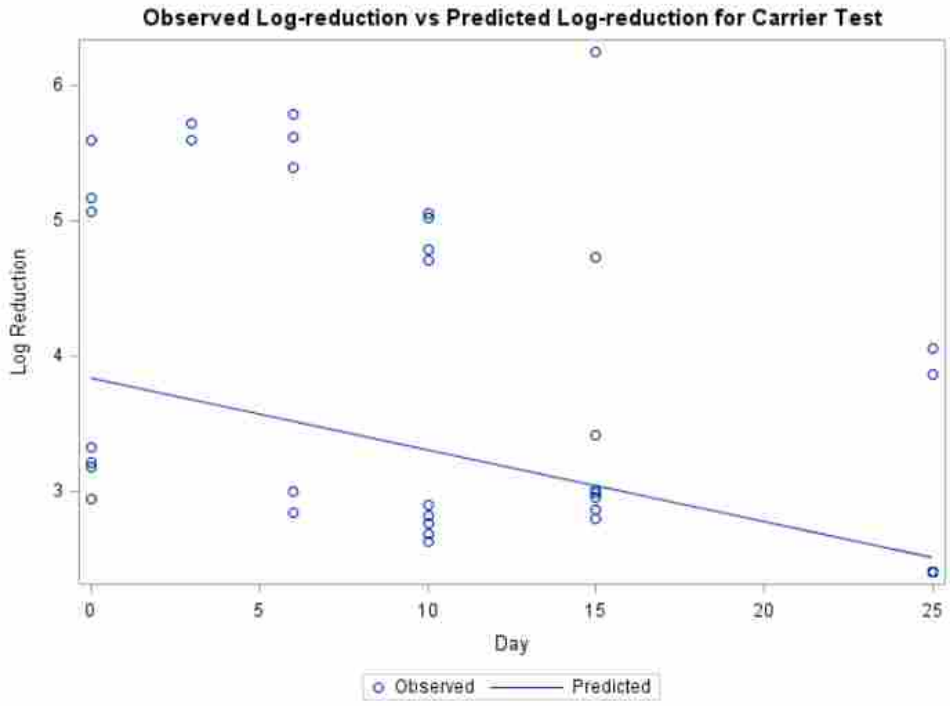


Figure 13. Predicted and observed log reduction values plotted against time(minutes) for A). the suspension method at all three contact times, and for B). the surface method at the 5-minute contact time. Lines denote predicted values and circles the observed values. Predicted values were obtained from the SAS PROC GENMOD statistical analysis software.

Discussion

Understanding the benefits and weaknesses of each test method is important in assessing which test provides the best evaluation of each disinfectant product. Studies have previously performed statistical analyses of current FDA approved methods (72), including their intra-laboratory reproducibility (73, 74), but have not done any analyses comparing the methods head-to-head. This study presents a novel statistical model to test the efficacy of two established testing methods.

Figures 11 and 12 show how the suspension and surface test methods predict sporicidal activity of a solution as its activity declines with age. The suspension method produces expected curves, showing the slow but predictable degradation of the disinfectant over time, with its corresponding decreases in log reduction of the *C. difficile* spores. Data from the surface method showed some unexpected trends (Fig 12). The initial increase in activity over the first 3 days was unexpected. The slight increase in activity after day 10 was also unusual. As the suspension method did not show these same trends, these anomalies could be due to the variability inherent in this method. As stated previously, there may be differences in the individual disks, or the way in which the spores dried on them, which allowed spores on these surfaces to resist, or be to protected from, the disinfectant due to interference from other spores in large clumps. It may be that some spores are able to reside safely in small marks and defects in the metal, thus being protected from full contact with the disinfectant.

Our statistical analysis provides a prediction of the change in log reduction over time according to method and contact time. This model predicts the suspension test log reductions well (Fig 13A). However, the initial change from day 0 to day 1 did not correlate with predicted

values for the 1 and 2-minute contact times. This may be explained in part by the drastic drop in efficacy of the disinfectant 24 hours after activation. These linear models are helpful for initial studies, and when considering a more complex models where additional covariates are included. They provide a framework to understand the pattern of the test and the disinfectant together over time.

For the surface test, when we compare the log reduction observed at day 0, which is 4.659, the predictive model underestimated this value by 0.832 (Fig 13B). In addition, as indicated before, the log reduction values obtained from the surface method actually increased during the first 3 days. This was not expected. These values then decreased steadily until day 10, at which point the values increased slightly until day 15. After day 15, there was a steady decrease in activity with time until the termination of the experiment. Overall, the predicted values were not as close to the observed as in the case of the suspension method. The linear model using log reduction values may not be the best fit to model the data, but it does provide relatively good predictions of how a disinfectant reduces the number of viable spore. There could be other issues that alter the effectiveness of the linear model, such as, it is difficult to calculate accurately the number of viable spore due to certain experimental limitations. Increasing the test days and gathering more data points would make the linear model more robust. Future non-linear models could also be helpful. As this is a new approach, the feedback and results from similar publications in this area would benefit this hypothesis.

As mentioned previously, another reason for the lack of predictable trends in the surface test may be due to the kinetics of the process. The disinfectant may not have full physical access to the spores for the full contact time since it must cut through any biofilm and

extracellular build up. This requires the disinfectant to complete two kinetic events before the spores are killed. Due to this two-part kinetic activity, the surface test possesses additional complications not present in the suspension test and does not represent a pure reaction of the disinfectant against the spores.

Conclusion

We have developed a statistical model that compares the two methods, suspension and surface, by the log reduction values produced over time. We have shown that the suspension test produces log reduction values closer to the predicted values of the model compared to the surface test. We conclude that the suspension test is better able to produce predictable data, and is therefore more reliable in modeling disinfectant kill.

Author Contributions

Joseph Thiriot and Marcus Jackson performed all experimental testing. Jie Wang developed the statistical model with assistance from Dennis Tolley. Joseph Thiriot wrote the thesis, with assistance from Jie Wang for the statistical analysis. Richard Robison oversaw all experimental design, performance of experiments, and editing of the thesis.

Conflict of Interest Statement

No conflicts of interest have been stated by the authors in conducting and reporting this research.

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Appendix

Statistical Analysis of Disinfectant Data

Abstract

In this report, we present the method that has been used to analyze the data collected for a certain brand of disinfectant.

1. The Problem Setting

The purpose of this study was to determine the sporicidal activity and post-activation use-life of a certain brand of disinfectant on C. difficile spores. Two methods had been used in order to accomplish the task.

A. Surface Test - An EPA regulated method.

Key covariate:

(1) Contact time: 5 minutes. This is a degenerated covariate because there is only one level for this variable.

(2) Days elapsed after opening the container: 0, 3, 6, 10, 15, 20.

B. Suspension Test - A comparable method.

Key covariate:

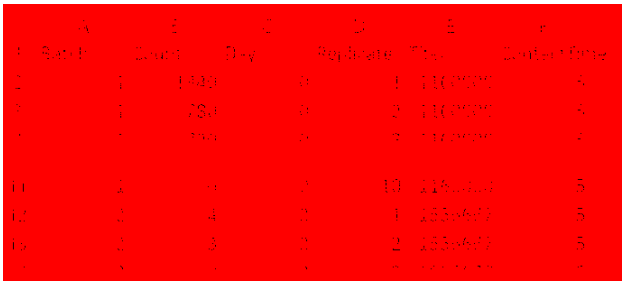
(1) Contact time: 1, 2, 5 minutes.

(2) Days elapsed after opening the container: 0, 3, 6, 10, 15, 20 days.

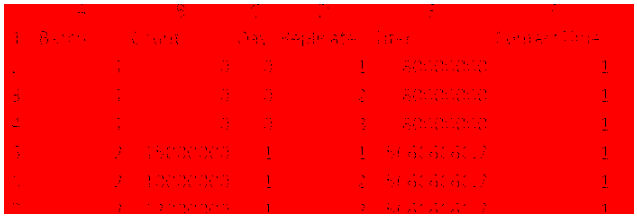
2. Method Used

The data has been divided into two sets and stored in two worksheets in Excel.

A. Surface Test Data



B. Suspension Test Data



2.1. Models of Data

We considered a log linear model with Poisson distribution to model the counts of colonies in each test setting. The model can be expressed as

$$Y_{ij} \sim \text{Poisson}(\mu_{Y_i})$$

and

$$\log(\mu_{Y_i}) = \log(E(Y_{ij})) = X_i' \beta + \log(n_{i0}),$$

where

- Y_{ij} is the count of the number of spore colonies after treatment (applying of the disinfectant on a certain day with a certain amount of contact time);
- μ_{Y_i} is the mean count of spore colonies under the corresponding treatment;
- X_i is the vector of the covariate(s);
- β is the vector of the coefficients for the covariates;
- n_{i0} is the initial count of spore colonies before treatment which is obtained through titer.

3. Results

3.1. Models

SAS PROC GENMOD has been used to fit the models. Codes can be found in the Appendix section. The outputs are presented below.

A. Surface Test Data

Analysis Of Maximum Likelihood Parameter Estimates							
Parameter	DF	Estimate	Standard Error	Wald 95% Confidence Limits		Wald Chi-Square	Pr > ChiSq
Intercept	1	-3.8275	0.0042	-3.8357	-3.8194	849650	<.0001
Day	1	0.0531	0.0002	0.0527	0.0535	65881.3	<.0001
Scale	0	1.0000	0.0000	1.0000	1.0000		

Model Information		
Data Set	WORK.CARRIER	
Distribution	Poisson	
Link Function	User	
Dependent Variable	Count	Count
Offset Variable	ln	

Number of Observations Read	60
Number of Observations Used	60

Parameter Information	
Parameter	Effect
Prm1	Intercept
Prm2	Day

Criteria For Assessing Goodness Of Fit			
Criterion	DF	Value	Value/DF
Deviance	58	77242.0906	1331.7602
Scaled Deviance	58	77242.0906	1331.7602
Pearson Chi-Square	58	76737.5437	1323.0611
Scaled Pearson X2	58	76737.5437	1323.0611
Log Likelihood		476036.0565	
Full Log Likelihood		-38774.3492	
AIC (smaller is better)		77552.6984	
AICC (smaller is better)		77552.9089	
BIC (smaller is better)		77556.8871	

$\log(\mu_{Y_i}) = -3.8275 + 0.0531\text{Day} + \log(n_{i0})$,

B. Suspension Test Data

$\log(\mu_{Y_i}) = -6.1891 + 0.1750 \times \text{Day} + 5.3503 \times 1\text{min} + 3.3710 \times 2\text{min} - 0.1419 \times \text{Day} * 1\text{min} - 0.0676 \times \text{Day} * 2\text{min} + \log(n_{i0})$

3.2. Comparing Two Models

In order to compare two models obtained above, we adopted the likelihood ratio test to determine which model is more likely to be a better estimate of the true model that fits data in general.

SAS macro Vuong has been used to generate the result, which is attached as follows.

3.3. Notes about Vuong Non-nested Model Test

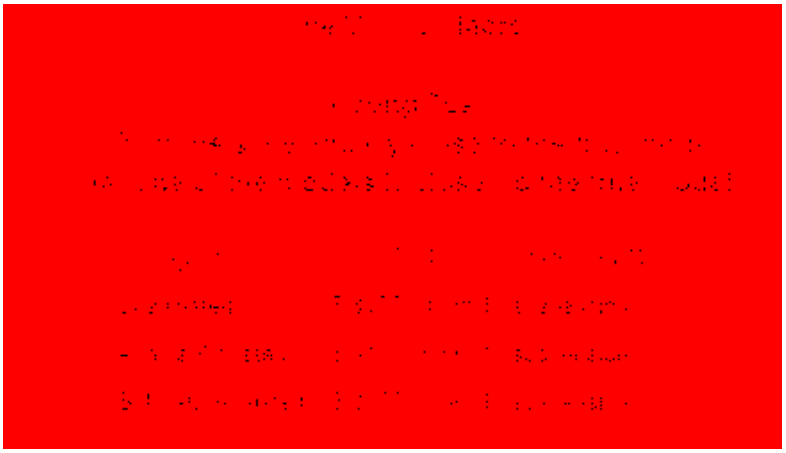
Vuong's test requires that both models are fit using exactly the same set of response values. However, in our experiment, we obtained two sets of response values, each under surface and suspension test conditions. In order to make Vuong's test available for our data, we used the models from Proc Genmod to fill the "missing" part of the response values so that we can get a single set of response values. This set of data can be found by run the SAS code in the Appendix section.

4. Conclusion

Since the hypotheses we tested are:

H_0 : Surface (1st) Model and Suspension (2nd) Model are equally close to the true model.

H_α : One of the models is closer to the true model.



Under the assumption that the null hypothesis is true, i.e. models are equally close to the true model, the test statistic, which is a standardized likelihood ratio, would follow a normal distribution. If the test statistic is negative and significant, the null hypothesis is rejected in favor of the second model being closer to the true model. According to the SAS output from the Vuong test macro, the test statistic is -5.8177. So we conclude that the model used to fit suspension test is better.

5. Appendix

proc import datafile="C:/Users/Jie/Documents/ BYU/Disinfectant/Data. xlsx"

```
out=Carrierdata dbms=xlsx replace ;  getnames= yes; sheet="Carrier ";

run ;  Data carrier ;

set Carrierdata ;  l n=l o g 1 0 ( T i t e r ) ;  run ;  proc print data=carrier ; run ;

proc import datafile="C:/Users/Jie/Documents/ BYU/Disinfectant/Data. xlsx"

out=Suspensiondata dbms=xlsx replace ;  getnames= yes; sheet="Suspension ";

run ;  Data suspension ;

set Suspensiondata ;  l n=l o g 1 0 ( T i t e r ) ;  run ;  proc print data=suspension ; run ;

proc genmod data=carrier plots=all ;

fwdlink link=log10( MEAN ); invlink ilink = 10**( XBETA ); model Count = Day / dist=poisson

/* link=log */ offset = ln obstats ;

output out=outcarrier p=pcarrier ; run ;

----
proc run ; proc

c l a s s  fwdlink link=log10( MEAN );  invlink ilink = 10**( XBETA );  model Count = Day
ContactTime Day*ContactTime/ dist=poisson  /* link=log */  offset = ln  obstats ;  output
out=outsusp p=psusp ;  run ;  proc  run ;  data  set  p c a r r i e r = 1 0 * * ( − 3 . 8 2 7 5 + 0 . 0 5 3
1 * D a y+ l n ) ;  run ;  proc print data=outsusp1 ;  run ;  data outcarrier1 ;  set outcarrier ;
psusp=10**(−6.1891+0.1750*Day+ln );  run ;  proc  run ;  data  set  run ;

print data=outcarrier ;

genmod data=suspension plots=all ; ContactTime ;

----
print data=outsusp ;

outsusp1 ; outsusp ;

print data=outcarrier1 ;

fits;  outcarrier1 outsusp1 ;

proc print data=f i t s ; run ;
```

%inc ”C:\Users\Jie\Documents\BYU\Disinfectant\vuong.sas”; %vuong(data=fits , response=Count,

model1=carrier , p1=pcarrier , dist1=poi , model2=suspension , p2=psusp , dist2=poi , nparm1=2, nparm2=6)