ESTABLISHMENT OF ANIMAL MODELS OF MYCOPLASMA PNEUMONIAE PNEUMONIA AND STAPHYLOCOCCUS ARUEUS OSTEOMYELITIS

THESIS

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INTRODUCTION

Mycoplasma pneumoniae and Staphylococcus aureus are two bacterium with significant clinical relevance leading to atypical pneumonia and osteomyelitis respectively. Our laboratory believes that we can develop animal models useful for the study of the pathogenicity of both of these bacterium as well as for the development of strategies for vaccination, prevention and treatment. The aims of this experiment will be to:

- Develop a model for observing Mycoplasma pneumoniae infection within humanized mice
- Create an experimental design for growing and confirming biofilm formation of Staphylococcus aureus on orthopedic pins

Mycoplasma pneumoniae and *Staphylococcus aureus* are two bacteria with significant clinical relevance. *M. pneumoniae* can infect the upper and lower respiratory tract causing "walking pneumonia", a form of atypical pneumonia caused by infection of the lungs with the bacterium, with symptoms that include fever, coughing, headaches along with pathology characterized by lung inflammation (26). Although *M. pneumoniae* is usually associated with community-acquired pneumonia, it is also known to exacerbate asthma within a host with the preexisting disease, possibly directly through its ability to damage cells or indirectly through the immune response generated by the host. *M. pneumoniae* can cause infections in several epidemiological settings, but the greatest burden is seen in closed community settings where outbreaks occur (17)**.**

In comparison to *M. pneumoniae*, *S. aureus* is one of the major causes of serious hospital and community-acquired bacteremia worldwide and is associated with high morbidity and mortality (28). Beyond bacteremia, it can also be responsible for other forms of invasive disease such as skin and soft tissue infections, sepsis, pneumonia, endocarditis and osteomyelitis (13).

At present, both pathogens have gaps in knowledge of either treatment or prevention that make them difficult organisms to address. *M. pneumoniae*, although treatable with antibiotics such as tetracyclines, currently has no effective vaccines available. The development of an effective commercial vaccine could reduce the incidence of milder infections as well as the outcomes of more severe infections (17). Similarly, treatment of *S. aureus* is becoming difficult. Over the course of being treated by beta-lactam antibiotics, there has been a rise of strains resistant to methicillins (Methicillin-resistant *S. aureus*). In such cases, glycopeptide antibiotics (vancomycin or teicoplanin) serve as a therapeutic alternative (28). Even when treated with antibiotics, *S. aureus* is capable of resisting the effects of antibiotic treatment as well as the host's immune response due to its ability to form biofilms, preventing clearance and leading to severe disease in the form of infective endocarditis or osteomyelitis (13).

As *M. pneumoniae* and *S. aureus* are prevalent within the community, there is a need to develop specific animal models to address the challenges presented by both of these pathogens. The focus of this thesis is the establishment of mouse models for *M. pneumoniae* – mediated pneumonia and *S. aureus* – biofilm formation on surgical wires as a prelude to establish an animal model of osteomyelitis. Both of these models will be useful for experiments into the prevention and treatment of both diseases.

MYCOPLASMA PNEUMONIAE

In humans, *M. pneumoniae* can cause the condition called "walking pneumonia" and is also capable of exasperating preexisting respiratory conditions through its own damage to the host or as a result of the immune response against it. *M. pneumoniae* causes the greatest burden within closed community settings due to outbreaks that can result (17). Community-Acquired Pneumonia is a sub-group that usually occurs within closed communities such as in hospitals or nursing homes; a significant setting since the elderly are at higher risk. The symptoms of an acute infection can include fever, chest pain, a productive or dry cough depending on age, lethargy and chills.

While there are simple methods for diagnosis, treatment options for *M. pneumoniae* vary due to factors like resistance and disease severity and options for prevention such as vaccines border on the absent. Diagnosis for an infection of *M. pneumoniae* can include PCR and serology for IgM production against the bacterium or chest x-ray to spot lung infiltrate. Treatment can vary by country or even by individual resistances to antibiotics toward pneumococci and the usual treatment includes the use of antimicrobials such as macrolides or doxycycline and beta-lactam antibiotics of increasing strength depending on the severity of the disease (32). Effective vaccinations could help in prevention of infection but as of yet none exist for the prevention of *M. pneumoniae*.

Mycoplasma differs from several other bacteria in that they lack a cell wall and instead have a cholesterol-containing lipid bilayer even though these are phylogenetically gram positive. The lack of a cell wall causes *M. pneumoniae* to be resistant to many antibiotics that target cell wall synthesis such as penicillin and other common beta-lactam antibiotics (4). Outside of its

ability to resist common antibiotic treatment, several strains of this bacterium have specific virulence factors that allow them to invade and persist within a host.

Virulence Factors

M. pneumoniae is a small, parasitic bacterium. Early in the pathogenesis of mycoplasma respiratory disease, *M. pneumoniae* adheres to the host's respiratory epithelium (31). *M. pneumoniae* has a tip organelle which is involved in adhesion to epithelial cells. P1 is the major adhesion protein found in the tip organelle that is expressed on the surface of *M. pneumoniae* (1, 14). Experiments with the M129 strain has shown that P1 binds to the glycoproteins present on host cells and tissues, and also serves as a target for antibody responses and can be bound by surfactant protein A, a bacteriostatic protein that is normally part of the host's pulmonary defense mechanisms (1, 9, 16). The interaction between the bacterium and epithelium can cause the *M. pneumoniae* to produce enzymes which can damage the host through oxidative stress by way of hydrogen peroxide production. Another virulence factor recently discovered is an ADP-ribosylating and vacuolating toxin produced by *M. pneumoniae* known as Community Acquired Respiratory Distress Syndrome (CARDS) toxin. CARDS toxin binds to alveolar surfactant protein A and is shown to be produced in previous murine models of infection inducing an asthma-like reaction within the animals (10, 16, 19). It can induce cytoplasmic vacuolization, a process by which affected cells will produce vacuoles that swell to a size and number the cell cannot recover from, resulting in cell death (30).

Biofilms are also a strategy by which the organism can persist *in vivo*. Biofilms are multicellular colonies of bacteria organized in layers on a surface and covered by an extracellular matrix of DNA and polysaccharides (14). This biofilm structure makes the *M. pneumoniae* more

resistant to stress, heat, desiccation, complement killing and antibiotics than planktonic cells (14, 18). Some bacteria can shift from the aggregate of biofilms to the free-moving form of the bacteria and can travel to other parts of the host body where it can manifest in extra-pulmonary areas such as the brain, blood, skin, and the urogenital tract (8, 17, 25). The UABP01 strain of *M. pneumoniae* was utilized during this project because it is effective in the production of biofilms.

Immune response to *Mycoplasma pneumoniae*

During a mycoplasma infection, the host's innate immune system responds first to protect against the pathogen. Upon infection of the host's lower respiratory system, *M. pulmonis* (*M. pneumoniae* may have similar action) is challenged by surfactant protein-A, an innate pulmonary defense utilized for neutralization of the bacterium and promoting phagocytosis by activating macrophages (16). During the innate immune response, inflammation-mediating cytokines and chemokines are produced in the respiratory tissues, recruiting lymphocytes, neutrophils, and macrophages toward the colonies of *M. pneumoniae*. This cytokine environment determines the adaptive immune response and whether this response ends up being beneficial or harmful to the respiratory system. The adaptive immune response is believed to play a partial role in protection against mycoplasma disease, while also another role in the pathology of mycoplasma disease (30). The adaptive immune system, activated by the presence of the *M. pneumoniae*, results in the proliferation of B and T lymphocytes as well as the upregulation of cytokines such as IL-2, IL-4 and TNF-α (8, 25). Cytokines like TNF-α, can cause inflammation in the respiratory tissues which in turn can exacerbate preexisting respiratory conditions such as asthma.

Mycoplasma vaccination

The development of effective vaccines could help prevent severe infections, as well as reduce the more moderate cases of the illness (17). At present, fluid treatment and antibiotics (e.g. tetracyclines, clarithromycin) are the only ways to control *M. pneumoniae* infections and the prevention of outbreaks is still a challenge for clinicians. Previous studies have developed live attenuated vaccines but have not reached clinical trials. These live vaccines were shown to be virulent or exacerbated preexisting respiratory conditions by way of inflammation caused by an activated Th17 immune response to the pathogen (17, 29). In the 60s and 70s, vaccines with inactivated organism were tested yet the suggested protective effect observed was not highly reinforced by the more sensitive diagnosis methods we use currently such as molecular diagnosis and serology methods (17). Based on these stalled investigations of vaccine treatment against *M. pneumoniae*, continued experimentation *in vivo* is a necessity to develop vaccines capable of preventing outbreaks without resulting in a harmful immune response within the recipient.

Animal models of *M. pneumoniae*

Mouse models are not only cost efficient but allow for a low upkeep burden. Mice are also extremely useful for testing drug efficacy and gene manipulation. As such, these animals are used to develop a wide variety of disease models, including *M. pneumoniae*. *M. pneumoniae* models have been used previously to examine the pathogen's pulmonary infection and cell-mediated immune response within mouse strains ranging from Balb/c to germ-free (7, 33). Studies with these models have provided insights into the acute phase of the immunological response to infection. However, to help better illuminate chronic phases within animals, more models for *M. pneumoniae* are desired.

Humanized mouse models specifically are highly valuable subjects for observing the impact of a human immune response on infection. These animals carry functional human gene, tissues or cells by way of specialized humanization protocols. In the case of studies focused on immunology, immunodeficient mice such as NOD or NSG strains are engrafted with hematopoetic stem cells derived from human donors. Over an observation period, the transplantation of these hematopoetic stem cells lead to a population of functional human T and B lymphocytes within the animal. As such, these mice model the human immune system and can be used for the *in vivo* study of the immunopathology of the human immune system against certain infectious diseases and even the therapeutic treatment of the disease such as in the creation of a vaccine that doesn't negatively exacerbate the immune system.

STAPHYLOCOCCUS AUREUS

S. aureus is a gram-positive facultative anaerobe that is capable of a stable relationship within a human host although, under certain circumstances, it can result in serious disease. *S. aureus* colonizes the skin, nares and gastrointestinal tract of humans (13). Although a commensal relationship within an immunocompetent individual allows *S. aureus* to persist without becoming pathogenic, a disruption of normal microbiota as a result of antibiotic or other therapies can lead to infection (27). This is one reason why *S. aureus* infections are often seen within hospital settings after patients are administered antibiotics for unrelated diseases. The antibiotics kill the primary disease causing bacteria but are also able to kill commensal colonies producing an environment that allows *S. aureus* to thrive and infect the host. *S. aureus* is one of the most problematic human pathogens, causing dangerous and costly infections worldwide. It is the second most common cause of hospital-associated bloodstream infections and is responsible for increased mortality and longer hospital stays (22).

Hospital treatments also provide a common invasion method for *S. aureus*, breaks in the skin, which can lead to skin or soft tissue infections. This potential introduction of the bacteria can lead to bacteremia, tissue invasion, and septic shock (6). These breaches in the skin can result in a variety of life-threatening conditions such as pneumonia, endocarditis, infected joint prosthesis, IV line infections and osteomyelitis. In the clinical setting, although often treatable with antibiotics, there are cases where their use may be ineffective. Additionally, an *S. aureus* infection can cause a secondary necrotizing pneumonia when it follows an influenza A viral infection (24).

Skin infections by *S. aureus* are usually diagnosed simply by visual evaluation of the patient while internal infection is usually discovered based on blood culturing. The usual treatment for such an infection includes the use of antibiotics but continued use has led to a rise of *S. aureus* resistant to antibiotics. For infections that reach bone in cases of osteomyelitis, the use of x-rays or MRIs can be used to assess the damage from the bacterium and surgical intervention to remove infected bone is useful for treatment.

Virulence factors

Along with the ability of some strains to avoid the antimicrobial actions of antibiotics, *S. aureus* has an array of other virulence factors. *S. aureus*'s pathogenicity can be attributed to its suite of toxins, exoenzymes, adhesins, and immune-modulating proteins that it can produce (6). An examples of one exoenzyme *S. aureus* can utilize is coagulase which can coat the bacterium and prevent it from being phagocytized. Despite its exotoxins and enzymes that afford *S. aureus* its pathogenicity, its ability to form biofilms is one of the major factors that make this pathogen difficult to treat.

Biofilms

A biofilm is an aggregate of cells that, after adhering to a surface or each other, embeds itself in a matrix of extracellular polymeric substance usually formed by polysaccharides, proteins, and extracellular DNA. The formation of a biofilm provides a colony of cells with a physical defense against bactericidal action from both antibiotic treatment and from the host's immune responses. Biofilms contain dormant bacterium that have suppressed any active metabolic action which bolsters its immunity. However, different strains or clinical isolates can vary in their ability to form biofilm. Thus, it is important to identify the ability of different *S.*

aureus to form biofilm. Without a way to be directly acted upon, the bacterial cells essentially are able to avoid killing. An infection with a bacterium capable of forming biofilms also poses a danger due to the ability of cells from the colony to slough off, releasing from the origin point of the biofilm and dispersing to other areas of the body where a new biofilm can form. These biofilms are formed normally when the bacteria are stressed under environmental conditions such as where iron or oxygen are limited, under high osmolality, or when the density of *S. aureus* cells is low (20). The biofilm is a lower metabolic state that reduces cell division intensely and allows for the bacteria to stay entrenched within the film to avoid antimicrobial factors (2).

Medical devices that penetrate the skin and mucosal surfaces are recognized as the most frequent causes of biofilm-associated infections due to Staphylococci (20). These biofilm infections are often difficult to treat with antibiotics and require the replacement of many of these penetrative medical devices in an attempt to control the infection. In hospital settings, there are several forms of *S. aureus* biofilm – mediated infections that can occur. Insertion of an IV line can cause bacteria to be introduced into the bloodstream of the patient or into the IV catheter where it can colonize. Introducing *S. aureus* into the bloodstream can lead to colonies settling in the lining of the heart valves or blood vessels leading to infective endocarditis. In addition, osteomyelitis, an infection and inflammation of the bone, can result from *S. aureus* contamination from a penetrating trauma (2).

Osteomyelitis

Osteomyelitis is an infection of the bone or the marrow caused by a bacteria entering through the bloodstream, a local area of infection, or penetrative trauma or surgery such as a joint replacement. Pathogenic microorganisms associated with osteomyelitis include

Enterococcus species (spp)., *Streptococcus spp.*, *Pseudomonas aeruginosa*, *Enterobacter spp.*, *Mycobacterium spp.*, but *S. aureus* accounts for the vast majority of chronic osteomyelitis infections in all age groups (2). In children, infection is seen in the long bones of the legs or arms commonly, while adults are observed with infections within the spine. Osteomyelitis can lead to bone death, impaired growth, septic arthritis, sclerosis or deformities.

After introduction of the bacterium and through the use of microbial-surface-component-recognizing adhesive matrix molecules (MSCRAMM) presented by *S. aureus*, the bacterium can attach, aggregate and mature to form a matrix of extracellular polymeric substance on bone or a device that has been implanted into the host (2, 3, 12, 20). Leukocytes, in an attempt to curb the infection, can end up lysing bone through the action of their own enzymes leading to pus that can cut off blood flow to the bone.

Most antimicrobials are designed to work against active, planktonic cells that are freely moving and colonizing within a host. This makes treatment of osteomyelitis resulting from *S. aureus* difficult to treat since the bacterium forms a biofilm that serves as a physical barrier protecting bacterium in a low metabolic state. Antibiotic treatments from drugs such as ceftazidime, ciprofloxacin, and vancomycin are commonly used in attempts to treat osteomyelitis. However, surgical interventions remain the most effective means of treatment of biofilm-associated infections meaning the debridement of all infected bone (2).

Antibiotic treatments

A majority of staphylococcal infections are treated with methicillin beta-lactam antibiotics that inhibit the synthesis of the bacteria's peptidoglycan cell wall. Other antibiotics such as macrolides, tetracyclines, & fluoroquinolones can be utilized in treatment as well (21).

However, continued use of these has led to the rise of a methicillin-resistant *S. aureus* (MRSA) strain. The enzyme beta-lactamase grants *S. aureus* resistance to beta-lactam antibiotics allowing it to break down the structure of the antibiotic and eliminate its antimicrobial properties. This strain, first discovered in 1960, was most often associated with healthcare facilities, but has since expanded to epidemic levels, capable of also being acquired from the community (3, 22).

Animal models of Osteomyelitis

There is precedent for the study of osteomyelitis using animal models. Models have been developed using rabbits, canines and rodents including mice. Mice in particular have had acute, chronic and periprosthetic osteomyelitis developed that utilized orthopedic pins and wires in reproducible capacities, including with the use of bioluminescent strains of *S. aureus* (23). With the existence of many models that study the efficacy of various treatments for *S. aureus* infection and little related to the study of the bacterium's biofilm formation, there is a clinical need to not only develop models for this highly relevant pathogen that are novel and necessary. Biofilm formation is an important feature of these infections and these animal models utilize isolates of *S. aureus* that form biofilms.

SUMMARY

The future of treatments for the human pathogens *Mycoplasma pneumoniae* and *Staphylococcus aureus* are inexorably tied to *in vivo* research with these bacteria. Both *M. pneumoniae* and *S. aureus* are clinically relevant pathogens that can infect a multitude of individuals under common environmental conditions from closed communities in the case of *M. pneumoniae* to the hospital settings where *S. aureus* has ample opportunity to infect patients with weakened immune systems. Their pathogenicity along with their ability to avoid treatment and clearance whether by binding to host epithelium or forming a physical layer to protect them from immune system and antibiotic action, make them consistently formidable bacterium to treat. Fortunately, by experimenting *in vivo* using animal models of both to observe immune reactions and test possible treatments, the opportunity to research and discover clinically beneficial ways of combating these pathogens is entirely within reach. The goal of this thesis, through such *in vivo* experimentation, is to begin the development and establishment of models useful for testing both of the pathogens outlined.

Chapter 2

INTRODUCTION TO THE *MYCOPLASMA PNEUMONIAE* STUDY

The purpose of this study is to establish a mouse model of *M. pneumoniae* pneumonia. We began by testing different strains of *M. pneumoniae*, to observe which would induce infection and subsequent disease in mice. A major hurdle of the creation of this model is the fact that *M. pneumoniae* is a human pathogen. Due to this, we tested different strains *M. pneumoniae*, which differ in their virulence factors, to find which strains would be successful in infecting and causing disease in mice. The mycoplasma strain that colonizes the animals and results in the development of inflammatory lesions and chronic disease will be the one our laboratory would utilize in the future. Once established, this model would further research into the immune responses against mycoplasma, and potentially be used in the development of beneficial vaccines and new therapies for human disease.

As mentioned, we compared the ability of three different strains of *M. pneumoniae* for their ability infect and cause disease in mice. These strains were M129, S1 and UABP01. The M129 strain (obtained from Duncan Krause, Ph.D.) is the reference strain often used in in vitro studies and *in vivo* murine infections. It has been utilized in a number of studies to examine virulence mechanisms, including the assembly and function of the P1 adhesion protein (14). The P1 being a protein that is crucial to the integrity attachment organelle which allows the bacterium to colonize the respiratory epithelium. The S1 strain (obtained from Joel Baseman, Ph.D.) is a clinical isolate of *M. pneumoniae* from a 1993 outbreak of respiratory infections in San Antonio, TX. S1 can produce CARDS Toxin, a toxin capable of causing more severe disease in mice compared to M129 (10). Lastly, the UABP01 strain (obtained from Kevin Dybvig, Ph.D.) is

capable of forming robust biofilms in vitro (26). The S1 and M129 strains was used previously in murine models of infection and disease (9, 29), but the UABP01 strain has yet to be examined for its ability to infect animals.

Balb/c mice were used to determine the ability of the different mycoplasma strains to infect mice. We chose Balb/c mice as they are expected to be susceptible to *M. pneumoniae* infection since another mycoplasma species, *M. pulmonis*, readily infects and causes disease within Balb/c mice (11, 15). They were also used in other studies with *M. pneumoniae* (33). Although *M. pneumoniae* is a human pathogen not readily adapted to animals, the vulnerability of the Balb/c mice to mycoplasma species shown in previous studies makes it valuable for our study.

Materials and Methods

Mice: Female BALB/c, Nod ShiLt, Nod.Cg-Prkdc<scid>, & CD34FL mice were received from either Harlan™ and Jackson™ Laboratories. Mice were housed in sterile cages and fed according to the Institutional Animal Care and Utilization Committee (IACUC) of the University of North Texas Health Science Center in the biosafety level II facility. The mice were aged 5 – 10 weeks. Before the intranasal inoculation and sacrifice, the mice were anesthetized with an intramuscular injection of ketamine/xylazine solution.

For our time point experiments, 30 female BALB/c mice were received from Harlan™ Laboratories. Mice were stored in sterile cages and given food, water and bedding to utilize at their own leisure. The mice were aged 5 – 7 weeks. 5 mice were left uninfected and sacrificed at the 0 day time point.

***M. pneumoniae*:** Three strains of *M. pneumoniae* were used in all experiments: S1, M129, and UABPO1. Stock cultures were grown in SP4 medium for mycoplasmas and frozen in 1 mL aliquots at – 80ºC & checked for their CFU/ml concentration. Before inoculation, the stocks were thawed and incubated at 37ºC for one hour in later experiments. The mice were given nasal-pulmonary inoculations of 20 µl of mycoplasma.

Lung isolation and scoring: Mice were anesthetized and euthanized by cervical dislocation. The lungs were removed aseptically from the chest cavity and rinsed in phosphate buffer saline (PBS). Lungs were scored for gross lesions present on 5 different lobes of the lung: the right lower lobe, right upper lobe, right middle lobe, left lobe, and the azygous.

Lung processing for CFUs: Lungs were placed into gentleMACS™ tubes pre-filled with 5 ml of sterile SP4 medium and placed on ice. Tubes were homogenized using the gentleMACS™ Dissociator unit on the setting Lung 2. The lung homogenate was serially diluted. 20 µl of each dilution were plated onto sections of SP4 agar plates and left to dry before covering and incubating at 37ºC for about 10 - 14 days. Hemolytic agar (Cole & Ward) was used to cover the SP4 plates at the end of the incubation period and left to set before returning to incubation for about 2 days. Plates were removed and CFUs per ml (as identified by hemolysis) were counted and recorded.

Quantitative PCR: We adapted a quantitative PCR method from Joel Baseman (University of Texas Health Science Center San Antonio). Utilizing the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN), DNA from *M. pneumoniae* strain S1 was isolated. DNA concentrations were quantified using a BioTek® Synergy™ HT Take3 Micro-Volume Plate. DNA was stored at 2 – 4ºC before PCR. PCR primers for the detection of *M.*

pneumoniae were: MP141 Forward: 5' -CCAACCAAACAACAACGTTCA - 3'; MP141 Reverse: 5'-ACCTTGACTGGAGGCCGTTA - 3'. Primer were provided by Dr. Joel Baseman and his laboratory. Each PCR reaction included 12.5 µl Sybr Green Master Mix (Qiagen, Germantown, MD), 0.5 µl of each primer, 5 µl of DNA solution, and 6.5 µl sterile PCR grade water for a final volume of 25 µl per reaction. Amplification conditions included one preincubation cycle of 3 min/94ºC, a 3 step amplification of 45 cycles of 30 sec/94ºC, 30 sec/60ºC, 30 sec/68ºC, a melting step of 5 sec/95ºC, 60 sec/65ºC, 5 second continuous acquisition/95ºC and one final cycle of 3 min/94ºC. A Roche LightCycler® 96 was used for all PCR assays.

Statistical analysis: Graphpad Prism 6 software was used to perform evaluation on data using ANOVA. A p value ≤ 0.05 was considered statistically significant.

Results

No significant weight change or clinical symptoms after infection with 1 x 10^4 CFUs of *M. pneumoniae*. Groups of Balb/c mice were infected with our *M. pneumoniae* strains (S1, M129 and UABPO1). The mice were examined for clinical signs of infection each day and their weights were measured over the course of 14 days. One group of mice was given sterile SP4 medium to serve as a control. Lungs were collected at the end of the 14 day observation period, washed in PBS and scored for the presence of gross lesions. Collected lungs were then homogenized and the number of Mycoplasma colony forming units (CFU) was determined.

No clinical signs of disease were apparent within any group infected with any of our mycoplasma strains during the observation period. Over the course of the 14 days, there was a steady increase in body weight in all groups (Figure 1). Furthermore, gross lesions were not

present on the lungs from any infected group. Lastly, no CFUs were recovered from the lungs. Thus, mice inoculated with ~1 x 10^4 CFU of *Mycoplasma pneumoniae* strains did not develop disease. One possibility for this result was that the inoculum dose concentration was too low to cause severe, observable symptoms of disease or acute or chronic infection within the Balb/c mice in comparison to the higher doses used in previous studies.

Gross lesions are present at day 14 post-infection with increased inoculum concentration. The inoculum with a concentration of ~1 x 10^4 CFU per dose did not cause quantifiable disease or a recoverable bacterial burden at 14 days after infection regardless of the *M. pneumoniae* strain. In order to see if an increase in the dose concentration could cause infection, each strain was cultured to yield a concentration higher than the ~1 x 10^4 CFU per dose utilized in the previous experiment. The strains (S1, M129 and UABPO1) were cultured to ~2.0 x 10^4– 1. 0 x 10^5 CFU per dose and used to intranasally infect Balb/c mice.

Over the 14 day time period, there was a steady increase in weight within the animals among all test groups (Figure 2). However, gross lesions were found in 1 of the 4 mice within the group infected with the UABP01 strain but no other group. No CFUs were found on the SP4 agar plates with dilutions of the lung homogenate. These data suggest that at least one strain of Mycoplasma (UABPO1) showed the capability to cause disease within mice. However, from our results, certain factors in the growth of our cultures or the concentration of our intranasal dose might be insufficient to cause severe disease

Actively growing bacterium pre-inoculation results in higher total gross lesions. The previous experiment showed that gross lesions did indeed begin to manifest once the concentration of *M. pneumoniae* was increased. The condition or concentration of the inoculum

was still insufficient in terms of causing signs of severe disease. To address this possibility, the *M. pneumoniae* strains were cultured to increase the infectious dose concentrations with S1 at ~7.3 x 10^5 CFU/dose, M129 at ~1.3 x 10^7CFU/dose, and UABPO1 at ~ 5.5 x 10^6CFU/dose. Before inoculation, the thawed stocks were incubated for ~1 hour at 37ºC. This was done to increase the actively growing population and virulence factor presentation that may have been lost during the freeze-thaw process. The experimental animals were also aged 8 – 9 weeks before inoculation. The strains were used to intranasally infect Balb/c mice with a volume of 40 µl instead of 20 µl used in previous experiments.

An increase in body weight within all test groups of animals was observed again (Figure 3). However, gross lesion incidence was high within *M. pneumoniae* infected groups (Figure 4). No CFUs were recovered from the lungs.

These data show that the use of highly concentrated and actively growing bacteria was sufficient to cause gross lesions within the test animals. However, CFUs continued to be elusive in plated lung homogenate from infected groups. This indicates that the bacteria had been cleared by the immune system by the end of the 14-day period, and/or that the amount of bacteria remaining within the animal was undetectable by the quantification method we utilized. Thus, a need to observe the bacterial burden at time points during the observation period was necessary, as was a separate method for detecting the bacterial burden within the test animals.

Bacterial clearance begins at around 3 days but high lesion scores persist at late time points. Although in the previous experiment the appearance of lesions did indicate infection within the test animals, the absence of CFUs showed that the bacterial burden was possibly being cleared by the end of our 14 day observation period. Thus, an experiment to

monitor the bacterial burden within the animals at separate times within the 14 days was necessary. In these studies, the time points used were 0 days, 1 day, 3 days, 7 days, 10 days, and 14 days post infection. The S1 strain was used to intranasally infect 25 Balb/c mice with 5 more mice left uninfected and used as the mice harvested for the day 0 time point. Mice were weighed and observed for clinical signs of infection, and 5 mice were harvested at each time point and their lungs were scored and processed to collect CFUs.

Body weights within the test animals remained similar to starting weights until day 8 when weights began to drop (Figure 5). Gross lung lesions showed a steady rise starting from day 1 to day 14 (Figure 6). CFUs were recovered from the lungs of animals starting from and peaking at day 1 before dropping by day 3 and becoming unrecoverable through CFU plating by day 10 (Figure 7).

To determine if the culture method limited our ability to detect organisms, 1mL aliquots of the time point experiment lungs were used for bacterial DNA extraction. Purified DNA was quantified and PCR amplified alongside an *M. pneumoniae* DNA standard. Genomes/µg DNA were calculated and compared with the CFU concentrations calculated from the same lungs (Figure 8) which showed a decrease in both concentrations over the course of the time point experiment, but we were able to detect genomes on day 10, where CFUs were undetectable.

These data suggest that there is a significant bacterial burden within animals infected with *M. pneumoniae* that is cleared by the immune system between the third day and tenth day of infection. Although, bacterial genomes were still detected indicating that there was still a presence of bacterial infection within the animals that was unobservable from CFU plating methods. The persistence of lung lesions beyond the point where it seemed that the bacteria were

cleared according to CFU plating suggests that the appearance of lesions may depend heavily on the immune response mounted against *M. pneumoniae* infection.

Immune competent mice show higher gross lesion percentage and incidence compared to immune deficient but no significant difference in bacterial load. To determine the effect of the adaptive immune response on bacterial burden and symptoms of chronic infection, seven immune competent mice (Nod.ShiLtJ) and seven immune deficient mice (Nod.Cg-Prkdc <scid>) were obtained. Our Nod.Cg-Prkdc <scid> mice in particular were important to future studies because they are the background strain used to form the humanized mice we utilized in upcoming experiments.

These animals were infected intranasally with the S1 strain of *M. pneumoniae* and observed for 14 days with weight changes and clinical symptoms documented. Mice lungs and spleens were harvested at the end of the 14 day time point. Lung lesion scores were counted before processing to collect CFUs.

Body weights between immune competent and immune deficient mice remained similar between groups with weight gain occurring over the course of the 14 day time period (Figure 9). Lungs harvested on day 14 showed evidence of gross lesions only within the immune competent group of mice; the total gross lesion percentage being significantly higher than the immune deficient group which were absent of gross lesions (Figure 10). Within both groups, CFUs were recovered from processed lungs. Analysis of the CFU/ml from processed lung showed no significant difference in the bacterial burden between the immune competent and immune deficient groups of animals (Figure 11).

Humanized mice show a low gross lesion percentage and low bacterial load at the end of 14 day time point. To test the effect of an immune response from human immune cells on chronic infection and lung damage, eight humanized mice (CD34FL) were inoculated intranasally with the S1 strain of *M. pneumoniae* at 2 concentrations: Group A was dosed with ~1.9 x 10^7CFU/mL and Group B was dosed with ~1.34 x 10^8CFU/mL. Mice were observed over the course of 14 days with body weights and observable clinical signs of infection documented each day. Lungs were harvested at the end of the 14 day experiment. Lung lesion scores were counted before processing to collect CFUs.

Body weights varied little between the two groups and over the course of the experiment (Figure 12) and although 2 mice within Group A showed evidence of lung damage, the total lesion score was not significantly different from Group B where no lesions were observed (Figure 13). CFUs from the processed lungs were collected, however each the bacterial burden within both groups was close to or below the limit of detection (Figure 14).

DISCUSSION

The goal of this study was to develop a mouse model of *M. pneumoniae* pneumonia. We began with the comparison between three *M. pneumoniae* strains to find which would result in infection and subsequent disease in mice. Those three strains of *M. pneumoniae* were: S1, M129, and UABPO1; with S1 being a strain that produces CARDS toxin, M129 being a reference strain used in many previous *M. pneumoniae* experiments, and UABPO1 being a strain that specializes in the formation of robust biofilms. Ultimately, the ability of the S1 virulence factor to produce more potent signs of infection and disease led to it being chosen as the bacterium that would be used in the further experiments.

A major finding within our experimentation that both higher doses of the strains as well as use of actively growing pre-inoculum by incubation was necessary to establish infection and that the presentation of gross lesions within the lungs of treated mice corresponded with clearance of the bacterium by the immune system. High gross lesion scores began to present after the combination of both incubation and dose increase to levels that indicated sufficient infection. In turn, lesions persisted after clearance of the bacterial load suggesting that not only does *M. pneumoniae* contribute to lung damage but the immune reaction to the infection also contributes as well resulting in the lesion presentation post bacterial clearance. However, a high discrepancy did appear between the weight changes for these experiments. Beyond external factors, we can count human error and inconsistencies in technique. These factors could result in one or more animals having more obvious disease presentation in comparison to others where technique may have been lacking. Also, due to variations in growing and harvesting methods between the strains of bacterium, our experiments had difficulty obtaining clearly consistent dose concentrations between the test strains used for the various groups.

Another major finding within our experiment was that immunodeficient mice had less pathology than their immunocompetent counterparts. This indicates that, similar to previous experiments with *M. pulmonis*, what contributes most to host pathology is the impact of the hosts own immune system rather that damage from the bacterium itself. This finding is supported by the previous portion of our overall experiment that showed a persistence of lesions post clearance of the bacterial load.

Through the final experiment, we found that humanized mice could be further developed as host for examining human immune responses against *M. pneumoniae*. With the presence of some gross lesions, despite a low percentage, as well as bacterial clearance a similar trend of

disease presentation through lesions caused by host immune system rather than bacterial action seen in previous experiments can indeed be observed within mice with human immune cells.

In the future, it will be important to continue repeated testing with the humanized mice to uncover the finer points of both the immune response and how it causes damage to the host. Through observation of the immune cell and cytokine production at different stages of infection, it will be possible to gain a clearer picture on how the human immune system contributes to its own pathology in the presence of *M. pneumoniae* infection.

Through these experiments, we've already outlined the clear possibility of immune pathology being a major factor in the symptoms of *M. pneumoniae* infection. With finer detail on the immune cascade produced in response to *M. pneumoniae*, we can start to outline alternatives for previous vaccines; an actual way to subvert immune pathology while still inducing protection from the bacteria for the treated patients.

Chapter 3

INTRODUCTION TO THE *STAPHYLOCOCCUS AUREUS* STUDY

The purpose of this study is to begin to establish *S. aureus* biofilm – mediated osteomyelitis in a mouse model. Orthopedic surgeries can provide an entry for *S. aureus* which can lead to the bacterium attaching and growing on fracture fixings or joint replacements. So the use of these pins in our model is appropriate. This mouse model will be advantageous for future drug trials for osteomyelitis treatment. Experimental drugs could be utilized at different concentrations and at different points within an infection to test if the bacterial load could be reduced or even prevented altogether. These studies were to determine the ability of multiple strains of S. aureus to form biofilm on orthopedic pins (Figures 15 & 18). Our experimental strains have been utilized frequently in studies of other biofilm models including on plastics and in an endocarditis model. Some strains used were altered to present luminescence for the purposes of visualizing the biofilm (Table 1).

Bacterium on biofilms are often more resistant to antibiotics, which impairs treatment. To confirm if our strains form biofilms, we focused on the drug vancomycin. Vancomycin is a first line glycopeptide antibiotic used in the treatment of infections derived from MRSA strains (27). The antibiotic works through bactericidal action, preventing cross-linking in the formation of the peptidoglycan layer of the bacteria (12). Although it is effective against planktonic, actively growing and metabolizing colonies, it has a low efficacy against biofilm structures. For the purposes of our experiment, an observable persistence of bacterial colonies despite treatment with the drug beyond the minimum inhibitory concentration would indicate bacterial resistance, ergo biofilm formation.

Materials and Methods

Orthopedic Pins: Two sizes of orthopedic pins were purchased from Teleflex® KMedic® which were .028 mm and .062 mm in diameter. Pins were clipped with wire cutters to form 1 centimeter pieces and each was placed into sterile 1.5 ml Eppendorf tubes for later use. When confirming the formation of a biofilm, only the orthopedic pins of .028 mm diameter were utilized.

Staphlococcus: Six strains of *S. aureus* were used (Table 1): UNT 005-4, UNT 111-3, UNT 020-4, UNT 021-4, UNT 022-4, & UNT 023-4. Bacteria were streaked on Tryptic soy agar (TSA) plates and incubated at 37ºC for 18 – 20 hours. When confirming the formation of a biofilm, only the UNT 005-4 strain of *S. aureus* was used.

Pin Cultures: Tryptic soy broth supplemented with 0.9% NaCl and 0.25% glucose (TSBGN) was used to suspend bacteria on the plates removed from incubation to an OD of 1 at 600 nm (~1.0 x 10^9 CFU/ml). The suspension serial was diluted to 1:1000 in TSBGN (~1.0 x 10^6 CFU/ml). 1 ml of the 1:1000 dilution pipetted into 25 tubes of each pin size for all strains. Pins were incubated in the inoculum at 37ºC for 120 hours.

CFU processing: 5 tubes were removed for each pin size and each strain at 24, 48, 72, 96 and 120 hours post inoculation. Pins were aseptically transferred from culture tubes to 1.5 ml Eppendorf tubes containing 1ml of 1x phosphate buffer saline supplemented with 20% Tween-80. The tubes were vortexed in a multi-vortexing unit for 5 minutes. Using a 96-well round bottom plate with wells of 180 µl of 1xPBS+20%Tween-80, the tubes were 10-fold serial diluted and 8 µl of the dilutions were spot plated onto brain heart infusion agar (BHI) supplemented with

5% activated charcoal and mannitol salt agar (MSA). Agar plates were incubated for 18 – 24 hours and colony counts were obtained to determine the CFU/pin segment.

Vancomycin Experiment Pin Cultures: Tryptic soy broth supplemented with 0.9% NaCl and 0.25% glucose (TSBGN) was used to suspend bacteria on the plates removed from incubation to an OD of 1 at 600 nm (~1.0 x 10^9 CFU/ml). The suspension was serial diluted to 1:1000 in TSBGN (~1.0 x 10^6 CFU/ml). 1 ml of the 1:1000 dilution is pipetted into 20 tubes of the pins. Pins were incubated in the inoculum at 37ºC for 72 hours.

Vancomycin Pre-treatment CFU processing: 5 tubes were removed at 72 hours post-inoculation. Pins were aseptically transferred from culture tubes to 1.5 ml Eppendorf tubes containing 1ml of 1xPBS supplemented with 20% Tween-80. The tubes were vortexed in a multi-vortexing unit for 5 minutes. Using a 96-well round bottom plate with wells of 180 µl of 1xPBS+20%Tween-80, the tubes were 10-fold serial diluted and 8 µl of the dilutions were spot plated onto brain heart infusion agar supplemented with 5% activated charcoal and mannitol salt agar. Agar plates were incubated for 18 – 24 hours and colony counts were obtained to determine the CFU/pin segment.

Vancomycin Treatment and CFU Processing: At 72 post-inoculation, pins were aseptically transferred into 1.5 ml Eppendorf tubes filled with 1ml of the separate treatment conditions: 5 tubes of 0.4 mg/ml vancomycin solution, 5 tubes of 4 mg/ml vancomycin solution, and 5 tubes of vehicle (sterile water for injection). The pins were incubated in each treatment for 18 hours. At 18 hours, the treatment solutions were pipetted out and the pins washed twice with sterile 1X PBS supplemented with 20% Tween-80. The PBS volume was removed and 1 ml of Mueller-Hinton broth supplemented with 2.5% universal neutralizer for biocide testing was pipetted into

the pin tubes. The pin tubes were incubated at 37ºC for 24 hours. After 24 hours, the Mueller-Hinton broth + neutralizer medium was removed and replaced with 1 ml of 1x PBS + 20% Tween-80. The tubes were vortexed in a multi-vortexing unit for 5 minutes and sonicated for 10 minutes. Using a 96-well round bottom plate with wells of 180 µl of 1xPBS+20%Tween-80, the tubes were 10-fold serial diluted and 8 µl of the dilutions were spot plated onto Brain Heart Infusion agar supplemented with 5% activated charcoal and Mannitol Salt agar. Agar plates were incubated for 18 – 24 hours and colony counts were obtained to determine the CFU/pin segment.

Statistical analysis: Graphpad Prism 6 software was used to perform evaluation on data using ANOVA. A p value ≤ 0.05 was considered statistically significant.

Results

CFU counts show growth and adherence to orthopedic pins within all sets of test strains. To test the growth and adherence of multiple *S. aureus* strains on the surface of the orthopedic pins (Figure 15), 6 separate strains of *S. aureus* were grown and diluted to 1.0×10^6 CFU/ml in tryptic soy broth supplemented with sodium chloride and glucose: UNT 005 - 4, UNT 111 – 3, UNT 020 – 4, UNT 021 – 4, UNT 022 – 4, and UNT 023 – 4. Each strain inoculum was used to fill 25 tubes with a clipped pin section for each diameter size (.028 mm and .062 mm). The pins were incubated at 37ºC and 5 pins were removed for each time point: 0, 24, 48, 72, 96, and 120 hours and the pins were processed to shake off the bacterial growth before dilution plating on both BHI agar supplemented with 5% activated charcoal and mannitol salt agar. In comparison to BHI agar, the mannitol salt agar is specific for *S. aureus* and can grow little else. By using both we can confirm if any fungal or non-essential bacterial contamination might exist

within the cultures when comparing the populations that grow on both. Plates were incubated at the same temperature and CFU/ml were counted after 24 hours and recorded as a log10 value.

After 24 hours, a slight rise in bacterial growth was observed followed by a steady decline and plateau (Figure 16 & 17). This trend was similar across all groups test strains with no significant difference between the strains. Recovery of the bacterial colonies indicated that colonies formed on the orthopedic pins and the trend seen may indicate that the bacteria may be entering a stressed stationary state, ripe for forming biofilms on the material.

CFU presence at hour 72 pre and post-treatment with vancomycin show adherence and establishment of biofilm on orthopedic pins. To test whether the colonies forming on the orthopedic pins were indeed forming a biofilm (Figure 18), the commonly used osteomyelitis antibiotic, vancomycin, was used at 10 and 100 times its minimum inhibitory concentration to treat the inoculated pins. The UNT 005 – 4 strain of *S. aureus* diluted to 1.0 x 10^6 CFU/ml was used to inoculate 20 clipped size .028 mm diameter pins. The inoculated pins were incubated at 37ºC for 72 hours. At the end of the time period, 5 pins were removed, processed and plated on BHI+5% activated charcoal and MSA to serve as the pre-treatment time point. The plates were incubated for 24 hours before obtaining the colony counts. The remaining 15 tubes were removed to be used for the separate drug treatments. 5 pins were transferred into tubes with 1 ml of vehicle, 5 pins were transferred into 1 ml of 0.4 mg/ml of vancomycin, and 5 pins were transferred into 1 ml of 4 mg/ml vancomycin. The treated pins were incubated for 18 hours before being removed, washed with PBS and placed into tubes with 1 ml of Mueller – Hinton broth supplemented with neutralizer and incubated again for 24 hours. At the end of the incubation period, the tubes were processed and processed and plated on BHI+5% activated

charcoal and MSA to serve as the post-treatment group. The plates were incubated for 24 hours before obtaining the colony counts.

These data revealed that in all post-treatment groups a high yield of CFUs occurred, in comparison to the pre-treatment group (Figure 19). This persistence of bacterial growth on pins treated with the antibiotic indicates that since significant bacterial killing is not occurring, the bacteria must be forming a protective biofilm.

DISCUSSION

We were able to see a stable growth of the bacterial colonies in our initial assay (Figure 16 & 17). This observation among the pins that were .028 mm in diameter is particularly advantageous for the purposes of our mouse model. The smaller size is suitable for implantation into the bones of mice. These data were also intriguing because of the similarities between the luminescent and non-luminescent strains. If the luminescent strains show biofilm formation, we can visualize infection and biofilm formation within our osteomyelitis model.

The UNT 005 – 4 strain formed a biofilm on our orthopedic pins despite vancomycin treatment. With the minimum inhibitory concentration of this UNT 005 – 4 strain being 4 µg/mL, our experiment at 100x and 1000x the minimum inhibitory concentration shows the resistance of UNT 005 – 4 (Figure 19). This high resistance suggests that the bacterial strain formed the protective physical layer of biofilm to persist even despite being in the presence of the treatment drug at levels far beyond what is required to suppress and counteract infection.

With our observation within the primary experiment that showed adherence to the biofilm for all tested *S. aureus* strains, the next step within our overall experiment is the investigation into whether this adherence means the biofilm formation of the other strains is a necessity. Each

strain has their particular minimum inhibitory concentration at which they can no longer withstand the efforts of inhibitory treatment drugs. By repeating our second experiment we will be able to ascertain whether all of the bacterial adherence also indicates the formation of biofilm for each strain. In the future we will be utilizing this experimental design to observe this biofilm formation in vivo within mice fitted internally with the surgical pins utilized previously.

With this *S. aureus* study, we were indeed able to establish a method for sufficiently proving the formation of biofilm on pins used in orthopedic surgery. This discovery is key in further investigation into bacterial behavior and response to treatment *in vivo* once pins are actually placed into our test animals. This is only the first step into finding a sufficient way to treat and possibly even pre-prepare the tools utilized in orthopedic surgery from bacterial adherence and the detrimental production of robust biofilm at vital areas of a patient's musculoskeletal system.

APPENDIX

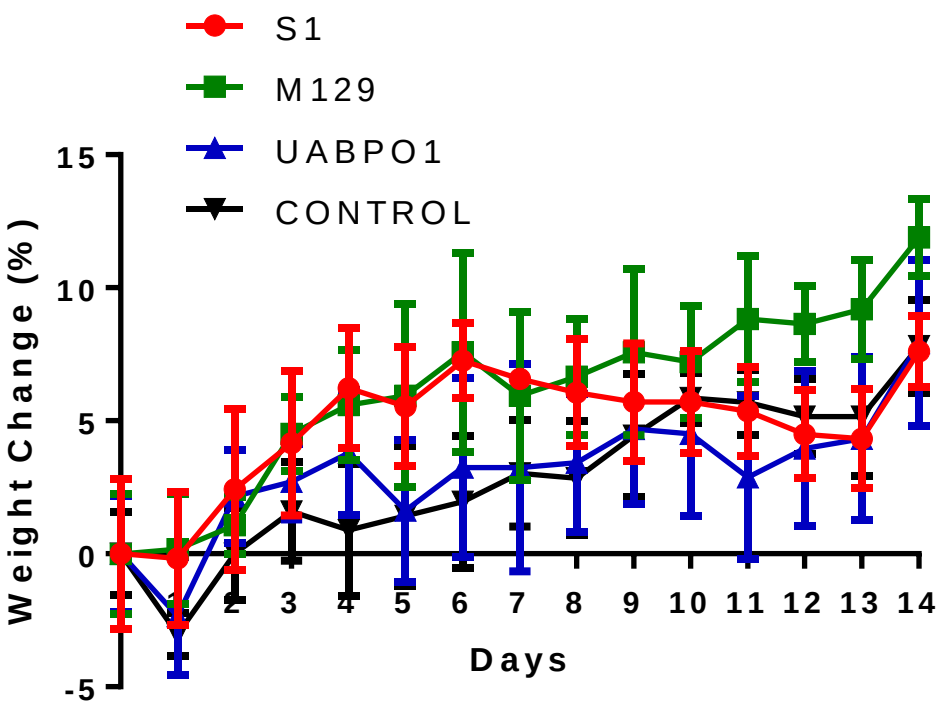


Figure 1: No significant weight change due to infection at 1 x 10^4 . – Calculated percent weight gain or loss between mice dosed with neat inoculum **(A)** and inoculum adjusted to 1 x 10^4 CFUs **(B)** of. *M. pneumoniae*. These mice were received from Harlan Laboratories at 5-6 weeks of age. Two-way ANOVA performed on data. Data represent mean ± SD (*n* = 4 mice/group from two independent test groups).

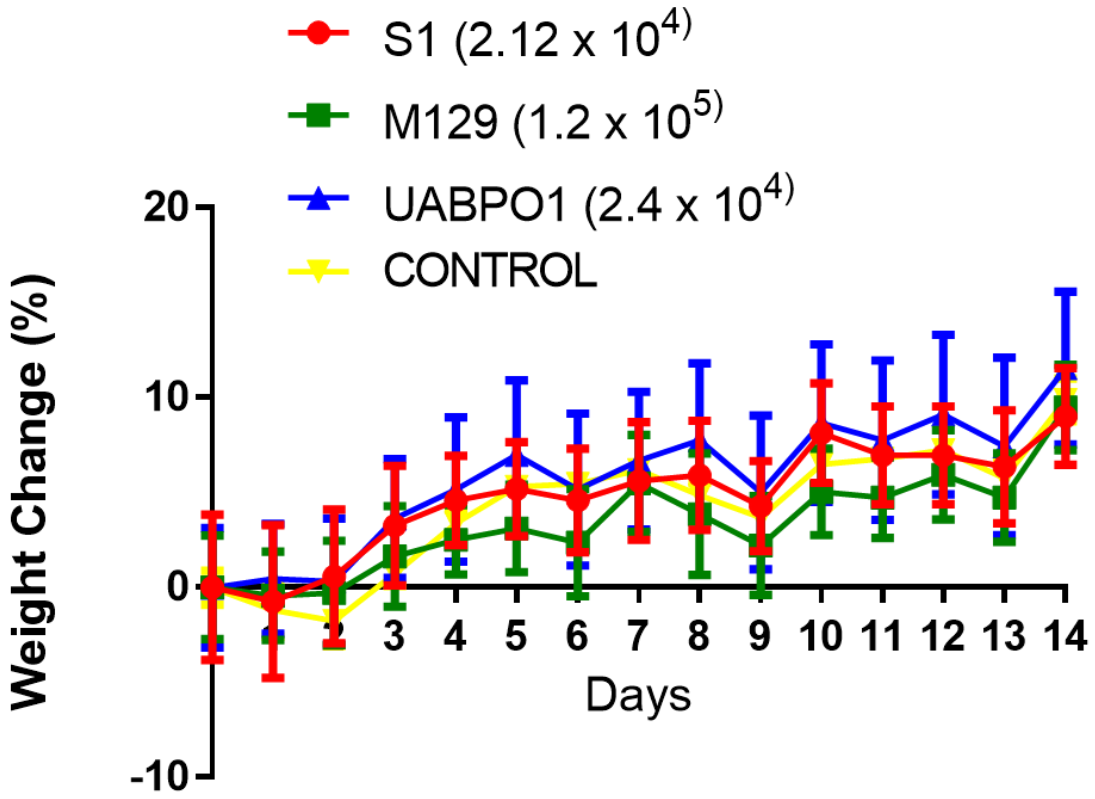


Figure 2: Percent Weight Change - Calculated percent weight gain or loss in mice dosed with either SP4 medium or neat inoculum (~2.0 x 10^4– 1. 0 x 10^5 CFU) on Day 0 of experiment. These mice were received from Harlan laboratories at 6-7 weeks of age. Two-way ANOVA performed on weight data. Data represent mean ± SD (*n* = 4 mice/group).

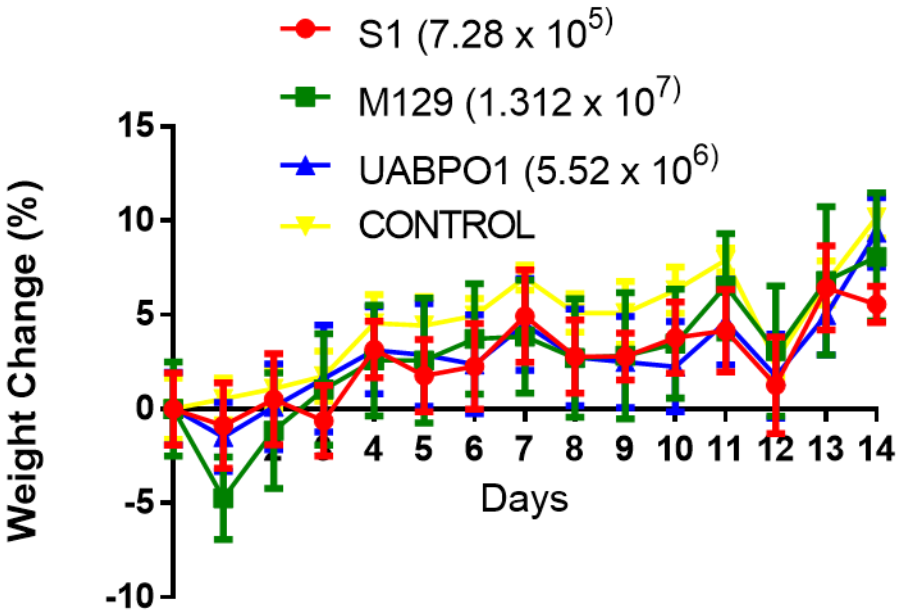
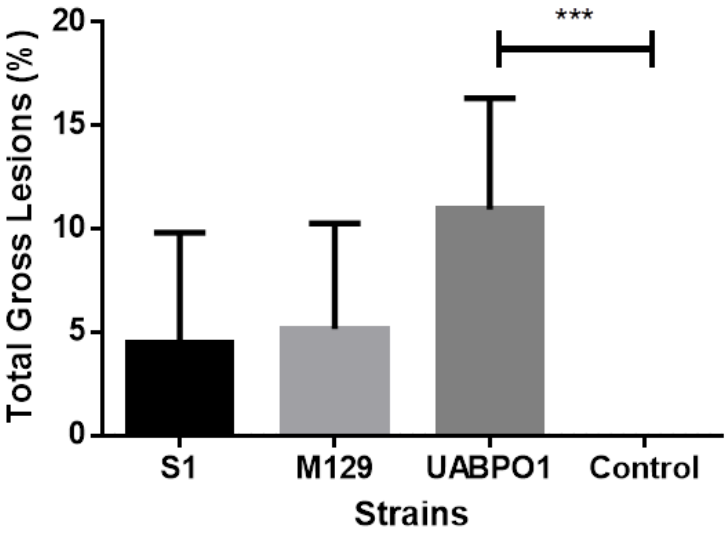


Figure 3: Actively growing bacterium pre-inoculation did not result in significant weight loss. - Calculated percent weight gain or loss in mice dosed with either SP4 medium or neat inoculum (S1: ~7.3 x 10^5 CFU/dose, M129: ~1.3 x 10^7CFU/dose, UABPO1: ~ 5.5 x 10^6CFU/dose) on Day 0 of experiment. These mice were received from Harlan Laboratories at 8 – 9 weeks of age. Two-way ANOVA performed on weight data. Data represent mean ± SD (*n* = 8 mice/group).



Gross Lesion Incidence	
S1	3/4
M129	4/4
UABPO1	4/4
CONTROL	0/4

Figure 4: Actively growing bacterium pre-inoculation results in higher total gross lesions. - Total gross lesion percentage in mice dosed with either SP4 medium or neat inoculum (S1: ~7.3 x 10^5 CFU/dose, M129: ~1.3 x 10^7CFU/dose, UABPO1: ~ 5.5 x 10^6CFU/dose) on Day 0 of experiment. These mice were received from Harlan Laboratories at 8 – 9 weeks of age. One-way ANOVA performed on data. Data represent mean ± SD (n = 8 mice/group). *** $p < 0.001$.

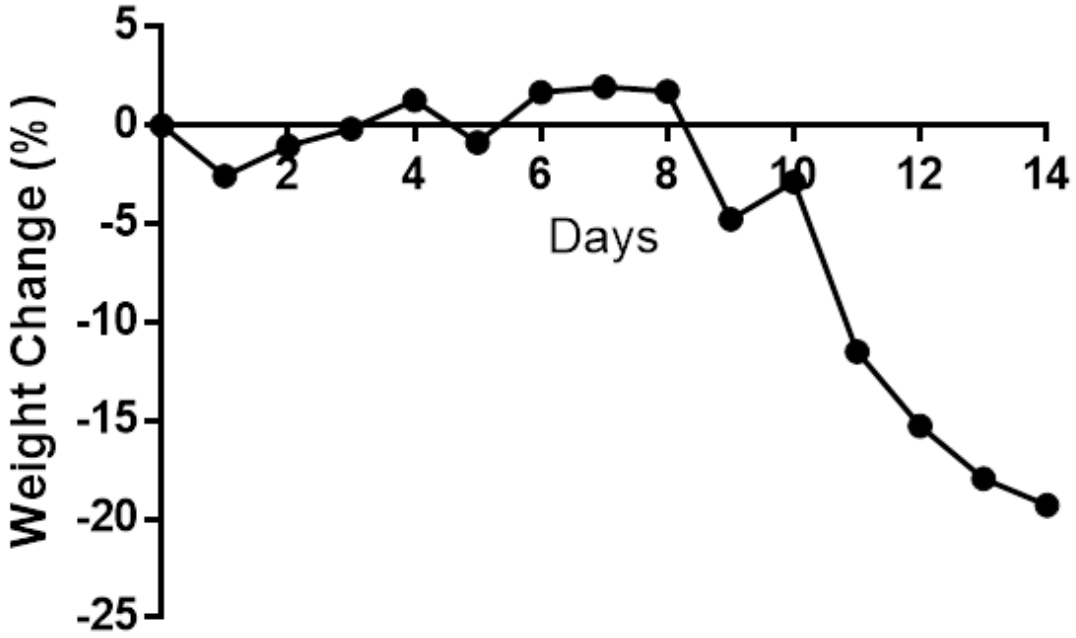


Figure 5: Weight loss begins at 8 days within infected mice. - Calculated percent weight gain or loss in mice dosed with either SP4 medium or neat S1 inoculum (3.44 x 10^5) on Day 0 of experiment. These mice were received from Harlan Laboratories at 5 – 7 weeks of age.

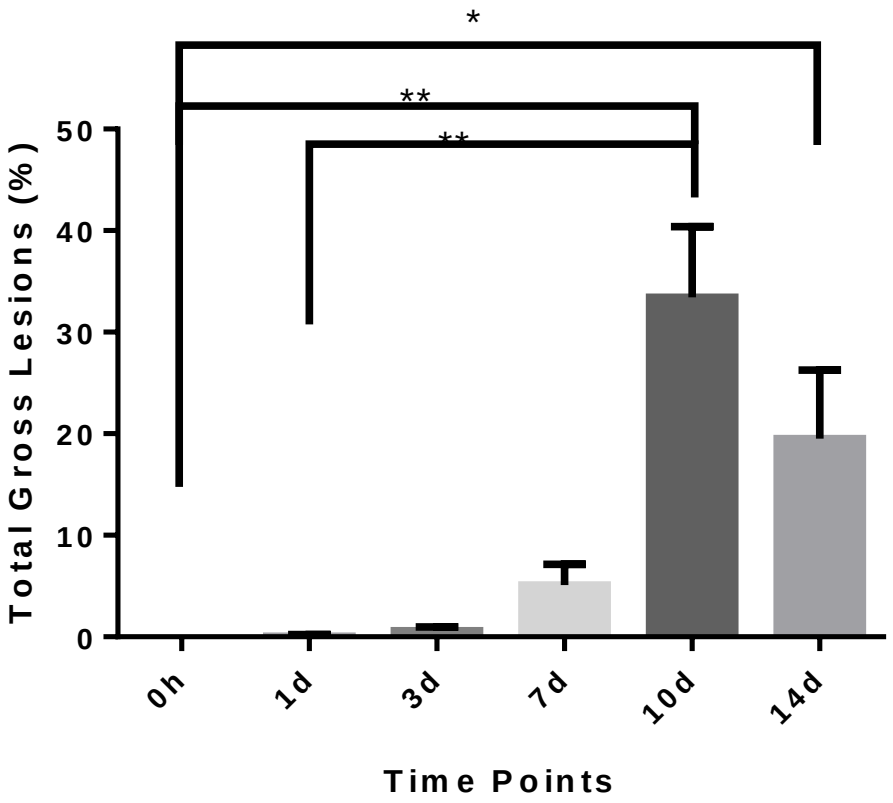


Figure 6 – High lesion scores appear at latter time points. - Total gross lesion percentage in mice dosed with either SP4 medium or neat S1 inoculum (3.44 x 10^5) on Day 0 of experiment. These mice were received from Harlan Laboratories at 5 – 7 weeks of age. One-way ANOVA performed on data. Data represent mean ± SD (*n* = 5 mice/group). * $p \leq 0.05$, ** $p \leq 0.01$

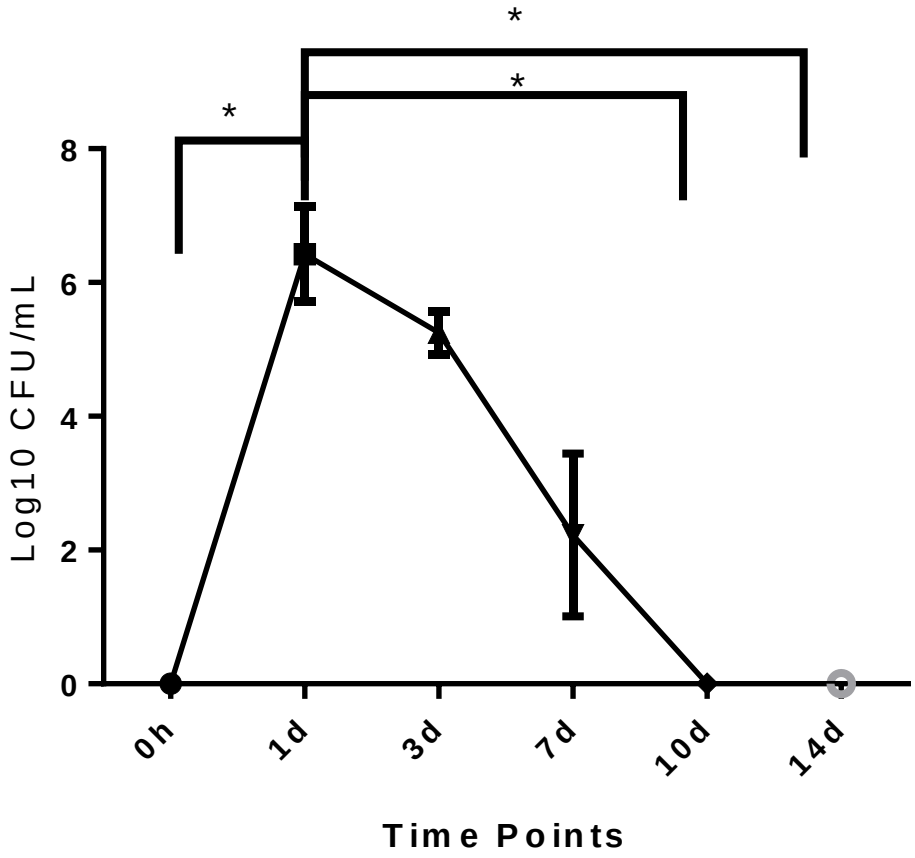


Figure 7: CFU testing shows a decline of colonies at around ~2-3 days. – Logarithmic representation of CFU/mL per time point in mice dosed with either SP4 medium or neat S1 inoculum (3.44 x 10^5) on Day 0 of experiment. These mice were received from Harlan Laboratories at 5 – 7 weeks of age. One-way ANOVA performed on data. Data represent mean ± SD (*n* = 5 mice/group). * $p \leq 0.05$.

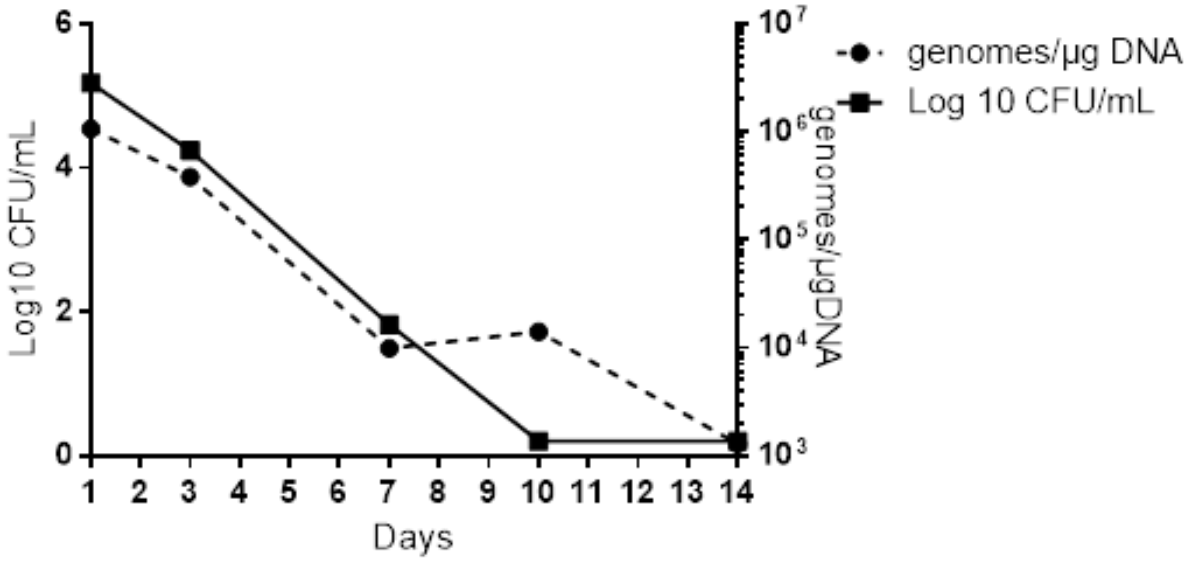


Figure 8: Time point overlay comparison of detected bacterial genomes and CFU counts. – Logarithmic representation of CFU/mL per time point in mice dosed with either SP4 medium or neat inoculum (3.44 x 10^5) on Day 0 of experiment compared with genomes/µg DNA calculated from PCR data. These mice were received from Harlan Laboratories at 5 – 7 weeks of age. One-way ANOVA performed on data. Data represent mean ± SD (*n* = 5 mice/group). * $p \leq 0.05$.

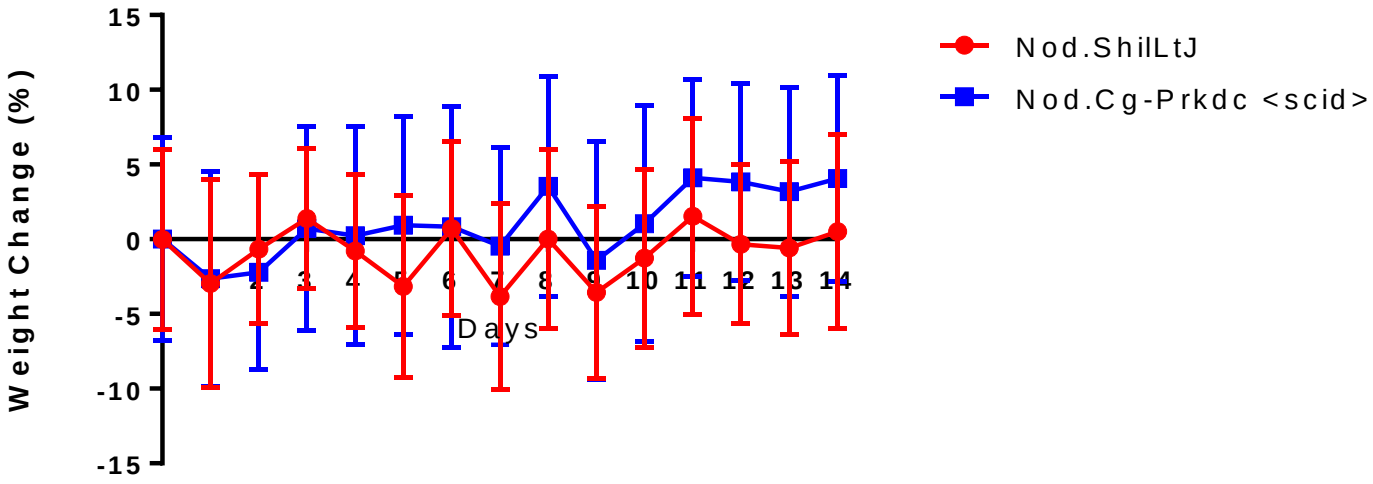
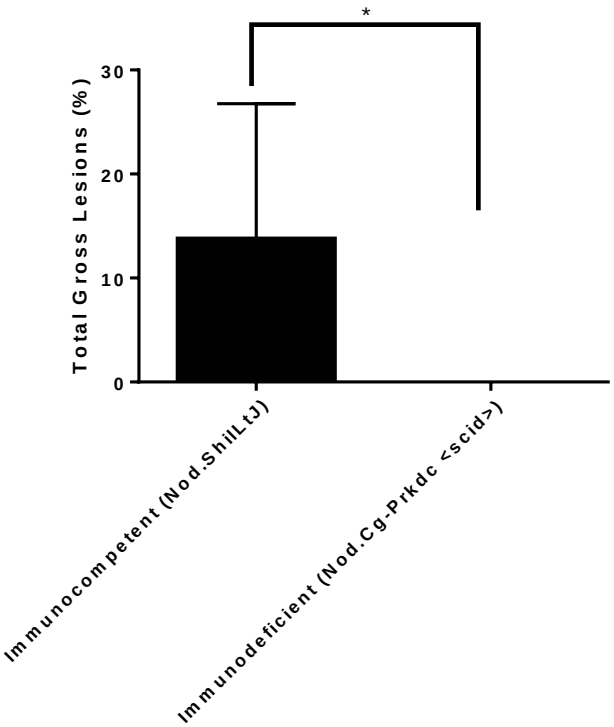
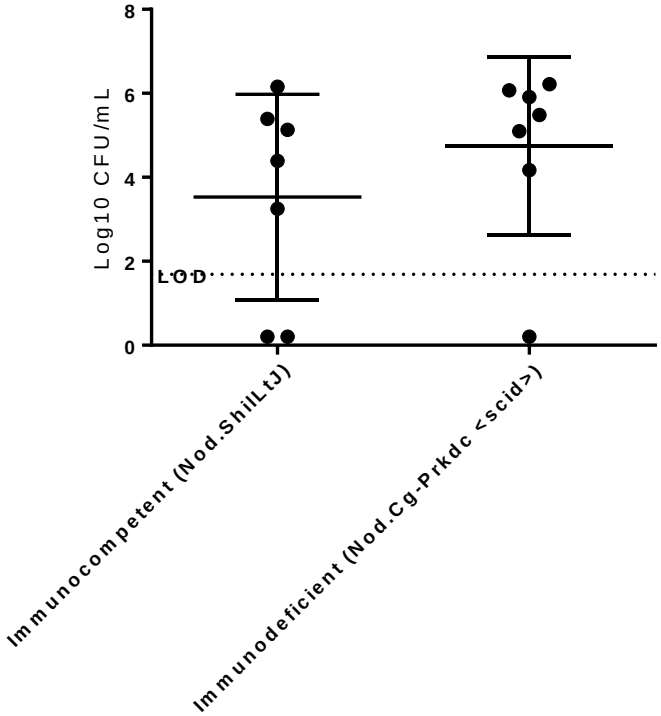


Figure 9: No significant weight loss between immune competent and immune deficient mice. - Calculated percent weight change in immune competent and immune deficient mice dosed with neat S1 inoculum (4.8 x10^6 CFU) on Day 0 of experiment. These mice were received from Jackson Laboratories at 9 – 10 weeks of age. Two-way ANOVA performed on weight data. Data represent mean ± SD (*n* = 7 mice/group).



Gross Lesion Incidence	
Immunocompetent (Nod.ShilLtJ)	5/7
Immunocompetent (Nod.ShilLtJ)	0/7

Figure 10: Immune competent mice show higher gross lesion percentage and incidence compared to immune deficient mice. - Total gross lesion percentage and incidence in immune competent and immune deficient mice dosed with neat S1 inoculum (4.8 x10^6 CFU) on Day 0 of experiment. These mice were received from Jackson Laboratories at 9 – 10 weeks of age. Two-way ANOVA performed on data. Data represent mean ± SD (n = 7 mice/group). * $p < 0.05$.



Test Groups	CFU Incidence	CFU Range (CFU/mL)
Immunocompetent (Nod.ShilLtJ)	5/7	0 - 1.45×10^6
Immunodeficient (Nod.Cg-Prkdc <scid>)	6/7	0 - 1.68×10^6

Figure 11: No significant difference in bacterial load between immune competent and deficient mice. – Logarithmic representation of CFU/mL and incidence in immune competent and immune deficient mice dosed with neat S1 inoculum (4.8×10^6 CFU) on Day 0 of experiment. These mice were received from Jackson Laboratories at 9 – 10 weeks of age. Two-way ANOVA performed on data. Data represent mean ± SD (*n* = 7 mice/group).

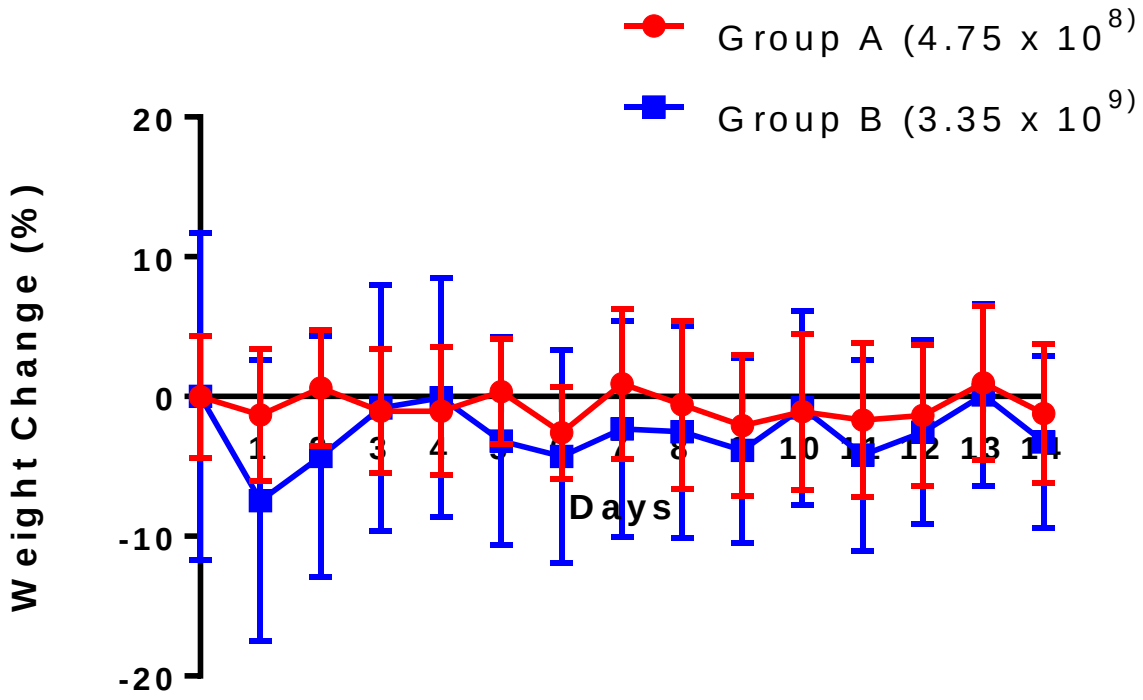
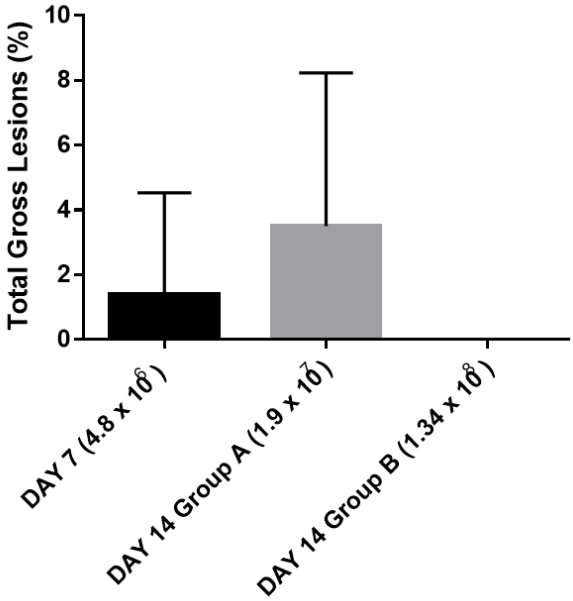
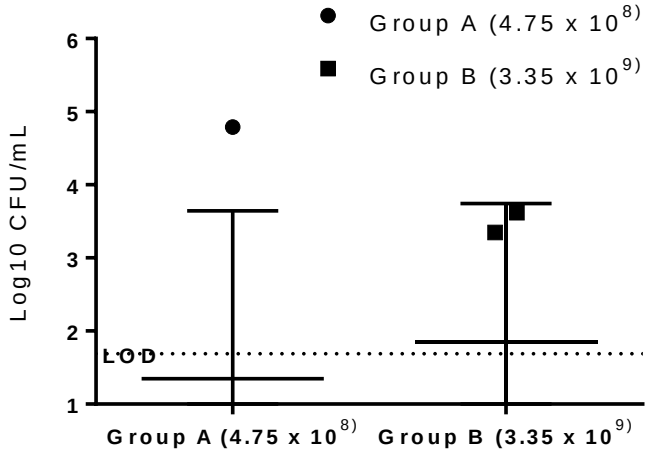


Figure 12: No significant weight loss between dosed groups of humanized mice. - Calculated percent weight change in humanized dosed with neat S1 inoculum (Group A: ~4.7 x 10^8CFU/mL, Group B: ~3.3 x 10^9CFU/mL) on Day 0 of experiment. These mice were received from Jackson Laboratories at 21 weeks of age. Two-way ANOVA performed on weight data. Data represent mean ± SD (*n* = 4 mice/group).



Gross Lesion Incidence	
DAY 7	1/5
DAY 14 Group A	2/4
DAY 14 Group B	0/4

Figure 13: Humanized mice show a low gross lesion percentage and low bacterial load at end of time point experiments. - Total gross lesion percentage in humanized dosed with neat S1 inoculum (Group A: ~1.9 x 10^7CFU/mL, Group B: ~1.34 x 10^8CFU/mL) on Day 0 of experiment. These mice were received from Jackson Laboratories at 21 weeks of age. Two-way ANOVA performed on weight data. Data represent mean ± SD (*n* = 4 mice/group).



Test Groups	CFU Incidence	CFU Range (CFU/mL)
DAY 7 (4.8 x 10^6)	3/5	0 - 1.5x10^4
DAY 14 Group A (1.9 x 10^7)	1/4	0 – 6.25x10^4
Day 14 Group B (1.34 x 10^8)	2/4	0 – 2.25x10^3

Figure 14: CFU counts at multiple time points and incidence – Logarithmic representation of CFU/mL in humanized dosed with neat S1 inoculum (Group A: ~1.9 x 10^7CFU/mL, Group B: ~1.34 x 10^8CFU/mL) on Day 0 of experiment. These mice were received from Jackson Laboratories at 21 weeks of age. Two-way ANOVA performed on weight data. Data represent mean ± SD (*n* = 4 mice/group).

STRAIN DESIGNATION	INFORMATION	LUMINECENCE
UNT005 – 4 (ATCC 6538)	Methicillin - sensitive	No
UNT111 – 3	Valve endocarditis isolate	No
UNT020 – 4	Validated in biofilm models	Yes – Xen 29 Single copy lux operon
UNT021 – 4 (ATCC 12600)	Parent strain of UNT020 - 4	Yes
UNT022 – 4	Validated in biofilm models	Yes - Xen 36 Single copy lux operon
UNT023 – 4 (Wright strain)	Parent strain of UNT022 - 4	Yes

Table 1: *Staphylococcus aureus* strains – Table of the various *S. aureus* strains utilized in the experiments.

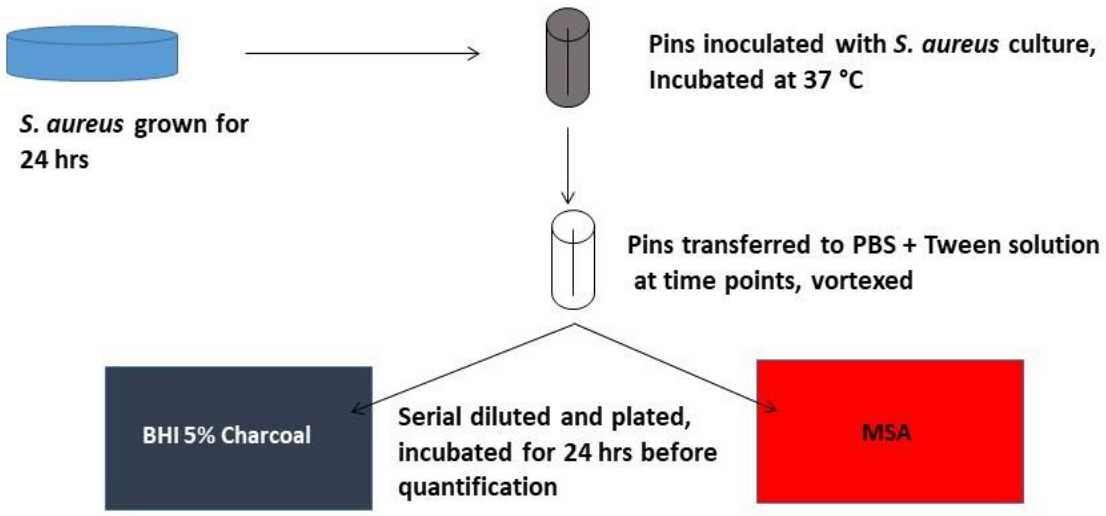


Figure 15 - Experimental Design for Testing Multiple S. aureus strains. Diagram of the steps for an experiment to observe S. aureus population of one or more strains on orthopedic pins.

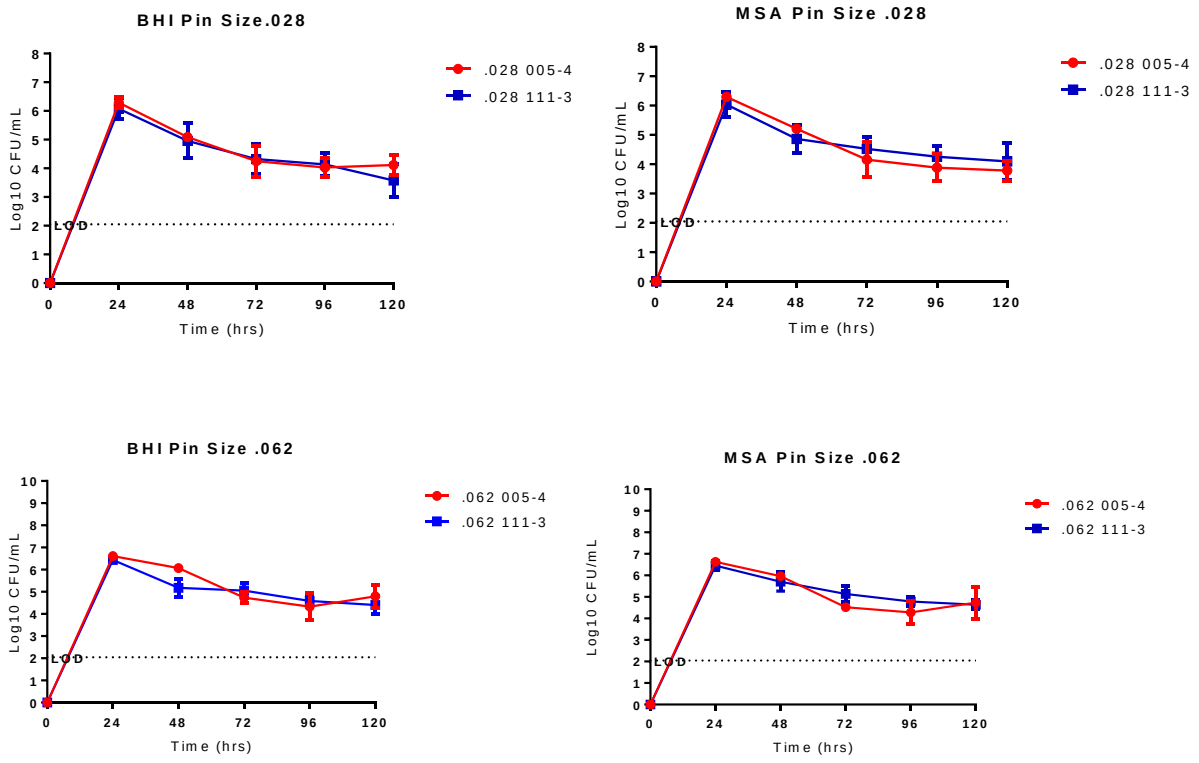


Figure 16 - CFU counts show growth and adherence to orthopedic pins within first set of test strains. Logarithmic representation of CFU/mL per time point on .028 pin size and .062 pin size on BHI+5% activated charcoal agar and Mannitol Salt Agar. Data represent mean ± SD (*n* = 5 pins/group). Limit of detection is $\log_{10}$ 2.05.

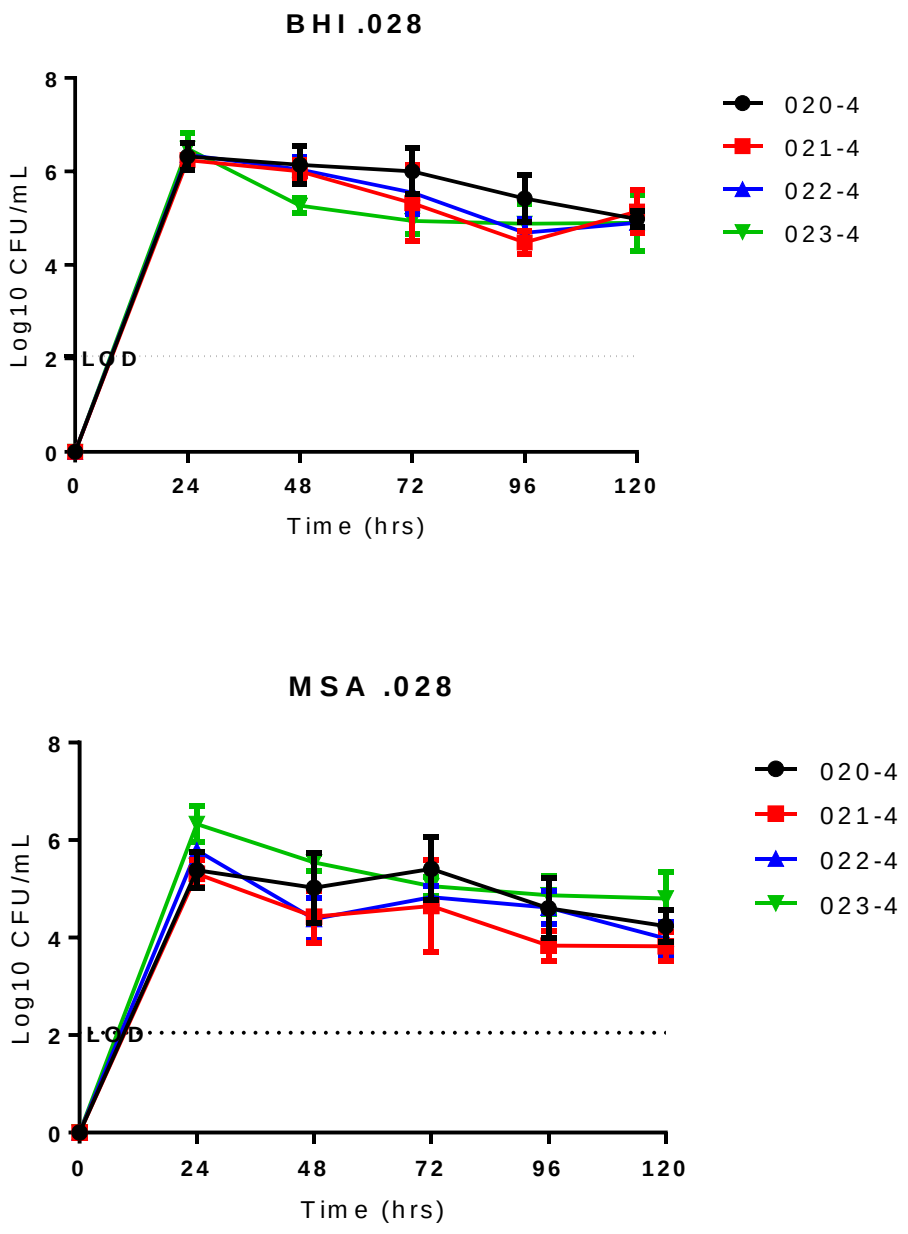


Figure 17 - CFU counts show growth and adherence to orthopedic pins within second set of test strains. Logarithmic representation of CFU/mL per time point on .028 pin size on BHI+5% activated charcoal agar and Mannitol Salt Agar. Data represent mean ± SD (*n* = 5 pins/group). Limit of detection is $\log_{10}$ 2.05.

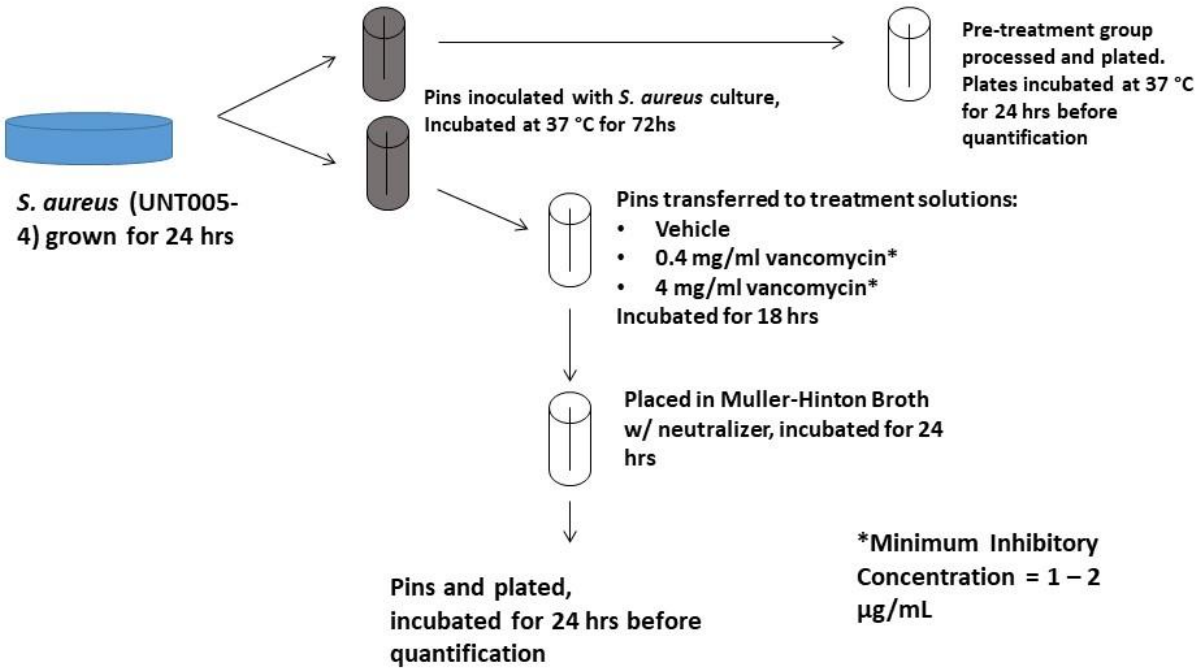


Figure 18 - Experimental Design for Confirming S. aureus Biofilm Formation. Diagram for an experiment to confirm the formation of biofilm by S. aureus colonies on orthopedic pins.

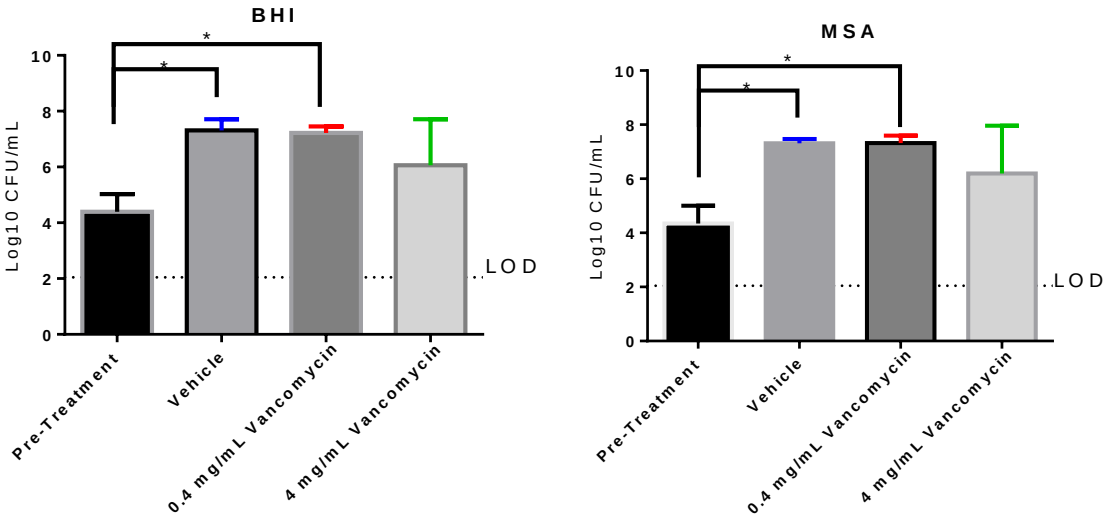


Figure 19 - CFU counts at 72 hours pre and post antibiotic treatment show adherence to and establishment of biofilm on orthopedic pins. Logarithmic representation of CFU/mL at 72 hours on .028 pin size on BHI+5% activated charcoal agar (A) and Mannitol Salt Agar (B). Data represent mean ± SD (n = 5 pins/group). Limit of detection is $\log_{10} 2.05$. Minimum inhibitory concentration of vancomycin is 4 ug/mL. * $p \leq 0.01$.

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