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Polymicrobial biofilm has been linked to chronic wounds, in recent years. Wounds such as pressure ulcers, diabetic foot ulcers, and venous stasis ulcers, can not heal properly because of the persistent nature of the biofilm in the wounds. Biofilms are harder to eradicate than plaktonic bacteria because they are high density colonies of bacteria that excrete a high volume of extracellular polymeric substance (EPS), up regulate genes that produce efflux pumps, and contain cells with little to no metabolic activity. The need for a topical antimicrobial to help eradicate the biofilm is necessary so the chronic wounds can heal. Healthpoint, Ltd. currently has *in vivo* gram positive bacteria models to evaluate novel topical antimicrobials. Since clinical biofilms are polymicrobial, this *Pseudomonas aeruginosa* 27312 biofilm in a murine model would further assist evaluate novel topical antimicrobials. The data from the model showed the wounds sustained a high level infection, and that a biofilm was established.

ESTABLISHING a *Pseudomonas aeruginosa* BIOFILM IN A MURINE MODEL

INTERNSHIP PRACTICUM REPORT

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MASTER'S IN BIOMEDICAL SCIENCES

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CHAPTER 1

INTRODUCTION TO THE STUDY

The scientific aim of Healthpoint, Ltd. (HP) is the treatment of traumatized and diseased skin. In recent years, bacterial biofilm formation has been linked as one of the contributors of chronic wounds and infections such as pressure ulcers, diabetic foot ulcers, and venous stasis ulcers (7, 10, 19). Therefore, targeting biofilms is one strategy to reduce the chronic nature of wounds. Healthpoint, Ltd. has both *in vitro* and *in vivo* models for gram-positive bacteria biofilms from *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA), to test their novel antimicrobial agents. However, bacterial biofilms can consist of both gram-negative and gram-positive bacteria (19). Thus, to successfully create novel topical antimicrobial agents against clinical bacterial biofilms, Healthpoint, Ltd. also needed to develop a gram-negative *in vivo* model.

This research project established and validated a *Pseudomonas aeruginosa* (Pa) 27312 biofilm in a superficial wound murine model. The model allowed Healthpoint, Ltd. to test their novel antimicrobial agents against bacterial biofilms *in vivo*. The project consisted of two main sections. The first section consisted of *in vitro* studies devoted to characterizing the strain, Pa 27312. *In vitro* growth curves were generated for Pa 27312 to establish which media broth supported the growth of the greatest density of bacteria in an overnight period. Minimum inhibitory concentrations (MIC) studies were also performed to compare the antibiotic sensitivity of the test wound isolate Pa 27312, to a control Pa strain, Pa 27853, isolate. The second section consisted of *in vivo* studies

devoted to verifying the presence of the biofilm in the wounds. Verification of the biofilm was demonstrated, in part, by generating *in vivo* growth curves to show that the wounds sustained a high level infection and to provide biopsy samples for histology purposes, so the biofilm could be visualized. Verification of the biofilm was also demonstrated through *in vivo* treatment studies, which quantified bacterial colonies after treatment, to show the reduced effectiveness of antimicrobials once a biofilm was established. Photographs were also taken of the wounds to demonstrate the reduced effectiveness of the antimicrobials once the biofilm was established. After the murine model was established, the model was used to evaluate the efficacy of a novel agent antimicrobial agent at reducing a Pa 27312 biofilm in a wound.

CHAPTER 2

INTERNSHIP SUBJECT

Background

Bacterial biofilms are organized, high-density, multi-layered aggregates of microbes that attach to a surface and each other through extracellular polymeric substances (EPS) (18, 19). Biofilm formation begins with a few planktonic bacterial cells attaching to a hard surface with a liquid interface. The newly adhered cells attach themselves to the surface and excrete EPS. The EPS generates layers by accumulating more bacterial cells. As maturation and quorum sensing occurs, bacterial cells begin to up-regulate EPS producing enzyme genes, that increase EPS, producing even thicker colonies of bacterial cells, and other genes that produce proteins that phenotypically change the bacterial cells in the biofilm (5, 18).

The EPS, the multilayered high density of bacterial cells and gene up-regulation all contribute to the resistance of biofilms to host defenses and antimicrobial agents. The thick EPS may hinder the penetration of some antimicrobial agents and antibodies, leaving them ineffective. Some biofilms express genes that produce efflux pumps that remove the antimicrobial back out of the bacteria. The thick multilayered biofilm contains cells with reduced metabolic rates that are too slow for some antimicrobials to be effective (6). These biofilms also contain persister cells, which individually enter into a dormant, non-dividing state and over express some genes, which give persisters a transient phenotype that is resistant to lethal concentrations of antibiotics (22, 23). Recent

research indicates that biofilms contain large numbers of persisters compared to planktonic bacteria which contribute to biofilms' antibiotic resistance (22, 23). Thus, the intrinsic antimicrobial resistance of biofilms allows the bacterial cells to persist over long periods of time.

Recent evidence suggests that polymicrobial biofilms are present in chronic wounds. (14). A statistically significant difference in biofilm formation in chronic wounds when compared to acute wounds has been demonstrated: 60% of chronic wound samples contained biofilms while only 6% of acute wound samples contained biofilms (12, 14). When chronic wounds, such as diabetic foot ulcers, pressure ulcers and venous leg ulcers were sampled in the study by James et al., they found *Staphylococcus, Enterococcus, Pseudomonas,* and *Proteus* were the most prominent bacteria in the wounds (12). Additional research has suggested that chronic ulcers do not heal due to *P. aeruginosa* biofilms (20). Scheierele et al. recently provided evidence that showed that bacterial biofilms contribute to delayed murine wound healing (19). Overall, there is growing evidence and support for the view that biofilms contribute to the chronic nature of wounds (14).

Therefore, it is important to establish models that will allow researchers to test the efficacy of antimicrobials against biofilms, so they can eventually be brought to the market. A useful *in vivo* model for investigating the efficacy of antimicrobials against biofilms involves reducing immune system function to limit immune influence on antimicrobial efficacy. Zuluaga et al. demonstrated that two injections of low dose cyclophosphamide at four days and one day prior to infection induced neutropenia in outbred mice that lasted at least three days (21). Studying the efficacy of antimicrobials

in a mouse with a compromised immune system is clinically relevant as patients that will be treated with antimicrobials often have a weak immune system due to chronic conditions such as diabetes mellitus. Useful models should also be able to sustain biofilm infection. The SKH1E hairless mice have been routinely used in wound healing, infectious and dermatological research because their skin is easily manipulated and topical creams are easily applied (3).

Specific Aims

The overall goal of the project was to establish and validate a Pa 27312 biofilm in a superficial wound murine model so Healthpoint, Ltd. could evaluate their novel topical antimicrobial agents. The research project consisted of two main sections, with two specific aims.

The aim of the first section was to characterize the test strain, Pa 27312. This section consisted of only *in vitro* studies: minimum inhibitory concentration (MIC) studies and *in vitro* growth curve studies. The goals of the two MIC studies were to compare the antibiotic sensitivity of the test wound isolate Pa 27312, to a control Pa strain, Pa 27853, isolate. The MIC assays were performed with Piperacillin, Tetracycline, Erythromycin, and Gentamicin to ensure Pa 27312 was inhibited within the expected concentration range of the control strain, Pa 27853. The results of this study also helped determine which established topical antimicrobial to use in the later *in vivo* studies. Two *in vitro* growth curve studies, for Pa 27312, were also performed to characterize the strain. The goals of these studies were to determine which media broth supported the growth of the greatest density of Pa 27312 in an overnight culture. Four different media broths: tryptic

soy broth (TSB), tryptic soy broth plus 4.5% glucose, cation adjusted Muller Hinton broth (CAMHB), and cation adjusted Muller Hinton broth plus 4.5% glucose were evaluated for the *in vitro* growth curve studies. The media broth that demonstrated to support the greatest density of Pa 27312 was used for inoculum preparation in later *in vivo* studies.

The aim of the second section of this project was to verify the presence of the biofilm in the wounds of the murine model. This section consisted of two types of *in vivo* studies: *in vivo* growth curve studies and *in vivo* treatment studies. A bacterial biofilm is generally characterized *in vivo,* through a large colonization of the bacteria, and the reduced effectiveness of antimicrobials once the biofilm has formed. So, verification of the presence of the Pa 27312 biofilm was accomplished by verifying that the model allowed the Pa 27312 to sustain a high level of infection, and by demonstrating that antimicrobials were less effective once Pa 27312 was given time to establish a biofilm. Three *in vivo* growth curve studies were performed with the goal of demonstrating that the wounds from the model sustained a high level Pa 27312 infection. Five *in vivo* treatment studies were conducted with the goal of demonstrating the reduced antimicrobial efficacy once a biofilm was established in a wound.

Significance

Chronic wounds such as diabetic foot ulcers, venous leg ulcers, and pressure sores have been linked to bacterial biofilms (7, 18, 19). These wounds decrease quality of life and lead to disabilities. In fact, nearly 25% of diabetic patients who develop lower extremity ulcers will undergo amputation due to inadequate healing (12). These chronic wounds cost billions of dollars every year to treat, and make up over half of the skin disease costs annually (14). Thus, in conjunction with current Healthpoint, Ltd. *in vivo* models, this *P. aeruginosa* biofilm in a murine model will aid Healthpoint, Ltd. in developing a novel topical antimicrobial that is effective against a clinical bacterial biofilm that contains both gram-negative and gram-positive bacteria. This novel agent, in turn, may lead to reduced healing times and reduced amputations, resulting in a lower healthcare costs for patients and (payors) medical insurance companies.

Materials and Methods

Mice:

All *in vivo* studies were conducted with outbred, female, SKH1E hairless mice because there was no need to have genetically similar mice. Hairless mice also allowed for easy manipulation and visualization of the skin. The outbred SKH1E mice were also easier and cheaper to acquire than other strains of hairless mice. Female 19-21g hairless SKH1E mice were obtained from Charles River Laboratories. Mice were housed and cared for in accordance with the "Guide for the Care and Use of Laboratory Animals". All studies were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center (UNTHSC).

Sampling and data collection for the *in vitro* growth curve studies:

Pa 27312 was streaked on a rich media plate, and grown overnight in a 37° C incubator. A 10 ml, direct suspension of phosphate buffer saline (PBS) with $1x10^9$ colony forming units/ml (cfu/ml) of Pa 27312 was generated to inoculate each media broth to a initial concentration of $1x10^6$ cfu/ml of Pa 27312. To obtain a final concentration of $1x10^9$ (cfu/ml) of Pa 27312 in the direct suspension, a spectrophotometer was used, and the assumption that an absorbance of 1.0 is about $1x10^9$ cfu/ml. Calculations were then made to insure an initial, time 0 hour, Pa 27312 concentration of $1x10^6$ cfu/ml in 20 ml of the 4 individual broths: Cation Adjusted Muller Hinton broth (CAMHB), CAMHB plus 4.5% glucose, Tryptic Soy Broth (TSB), and TSB plus 4.5% glucose. The inoculated broths were placed in a 37° C shaking incubator. For the first study, 1 ml samples of each broth were taken in duplicate at 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 12 hours and 24 hours; 1:10 dilutions were performed for each sample and the optical density (OD) was measured at A600nm on a spectrophotometer. For study 2, 1 ml samples of each broth were removed in duplicate at 1 hour, 2 hours, 4 hours, 5 hours, 6 hours, 7 hours, 11 hours and 24 hours; 1:10 dilution were performed for each sample and the optical density (OD) was measured at A600nm on a spectrophotometer. The cfu/ml was calculated for each time period.

Sampling and data collection for minimum inhibitory concentration (MIC) studies:

The Clinical Laboratory Standards Institute (CLSI) standard method was used. Pa 27853 was used as a control strain to compare to the test, wound isolate strain, Pa 27312. Two MIC studies were conducted. Each study consisted of two MIC assays with its own 96-well test plate: one plate for Pa 27853 and the other for Pa 27312. Tetracycline,

Gentamicin, Piperacillin, and Erythromyocin were tested under the same concentration ranges for both Pa strains.

For each study, a stock solution of each antimicrobial was produced at 40x the final desired concentration. The stock solution of each antimicrobial was then diluted to 2x the desired final concentration, and 100 µl of that antimicrobial was added to column 1 of the two, test 96-well plates. 50 µl of Cation-Adjusted Muller Hinton Both (CAMHB) was added to the columns 2-12 of the two, test 96-well plates. Then serial 1:2 dilutions were performed by adding 50 µl from column 1 to column 2, and so on, until column 11, for rows A-H. Column 12 was a positive growth control. Overnight cultures were generated for Pa 27312 and Pa 27853. The next day, inoculums were prepared in CAMHB, for Pa 27312 and Pa 27853, to a final concentration of $1x10^6$ cfu/ml. 50 µl of the inoculums were added to all the wells of the 96 well plates that contain the antimicrobials, except wells 12D-12H, which serves as a sterile control of CAMHB. The plates were then put in a 37°C incubator overnight, and then evaluated the next day.

Cytoxan preparation for intraperitoneal (IP) injection:

For each *in vivo* study all the mice were given a 150 mg/kg dose of Cytoxan through an intraperitoneal (IP) injection. The mice were weighed, and then the average weight was calculated. Then, the 500 mg vial of Cytoxan was diluted with bacteriostatic water, so the injection volume of 0.2 ml was equivalent to a concentration of 150 mg/kg of Cytoxan, when administered to each mouse.

Inoculum preparation for the *in vivo* growth curve studies and the *in vivo* treatment studies:

Pa 27312 was obtained from the American Type Culture Collection. The inoculums for the studies were generated by inoculating 20 ml of tryptic soy broth (TSB) + 4.5% glucose with Pa 27312 overnight in a 37°C shaking incubator. A streak of the inoculated broth was performed to insure purity of the broth. The following day, the overnight culture was centrifuged for 5 minutes at 4,000 x g. to create a pellet. The supernatant was decanted and the pellet resuspended into 5 ml of TSB medium to a final concentration of $2x10^9$ cfu/ml in a snap-cap centrifuge tube.

Superficial wounding and inoculation for the *in vivo* growth curve studies and *in vivo* treatment studies:

Transient neutropenia was induced by injecting a 150 mg/kg dose of Cytoxan (cyclophosphamide for injection) into the peritoneum of mice four days prior to the growth curve and treatment studies. Transient neutropenia was induced so the infection would set in easier. Mice were anesthetized by isofluorane inhalation; the backs were sterilized with povidone-iodine swab sticks, then the povidone-iodine was wiped off with alcohol swab sticks. Two 1 cm superficial wounds were generated, one on each side of the midline, each 0.5-1 cm off the midline, just down from the scapula of the mice, using a Dremel rotary tool with an isopropanol sterilized sanding attachment. To generate consistent wounds, a sterilized piece of plastic with a 1 cm circle cut out was placed on the sterile skin; the Dremel attachment was lightly touched in short intervals to the skin until it was red and glistening. The Dremel tool allowed for quick wounding, and was easy to handle and use. Wounding was done on the backs of the mice for easy access and

to prevent the mice from manipulating their own wounds. The sterilized piece of plastic was removed and the wounds were gently cleaned with saline to remove any debris. The wounds were inoculated with 10µl of the inoculum. The purpose of this was to uniformly inoculate each wound at a concentration of $2x10^7$ cfu/wound. Spot Band-Aids were moistened with saline and applied to each wound. The Band-Aids were moistened because *Pseudomonas aeruginosa* thrives in wet environments and allows a biofilm to establish. The wounds were then secured with a Surgilast sleeve and taped with a strip of Elastikon around the waist of the mice.

Antimicrobial treatment plan for the *in vivo* treatment studies:

Treatments were applied at two different occasions for each wound. Treatment was initiated either 4 hours or 24 hours (established biofilm) post inoculation; the second treatment was applied 24 hours after the first. The Elastikon strip, Surgilast sleeve, and spot Band-Aids were removed prior to treatment application. Each wound was gently cleaned with saline, and then the antimicrobial agent or saline (Moist Control) was applied using a three ml syringe to dispense 0.1 ml across the wound. Then a spot Band-Aid was moistened with saline and applied to each wound, the Band-Aid was secured with a Surgilast sleeve and a strip of Elastikon. For the second treatment, previous dressings were removed and the wounds were gently cleaned with saline prior to the second treatment application. The wounds were then dressed with a saline moistened spot Band-Aid, and secured with Surgilast sleeve and an Elastikon strip of tape.

Sample collection and processing for the *in vivo* studies:

24 hours after the last treatment, the mice were humanely euthanized through carbon dioxide inhalation and cervical dislocation; each wound was sampled in duplicate using

four mm biopsy punches. One of the punched samples was placed in 10% buffered formalin for histology. The second punched sample was placed into an individual tared sample tube containing 1 ml of 1x PBS and stored on ice until processed. Each sample was weighed to determine total weight, homogenized at 30,000 rpm, and then the homogenized sample was serially diluted 10-fold in a 96 well plate and then spotted onto a Charcoal agar plate and a *Pseudomonas* Selection Agar (PSA) plate. Charcoal agar was used in case there was drug carry over in the treatment studies. The plates were allowed to dry, and then incubated overnight at 37°C.

Data analysis for the *in vivo* studies:

For all *in vivo* studies, after the overnight incubation of the PSA and charcoal agar spot plates, the sample colonies were counted and cfu/gram tissue was calculated and then was converted to log units, for each sample.

After all the *in vivo* growth curve studies were completed, the results were pooled, and Normality was tested for with Sigma Plot 11. Once Normality was determined, data was evaluated using One-way ANOVA to determine if there was any significant difference in colony formation between the data points.

After all the *in vivo* treatment studies were completed, the results were pooled, and normal distribution was assessed using Sigma Plot 11. To determine if a biofilm was established, each treatment's log cfu/g of tissue was pooled, for both groups (early 4 hour group and 24 hour group), and a bar graph was produced in MS Excel illustrating the log reduction of Pa 27312 after treatment when compared to the Moist Control of that group (4 hour or 24 hour group). Reduced effectiveness of treatment in the 24 hour group indicated a biofilm formed. One-way ANOVA was used to determine if there was a

significant drop in bacterial colonies after treatment, when compared to the Moist Control of the group. One-way ANOVA was also used to determine if there was a significant drop in bacterial colonies after treatment, between the two groups.

Results

First Section: Characterization of Pa 27312

Minimum inhibitory concentrations (MIC):

Table 1: Minimum Inhibitory Concentrations (MIC) for *Pseudomonas aeruginosa* (Pa) 27312 and Pa 27853

Antimicrobial	Known MIC Range for Pa 27853	MIC #1 for Pa 27853	MIC #2 for Pa 27853	MIC #1 for Pa 27312	MIC #2 for Pa 27312
Gentamicin	0.5-2 µg/ml	0.5 µg/ml	0.5 µg/ml	2 µg/ml	4 µg/ml
Tetracycline	8-32 µg/ml	8 µg/ml	8 µg/ml	8 µg/ml	8 µg/ml
Piperacillin	1-8 µg/ml	0.5 µg/ml	2 µg/ml	0.5 µg/ml	0.5 µg/ml
Erythromycin	----------	----------	80 µg/ml	----------	40 µg/ml

Table 1: Shows the MIC results for test strain, Pa 27312, and the control strain, Pa 27853. Pa 273212 was sensitive to the antibiotics under the same concentration range as the control strain Pa 27853.

In order to compare the antibiotic sensitivity of Pa 27312 to a control Pa strain, Pa 27853, two minimum inhibitory concentration (MIC) studies were performed using the procedure set by the Clinical Laboratory Standards Institute (CLSI); Gentamicin, Tetracycline, Piperacillin, and Erythromycin were the antimicrobials evaluated. The known inhibitory concentration ranges for Pa 27853 were obtained from the CLSI

handbook. Erythromycin did not have a known MIC for Pa 27853 according to the CLSI. Table 1 depicts the results of both MIC studies. The first study indicated that Pa 27853 was inhibited within the known concentration ranges of the test antimicrobials. It also indicated that Pa 27312 was susceptible to all the antimicrobials, within the known inhibitory concentration range, except Erythromycin. For the first study, Erythromycin was tested from 8 μg/ml to 0.0156 μg/ml and neither strain was inhibited. The second study, again, indicated that Pa 27853 was inhibited by all the tested antimicrobials within the known concentration ranges of the antimicrobials. Pa 27312 was also susceptible to all the antimicrobials. For the second study, Erythromycin was tested from 160 μg/ml to 0.312 μg/ml, and both strains of *Pseudomonas aeruginosa* were susceptible at that range.

After the examination of the MIC studies, it was determined that Pa 27312 was a valid strain to continue with the *in vivo* studies because the tested antibiotics killed the planktonic version of Pa 27312 under the same concentration range as the control strain, Pa 27853. And after researching the topical antimicrobials used clinically against bacterial infections and biofilms, it was decided Gentamicin sulfate cream, Silvasorb gel (which uses silver ions as the antimicrobial) and Neosporin (which contains neomycin, polymyxin B, and bacitracin) were to be used as the known antimicrobial agents in the *in vivo* treamtment studies.

In vitro growth curve:

In order to determine which media broth supported the growth of the greatest density of Pa 27312 in an overnight culture, an *in vitro* growth curve was produced. The media broth that supported the growth of the greatest density of Pa 27312 was used during the inoculation preparation in the *in vivo* studies. An initial streak plate of Pa 27312 was

grown on PSA overnight in a 37°C incubator to insure purity of the bacteria. A direct suspension of phosphate buffer saline (PBS) with $1x10^9$ cfu/ml of Pa 27312 was generated to inoculate each media broth to a initial concentration of $1x10^6$ cfu/ml of Pa 27312. This was done to insure all samples started with the same initial growth point. The direct suspension and the results were generated with the aid a spectrophotometer. For each time point, the absorbance of samples (1:10 diluted) was measured and the bacteria quantified by working under the assumption that a 1.0 absorbance reading was equivalent to $1x10^9$ cfu/ml, then multiplied by the dilution factor. For both studies, each time point absorbance was measured in triplicate. Figure 1 depicts the averaged results for each time point.

Figure 1: *In Vitro Pseudomonas aeruginosa* 27312 Growth Curve

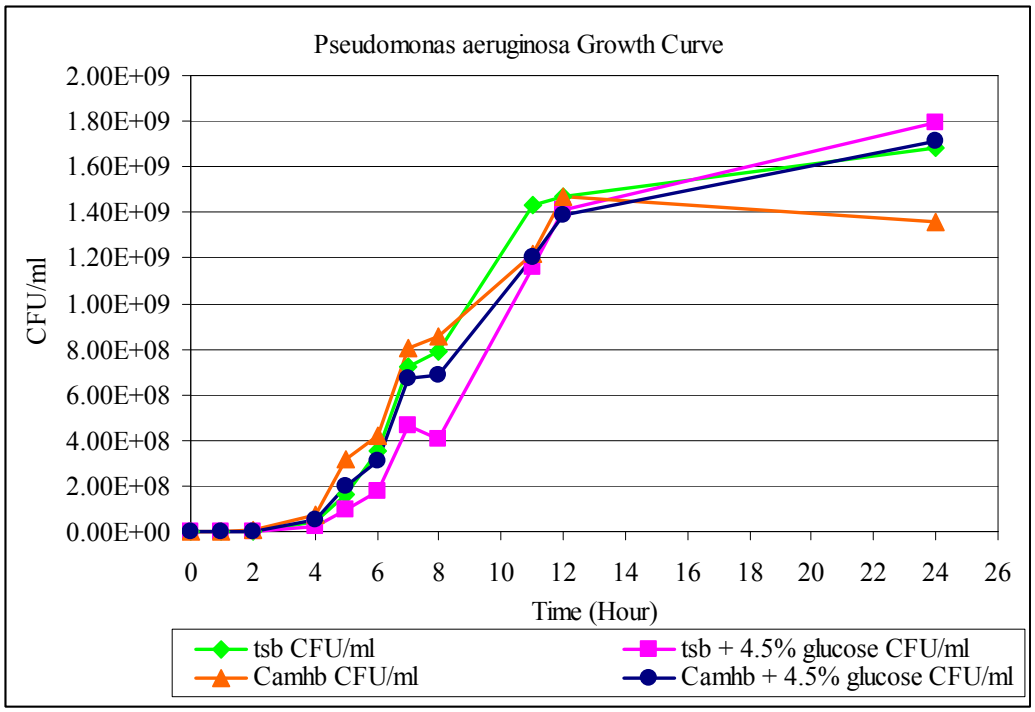

Figure 1: Abbreviations: tsb = Tryptic Soy Broth; Cambh = Cation-Adjusted Mueller Hinton Broth; CFU = Colony Forming Units. This figure illustrates the average cfu/ml generated from the two growth curve studies. TSB + 4.5 glucose supported the greatest density of growth over the 24 hour period.

Before the *in vitro* growth curve studies were performed, literature research was conducted and based on previous *in vitro* and *in vivo Pseudomonas* models, Cation-Adjusted Mueller Hinton Broth (Camhb), Camhb + 4.5% glucose, Tryptic Soy Broth (TSB), and TSB + 4.5% glucose was determined to be the best broths to test. Two growth curve studies were conducted with those broths. Figure 1 illustrates the average colony forming units/ml (cfu/ml) generated from the two growth curves, per media broth. TSB + 4.5 glucose had the greatest density of Pa 27312 after the 24 hours with $1.79x10^9$ cfu/ml. TSB + 4.5 glucose also had the greatest growth rates, from 12 hours to 24 hours. The time period of 12-24 hours was of special interest because, due to an array of circumstances, the overnight culture for the *in vivo* studies may not be able to be started 24 hours before surgery and inoculation of the wound, but would always be started at least 12 hours before surgery and inoculation of the wound. Figure 1 also shows TSB + 4.5 glucose tended to support the greatest rate of growth for the 12-24 hour time period, by increasing the absorbance and therefore, cfu/ml, by an average of $5.08x10^8$ cfu/ml. Therefore, although most of the media broths supported a great density of Pa 27321, it was determined that TSB + 4.5% glucose tended to support the greatest density of Pa 27312 in the overnight culture, and therefore, used to prepare the inoculum for all the *in vivo* studies.

The MIC studies and the *in vitro* growth curve studies were performed to characterize the strain, Pa 27312. The MIC studies showed that Pa 27312 was inhibited at the same concentration ranges of various antibiotics, when compared to the control strain, Pa 27853. Therefore, Pa 27312 was shown to be a valid strain to use in the *in vivo* biofilm studies. And from the results with the Gentamicin MIC and literature research, it was determined Gentamicin cream, Silvasorb gel, and Neosporin were to be used in the *in vivo* treatment studies. The *in vitro* growth curve demonstrated that TSB +4.5 % glucose supported the growth of the greatest density of Pa 27312 in an overnight culture. Therefore, TSB + 4.5% glucose was used to prepare the inoculum for all the *in vivo* studies.

Second Section: Verification of the Presence of the Pa 27312 Biofilm in the Murine Model

The purpose of generating the Pa 27312 biofilm in a murine model for Healthpoint, ltd., was to evaluate novel agents against bacterial biofilms. But, in order to test novel agents, verification that a biofilm was generated in the model was needed. So, two types of *in vivo* studies were performed, in *vivo* growth curve studies and *in vivo* treatment studies, with the overall aim of verifying the presence of a bacterial biofilm in the model. A bacterial biofilm is generally characterized *in vivo,* through a large colonization of the bacteria, and the reduced effectiveness of antimicrobials once the biofilm has formed. The purpose of the *in vivo* growth curve studies was to demonstrate the murine model could sustain high bacterial infection. The purpose of the *in vivo* treatment studies was to demonstrate the reduced effectiveness of antimicrobials once a biofilm was established in the wound.

In vivo growth curve:

To determine if the murine model could sustain a high bacterial infection in the wounds, two, 4 mm biopsy samples were taken from each wound at certain time points, one for histological examination and the other for enumeration of bacteria on the wound. The biopsy sample for enumeration was quantified by homogenizing the sample; placing it in 96-well plates for serial dilution, spotting the sample on *Pseudomonas* selection agar and charcoal agar, for a more accurate count of bacterial colonies in the wound. The colonies were counted for each sample and quantified in colony forming units/gram of tissue (cfu/g), then converted to the logarithmic form, so they could be easily displayed. The biopsy sample for histology purposes was stored in formalin for preservation, and later processed by Healthpoint employee, Sarah Ramsey. Histology was used to provide visual evidence of the bacterial biofilm infection in the wound.

Figure 2: Mean *Pseudomonas aeruginosa* 27312 Count from the Biopsy Samples

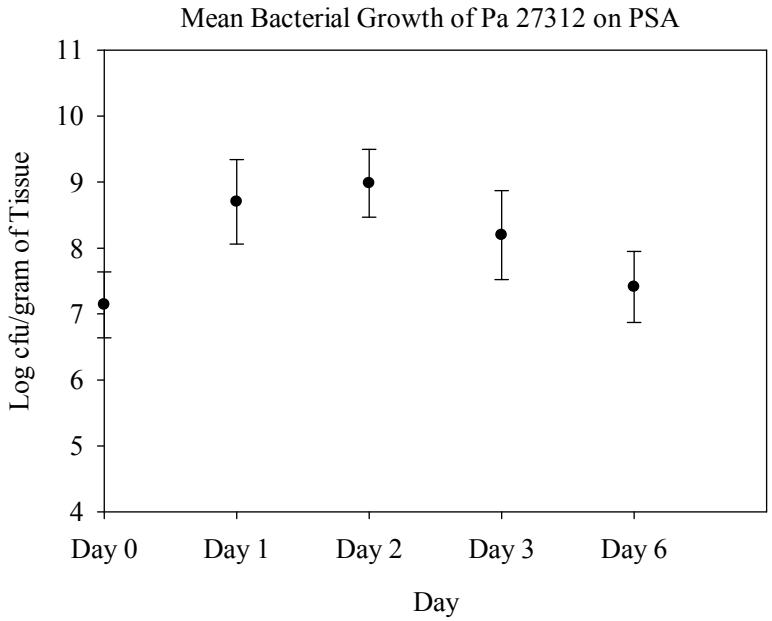

Figure 2: This figure represents the *in vivo* growth curve of Pa 27312. Biopsy samples were taken at certain time points for each study. Then, those time points were pooled and averaged. The graph depicts the average cfu/g of tissue for each time point. Over three days, a high bacterial infection was sustained in the wounds.

The data of all the *in vivo* growth curve studies were pooled and the Shapiro-Wilks normality test was run. The data collected was shown to have a normal distribution, so the mean instead of median was used to compare bacterial counts and the stability of the infection; instead of Kruskal-Wallis One Way Analysis of Variance on Ranks, One-way ANOVA was used to analyze the difference in means between the time points. Figure 2 illustrates the pooled, mean bacterial log colony forming units per gram of tissue (cfu/g) for each time period grown on *Pseudomonas* selection agar (PSA). Data points were taken at day 1, day 2, day 3, and day 6. Day 0 corresponds to the average challenge inoculum placed on the wounds. Day 2 would correspond to biopsy samples taken from the early (4 hour) treatment group and day 3 would correspond to biopsy samples taken from the late 24-hour treatment group, in later *in vivo* treatment studies. The day 6 time period was added to the study to determine how much the infection would decline after most of the Cytoxan was out of the mice's system. In theory, the bacterial colonies should drop once the immune system is at full strength; however it should still be high if a biofilm is present. Bacterial colonies grown on PSA for the day 1 biopsies had an average of 8.699 log cfu/g of tissue, day 2 biopsies had an average of 8.982 log cfu/g of tissue, day 3 biopsies had an average 8.195 log cfu/g of tissue, and day 6 biopsies had an average 7.410 log cfu/g of tissue. There was no statistical significant difference between

day 1 and day 2, and day 1 and day 3 bacterial colonies ($p < 0.05$). There was a significant difference between day 2 and day 3 ($p < 0.05$). However, this difference was evident because the mean for day 2 was higher than any other day, and day 3 had the second lowest bacterial colonies, which resulted in a greater difference in mean between day 2 and day 3. So, it was concluded that the high bacterial infection was maintained over three days without a significant drop in the Pa 27312 colonies. Day 6 bacterial counts had a statistically significant lower difference when compared to the first three days ($p < 0.05$). However, the day 6 bacterial count was still higher than the challenge inoculum of 7.140 log cfu/g of tissue. This infers that although the day 6 colonies were significantly lower than the first three days, the average was 7.410 log cfu/g of tissue, which is still a highly colonized infection.

All the previous information indicates that this murine model did maintain a stable, high bacterial infection, which is an indicator of a bacterial biofilm. Over the entire six days, the average Pa 27312 infection was log 8.38 cfu/g of tissue, and the maximum difference in means was 1.57 log cfu/g of tissue. Another indicator that a bacterial biofilm is present is the reduced effect of antimicrobials in killing the bacteria once the biofilm has been established. The following treatment studies assessed this issue.

In vivo treatment studies:

In order to further determine if a Pa 27312 biofilm was established in this murine model, the wounds were treated with three antimicrobials, shown clinically or *in vitro,* to be effective at the killing planktonic, gram negative bacteria, including, *Pseudomonas aeruginosa.* The treatments tested were Neosporin, Gentamicin cream, Silvasorb gel, and a Moist Control to which the three treatments were compared. Neosporin was chosen

because it is a commonly used antimicrobial to prevent infection in wounds. Gentamicin was chosen as a treatment because it was shown to be effective at eliminating the planktonic version of Pa 27312. Silvasorb gel was chosen as a treatment because it is one of the silver ion products clinically used to prevent infection in burn and open wound patients. Treatment plans were split into two groups, the first was the early treatment group (4 hour) and the other group was the late established biofilm treatment group (24 hour). These two groups helped verify if a biofilm was present in this model, by allowing the quantification of the reduced effectiveness of treatments once Pa 27312 was given time to fully colonize and establish a biofilm. The 4 hour group had their treatment start 4 hours post inoculation, which mimics a wound that has yet to establish a biofilm, therefore, assessing if the treatment was effective at killing Pa 27312 and by how much, when compared to the Moist Control log cfu/g of tissue for the 4 hour group. The 24 hour group had their treatment start 24 hours post inoculation, which gives the bacteria enough time to fully colonize and establish the Pa 27312 biofilm, therefore, assessing if the treatment was effective at killing Pa 27312 biofilm and by how much, when compared to the Moist Control log cfu/g of tissue for the 24 hour group. More importantly for this section of the research, the 24 hour group showed how much the antimicrobials lost effectiveness at reducing the colonies of Pa 27312 when compared to the 4 hour group; indicating that indeed a biofilm was established in this murine model. A 4 mm biopsy sample was taken from each wound, then processed and quantified though homogenization of sample in phosphate buffered saline, serial dilution in a 96-well plate, and spot plating on *Pseudomonas* selection agar (PSA) and Charcoal agar, to ensure there was no drug carry-over. Results of the reduced effectiveness of antimicrobials are shown

in figure 4 only for PSA because no drug carry-over was evident. Photographs of the wounds were also taken to provide visual evidence of the infection.

Figure 3: Mean *Pseudomonas aeruginosa* 27312 Count on *Pseudomonas* Selection Agar (PSA) From the Biopsy Samples After Antimicrobial Treatment

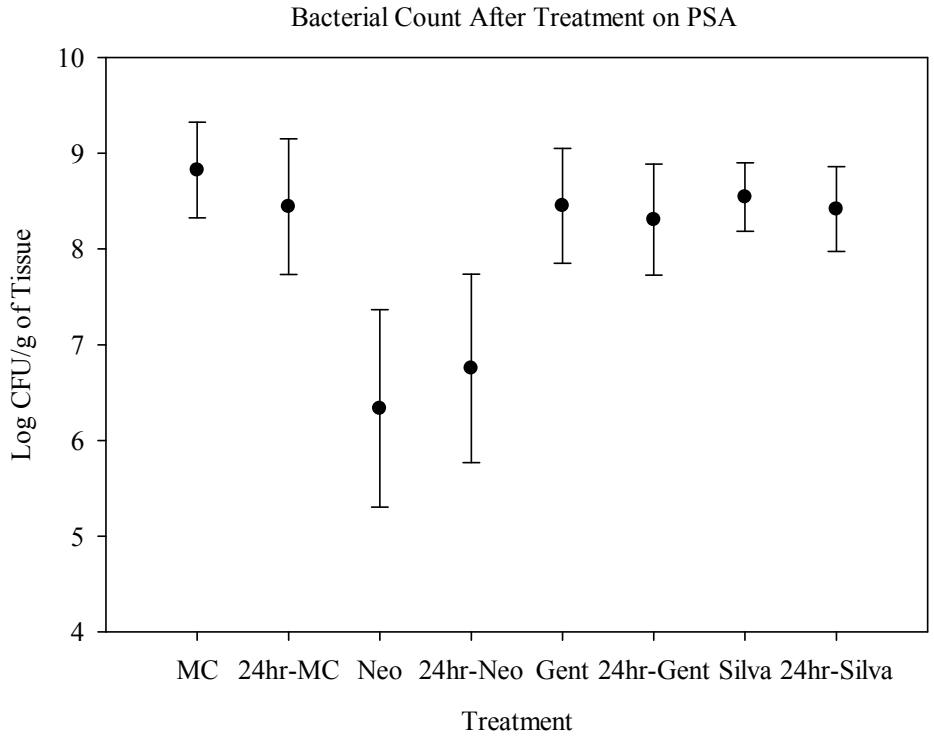

Figure 3: Values are the pooled, mean +/- standard deviation for all treatments. Y-axis = log cfu/g Abbreviations: Moist Control = MC; Neosporin = Neo; Gentamicin = Gent; Silvasorb = Silva; 24hr = 24 hour, established biofilm group. The figure represents the bacterial colonies grown from the biopsy samples, of each wound, after antimicrobial treatment. Neosporin showed reduced Pa 27312 colonization before and after a biofilm was established.

Table 2: Mean and Standard Deviation of *Pseudomonas aeruginosa* 27312 Colonies after Treatment from the Biopsy Samples

	Moist Control (MC)	24hr-MC	Neosporin (Neo)	24hr-Neo	Gentamicin Cream (Gent)	24hr-Gent	Silvasorb gel (Silva)	24hr-Silva
Mean (Log cfu/g of tissue)	8.82	8.44	6.33	6.75	8.45	8.30	8.54	8.42
Standard Deviation	0.50	0.71	1.03	0.98	0.60	0.58	0.36	0.44
Sample size	15	14	13	13	14	14	9	9

Table 2: This table is the numerical representation of Figure 3; the bacterial colonies grown from the biopsy samples, of each wound, after antimicrobial treatment.

Figure 4: Log Reduction Results after Treatment on PSA

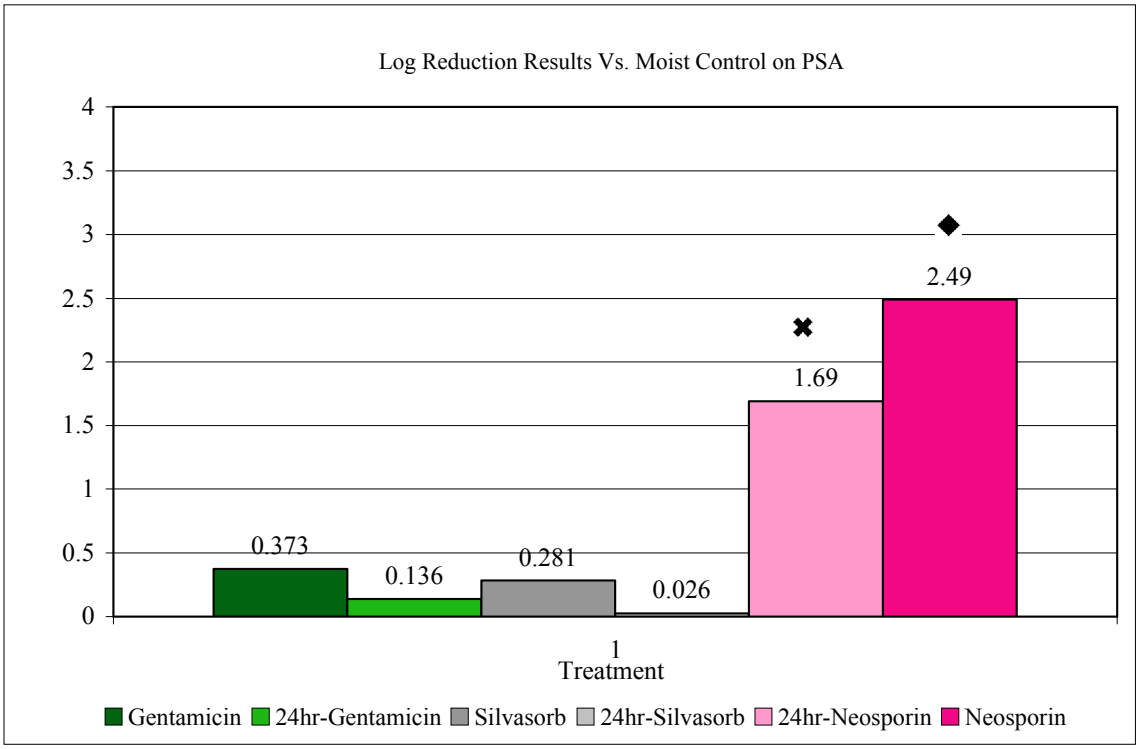

Figure 4: This figure illustrates the log reduction of cfu/g of tissue for each treatment compared to the Moist Control, for the early treatment group (4 hour) and the 24 hour established biofilm treatment group (24 hour). Neosporin is the only treatment that had a significant reduction in bacterial colonization before and after the establishment of the biofilm. There was a reduced effectiveness of Neosporin after the biofilm was established in the wound. ✕ = Represents statistical significance ♦ = Represents statistical significance

Figure 3 in conjunction with Table 2 show the mean bacterial colonies after treatment plan, for each of the antimicrobials, for both the early treatment groups and the established biofilm treatment groups. For both treatment groups, over the course of the treatment period, there were stable Pa 27312 infections for each treatment, other than Neosporin. The mean bacterial colonies, for the Moist Control treatment, had a 0.382 log cfu/g of tissue difference between the 4-hour group and the 24-hour group. Both means were within the 99% confidence interval, for all Moist Control treatments. The mean bacterial colonies for the Gentamicin treatments had a 0.145 log cfu/g of tissue difference between the early treatment and the late treatment. The mean for the Silvasorb gel treatments had 0.127 log cfu/g of tissue difference between the two treatment groups. Thus, over the two and three days of treatment, corresponding to the early treatment and late treatment groups, respectively, a stable bacterial infection was maintained.

Figure 4 illustrates the log reduction of cfu/g of tissue for each treatment compared to the Moist Control, for the early treatment group (4 hour) and the 24 hour established biofilm treatment group (24 hour), on PSA. The 4 hour group is the darker shade of color

for each of antimicrobial treatments depicted on the figure. Gentamicin had a log reduction of 0.373 cfu/g and Silvasorb had a log reduction of 0.281 cfu/g, when compared the Moist Control. Both of the antimicrobials reduced bacterial colonies, but they were not statistically significant. Neosporin was the only treatment that significantly reduced the bacterial colonies when compared to the Moist Control with a 2.49 log cfu/g reduction. For the 24 hour treatment group, Gentamicin had a log reduction of 0.136 log cfu/g and Silvasorb had a log reduction of 0.026 log cfu/g when compared to the Moist Control, which were both statistically insignificant. The Neosporin had a statistically significant ($p<0.05$), 1.69 log cfu/g reduction when compared to the Moist Control.

A comparison between the 4 hour group log reduction results and the 24 hour group log reduction results revealed that each of the three treatments were not as effective once the biofilm had time to establish. There was a 0.24 log cfu/g reduction in effectiveness for Gentamicin, a 0.26 log cfu/g reduction in effectiveness for Silvasorb, and 0.8 log cfu/g reduction in effectiveness for Neosporin, when comparing the log reduction results of the 4 hour group to the 24 hour group. Although Gentamicin and Silvasorb did not have significant log reduction when compared to the Moist Control in the early treatment group, they both were not as effective once the biofilm had time to establish. Neosporin was also not as effective once the biofilm was established. The decline of log reduction results for all three treatments and the sustained levels of Pa 27312 colonies among the non-effective treatments indicate a biofilm was formed.

The *in vivo* studies showed the model could sustain a high level of infection and that antimicrobials lose effectiveness once the biofilm formed.

Histology of biopsy samples to verify the presence of biofilm

In order to visualize the presence of the Pa 27312 infection in the wounds and to further provide evidence a biofilm was generated, gram staining was performed.

Illustration 1: Gram Stain: Three Days Post Inoculation

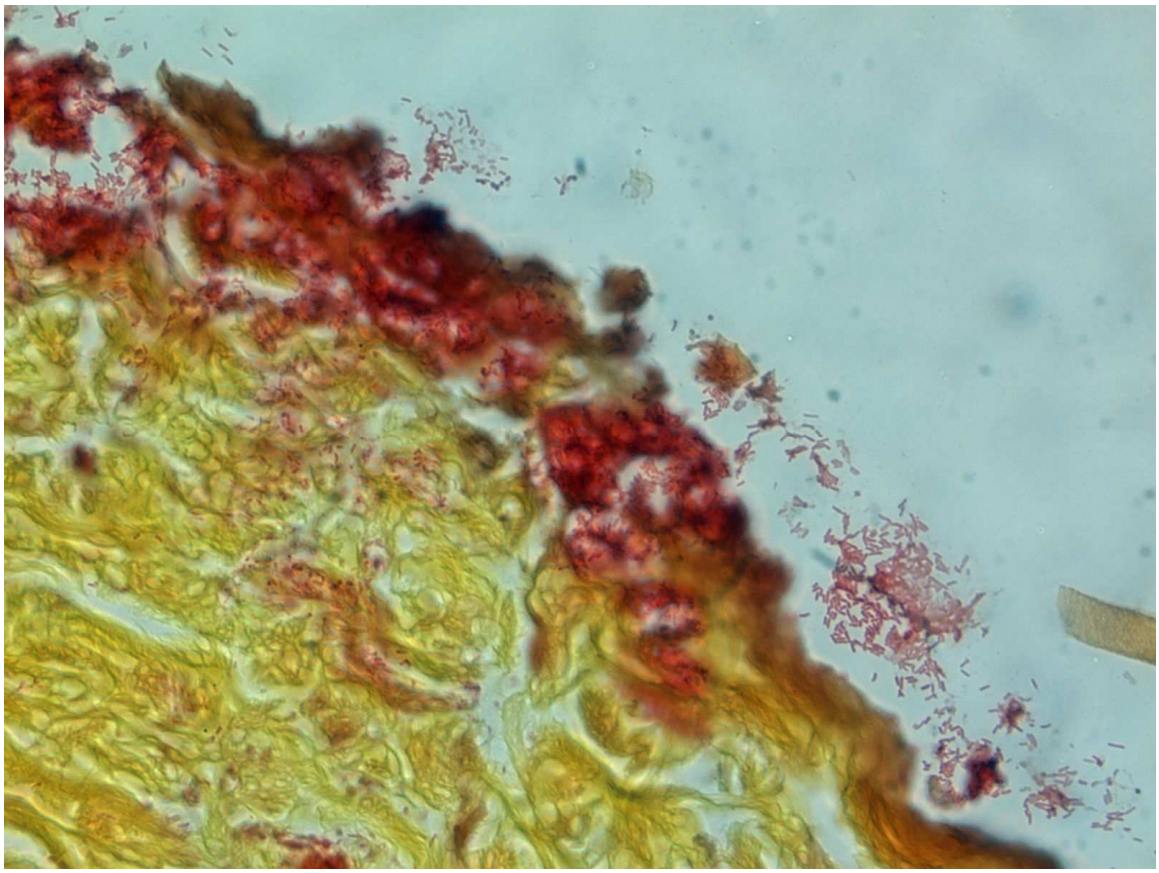

Illustration 1: This picture depicts a gram stained biopsy sample after three days of Moist Control treatment. The red stains on the cells are the Pa 27312 biofilm that was present on the wound.

Not pictured; the biopsy samples were processed with H&E in order to visualize the cellular structure/arrangement of the samples. If the sample were not sectioned properly, the procedure would not continue. However, the stain displayed promising results of a

potentially thick biofilm, so gram staining was performed. The biopsy sample was gram stained and showed thick bacterial biofilm just on the epidermis of the wound. All the red stain in the photo is the Pa 27321 biofilm. The darker red within the thick biofilm seemed to be a greater density of Pa 27312. The red stained rods are individual Pa 27312 cells. Basically, this picture shows a definitive Pa 27312 biofilm did form in wounds of this model.

Photographic evidence of the reduced effectiveness of antimicrobials once a biofilm was established in the wounds:

In order to further verify a biofilm did develop, visual evidence of the reduced effectiveness of the treatment was produced. After the completion of the treatment cycle, for both groups, photographs were taken before the 4mm biopsy samples were taken.

Illustration 2: Reduced Effectiveness of Treatment Before and after the Establishment of *Pseudomonas aeruginosa* 27312 Biofilm: Neosporin and Moist Control

A) 4 hour Moist Control

B) 24 hour Moist Control

:

C) 4 hour Neosporin

D) 24 hour Neosporin

Illustration 2: These pictures illustrate the reduced effectiveness of Neosporin once the Pa 27312 biofilm was established. 2B showed a wound that was not healed and typical Pa 27312 biofilm infection; a slimy green topical layer, with a well defined wounded area. (Note that 2A also had a biofilm infection, but the wound was red, because the spot Band-Aid fell off during the treatment course). 2C showed how effective Neosporin was if initiated before a biofilm was formed; the wound area was not visible, and there was no visual infection. 2D showed how Neosporin losses effectiveness once the Pa 27312 biofilm was established; there was visible wound area and a topical slimy Pa 27312 biofilm on the wound.

Illustration 2 depicted the wounds after treatment with Moist Controls and Neosporin. 2A and 2B showed how wounds look after being treated with Moist Control, 4 hours post inoculation and 24 hours post inoculation respectively. Both 2A and 2B had a visible biofilm infection in the wound, but 2A was a bit dried out due to the spot Band-Aid falling off. 2B showed what a typical Pa 27312 biofilm looked liked; it was slimy, slightly green presence on the wound. 2C showed how the wound looked after treatment was initiated 4 hours post inoculation with Neosporin; the treatment reduced the bacterial colonies to the point that the wound almost completely healed, and the infection was not visually evident. 2D showed how wounds looked after Neosporin was initiated 24 hours post inoculation; there was a visual Pa 27312 biofilm on the wound with a well defined wound area and a slimy topical layer on the wound. Overall, illustration 2D depicted the reduced effectiveness of an antimicrobial (Neosporin) once a biofilm was established.

Testing novel antimicrobial treatments against *Pseudomonas aeruginosa* 27312 biofilm in a murine model:

To demonstrate the effectiveness of this model at evaluating novel agents, Novel Agent #1, provided by Healthpoint, Ltd., was tested. Healthpoint wanted to know if Novel Agent #1 was capable of reducing Pa 27312 colonies once a biofilm had formed. Biopsies of wounds were taken after treatment with Novel Agent #1. Bacterial colonies were quantified after the two days of treatment, for the group with treatment initiated 4 hours post inoculation of wound (4 hour group), and for the group with treatment initiated 24 hours post inoculation of wound (24 hour group). Novel Agent #1 was then compared to a Moist Control, for each group.

Figure 5: Log Reduction Results vs. Moist Control for Novel Agent #1

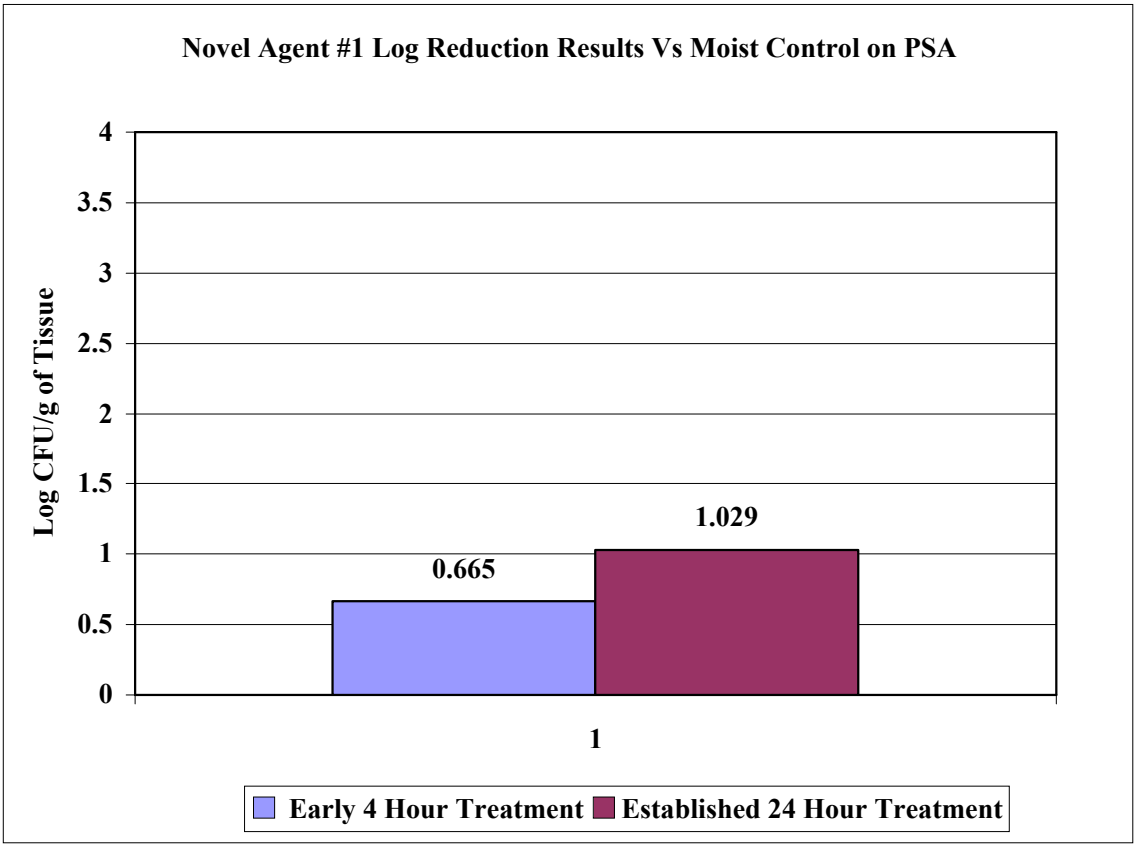

Figure 5: This figure depicts the log cfu/g reduction results of Novel Agent #1 when compared to the Moist Control, when Novel Agent #1 was initiated either 4 hours post inoculation or 24 hours post inoculation of the wound. There was no significant drop in colonization in either group.

Figure 5 illustrates the log cfu/g of tissue reduction results of Novel Agent #1 when compared to the Moist Control, when Novel Agent #1 was initiated either 4 hours post inoculation (blue bar) or 24 hours post inoculation (purple bar). Using One-way ANOVA, Novel Agent #1 was shown to not significantly kill Pa 27312 colonies before or after a biofilm had formed. However, the antimicrobial showed some promise by

demonstrating more than a 0.5 log kill in both situations it was tested. As a result of this evaluation, Healthpoint, Ltd reformulated Novel Agent #1. Now, the model is being used to evaluate the new formulation of the Novel Agent, which demonstrates the usefulness of the model at evaluating novel agents to Healthpoint.

Discussion and Conclusion

A bacterial biofilm is a highly organized aggregate of bacterial colonies that reduce the effectiveness of antimicrobials, are very persistent, and prevent healing in wounds. They have been demonstrated to be present in 60% of chronic wounds (12, 14). And those chronic wounds costs billions of dollars annually to treat (14). These polymicrobial biofilms, in wounds, have been found to primarily be composed of *Staphylococcus, Enterococcus, Pseudomonas,* and *Proteus* (12). Healthpoint, Ltd. already had a MRSA biofilm in a murine a model to evaluate novel antimicrobials. The overall goal of the project was to establish and validate a Pa 27312 biofilm in a murine model so Healthpoint, Ltd could evaluate novel antimicrobial agents against polymicrobial biofilms.

This goal was accomplished by verifying the presence of the Pa 27312 biofilm in the wound of the model. The Pa 27312 biofilm was verified in the wound by demonstrating the two main characteristics of a bacterial biofilm, a high bacterial infection and a reduced effectiveness of antimicrobials, and through the photographs taken of the wounds after treatment and gram staining of the wound. The *in vivo* growth curve studies demonstrated a Pa 27312 infection could be sustained at high levels in the wounds. Biopsy samples from these studies were gram stained, and showed a visual biofilm was

present in the wounds. The *in vivo* treatment studies demonstrated that the established Pa 27312 biofilm in the wounds reduced the effectiveness of antimicrobials. Photographs from these studies also visually showed the reduced of effectiveness of Neosporin once a biofilm was established.

The established Pa 27312 biofilm in a murine model demonstrated it was useful at evaluating novel antimicrobial agents, could be useful at evaluating reformulated antimicrobial agent, and is useful because it mimics clinical patients that would have chronic wounds with biofilms. Novel Agent #1 was evaluated using this model, and was shown to be ineffective at reducing the Pa 27312 biofilm. Therefore, Novel Agent #1 was reformulated. The reformulated agent will be re-evaluated using this model.

The model could also be used to evaluate other novel agents, so they could also be reformulated like Novel Agent #1. The novel agent could be almost any consistency. During the establishment of the model, antimicrobials with different consistencies were used; Gentamicin was a cream with a thick consistency, Silvasorb and Neosporin were a gel with a thick consistency, Moist Control was saline, and Novel Agent #1 was a gel with a really thick consistency. The different consistencies that could be tested with this model allow Healthpoint, Ltd. to broaden their range in antimicrobial selection.

The model is also useful because it allows the antimicrobials to be evaluated in an outbred SKH1 mouse model with a reduced immune system. This is useful because most patients with chronic wounds that contain biofilms usually have chronic conditions with reduced immune systems, and are not related. The outbred mice allow for the evaluation of antimicrobials in genetically different mice, which mimic different patients. Hairless mice allow for easy wounding and evaluation of the biofilms before and after treatment.

The Cytoxan injection weakens the immune system of the mice during the antimicrobial treatment, which mimics the weaken immune system of patients.

A Pa 27312 biofilm in a murine model, in conjunction with other *in vitro* and *in vivo* models, allows Healthpoint, ltd. to properly evaluate topical antimicrobials agents against the clinically found polymicrobial biofilms. The potential novel agent developed with these models will help eradicate bacterial biofilms and help heal chronic wounds. Thus, the Pa 27312 biofilm in a murine model would be used to bring a novel agent to market, which would help Healthpoint, Ltd. towards their goal of advancing the science of wound care.

Limitations

The small sample sizes per antimicrobial treatment may limit the statistical significance of the results

CHAPTER 3

INTERNSHIP EXPIERENCE

Internship Site

Healthpoint, Ltd. is a pharmaceutical company dedicated to advancing the science of wounds care. One of their main scientific goals is the prevention and treatment chronic wounds. In 1992, DFB pharmaceuticals created Healthpoint, Ltd. to further develop wound care products. Since then, Healthpoint, Ltd. has enhanced their research and development division to become leaders in the wound care management market.

Journal Summary

I read journal and review articles that pertained to my research project, such as, biofilms, wound care, *in vivo* murine studies, and statistical reviews. I also read previous *in vivo* MRSA biofilm projects performed by Healthpoint, Ltd. Then, I designed and wrote the research proposal for my project. I collected and prepared all the test material needed for each study before I started the study. I preformed all the studies of the research project. I analyzed all the data, and presented it in bi-weekly group meetings. I wrote my thesis. In between studies, I gained more *in vivo* experience by assisting Healthpoint, Ltd. employees with their animal studies. For the day-to-day log of activities, refer to appendix A: Internship Journal.

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Appendix A: Internship Journal

8-25-09

We had a general orientation with our advisory committee at Healthpoint. It consisted of the expectations UNTHSC has of us as interns, the projects we were assigned, and the general Healthpoint (HP) policies and procedures.

8-26-09

Paul Renick, my on-site mentor, gave me a tour of the facility and introduced me to other non-R&D employees. We also discussed some of the projects I will be participating in and I received posters that HP recently presented at conferences to read. I began safety training and read the standard operating procedure (SOP) for general lab activities. I started reading background information on biofilms.

8-27-09

I continued watching the required safety videos. The company had an awards luncheon for all HP employees. I continued reading the background information on biofilms, in particular, the swine/mouse models for *Staphylococcus aureus* biofilms that HP had previously worked on.

8-28-09

I continued reading journal articles and reviews about antimicrobial treatments combating biofilms *in vivo*. I also began reading the CLSI recommended method for antimicrobial disk susceptibility test. I took a tour of the office, lab, and animal room that HP has at

UNTHSC. I also received bio-safety lab-2 training at UNTHSC to prepare for my research project.

8-31-09

I recorded vitals and observed Paul Renick's porcine model for full thickness biofilms. I also attended a group meeting in which individuals reported the progress of their projects. I began reading Healthpoint's minimum zone of inhibition (MZOI) SOP.

9-1-09

I started a project to evaluate the efficacy of 7 topical wound treatments against *in vitro P. aeruginosa* biofilms formed on a collagen matrix. For that project, I prepared an overnight culture of *P. aeruginosa*. I cut gauze and collagen matrix that will go on Tryptic soy agar (TSA) plates. I also started a saline viability study to test which *S. Aureus* strain would grow best in saline; I did a direct suspension of three strains of *S. Aureus* in saline, diluted each sample to a final concentration of $1x10^6$ cfu/ml, and measured OD at 600nm. Then, I did a 1:10 serial dilution and spot platted on Mueller Hinton agar to quantify *S.Aureus* at 0 hours. Then, I incubated the three $1x10^6$ saline solutions. I also assisted Catherine in one of her studies to test an antimicrobial dressing; she preformed a direct suspension, a concentration dilution, a serial dilution and spot plated to quantify the bacteria at hour zero of treatment.

9-2-09

I continued the *in vitro P. aeruginosa* biofilm study by assembling the biofilm on TSA plates. Then, I preformed a dilution of the overnight *P. aeruginosa* culture to a concentration of $2x10^8$ cfu/ml. I inoculated the biofilm assembly on the 6 TSA plates. I put the 24 hour bacterial growth plate in the incubator, and preformed the treatment plan on the 0 hour plates. I quantified the *P. aeruginosa* for non-treated growth control assembly for 0 hour by spot platting. I continued the vitality study by counting the spot plates of *S. aureus* for 0 hour. I took out the three saline test tubes from the incubator, so I could quantify the bacteria after the 24 hrs of incubation; I preformed the serial dilution and spot platted. Nancy and I had a meeting with two employees in the regulatory affairs division of HP to discuss what their job entailed.

9-3-09

I completed the saline viability study. I continued the biofilm study; I added treatment to the 24 hour growth of biofilm, spot platted the 0 hour biofilms after their 24 hours of treatment, and quantified the cfu/ml of the non-treated growth control for the 0 hour biofilm. I also attended the R&D journal club meeting to discuss a journal and review article on persisters. I also observed Catherine perform a MIC assay for two unknown drugs. Nancy and I had a meeting with another employee of HP to discuss what he does for the company.

9-4-09

I continued the *in vitro* biofilm study; I quantified the cfu/ml for all the 0 hour treated biofilms and spot platted all the 24 hour biofilms after their 24 hours of treatment. Catherine concluded her MIC assay. In the afternoon, Paul and I set up for the first phase of my research project: the establishment of a *P. aeruginosa* biofilm growth-curve. We preformed an IP injection of Cytoxan into 12 mice to induce neutropenia.

9-8-09

Paul Renick and I wounded and inoculated the mice with *P. aeruginosa*, in hopes to establish a biofilm. We had 12 mice; 3 in group A, 4 in group B and 4 in group C; 1 mouse was euthanized during the wounding procedure due to tool malfunction. We will quantify the growth of the bacteria over 3 days by biopsing the wounds, then spot plating the samples to determine cfu/gram of tissue. We also set up the materials we will need to process the samples we get from mice. Nancy and I had a meeting with Michael Michaels about quality assurance and quality control.

9-9-09

I was out sick.

9-10-09

Paul and I changed the dressings on the 4 mice in group C. We euthanized the 4 mice of group B. I learned how to take a biopsy of the wound, and how to process the samples:

homogenize sample, do serial 1:10 dilution of sample, spot plate, count colonies to determine cfu/ gram of tissue.

9-11-09

Shannon and I took pictures of the guinea pigs she was using for one of her studies. We continued my pilot mouse study; euthanized the 4 mice in group C, took biopsies of the wounds, homogenized samples, serial diluted, spot plated. Nancy and I had a meeting with the project management group.

9-14-09

I assisted Paul and Shannon with their MRSA pig study.

9-15-09

Today, I made tryptic soy agar and oxacillin resistance screen agar for Paul's pig study. I also prepared the processing tubes for Paul's study. I counted the colonies for the three-day growth of *P. aeruginosa* in the superficial wound of the mice. I completed the calculations of the pilot study growth curve for day 1-3 growth of *P. aeruginosa* in superficial wound of mice to assess the presence of a biofilm.

9-16-09

I worked on my research proposal.

9-17-09

I worked on my research proposal and set up the sample processing tubes for Paul's pig study.

9-18-09

I analyzed the data for the pilot study. I also started the second growth curve study by performing the IP Cytoxan injections.

9-21-09

I prepared for wounding and inoculation by getting papers and protocols ready to go. I started the overnight culture of *P. aeruginosa.* I also assisted Paul and Shannon on their pig study.

9-22-09

I prepared the inoculum for the study. I wounded and inoculated 12 mice. I prepared charcoal agar plates, and the sample collection tubes.

9-23-09

I changed the dressings on 8 mice and collected samples from 4 mice. I processed the samples taken and plated them on *Pseudomonas* selection agar (PSA) and charcoal agar. I also assisted Paul and Shannon with their pig study.

9-24-09

I changed the dressings on 6 mice and took day + 2 samples from 2 mice. I processed and plated the samples on PSA and charcoal agar. I also counted the colonies from day + 1 samples to obtain cfu/g tissue.

9-25-09

I changed the dressings on 4 mice and took day + 3 samples from 2 mice. I processed and plated the samples on PSA and charcoal agar. I also counted the colonies from day + 2 samples to obtain cfu/g tissue.

9-26-09

I counted the colonies from day + 3 samples to obtain cfu/g tissue. I also checked the status of the mice at the UNTHSC vivarium.

9-28-09

I collected the samples for day + 6 mice. I processed and plated the samples on PSA and charcoal agar. I also participated in the staff research update meeting.

9-29-09

I counted the colonies from day + 6 samples to obtain cfu/g tissue. I worked on my research proposal and collected data to perform my *in vitro* growth curve and MIC.

9-30-09

I researched which antibiotics to use and what control strain to use for the MIC studies. I also researched which media to culture Pa 27312 in for the *in vitro* growth curve. Then, I made those medias. I prepared the antibiotic stock solutions for the MIC study. I prepared a streak plate of Pa 27312 and Pa 27853 for the growth curve study and MIC study.

10-1-09

I started the characterization of strain studies: the *in vitro* growth curves studies, and the MIC studies. The *in vitro* growth curves consisted of *P. aeruginosa* grown in four different medias; Tryptic Soy Broth (TSB), TSB+4.5% glucose, Cation Adjusted Mueller-Hinton Broth (CAMHB), and CAMHB+4.5% glucose. The four medias were inoculated with *P.aeruginosa* in order determine which media supported the most robust overnight culture. I collected samples at various time periods and measured the absorbance at 600nm to determine the cfu/ml. The MIC studies were done to compare the antibiotic sensitivity of two *P.aeruginosa* strains. I prepared a streak plate of Pa 27312 and Pa 27853 for the second growth curve, and second MIC study.

10-2-09

I took the 24 hour time sample for the first *in vitro* growth curve. I also assessed the MIC plates. In addition, I started the second *in vitro* growth curve and second MIC study. I weighed the 12 mice for my third study and prepared the Cytoxan for the IP injection. Then, I gave an IP injection to all 12 mice.

10-3-09

I assessed the MIC plates #2 and took the 24 hour samples for the *in vitro* growth curves.

10-5-09

I assisted Paul and Shannon with their pig study. I started the overnight culture of Pa 27312 for my third study. I also prepared the material needed for the wounding and inoculation tomorrow.

10-6-09

I wounded and inoculated the mice for my third study: *in vivo* growth curve. During the surgery 6 mice died, so I modified the study and took out the treatments. I worked on my research proposal and analyzed the data from the growth curves.

10-7-09

I explanted and processed the samples for day + 1 mice. I worked on my research proposal and analyzed the data from the *in vitro* growth curves and MIC studies.

10-8-09

I explanted and processed the samples for day + 2 mice. I worked on my research proposal and analyzed the data from the *in vitro* growth curves.

10-9-09

I explanted and processed the day + 3 samples. I also made charcoal agar plates for future studies. I worked on my research proposal.

10-12-09

I worked on my research proposal. I made the drug stocks to be used for the MICs. I streaked Pa 27312 and Pa 27835 on tryptic soy agar (TSA) plates for the growth curve and MIC studies. I made Cationic Adjusted Mueller-Hinton Broth (CAMHB). I worked on my lab notebook.

10-13-09

I started the overnight growth of Pa 27312. I also started the MICs for Pa 27312 and Pa 27853. I worked in my research proposal. I worked on my lab notebook.

10-14-09

I continued taking samples for the growth curve. I analyzed the MIC results. I worked on my research proposal.

10-15-09

I worked on my research paper and prepared the paper work for study 4.

10-16-09

I worked on my research paper. I also prepared for study 4 by weighing the mice and giving them an IP injection of Cytoxan.

10-19-09

I prepared all the tubes necessary for the collection of the skin samples for study 4. I gathered all the antimicrobial treatment creams needed for the study. I made sure I had all the necessary equipment for the surgery and treatment applications.

10-20-09

I successfully wounded and inoculated all 12 mice. They were all responsive after the surgery. 4 hours after inoculation, I initiated treatment on 6 mice. I applied Gentamicin sulfate cream, Neosporin, Bactroban (mupirocin) and a saline Moist Control. Bactroban was used a negative control.

10-21-09

I initiated the treatment for the 24 hour biofilm established mice. I used the same 4 antimicrobial creams. I applied the second treatment on the 4 hour mice.

10-22-09

I applied the second treatment on the 24 hour mice. I euthanized the 4 hour mice and took biopsies of all the wounds. I processed the samples, serial diluted them and spot plated them to obtain the cfu/g.

10-23-09

I euthanized the 24 hour sample mice and collected biopsy samples of all the wounds. I processed the samples; serial diluted and spot plated the samples to obtain the cfu/g.

10-24-09

I counted the colonies and calculated the cfu/g.

10-26-09

I analyzed the data and found that, as expected, Bactroban was not effective at reducing the bacterial infection in neither the 4 hour nor the 24 hour samples. Gentamicin was slightly effective at 4 hour at reducing the bacterial infection and was not effective when there is an established biofilm. Neosporin was the most effective at reducing the bacterial infection at 4 hour samples and 24 hour samples. However, the 24 hour did not have a significant reduction in bacterial infection. I presented some of my results in a staff meeting.

10-27-09

I finished my proposal draft and sent it to Dr. McClain and Dr. D. I read current articles on biofilms.

10-28-09

I assisted Shannon, Eric and Paul with the partial thickness wound pig study.

10-29-09

I assisted Shannon, Eric and Paul with the partial thickness wound pig study.

10-30-09

I assisted Shannon, Eric and Paul with the partial thickness wound pig study. I started my fifth study for my research project. I weighed all 12 mice and prepared the Cytoxan for injection. I gave each mouse an IP injection of 250 mg/kg Cytoxan.

11-2-09

I assisted Shannon with the partial thickness wound pig model. I prepared all the paper work and collected all the material needed for the rest of study.

11-3-09

I prepared all the sample tubes for the study. I preformed the surgery and inoculation. 4 hours post inoculation, I initiated treatment; I applied Neosporin, Gentamicin, Silvasorb, and a Moist Control to the wounds.

11-4-09

I initiated treatment of the 24 hour established biofilm wounds with Neosporin, Gentamicin cream, Silvasorb gel, and Moist Control. I applied the second course of treatment to the 4 hour group. I had a meeting with Eric and Paul about my project after Paul gets the new job.

11-5-09

I applied the second treatment to the 24 hour established biofilm group. I euthanized mice with the 4 hour wounds, and collected the wound samples. I processed the samples so I can calculate the cfu/g.

11-6-09

I collected and processed the samples of the 24 hour established biofilm. I calculated the cfu/g of the 4 hour samples. I attended journal club.

11-7-09

I calculated the cfu/g of the established biofilm samples.

11-10-09

I continued to write my research proposal and analyzed the data from my previous studies.

11-11-09

I continued to write my research proposal and analyzed the data from my previous studies.

11-12-09

I continued to write my research proposal and analyzed the data from my previous studies. I helped Shannon take inventory of the chemicals at the HP UNTHSC lab.

11-13-09

I continued to write my research proposal and analyzed the data from my previous studies.

11-16-09

I took inventory of the supplies in the CBH laboratory.

11-17-09

I organized the supplies in the CBH laboratory

11-18-09

I organized the supplies in the CBH laboratory

11-19-09

I gathered all the supplies I needed for my 2 studies in December.

11-20-09

I made PBS, sterile water, TSB, charcoal plates, and PSA plates for my 2 studies.

11-30-09

I assisted Shannon with her pig studies.

12-1-09

I assisted Shannon with her pig studies.

12-2-09

I assisted Shannon with her pig studies.

12-3-09

I assisted Shannon with her pig studies.

12-4-09

I assisted Shannon with her pig studies. I weighed the mice and gave them an IP injection of Cytoxan.

12-7-09

I assisted Shannon with her pig studies. I started the overnight growth of Pa 27312 for the sixth study.

12-8-09

I started my sixth study; I wounded and inoculated the mice. I, then, treated group A, the 4 hour post inoculation mice. The treatments are Neosporin, Gentamicin cream, moist control, and a novel HP antimicrobial. I prepared the sample tubes for sample processing

12-9-09

I applied the second treatment to the 4 hour mice. Then, I applied the first treatments to group B, the 24 hour established biofilm.

12-10-09

I applied the second treatment to group B. I euthanized and took samples from all the mice of group A. I homogenized, serial diluted, and spot plated each sample on PSA plates and Charcoal agar plates.

12-11-09

I euthanized and took samples from all the mice of group B. I homogenized, serial diluted, and spot plated each sample on PSA plates and Charcoal agar plates.
I counted the colonies on the PSA and Charcoal plates and calculated cfu/g for each sample. I weighed the mice and gave them an IP injection of Cytoxan for study 7.

12-12-09

I counted the colonies on the PSA and Charcoal plates and calculated cfu/g for each sample.

12-14-09

I assisted Shannon with her pig studies. I started the overnight growth of Pa 27312 for the 7th study.

12-15-09

I started my 7th study; I wounded and inoculated the mice. I, then, treated group A, the 4 hour post inoculation mice. The treatments are Neosporin, Gentamicin cream, moist control, and a novel HP antimicrobial. I prepared the sample tubes for sample processing

12-16-09

I applied the second treatment to the 4 hour mice; then applied the first treatments to group B, the 24 hour established biofilm.

12-17-09

I applied the second treatment to group B. I euthanized and took samples from all the mice of group A. I homogenized, serial diluted, and spot plated each sample on PSA plates and Charcoal agar plates.

12-18-09

I euthanized and took samples from all the mice of group B. I homogenized, serial diluted, and spot plated each sample on PSA plates and Charcoal agar plates.
I counted the colonies on the PSA and Charcoal plates and calculated cfu/g for each sample.

12-19-09

I counted the colonies on the PSA and Charcoal plates and calculated cfu/g for each sample.

12-21-09

I assisted Shannon with her pig studies. I updated my lab notebook.

12-22-09

I assisted Shannon with her pig studies. I cleaned the tools from the previous two studies.

12-23-09 through 1-3-09

Christmas and New Years vacation

1-4-10

I attended a group meeting and analyzed the data I have accumulated.

1-5-10

I worked on my thesis and put the paper work together for my 8^{th} study.

1-6-10

I worked on my thesis.

1-7-10

I worked on my thesis.

1-8-10

I worked on my thesis. I weighed all the mice and gave them an IP injection of Cytoxan.

1-11-10

I started the overnight growth of Pa 27312 for the 8th study. I autoclaved all the material needed for the study and made sure I had all the material for surgery.

1-12-10

I started my 8th study; I wounded and inoculated the mice. I, then, treated group A, the 4 hour post inoculation mice. The treatments are Neosporin, Gentamicin cream, moist control, and Silvasorb gel. I prepared the sample tubes for sample processing.

1-13-10

I applied the second treatment to the 4 hour mice. Then, I applied the first treatments to group B, the 24 hour established biofilm.

1-14-10

I applied the second treatment to group B. I euthanized and took samples from all the mice of group A. I homogenized, serial diluted, and spot plated each sample on PSA plates and Charcoal agar plates

1-15-10

I euthanized and took samples from all the mice of group B. I homogenized, serial diluted, and spot plated each sample on PSA plates and Charcoal agar plates.
I counted the colonies on the PSA and Charcoal plates and calculated cfu/g for each sample.

1-16-10

I counted the colonies on the PSA and Charcoal plates and calculated cfu/g for each sample.

1-18-10

I analyzed the data from the study. I cleaned the instruments used for the study. I went to a group meeting to discuss HP's plan for the rest of the year. I assisted Nancy with one of her projects.

1-19-10

I started to analyze all the data I accumulated to date.

1-20-10

I continued to analyze the data. I continued to write my thesis.

1-21-10

I continued to analyze the data. I continued to write my thesis.

1-22-10

I continued to analyze the data. I continued to write my thesis. I assisted Nancy with an Elisa assay.

1-25-10

I continued to analyze the data. I continued to write my thesis.

1-26-10

I continued to analyze the data. I continued to write my thesis.

1-27-10

I continued to analyze the data. I continued to write my thesis.

1-28-10

I continued to analyze the data. I continued to write my thesis.

1-29-10

I continued to analyze the data. I continued to write my thesis. I observed the sectioning process for histology.

2-1-10

I continued to analyze the data. I continued to write my thesis. I went to a group meeting.

2-2-10

I continued to analyze the data. I continued to write my thesis

2-3-10

I continued to analyze the data. I continued to write my lab practicum.

2-4-10

I continued to analyze the data. I continued to write my lab practicum. I also started the processing/fixed process for histology.

2-5-10

I embedded my histology samples in paraffin. I continued to analyze the data. I continued to write my lab practicum.

2-8-10

I continued to analyze the data. I continued to write my lab practicum.

2-9-10

I sectioned my samples, and placed them on slides for analysis. I discussed my practicum with Eric Roche. I rearranged the practicum.

2-10-10

I stained the samples by dissolving the paraffin with xylene, rehydrating the sample through a gradual submersion in 100%, 95%, 70% ethanol, and eventually submerging it in water. Then, the samples were stained with hematoxylin and eosin. Then, the slides were dehydrated through a gradual submersion in 70%, 95% and 100% ethanol and then,

xylene. The slides were allowed to dry, and then a cover slip was put on. I viewed the samples under the microscope.

2-15-10

I worked on my lab practicum.

2-16-10

I worked on my lab practicum. I prepared data for a meeting. I went to the meeting and presented the results from previous studies.

2-17-10

I had a meeting with Eric and Shannon about HPs upcoming study to evaluate a novel agent against a MRSA biofilm.

2-18-10

I helped get material ready for the upcoming study. I worked on my practicum.

2-19-10

I weighed the mice, and prepared the Cytoxan for the IP injection. I finished my lab practicum draft, and sent it to Dr. Gwirtz. I made charcoal and oxacillin resistant selection agar (ORSA) plates.

2-22-10

I finished my lab practicum draft and sent it to Dr. Gwirtz.

2-23-10

I assisted Shannon with the study to evaluate a novel agent against MRSA biofilm. We performed the surgery and inoculation.

2-24-10

I assisted Shannon with her study by applying the treatments.

2-25-10

I assisted Shannon with her study by applying the treatments. I prepared all the tubes for explant sampling and histology.

2-26-10

I assisted Shannon with her study by explant sampling the wounds.

3-1-10

I started to arrange the date and time for my defense. I researched jobs. I had a short meeting with Eric Roche. I went to a group meeting.

3-2-10 to 4-12-10

I finished the lab practicum and prepared for the public seminar/defense.

4-13-10

I successfully defended my internship project.