AWARD NUMBER:
W81XWH-19-1-0051
PR180978

TITLE: A NOVEL SCREEN FOR BIOFILM INHIBITORS IN ANIMALS

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REPORT DATE: MARCH 2020

TYPE OF REPORT: ANNUAL REPORT

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MARCH 2020	Annual	02/15/19 - 02/14/2020

4. TITLE AND SUBTITLE
A Novel Screen for Biofilm Inhibitors in Animals

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0051

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

MICHIGAN STATE UNIVERSITY

EAST LANSING, MI 48824

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Over 37,000 military personnel have been wounded in Operation Iraqi Freedom. Other than the wound itself, the greatest threat to these personnel is infection, which strikes one in three wounded individuals during initial hospitalization. Although antibiotics are crucial tools in the treatment of the wounded, microbial pathogens form communities known as biofilms that resist antibiotics, and greatly complicate the treatment of wounds. In addition to threatening the lives of the wounded, biofilms increase hospitalization times and delay return to duty. Biofilms also attack orthopedic and other implants, as well as catheters. Thus, the Department of Defense has targeted biofilm inhibition and treatment as a goal, specifically stated in the announcement. Despite decades of research in biofilms, no compound has been identified that prevents or destroys these structures in wounds or other infection sites. We propose to address this problem by developing a novel system of screening wound and implant models in animals and in biofilm cultures. We will employ the technique of in vivo **bioluminescence imaging (BLI)** to establish and test this system. BLI uses recombinant pathogens that emit light. We will use recombinant bioluminescent *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are both known as important wound and implant pathogens, and which form distinct biofilms. Should we be able to identify even one new treatment, many military lives will be saved, and hospitalization times and time to return to duty will be reduced, and our goal is to identify many such compounds. In addition, the capability to respond rapidly to new and more dangerous infections will be greatly enhanced. The overall goal of this project is to establish the system rather than to develop the selected plant derived substances themselves as treatments.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	**21**	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			

Standard Form 298 (Rev. 8-98)

TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

This project proposes a new method of assessing anti-biofilm treatments, employing bioluminescent bacterial biofilms in culture and in vivo bioluminescence imaging in mice. Candidate substances are first screened by measuring bioluminescence over time in biofilm cultures and then promising formulations are tested with the same technology in wound and implant models in animals. The goal is to be able to rapidly screen new anti-biofilm therapeutics.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Antibiotic; biofilm; bioluminescence; wound infection; implant; therapeutic

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The project has three main goals:
1. To establish bioluminescence-based culture systems for *Staphylococcus* and *Pseudomonas* biofilms
2. To test plant-derived substances used to treat wound infections for efficacy in the above cultures
3. To use in vivo bioluminescence wound and implant models in mice to test the best candidates of these substances for efficacy

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Statement of Work Part 1. Establish bioluminescence-based biofilm models in culture. We have established bioluminescence models of biofilms in culture for both bacterial pathogens. These experiments involved extensive testing different growth conditions, substrates, media, and timing of the cultures in order to optimize the process and provide reproducible bioluminescence signals (**bioluminescence is henceforth abbreviated BL and in vivo bioluminescence imaging abbreviated BLI**). One of the important considerations was that these two pathogens, *Staphylococcus aureus* (SA; strain Xen36) and *Pseudomonas aeruginosa* (PA; strain Xen5) exhibit completely different biofilms, requiring different media and growth substrates. We have used these bioluminescent pathogens for previous studies (), but had not grown them in biofilm form in culture until we undertook these experiments.

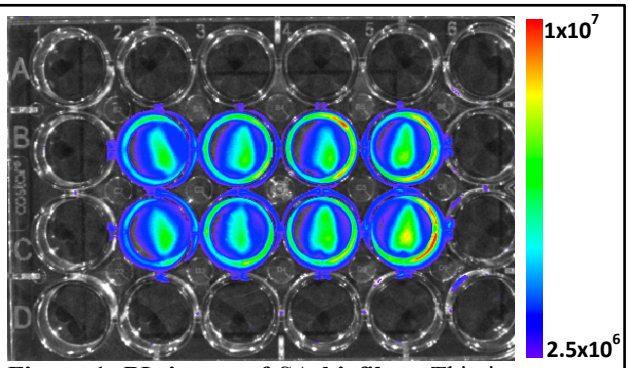

Figure 1. BL image of SA biofilms. This image was acquired after two days of growth in a 24-well plate. The medium was removed and the biofilms washed before imaging in the IVIS instrument.

Figure 1 shows an example of BL measurement of SA biofilms in a 24-well format. As can be seen, the biofilms are not uniform, as is reported in the literature using other methods such as methylene blue staining. However, this does not prevent the assessment of anti-biofilm substances because the IVIS system is fully quantifiable using Living Image Software (Perkin Elmer, Inc.). The color bar on the right side of the figure displays the false-color light levels in photons/second (p/s). False-color levels are selected for visual display and for analysis of distribution. The p/s is measured using regions of interest (ROI) and is a fixed measurement.

For quantitative measurements, Living Image allows grid formulations of ROIs that correspond to 6, 24, 48, 96 and 312 wells. Note that the center wells are used, because the outer wells show greater bacterial growth and therefore complicate the analysis. For SA, we have determined that 24-well plates produce the greatest amount of biofilm per unit area (data not shown), and so this format is used when performing analysis of biofilms that requires the removal of the biofilm from the plate. Other formats do produce biofilms and can be used for analysis of antibiofilm substances by BL, but we use this format for biofilm recovery from the plate. **Figure 2** shows a darkfield microscopy image of an SA biofilm on a 24-well plate. These images show the expected morphology of SA cocci embedded in a matrix. As can be seen by the images, much of the biofilm forms at the edges of the well, which is not unexpected because there are two surfaces for the bacteria to adhere to at the edge, the bottom and the side wall. Nonetheless, the images below are of tightly adherent structures that resist washing and possess the visual features of biofilms including the extracellular matrix (ECM).

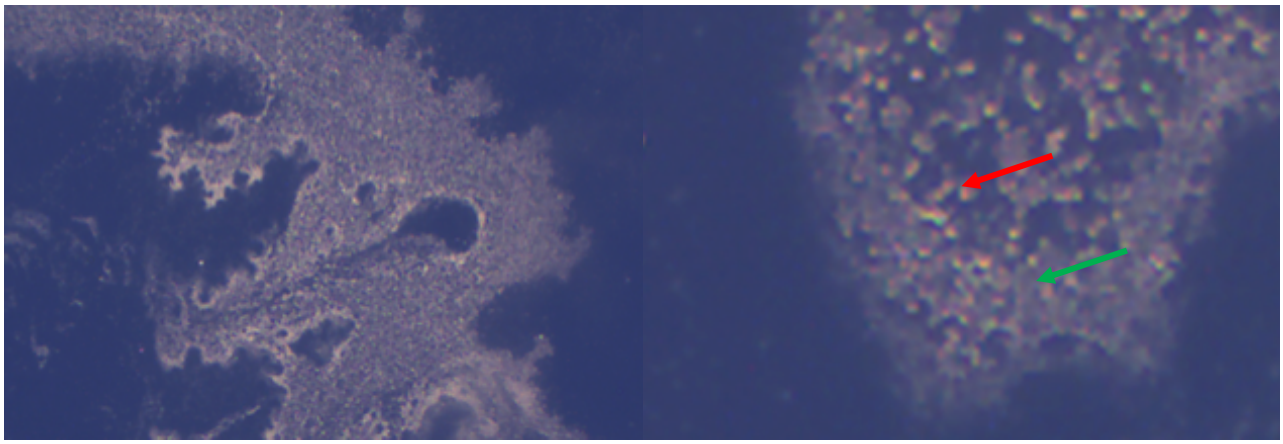

Figure 2. Micrograph of SA biofilm. Left; low magnification. Right; high magnification. Red arrow, SA cocci; green arrow, extracellular matrix (ECM).

We also compared the efficiency of different media in the formation of biofilms. In **Figure 3**, data is shown for such a comparison for SA biofilms in the 24-well format. Luria-Bertani (LB) medium was found to be slightly superior to brain heart infusion (BHI) and Todd Hewitt Broth (THB). There were many other details that had to be optimized, such as the timing of the biofilm and whether the culture should be oscillated. We determined that biofilms formed best after three days of incubation and that oscillation adversely affects biofilm variation between the wells.

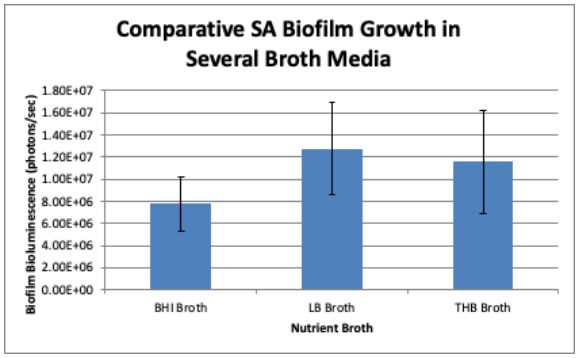

Figure 3. SA biofilm broth comparison. Biofilms were grown in the indicated broth cultures in 24-well plates and imaged 3 days later with the IVIS instrument as described. Identical regions of interest (ROIs) were measured in triplicate wells. BHI, brain heart infusion broth; LB; Luria-Bertani broth; THB, Todd Hewitt broth.

We reasoned that oscillation of the plates might aid in biofilm formation because some systems actually require oscillation for extensive biofilms to form. Although this has not been shown in the literature to be required for SA biofilms, we tested the possibility. **Figure 4** shows the results of such an experiment, indicating that oscillation at 20 revolutions per minute (RPM) did indeed increase biofilm formation, but that variation between wells also increased significantly.

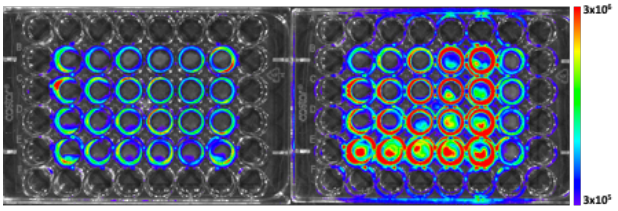

Figure 4. Effect of oscillation on SA biofilms. The plate on the left was incubated without oscillation, the plate on the right was oscillated at 20 RPM. Both plates were incubated for 24 hours. The images are normalized to the same scale as shown in the color bar on the right.

For PA biofilms, an entirely different set of parameters were necessary to establish and optimize, because this organism forms biofilms quite differently from SA.

PA biofilms do not form at the bottom of the well, but rather tend to localize at the liquid-air interface, and cannot therefore be grown and analyzed in the same manner as SA biofilms. PA requires a specialized plate for this purpose. We selected the MBEC Biofilm Inoculator (innovotech, Inc.). This plate features posts that protrude down into the well from the lid of the plate. The biofilms grow on these pegs, which can be broken off for analysis. PA is notorious for the formation of mucoid biofilms, including those that form in the lungs of cystic fibrosis patients. It was therefore important to treat these biofilms with great care such that a quantitative multi-well BL system could be established. To illustrate some of the problem associated with PA, **Figure 5** shows the lid of a plate that has exhibited extensive mucoid biofilm formation and the strands of biofilm are clearly visible, having stretched between wells and made any analysis useless. Incubation of the cultures for no more than three days and careful removal of the lid of the plate were among the techniques and parameters that needed to be optimized in order to establish useful, reproducible and quantitative BL biofilm cultures of PA. In addition, the outer wells exhibit more signal, presumably due to higher access to oxygen at the edge of the plate. These wells need to be excluded from the analysis. This is the case for SA biofilms also.

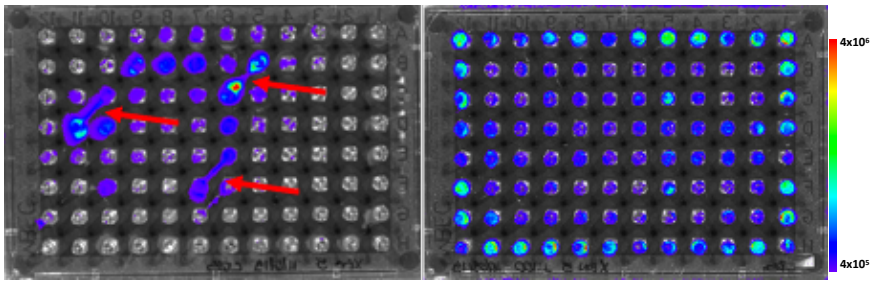

Figure 5. Complications of PA biofilms. Left, strands of mucoid biofilm on lid posts. Right, greater BL signals in the wells at the edge of the plate.

Another factor that complicated PA biofilms was the verification of the biofilm, because on the post, microscopy is uninformative. This fact is not of critical concern,

because PA spontaneously forms biofilms in almost all extended cultures and so the adherence to the post upon washing as well as the formation of the mucoid strands strongly indicates that a biofilm is forming. Nonetheless, we wanted to compare planktonic (free swimming) and biofilm cultures of both PA and SA using several methods. These comparisons are still ongoing, but much progress has been made. One example is mass spectrometry, which is very useful for the characterization of bacterial cultures. PA produces many substances in biofilms, including alginate, DNA, polysaccharides and glycoproteins. One of the substances that is elevated in biofilms is the virulence determinant **pyocyanin**. This substance has unique qualities that make it a good target for analysis by mass spectrometry. Pyocyanin is a blue pigmented compound that binds iron and is abundant in PA biofilms. Its production is regulated by the concentration of cyclic-di-GMP, a signaling molecule important in the transition between planktonic, motile state and the non-motile biofilm state. Among other compounds, we analyzed pyocyanin in planktonic cultures and from biofilms. The results of this analysis are shown in **Figure 6**. Here, we have compared three PA strains for pyocyanin content of planktonic and biofilm cultures. The laboratory strains PA01 and PA014 are well studied, and served as controls. As can be seen, PA01 and PA014 exhibited modest increases in pyocyanin, whereas the bioluminescent strain Xe5 employed in our studies showed increases in pyocyanin of over 6-fold. These studies are continuing in collaboration with Todd Lydic of MSU, who is performing the mass spectrometry at very reasonable cost as part of a long-standing collaboration.

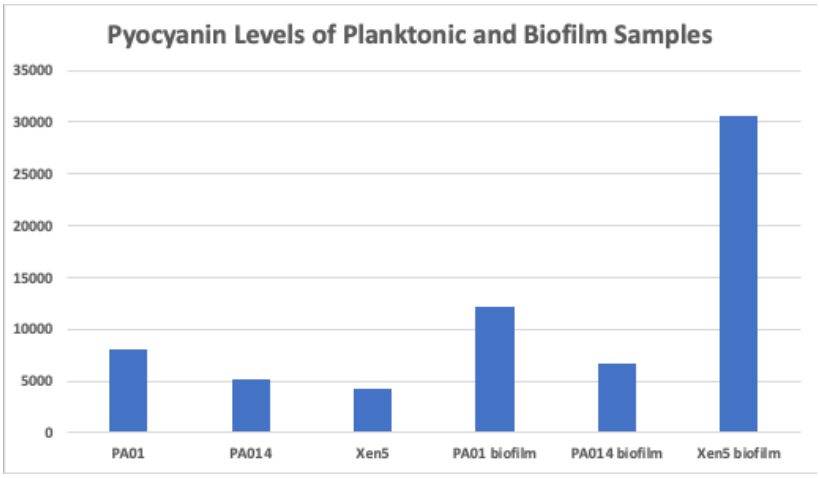

Figure 6 mass spectrometry of PA cultures. Samples of the indicated strains were analyzed by mass spectrometry for pyocyanin content.

The data generated using mass spectrometry is rich, and other pigments were measured as well (data not shown). Pyocyanin showed the greatest increase so far and because this molecule is known to be associated with biofilm production, we are encouraged. Ongoing analysis of both PA and SA biofilms by mass spectrometry will be very informative.

Another characterization of biofilms currently underway is RNA sequencing of the cultures. We have not yet performed this analysis but have ordered and received all the reagents and are now prepared to proceed. The reason for the delay in this process is because we wanted to be certain our samples were truly biofilms and that we would be analyzing RNA at the correct timing to observe relevant changes in gene expression. While the preparation of RNA is not expensive, the sequencing and analysis of the samples represents a significant cost. We therefore want to be certain to deliver not only RNA of high quality, but also obtain relevant and informative results. For this purpose, we have consulted with Drs. Christopher Waters and Neal Hammer of MSU for methods. We will employ a specialized lithium chloride lysis buffer and RNA purification using kits from Qiagen Inc., in a procedure developed for isolation of RNA from SA, which is difficult because it is a Gram-positive organism with a strong cell wall. For lysis, a bead beater is used. We have purchased a BeadBug 6 (Benchmark Inc.) and custom 2 ml tubes pre-filled with 1.5 mm high impact zirconium beads. The BeadBug was not purchased with CDMRP funds, but rather using other funding sources, as it will be used for many projects in the laboratory. We are confident that quality RNA will be produced using this method.

One of the important goals of this project is the use of biofilm-colonized implants. We have established this goal also, and are continuing to pursue these efforts. This process requires careful analysis because many substances do not readily support biofilms without some kind of coating or treatment that permits bacterial adhesion. In the body, implants are in an environment where many bodily fluids and other biological material such as complement have access to the implant and may facilitate biofilm infection. We began our studies by comparing two different intravenous catheter materials, Optiva Radiopaque IV Catheters (catalog number 5042, Jelco Inc.) and vinyl catheter tubing (catalog number 0007760, Durect Corporation). Untreated catheters were subjected to SA cultures for three days, washed, and imaged to measure biofilm accumulation. We observed colonization of the vinyl tubing but not the Optiva catheters (**Figure 7**). Although these results demonstrate the feasibility of implanting biofilms on the vinyl tubing, we are proceeding to attempt to coat the catheters with poly-L-lysine, heparin, serum and other substances to increase bacterial attachment.

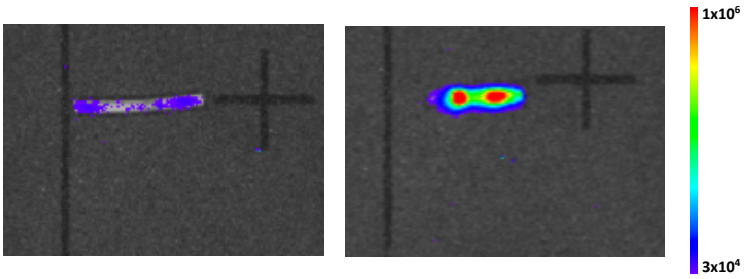

Figure 7. SA Biofilms on catheter tubing. Left, Optiva IV catheter; right, vinyl catheter tubing.

We are continuing these experiments with SA and PA, and fully expect to have well-colonized materials very soon. The vinyl tubing is already suitable for implantation into animals and we are expanding these studies to include metal orthopedic wire as well as other substances.

Statement of Work Part 2. Test plant-derived substances in biofilm cultures. We have now repeatedly tested five of the eight plant-derived substances against both SA and PA biofilms and are in the process of testing the remaining three.

Figure 8 shows the minimum inhibitory concentration of cinnamon oil on SA biofilms. The graph is presented as percent inhibition vs. dilution of the oil. The data shows that 1% cinnamon oil is completely effective against SA biofilms. **Figure 9** shows similar analysis for cinnamaldehyde.

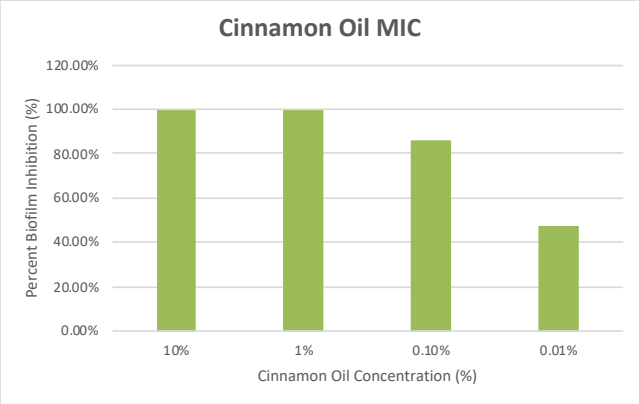

Figure 8. Minimum inhibitory concentration (MIC) of cinnamon oil against SA biofilms. SA biofilms were grown for 2 days, treated for 24 hours with the indicated concentrations of cinnamon oil and imaged.

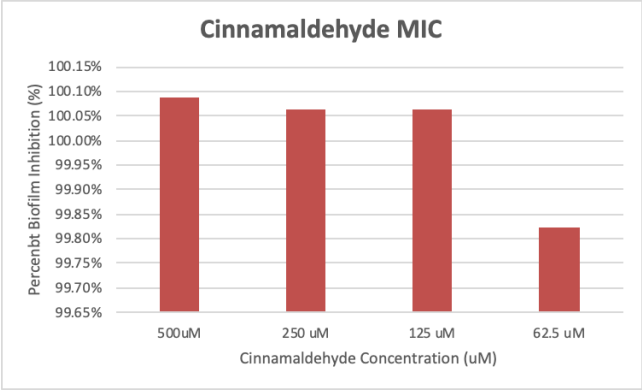

Figure 9. Minimum inhibitory concentration (MIC) of cinnamaldehyde against SA biofilms. SA biofilms were grown and treated as in **Figure 8**.

These data can be displayed in a variety of ways. For example, comparison of essential oil activity and that of the purified active ingredient is possible. This comparison, however, must be carefully considered because the oil is a complex mixture rather than a purified substance. One such comparison is shown in **Figure 10**. Here, eucalyptol, the main active ingredient of eucalyptus oil according to many studies, is compared to the oil itself against PA biofilms.

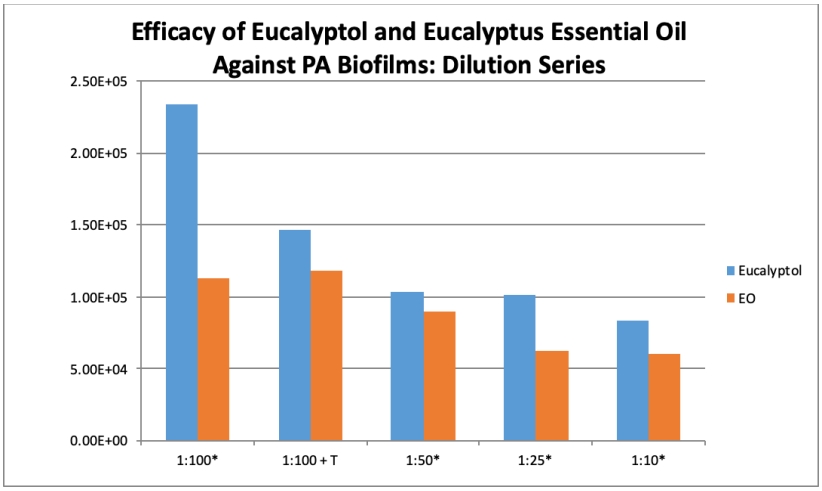

Figure 10. Comparison of eucalyptol and eucalyptus essential oil against PA biofilms. The indicated dilutions of the oil and the compound are shown. The +T sample has had Tween-80 added.

The system permits the rapid assessment of formulations as well. In Figure 10, Tween-80, a non-ionic detergent was added to the wells, to investigate possible enhancement of permeability through the addition of detergents. As can be seen in the graph, tween-80 does indeed enhance the antibiofilm effects of the eucalyptol, but not of the essential oil. Perhaps this difference is due to the enhanced penetration of hydrophobic oils into the biofilm matrix.

We have performed analyses of this kind for cinnamon oil, cinnamaldehyde, eucalyptus oil, eucalyptol, and berberine. By far, the most potent of these substances is cinnamaldehyde. This compound is remarkably effective against bot PA and SA biofilms and will be the first to be tested in the animal model. We have determined that eucalyptus essential oil is more effective against SA and PA biofilms than the reported active ingredient, eucalyptol.

Although we have obtained oils and active ingredients, two of the plant products, *Larrea* (the creosote or chaparral plant) and *Lantana*, a flowering plant native to Michigan, are available only in the form of the plant itself (**Figure 11**). We are currently processing *Larrea* and will obtain *Lantana* when it becomes available in the spring. The various plant-derived substances such as cinnamon and eucalyptus make for quite a fragrant laboratory.

Figure 11. *Larrea*, obtained from SPV Treasures, Benson AZ.

Statement of Work, Part 3: Establish wound and implant infection models for testing novel biofilm inhibitors. We have performed wound infection studies in animals and plan to use the system to evaluate biofilm inhibitor candidates identified in the culture studies described above very soon. To test the system and begin the imaging studies at Michigan State, we infected mice with SA derived from biofilms and planktonic cultures and imaged the animals using the in vivo IVIS system in the Imaging Facility in the IQ Building. An example of these studies is shown in **Figure 12**.

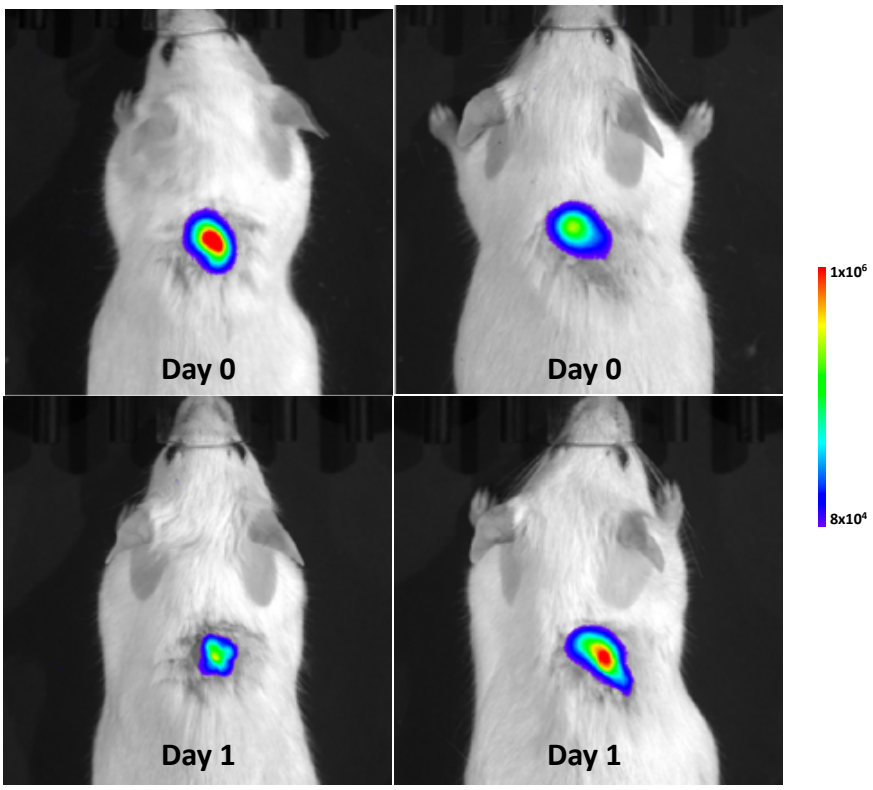

Figure 12. BLI of wound infection by SA. The mice were infected with either 10^7 colony forming units (CFU) of biofilm derived SA (left mouse) or 10^7 CFO of planktonic SA (right mouse) and imaged on the day of infection (day 0) and the next day (day1).

For the experiment shown in Figure 12, SA removed from a biofilm and SA from a planktonic culture were introduced into the wound to compare the ability of each to establish wound infection on the first day. The inoculum, imaged in the animals on day 0, showed that biofilm derived SA was actually brighter in the animal though the same number of bacteria were used. Care must be taken in the interpretation of the imaging of inocula, however, because of the variation in placement of the bacteria within the wound and other factors such as how long the cultures were kept at room temperature. Nonetheless, this is an interesting observation that should be repeated. On day 0, identical regions of interest (ROIs) show that the biofilm mouse had a BLI signal of $3.8x10^6$ photons/second/cm2/steradian (p/s/cm^2/str), whereas the planktonic mouse had a signal of $2.5x10^6$ p/s/cm^2/str. On day one, the planktonic mouse signal increased to $3.3x10^6$ p/s/cm^2/str. However, the biofilm derived infection decreased to $1.5x10^6$ p/s/cm^2/str. These results may have implications for the virulence of the two forms of SA. Perhaps the planktonic forms are more capable of initial attachment. We are very encouraged that BLI of infected wounds has been successful so far and now plan to execute the last of the aims; treatment of the wounds and implant infection with the selected plant products.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

This project has allowed three undergraduate student researchers at Michigan State, Michael Witte, Allison Kennedy and Lucy Richards, to perform experiments and further their scientific careers. These students were able to participate in this project as part of an advanced research class, MMG 499, which compensates them with college credits (they are not paid). This represents an invaluable opportunity for these young scientists, and has trained them in many aspects of research, from the practical procedures of handling BSL-2 pathogens to experimental design and data analysis and interpretation. In addition to these experiences, they are presenting their work as part of the University Undergraduate Research and Arts Forum. This project was part of their work, not the entirety, but the funding has supported their work on biofilms. We are most grateful to the CDMRP for providing this extremely valuable contribution to their hopefully long careers as scientists.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these

project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We plan to:
1. Expand the analysis of biofilms using mass spectrometry and RNA sequencing
2. Perform further tests on remaining plant products against biofilms in culture
3. Test the promising substances in the wound and implant models
4. Publish the results

Addendum added on 03/17/2020:

The recent coronavirus pandemic and restrictions placed on research by MSU, as well as restrictions suggested by the Federal Government, may adversely impact the timing of the above plans. These restrictions include the temporary shutdown of the MSU Genomics Facility, which performs RNA sequencing and analysis, as well as the discontinuation of all animal orders at MSU. These significant events will undoubtedly delay the project. We will be in close consultation with the CDMRP concerning these developments and may seek solutions such as a no-cost extension or other responses to these issues.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We have not yet sequenced RNA but have all the reagents and materials in hand to do so.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic*

information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

- Nothing to report

[illegible]

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Jonathan Hardy, Ph.D .
Principal Investigator
2 months effort over the past o
Contribution: Directed the project

Michael Bachmann, Ph.D.
Investigator
Contribution: Directed experiments by DeTomaso and Pereira Hicks, analyzed data

Angela DeTomaso
Technician
1 month effort
Contribution: Performed experiments and analyzed data

Cristiane Pereira Hicks
Technician (replaced Angela DeTomaso)
2 months effort
Contribution: Performed experiments and analyzed data

The undergraduates Michael Witte, Allison Kennedy and Lucy Richards contributed by performing many experiments and analyzing data. Their compensation was college credit.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.