AWARD NUMBER: CDMRPL-18-0-DM171034

TITLE: Targeted Sequencing toward Precision Wound Care

PRINCIPAL INVESTIGATOR: Nicholas A. Be

CONTRACTING ORGANIZATION: Lawrence Livermore National Laboratory
Livermore, CA

REPORT DATE: December 2020

TYPE OF REPORT: Final report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
December 2020	Final report	30Sep2018-30Sep2020

4. TITLE AND SUBTITLE

Targeted Sequencing toward Precision Wound Care

5a. CONTRACT NUMBER

5b. GRANT NUMBER
CDMRPL-18-0-DM171034

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Nicholas A. Be, Nisha Mulakken, James B. Thissen

E-Mail: be1@llnl.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Lawrence Livermore National
Laboratory
7000 East Avenue
Livermore, CA 94550

8. PERFORMING ORGANIZATION REPORT NUMBER

LLNL-TR-817867

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)
USAMRDC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Novel methods are needed to support precision wound infection care, which is limited by a lack of diagnostic information. The effort described in this report develops and tests a targeted sequencing platform for measuring microbial genomic determinants critical to wound healing. We applied a bioinformatics approach to design probes for capture of DNA sequence indicating species/genus present, for select wound-associated pathogens, in addition to genomic determinants for virulence and antimicrobial resistance. The panel was tested using control reference microbial genomes in samples within a simulated human background, demonstrating sensitive, specific detection. The panel was further tested using wound samples from combat injuries. Detection of microbial factors was demonstrated across a range of samples. Detected determinants associated with clinical parameters with utility for guiding care. The benefits of this panel to combat wound care are numerous and wide ranging, including more timely evaluation of critical wound infection properties.

15. SUBJECT TERMS
combat injury; wound infection; antimicrobial resistance; virulence; next-generation sequencing; targeted sequencing; genomics; metagenomics; pathogen detection

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

TABLE OF CONTENTS

1. INTRODUCTION:

Genomics and informatics analyses continue to advance at an accelerating pace and, in the modern age of precision medicine, will prove invaluable for exploring the role of microbial communities in healthy and disease states. These technologies hold potential for tremendous benefit in combat casualty care. The severely invasive nature of combat trauma creates massive regions of injury and infection, requiring specialized diagnostic and aggressive therapeutic approaches. Even in the hands of experienced surgeons, some wounds fail to heal due to a lack of actionable diagnostic information. More timely, informative evaluation of wound infection would reduce morbidity, shorten hospital stays, and improve rehabilitation for combat-wounded service members. Microbial metagenomic sequencing has the potential to provide this information through analysis of genomic factors such as antibiotic resistance; however, whole genome sequencing does not yield the coverage required for sensitively detecting and analyzing individual microbial genes due to background created by human genomic sequence in clinical samples. A new approach is needed to bring the utility of DNA sequencing to management of wound infections. The low limit of detection and high specificity delivered by an approach employing targeted sequencing would facilitate detection of microbial genomic determinants in clinical samples, even before they result in emergent phenotypes that are detrimental to the patient.

The current effort developed a targeted sequencing panel for capture and sequencing of microbial genomic determinants with relevance to wound colonization and healing. This platform selectively sequences thousands of microbial genomic regions, thereby facilitating high-confidence detection of critical microbial factors that are otherwise difficult or impossible to assess. These microbial factors included those associated with pathogen genus/species identity, antimicrobial resistance, and virulence. Profiling and delivering information on these factors could guide and influence subsequent therapeutic interventions.

2. KEYWORDS:

combat injury; wound infection; antimicrobial resistance; virulence; next-generation sequencing; targeted sequencing; genomics; metagenomics; pathogen detection; polymicrobial infection; bioinformatics; microbial genomics; military medicine; nosocomial pathogen; hospital-acquired infection

3. ACCOMPLISHMENTS:

Major Goals:

The major aims of this project were to 1) Select microbial genomic targets relevant to wound healing and construct a targeted sequencing panel, 2) Validate the sensitivity and specificity of the resultant panel using reference genome controls, and 3) Validate the panel for predicting select clinical parameters in combat injuries. These aims were achieved through the completion of six Tasks.

Task 1: Identify microbial gene targets – 100% complete

Task 2: Design and computationally optimize target probes – 100% complete
Task 3: Select control reference genomes and identify targets – 100% complete
Task 4: Validate panel with selected control genomes – 100% complete
Task 5: Apply targeted sequencing panel to experimental wound samples – 100% complete
Task 6: Assess association of target detection with outcomes – 100% complete

Activities, Objectives, Results, and Achievements:

Aim 1: Select microbial genomic targets and construct targeted sequencing panel

The goal of Aim 1 was to select microbial genes with an anticipated role in wound progression such that identified targets can be evaluated based on their anticipated impact on the wound healing process and employed for construction of a targeted sequencing panel.

Task 1: Identify informative microbial gene targets

Microbial genomic targets relevant to wound infection were identified using publicly available databases and pre-existing data. Such targets included a) microbial species/genus identity, b) virulence factors, and c) genomic signatures associated with antimicrobial resistance (AMR).

a) Species/genus identity targets: A subset of microbial genera/species known to be relevant to wound healing and hospital-acquired infections was selected based on pre-existing data and expertise (Table 1). DNA sequences conserved within, and unique to, these species were identified.

Bacteria	**Fungi**
Achromobacter sp.	*Absidia sp.*
Acinetobacter baumannii	*Aspergillus sp.*
Enterobacter cloacae	*Bipolaris sp.*
Enterococcus faecalis	*Candida sp.*
Enterococcus faecium	*Cunninghamella sp.*
Escherichia coli	*Fusarium sp.*
Klebsiella pneumoniae	*Lichtheimia sp.*
Proteus sp.	*Mucor sp.*
Pseudomonas aeruginosa	*Pythium sp.*
Pseudomonas putida	*Saksenaea sp.*
Staphylococcus aureus	
Stenotrophomonas maltophila	

Table 1. Wound infection-associated bacterial and fungal species selected for genomic target identification.

b) Virulence targets: Microbial virulence targets relevant to wound infection were identified and extracted from the Virulence Factor Database (VFDB).
c) AMR targets: Through previous collaborations, members of the team piloted an amplification-based targeted sequencing platform for detection of AMR. A modified,

supplemented list of the genes from this previous panel was finalized for hybridization capture probe design, including both bacterial and fungal resistance determinants.

Task 1 milestone/outcome: All genomic targets for the targeted sequencing panel were identified, compiling genomic signatures whose presence or absence will reflect parameters relevant to wound healing.

Task 2: Design and computationally optimize target capture probes

Following selection of microbial genomic targets, DNA probes for these determinants were designed for inclusion in the targeted sequencing platform for detection of microbial a) species/genus, b) virulence genes, and c) AMR gene signatures.

a) Species/genus probe design: Previous experience was employed to extract approximately 11,000 candidate hybridization probes for identification of microbial species and >400 for microbial genera. For genus-level detection, all probes were included to facilitate detection of fungi. A total of 40 species probes with maximal predicted breadth and sensitivity were identified by calculating hybridization probabilities.
b) Virulence probe design:
    i. Multiple sequence alignments were employed to identify conserved regions for each gene. This approach iteratively tested different dominant base frequency thresholds, and the highest frequency threshold yielding at least one region of a predetermined window size (free of indeterminate bases) was used to generate the consensus sequence.
    ii. When conserved regions could not be identified, a phylogenetic approach was used to identify two consensus sequences per gene.
    iii. Probe generation was performed using a publicly available tool (CATCH: Compact Aggregation of Targets for Comprehensive Hybridization, Broad Institute). Probes with more than three indeterminate bases, or predicted human sequence capture, were omitted.
c) AMR probe design:
    i. A subset of AMR-associated genes exhibit multifunctional domains, requiring a larger proportion of captured sequence to assign detection. In these cases, a gene tiling approach was taken.
    ii. For all genes not requiring a tiling approach, a minimum of two probes were selected.

Task 2 milestone/outcome: All capture enrichment probe design was completed, and probes were prepared for testing and evaluation.

Aim 2: Validate sensitivity and specificity of targeted sequencing panel using reference genome controls

The designed probe pool was synthesized commercially (Illumina). Reference genomes were selected and screened informatically to determine presence of targeted genomic loci are in each

reference genome. Samples containing genomic DNA from these references were experimentally evaluated.

Task 3: Identify presence of genomic targets in reference genomes

Reference genomes for *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were selected for testing due to their involvement in combat wound infection. To test a simulated polymicrobial bioburden, a mixed microbial pathogen gDNA sample (ATCC MSA-4000) was also selected, containing 10 human pathogen species (*Acinetobacter baumannii, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Neisseria meningitidis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes*). A mixture of non-pathogenic, commensal microbes (ATCC MSA-1005) from six species was also tested (*Acinetobacter johnsonii, Corynebacterium striatum, Micrococcus luteus, Cutibacterium acnes, Staphylococcus epidermidis, Streptococcus mitis*). This latter mixture is intended as a polymicrobial "negative control," assessing the detection response elicited from naturally occurring skin microbes.

Reference genomes for indicated species were screened for virulence and AMR genomic targets to create a "ground truth" table, indicating which genes should be expected. In rare cases where the sequence for the exact strain to be employed was not available, a closest neighbor strain was used. Mapping was performed via BLAST, as regions corresponding to AMR signatures were longer (~275 bp) than the probes themselves (80 bp). Virulence mapping was performed at the probe level via Bowtie to improve specificity for shorter sequences (80 bp). As anticipated, genomic content corresponding to virulence-targeted probes was substantially higher in pathogenic species relative to skin microflora, which exhibited very few hits to virulence probes.

> *Task 3 milestone/outcome:* Anticipated detection levels were characterized for each reference genome, providing a database of projected ground truth for validation samples.

Task 4: Experimental validation of panel using selected reference genomes

Sample preparation and data processing. Reference gDNA for *A. baumannii* and *P. aeruginosa* were spiked into human reference control background at 10, 100, 1,000, 10,000, and 100,000 genomic copies. Mixed polymicrobial controls were created for pathogen (100,000 copies) and skin commensal (10,000 and 100,000 copies) mixtures. Samples were subjected to hybridization capture using Illumina Nextera Flex for Enrichment and sequencing (Illumina NextSeq 500). Reads were aligned to target sequences corresponding to genus/species, virulence, or AMR content using either the capture probe sequence (for genus/species and virulence) or the full gene target sequence (for AMR). For assigning taxonomic identifiers to genus/species-assigned reads, the Livermore Metagenomics Analysis Toolkit was applied.

Genus and species detection performance. Read-level taxonomic assignment was performed for all validation samples. For samples spiked with *A. baumannii*, reads were captured, sequenced, and identified correctly (genus and species), at all tested copy numbers (Figure 1). For *P. aeruginosa*, correct genus and species-specific reads were identified in all samples except 100 copies. In both cases no taxonomic assignments were made to genera other than *Acinetobacter*

and *Pseudomonas*. These results indicate that genus/species-level detection of microbes by the panel is sensitive and specific to 10 genomic copies (lowest tested) in human background.

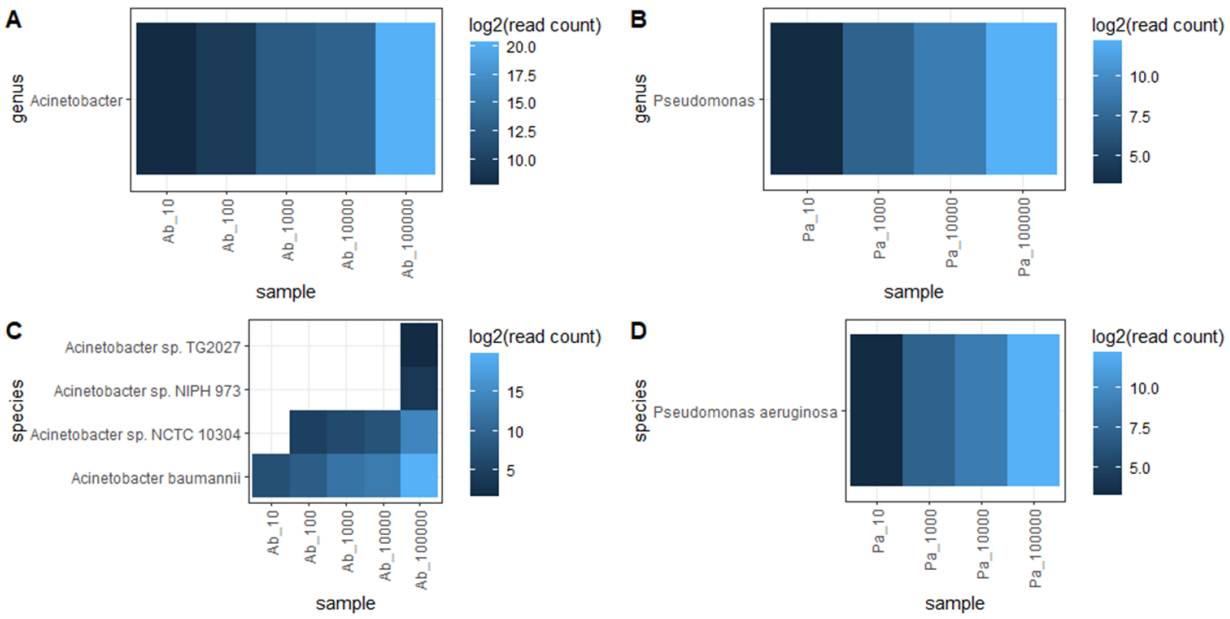

Figure 1. Number of reads detected following targeted capture sequencing of samples containing *A. baumannii* and *P. aeruginosa* in human reference background. Results are shown at the genus level for A. *A. baumannii* and B. *P. aeruginosa* and at the species level for C. *A. baumannii* and D. *P. aeruginosa.* No captured/assigned reads were detected for the sample containing 100 copies of *P. aeruginosa*.

For the polymicrobial pathogen mixture at 100,000 copies, the correct genus and species were detected for all microbes for which probes were designed (Table 2) (probes not designed for *Neisseria* or *Streptococcus*). No reads were assigned to *Streptococcus*. For *N. meningitidis*, 14 reads were detected at the genus level and one read at the species level, likely due to the elevated DNA content of this species. In further iterations, such detection events could be filtered through application of a read count threshold, according to needs of any given application. These results demonstrate sensitive detection of microbial species in a polymicrobial mixture.

species present in sample	% of mixture	probes designed?	Genus reads detected?	Species reads detected?
Neisseria meningitidis	28.9	NO	YES*	YES*
Streptococcus pneumoniae	28.9	NO	NO	NO
Klebsiella pneumoniae	14.4	YES	YES	YES
Staphylococcus aureus	15.1	YES	YES	YES
Streptococcus pyogenes	7.2	NO	NO	NO
Streptococcus agalacitae	2.9	NO	NO	NO
Escherichia coli	1.4	YES	YES	YES
Enterococcus faecalis	0.7	YES	YES	YES
Pseudomonas aeruginosa	0.3	YES	YES	YES

Acinetobacter baumannii	0.1	YES	YES	YES

Table 2. Microbial detection following targeted capture sequencing of samples containing a defined polymicrobial pathogen control genomic DNA mix. Green highlight indicates a species from which sequence was positively detected, and for which probes were specifically designed. *indicates < 20 sequence reads detected

For the skin commensal mixture at 10,000 copies, zero captured reads were assigned. At 100,000 copies, reads were assigned to one species (*Acinetobacter johnsonii*). These results indicate detection results would not be confounded by skin commensal presence. The exception of *A. johnsonii* detection due to the presence of probes from the related *A. baumannii* represents a beneficial observation, as *A. johnsonii* is also capable of causing hospital-acquired infection.

Virulence signature detection performance. Virulence genes were called as detected at a threshold of 50 assigned reads. Results were compared to expected detection based on reference analysis (Table 3). Correct gene calls were made with as few as 10 genome copies. At 100,000 copies, 98% of expected genes were detected for *A. baumannii* and 94% for *P. aeruginosa*. For *A. baumannii*, only one instance occurred where a probe region was not present in the reference but was detected experimentally (at 1,000 copies). For *P. aeruginosa*, three instances of such detection events were observed at 10,000 copies and four at 100,000 copies.

		Virulence probe analysis		
Sample copy number	**# genes expected based on reference**	**# genes detected and present in reference**	**# genes detected but not present in reference**	**# genes undetected but present in reference**
Acinetobacter baumannii				
10	46	14	0	32
100	46	25	0	21
1,000	46	39	1	7
10,000	46	39	0	7
100,000	46	45	0	1
Pseudomonas aeruginosa				
10	106	6	0	100
100	106	29	0	77
1,000	106	98	0	8
10,000	106	96	3	10
100,000	106	100	4	6

Table 3. Detection of virulence genes via targeted sequencing capture panel in control reference genomes at varying copy numbers. Comparisons are made to genes indicated as present vs. absent in the reference sequence of the corresponding strain via informatic analysis.

Analysis of virulence detection in the pathogen mixture showed that the targeted capture panel detected 85% genes expected based on the reference sequence. The capture panel detected 34 additional genes (8% of the 420 total detected genes) not indicated as present based on analysis of the reference sequence. Testing at 10,000 copies of skin commensals demonstrated that only

two total reads were captured and assigned to one virulence gene. At 100,000 copies, sequence data were mapped to only five genes.

These results show that a clear, unambiguous virulence signal is detected when a mixture of pathogens is present, but that minimal virulence signal is obtained when analyzing avirulent commensal microbes using the targeted panel. This indicates a low risk for false positive detection events.

AMR signature detection performance. To assign AMR detection events, the percent of normalized gene length covered at > 20X sequence read coverage was examined (normalized gene length = probe count * 80 bp). Coverage thresholds of 50%, 75%, and 100% were tested. Percent of the normalized length covered can exceed 100%, as probes can capture regions outside their 80 bp length. Detection of AMR genes in control genomes according to these thresholds was compared to expected detection based on reference sequence analysis (Figure 2). At all thresholds 12 out of 13 AMR genes predicted to be present in the reference were detected. The *blaADC-12* gene was not detected; however, several related beta-lactamases were detected. Correct AMR gene detection events were observed even at the lowest tested concentration of 10 genome copies. Four AMR-associated genes included in the panel were identified as present in the *P. aeruginosa* reference genome. All were correctly detected at all thresholds at a genome copy number of 1,000 copies and above (Figure 3).

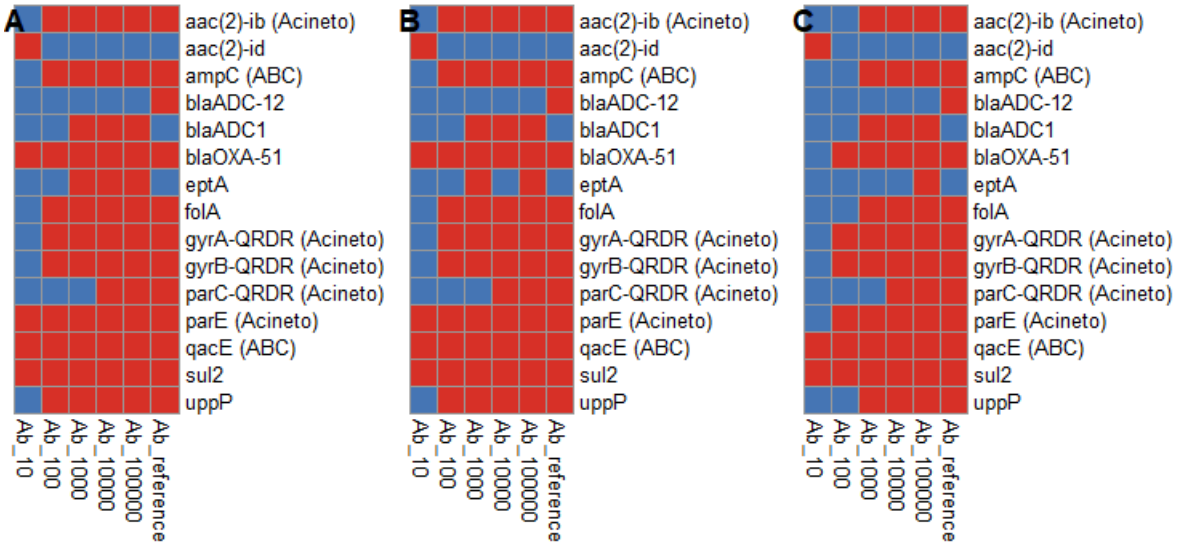

Figure 2. Detection of AMR-associated genes via targeted sequencing capture panel in control reference genomes for *A. baumannii* at increasing genome copy number, compared to expected genes based on reference sequence. Results are shown at coverage thresholds of A. 50%, B. 75%, and C. 100%. Red = present, blue = absent.

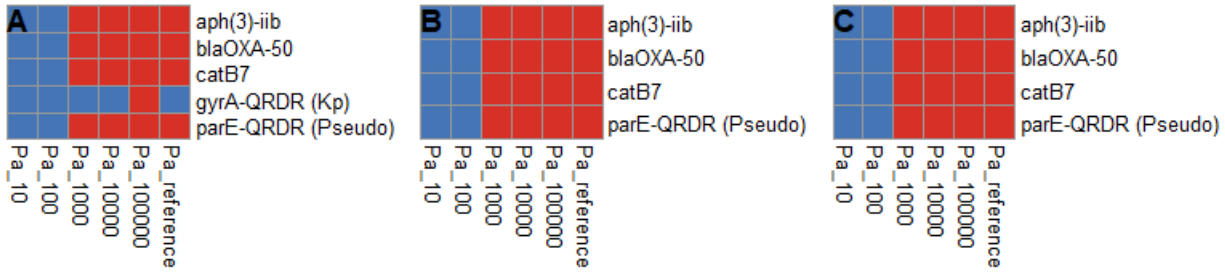

Figure 3. Detection of AMR-associated genes via targeted sequencing capture panel in control reference genomes for *P. aeruginosa* at increasing genome copy number, compared to expected genes based on reference sequence. Results are shown at coverage thresholds of A. 50%, B. 75%, and C. 100%. Red = present, blue = absent.

Detection results were also examined for pathogen and skin mixtures. For the pathogen mixture, at the highest threshold, the targeted capture panel detected 54/86 genes that were expected based on the reference sequence. For the majority of genes that were undetected, genes belonging to the same family were detected. It is therefore likely that sequence corresponding to these genes was indeed captured, but assigned to an alternate but closely related gene.

Analysis of reference genomes for species present in the skin commensal mixture identified the presence of six of the AMR-associated genes included in the capture panel. At a read count threshold of 100, 4 out of 6 of these genes were detected by the capture panel. Two additional genes were detected that were not anticipated to be present based on analysis of the reference sequences for each species. As anticipated, and in agreement with observations from analysis of virulence results, a much stronger AMR signal was observed from the pathogen mixture relative to the skin commensal mixture.

> *Task 4 milestone/outcome:* The targeted panel was validated using single and mixed microbial controls. Sensitive, specific detection of microbial taxa was achieved in both contexts. A clear, pathogen-specific virulence and AMR signal was observed through testing of polymicrobial samples.

Aim 3: Validate targeted sequencing panel for predicting select clinical parameters in combat injuries

Administrative Task: IRB exemptions for processing and analysis of samples were approved at both LLNL and Uniformed Services University, and each received HRPO concurrence.

Task 5: Apply targeted sequencing panel to experimental wound samples

Wound effluent samples were prepared for processing and subjected to the experimental workflow described above for the targeted capture sequencing panel. 176 wound effluent samples were processed, thereby meeting the projected sample processing quantity for Task 5. Pre-processing of the resultant sequence data was performed (Figure 4).

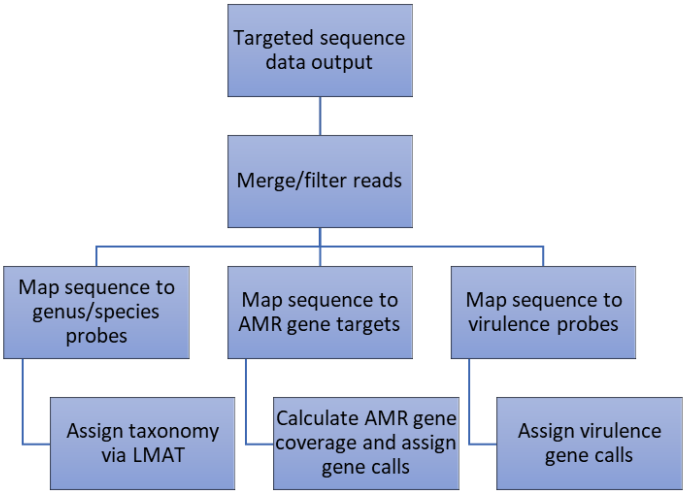

Figure 4. Workflow for pre-processing and analysis of sequence data from targeted sequencing panel.

Microbial taxonomy assignment for wound samples. Sequence reads mapped to genus/species probes were assigned microbial taxonomy using the Livermore Metagenomics Analysis Toolkit (LMAT) (Figure 5). Ten microbial genera (each included in the panel design) were detected across all samples.

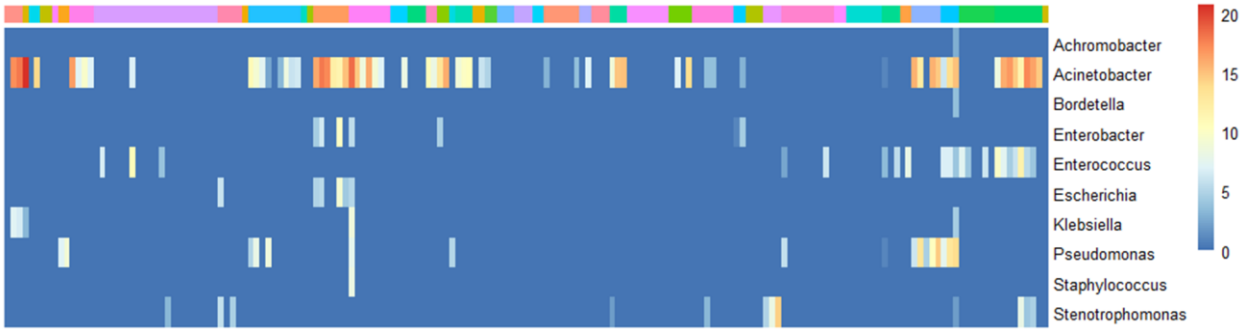

Figure 5. Microbial genera detected in wound samples through analysis with targeted sequencing panel. Samples are shown in columns and microbial genera in rows. Continuous numeric scale indicates $\log_2$(read count). Samples obtained from the same individual are shown adjacent, as indicated in the annotation row colors above the plot.

Virulence gene calling for wound samples. Virulence gene detection calls were made for wound samples according to the thresholds described above. Most samples did not exhibit detection of a large number of genomic virulence factors. A subset of samples were observed in clusters with a larger number of genes and virulence island components, and were subjected to further analysis in Task 6.

AMR gene calling for wound samples. The control validation data obtained via analyses in Task 4 were applied as guidance for selection of the AMR gene calling threshold. A coverage threshold of 50% was selected for downstream analyses. These methods were applied to

sequence reads mapped to AMR target regions and the previously described approach was employed to call presence or absence of genes in each sample.

> *Task 5 milestone/outcome:* All projected wound samples were processed using the designed targeted sequencing panel. Resultant data were processed and assigned to corresponding taxonomy, virulence genes, and AMR associated genes. These results demonstrate successful data generation from challenging samples using the proposed methods.

Task 6: Assess association of target detection with clinically relevant outcomes and treatment patterns

Clustering analyses were performed to determine whether panel detection events associate with clinical parameters. Three sample categories were available for analysis: initial (earliest sample available after injury), intermediate, and final (pre-closure). Wound outcomes examined included outcome overall (successful healing versus failed healing [dehiscence], when available) and critical colonization (microbial colonization above a defined threshold indicating clinical infection). Analysis focused on final samplings, as this timepoint is expected to most accurately reflect conditions relevant to wound outcomes. Hierarchical clustering of samples based on microbial taxa detection data show a defined cluster of critically colonized samples with positive taxonomic detection events from the targeted panel (Figure 6).

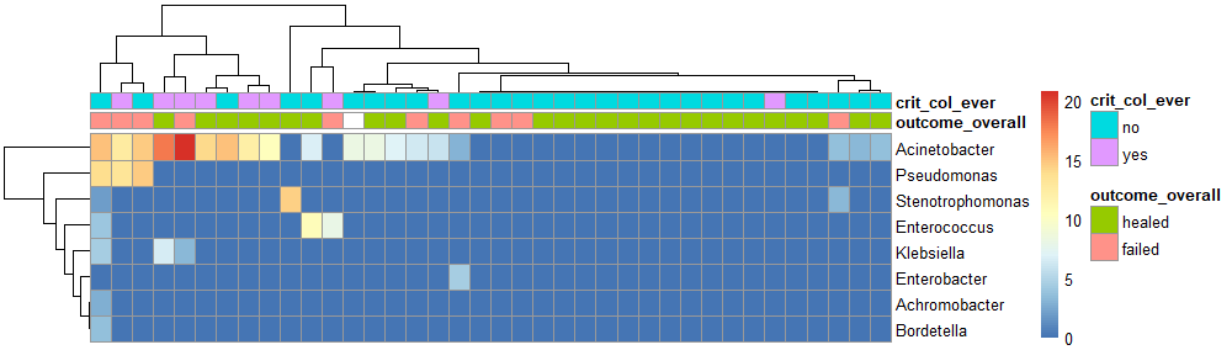

Figure 6. Microbial genera detected in wound samples by targeted sequencing panel. Samples are shown in columns and microbial genera in rows. Continuous numeric scale indicates $\log_2$(read count). Final samplings are shown. Critical colonization (crit_col_ever) is shown in the first annotation row and successful versus failed healing (outcome_overall) in the second annotation row.

To facilitate functional genomic comparison, virulence genes were binned into functional categories, and AMR-associated genes were binned into the antimicrobial category to which they confer resistance. Samples from final timepoints were again clustered and plotted based on the targeted sequencing detection profile for virulence and AMR.

Final wound samples were subjected to hierarchical clustering based on either detected virulence functional category (Figure 7) or detected antimicrobial resistance class (Figure 8). Final samples from wounds with critical colonization outcomes cluster together with positive detection events for both virulence and AMR. These results demonstrate that taxonomic, virulence, and AMR detection using the designed targeted sequencing panel associates with clinically relevant

parameters, including critical colonization. As such, detection data could predict and/or reflect whether microbial colonization has occurred at a sufficient level to cause an infection with downstream impacts on inflammation and wound resolution.

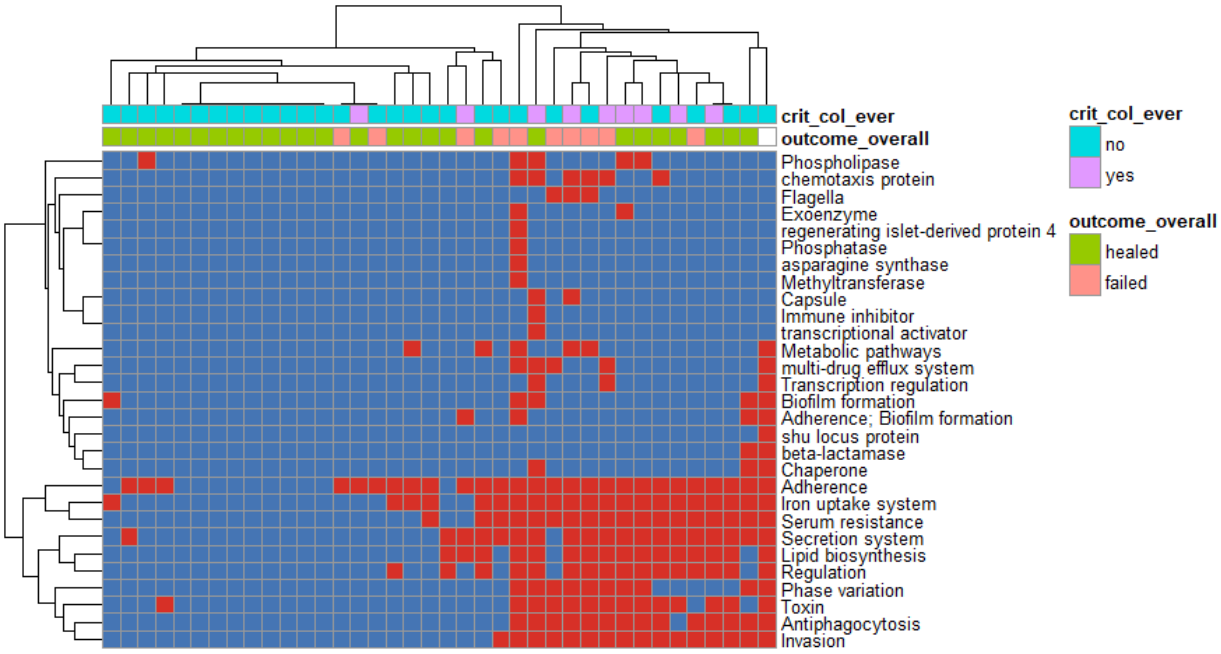

Figure 7. Virulence factors detected in wound samples obtained from the final sampling timepoint through analysis with targeted sequencing panel. Virulence genes were binned into functional virulence categories prior to plotting. Samples are shown in columns and detected virulence categories in rows. Critical colonization (crit_col_ever) is shown in the first annotation row and successful versus failed healing (outcome_overall) in the second annotation row. Red = present, blue = absent.

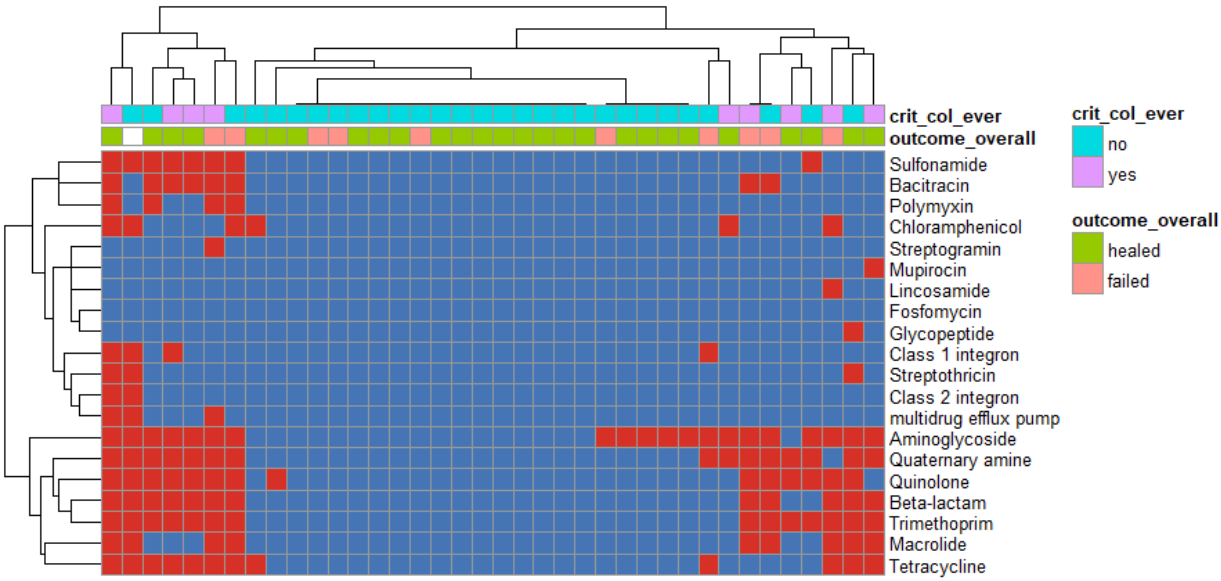

Figure 8. AMR categories detected in wound samples obtained from the final sampling timepoint through analysis with targeted sequencing panel. AMR-associated genes were binned into the

category of antibiotic to which they confer resistance prior to plotting. Samples are shown in columns and detected antibiotic categories in rows. Critical colonization (crit_col_ever) is shown in the first annotation row and successful versus failed healing (outcome_overall) in the second annotation row. Red = present, blue = absent.

Association testing was performed to determine whether detected microbial species associate with specific virulence/AMR detection events and whether these observations differed across distinct wound outcomes. This analysis was performed using the Hierarchical All-against-All (HAllA) analysis tool (Rahnavard, Huttenhower, et al., Harvard School of Public Health). Binary data were input to HAllA using normalized mutual information (NMI) as an indicator of similarity between species and genes, as this metric is amenable to both categorical and continuous data.

Significant associations were identified between detected AMR gene classes and detected species, with distinctions between failed and successfully healed wounds (Figure 9). *Acinetobacter baumannii* were detected in both healed and failed wounds; however, a larger number of significant associations of *A. baumannii* with AMR gene categories was observed in samples from failed wounds. Associations with *Pseudomonas* species was observed in samples from failed wounds, but not healed wounds. These species associated with detection of AMR gene categories, including quinolones, aminoglycosides, beta-lactams, and chloramphenicol. The graphical representations of the NMI analysis do not illustrate *all* detection events; instead they represent detection events with statistically significant associations for the categories of detection events being compared in each analysis.

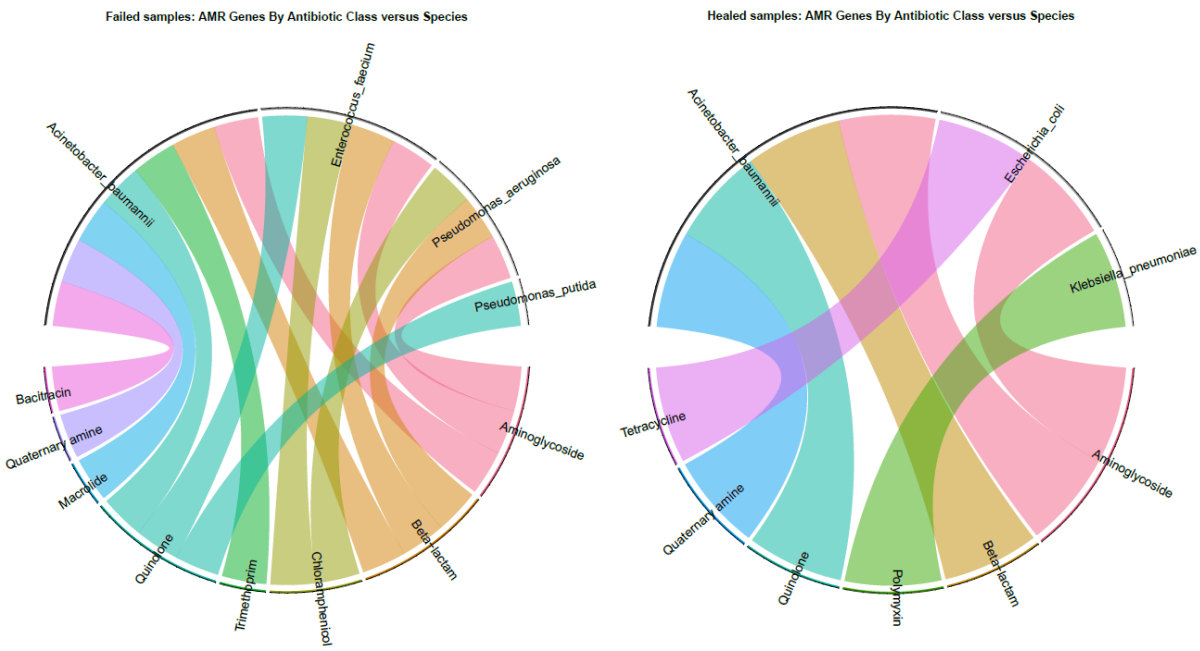

Figure 9. Associations between detected species and AMR genes in wounds with failed (left) versus successful (right) healing, as determined by mutual information analysis.

Analysis of healed versus failed wounds was also undertaken in the context of detected species and detected virulence genes (Figure 10). As was the case with AMR genes, a larger quantity of associations with virulence gene categories were detected across samples from failed wounds relative to those from healed wounds. In failed wounds, significant associations with virulence categories were identified for detection of *A. baumannii*, *E. faecium*, *P. aeruginosa*, and *P. putida*. Healed wounds exhibited significant associations for *A. baumannii* and *K. pneumoniae*; however, the number of virulence categories detected was lower in these samples.

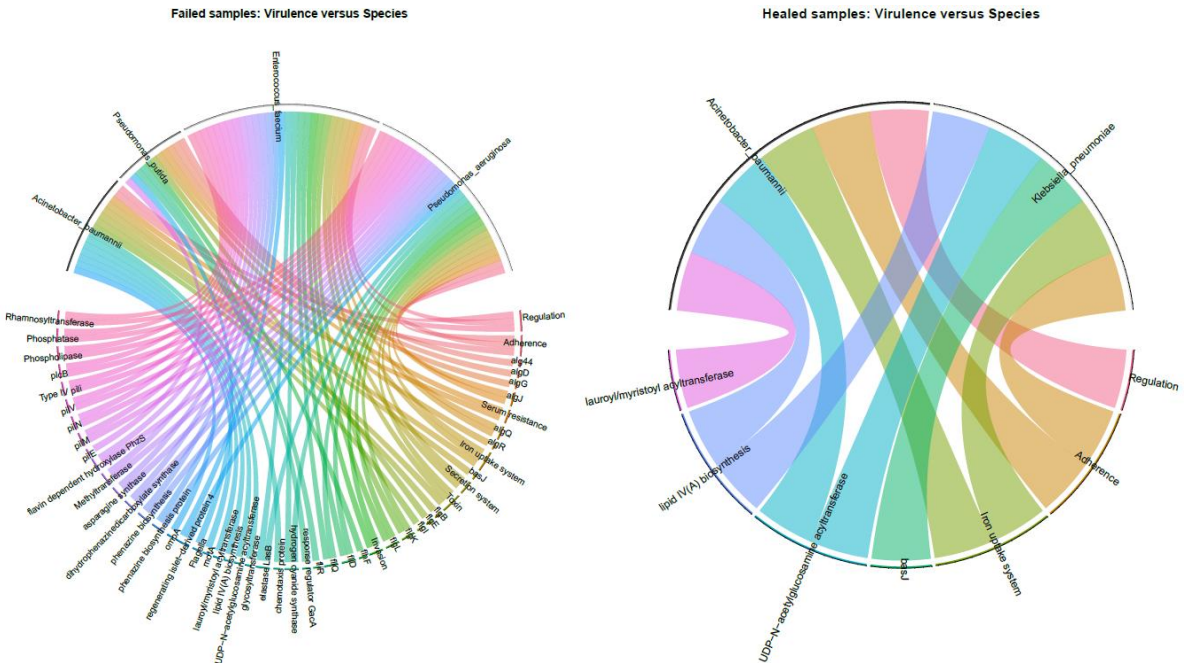

Figure 10. Associations between detected species and virulence genes in wounds with failed (left) versus successful (right) healing, as determined by mutual information analysis.

Task 6 milestone/outcome: Analysis of wound targeted sequence data showed that clustering of samples based on detection results from the designed panel yields cohesive clustering of samples derived from wounds with detrimental outcomes. These results indicate that such detection events would be clinically relevant and potentially predictive. Statistical association analyses demonstrated that an elevated quantity of significant associations are observed between microbial species and AMR/virulence genes when samples are derived from failed wounds. These results show that the designed panel could indicate cases where colonizing microbes exhibit functional potential for impeding healing, thereby providing data that could inform clinical decision support.

Training and professional development: Nothing to report.

Results dissemination: Results relevant to, and impactful for, the military medical scientific community were communicated at the 2019 Military Health System Research Symposium (MHSRS). An abstract containing these data was accepted for the 2020 MHSRS; however, the meeting was cancelled, thus the results could not be presented. As a substitute for this meeting, results from the abstract were communicated in a virtual meeting held by, and presented to, the Uniformed Services University community.

Activities in next reporting period: Final report

4. IMPACT:

Discipline-specific impact: More comprehensive assessment of wound colonization and bioburden can critically influence decisions that improve outcomes in combat injuries. Advances in metagenomic sequencing and analysis could address this need by expanding the ability to measure and track microbial populations as they relate to clinical outcomes. The effort in this report describes performance of a targeted enrichment panel designed to sequence genomic determinants with the highest degree of relevance to combat wound microbial colonization and subsequent healing, and therefore the highest potential to influence interventions. The described results indicate sensitive and comprehensive detection of microbial factors, which would be highly challenging to accomplish via other currently applied platforms. The ability to detect these microbial determinants was demonstrated in both control validation samples and highly relevant wound samples. Associations between distinct detection results and clinical outcomes were shown, including the increased association between microbial species and functional genomic indicators of infection. This demonstrated promise indicates that future use of this and other targeted sequencing panels in military medicine could allow for early detection of clinically impactful factors, facilitating a more precise approach to care of combat wound infections.

Outside impact in military medicine: The potential impact of these results and developed panel include applications relevant to battlefield injuries and beyond. More timely evaluation of infection properties and profiles could hold utility in many contexts, including infections in other body sites. Corresponding interventions would have the potential to reduce morbidity, shorten hospital stays, and improve rehabilitation for service members with traumatic injuries or other conditions associated with microbial infection. Currently, early evaluation of infectious parameters related to infection identity, virulence, and AMR, is challenging or unavailable, particularly evaluation of multiple such factors simultaneously. The advancement catalyzed by this effort has the potential for benefit throughout the Military Health System, including addressing hospital-acquired and surgical infections as a whole.

Societal impact: Infections resulting from resistant nosocomial pathogens represent a tremendous burden on the U.S. healthcare system. The factors selected for inclusion in the proposed panel are relevant to both wound and other infections throughout both military medicine and civilian healthcare. This includes infections residing in non-healing wounds, such as diabetic ulcers. The initial results described in this report lay groundwork for expansion of the utility of targeted sequencing throughout both military and civilian healthcare diagnostic infrastructures. Advantages of the targeted sequencing panel developed for this effort are wide ranging and apply broadly to evaluation of polymicrobial infection, including 1) low limit of detection, 2) enhanced depth, 3) increased coverage of any desired region, and 4) fractional cost and analysis burden. A particularly advantageous future impact of the proposed panel is the enabling of gene- and mutation-level analysis in near-point of care applications. Thus, while it is not described in the current report, any resultant data could potentially allow for downstream analysis of variation within these genes and impact on clinical decision making. This is

particularly important for accurate assessment of antimicrobial susceptibility profiles. Microbial parameters relevant to genus/species identification, resistance, and virulence are critical for clinical application; amplifying the ability to assess these factors has the potential to change the landscape of microbial profiling.

Technology transfer impact: Nothing to report.

5. CHANGES/PROBLEMS

Changes in approach: Nothing to report.

Actual or anticipated delays: All potential delays resultant from staffing changes and shelter-in-place orders were effectively mitigated, and all Tasks were completed within the period of performance.

Other changes: Nothing to report.

6. PRODUCTS:

Journal publications:

A manuscript describing this effort is in preparation.

Books: Nothing to report.

Conference abstracts/presentations:

Be NA, et al. Composition, function, and relevance of the microbial microenvironment in wounds from combat injuries. Military Health System Research Symposium. August 2019. Abstract # MHSRS-19-01833.

Thissen JB, et al. Capturing value through targeted sequencing of combat wound-relevant microbial genomes. August 2020. MHSRS-20-01138. *Conference cancelled.*

Thissen JB, et al. Capturing value through targeted sequencing of combat wound-relevant microbial genomes. Uniformed Services University Forum. August 2020.

Websites: Nothing to report.

Technologies or techniques: Nothing to report.

Inventions: Nothing to report.

7. PARTICIPANTS AND COLLABORATING ORGANIZATIONS:

Individuals working at least one month per year during period of performance:

Name:	Nicholas Be, Ph.D.
Project Role:	PI
Nearest person month:	2
Contribution to Project:	Project management, technical oversight, experimental design

Name:	Nisha Mulakken, M.S.
Project Role:	co-I
Nearest person month:	5
Contribution to Project:	Target identification, probe design, data analysis

Name:	James Thissen, M.S.
Project Role:	co-I
Nearest person month:	5
Contribution to Project:	Workflow design, experimentation/sequencing, data analysis

Change in active other support: The previously reported pending support indicated below became active during the performance period. No impact on effort for the subject of this project report was anticipated or experienced.

Sponsor: Defense Threat Reduction Agency
Project title: Development of CNS penetrating oxime countermeasures for nerve agent exposure: A path toward an IND application
Role: co-Investigator
Time commitment: 20%
Overlap: None

Other organizations:

Name: Uniformed Services University/Henry M. Jackson Foundation
Location: Bethesda, MD
Partner's contribution: collaboration, data analysis, and application guidance

8. SPECIAL REPORTING REQUIREMENTS

Quad chart submitted with attachments.

9. APPENDICES

No additional materials for attachment.