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Integrated Detection of Pathogens and Host Biomarkers for Wounds

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14. ABSTRACT

The increasing incidence and complications arising from combat wounds has necessitated a reassessment of methods for effective treatment. Infection, excessive inflammation, and incidence of drug-resistant organisms all contribute toward negative outcomes for afflicted individuals. The organisms and host processes involved in wound progression, however, are incompletely understood. We therefore set out, using our unique technical resources, to construct a profile of combat wounds which did or did not successfully resolve. We employed the Lawrence Livermore Microbial Detection Array and identified a number of nosocomial pathogens present in wound samples. Some of these identities corresponded with bacterial isolates previously cultured, while others were not obtained via standard microbiology. Further, we optimized proteomics protocols for the identification of host biomarkers indicative of various stages in wound progression. In combination with our pathogen data, our biomarker discovery efforts will provide a profile corresponding to wound complications, and will assist significantly in treatment of these complex cases.

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Wound infection, biomarker, nosocomial pathogen, host-pathogen interaction

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INTRODUCTION

Modern combat environments present many unique and difficult challenges to the evolution of medical treatment for the warfighter. Improvised explosive devices, increasing number and severity of injuries per casualty, longer transport times, and higher died of wound rates have necessitated a reassessment of our approach to wound treatment.

One of the most important determining factors in wound resolution is the degree to which infection is observed, and whether the infection manifests in an acute or chronic manner. Previous studies of wound infection have focused on a relatively small subset of well-characterized pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [1-3]. Recent studies, however, indicate that analysis of wound infection via standard microbiological techniques likely overestimates the importance of readily-cultured species in chronic wounds [4]. It is becoming clear that the community of microorganisms inhabiting the human body, collectively known as the human microbiome, represent tremendous breadth and microbial diversity, and that a much broader range of organisms likely plays a role in wound response. Many of these organisms may be difficult or impossible to culture using standard protocols, and their importance in infection may be previously unrecognized. A comprehensive approach is therefore needed in order to completely assess the role of microbial communities in wound pathology.

Also highly relevant to treatment of combat wounds are host response mechanisms. Biomarkers such as inflammatory cytokines and chemokines could provide informative indications of likely outcome and appropriate timing of wound closure [5]. Past studies examining the chemical immune response in serum and wound effluent demonstrated that such biomarkers are capable of predicting healing and identifying instances where immune deregulation results in healing failure [6]. This process also involves the regulation of matrix remodeling metalloproteinases, profiles of which have predicted outcome for traumatic war wounds [7].

Construction of a comprehensive panel of host response biomarkers, in combination with a microbial profile of corresponding wounds, would aid tremendously in clinical decision-making. We therefore assembled a collaborative and experienced team, combining the extensive clinical wound research expertise and the unique wound sample collection from the Naval Medical Research Center (NMRC), the advanced and proven bioinformatics and pathogen detection expertise from the Lawrence Livermore National Laboratory (LLNL), and the proteomic and biomarker research expertise from the University of California, Davis to conduct a comprehensive characterization of microorganisms and host proteins associated with negative or positive wound outcomes. A Cooperative Research and Development Agreement was established between the three institutions to perform this study.

We elected to analyze wound samples using the Lawrence Livermore Microbial Detection Array (LLMDA) [8], which is a highly cost-effective detection and discovery platform for the identification of a very broad range of microbes. For host factors, we optimized a 2-D difference gel electrophoresis (2D DIGE) system to identify protein biomarkers. Our unique approach of characterizing both host and pathogen profiles in wound healing will reveal unique aspects of these essential interactions and will improve the ultimate outcome and quality of life of wound victims.

BODY: RESEARCH METHODS AND RESULTS

Aim 1: Detection of microbial pathogens in wounds (LLNL)

Methods: Nucleic acid processing from wound samples

In order to assess microbial content of wounds using microarray technology, we first extracted DNA and RNA from tissue samples. A total of 28 individual muscle wound biopsy tissue samples from NMRC were first received by LLNL in November 2011. These samples represented patients who recovered without severe complications, as well as those who developed a severe inflammatory response. Sample identity was blinded prior to initial tissue processing. We extracted nucleic acid using the RNeasy Fibrous Tissue Kit (Qiagen). Briefly, tissues were homogenized via bead-beating, further lysed by incubation with proteinase K, and nucleic acid collected and purified using the manufacturer's provided centrifugation columns. We then amplified the extracted nucleic acid via reverse transcription followed by whole transcriptome/genome amplification using the Quantitect Whole Transcriptome Kit (Qiagen). We labeled resultant amplified DNA using Cy3 fluorescent dye (Roche) and hybridized to the LLMDA v.2 detection array. We analyzed data using the automated composite likelihood maximization method developed at LLNL [8]. We used a threshold of signal intensity above the random control probes at a confidence interval of 0.99. Eight probes were required to be detected in order to identify a "hit" organism, and these detected probes were required to comprise at least 20% of total expected probes.

Results: Microbial content in wound samples

We did not initially detect a significant bacterial presence in any tissues. We did, however, observe a number of samples containing human endogenous retrovirus and human herpesvirus, although the presence of human endogenous retrovirus was most likely due to the presence of human background DNA. We determined our apparent lack of sensitivity to be due to challenges with setting analysis thresholds in our processing algorithm. Due to the high level of background human DNA in samples, these thresholds are more difficult to empirically determine than in samples which are enriched for target DNA. The identities of organisms cultured from wound samples were, therefore, un-blinded by NMRC, so that proper thresholds could be identified for future analyses.

Following examination of the un-blinded data, we observed that the wound tissues under study had been previously identified infected exclusively with bacteria. We therefore determined that, for subsequent samples, reverse transcription would be omitted, and only whole genome amplification performed. This would preclude the possibility of reverse transcription bias for host over microbial mRNA. We reprocessed each of the 28 samples and hybridized to an updated array, the LLMDA v4, which contains a more comprehensive panel of bacterial and viral probes. We once again analyzed data using the composite likelihood maximization method, this time using relaxed detection thresholds: only four detected probes and 10% of the expected probes were required to be hit. Table 1 shows expected cultured organisms (NMRC) and array-detected organisms (LLNL). These results were filtered to include nosocomial pathogens, with non-specific hits removed. A complete list of detected organisms can be found in Appendix A.

Of the 10 tissues from which bacterial species were cultured by NMRC, we detected nosocomial pathogens in nine samples. In four of these samples we detected an organism identical to that which was cultured. In four additional samples, we detected nosocomial pathogens within tissues from which no organisms were successfully cultured. Several cultured organisms were not detected by the LLMDA; however, the array did not contain corresponding probes for a number of these bacterial species (See Appendix A). Our team has recently updated the LLMDA with newly sequenced viruses, bacteria, fungi, archaea and protozoa (LLMDA v5),

and it is very likely that these pathogens will be detected by the revised LLMDA v5, as well as pathogens from fungal infections in wounds that have not been previously characterized.

Table 1. Nosocomial pathogens detected in wound tissues by LLMDA.

Sample	Cultured by NMRC	Nosocomial pathogens detected by LLNL
CG531A	None	*Acinetobacter sp.* plasmid pRAY *Klebsiella pneumoniae* plasmid pKlebB
DM231B	None	None
EB471A	*Acinetobacter baumannii*	*Acinetobacter SUN* plasmid pRAY *Stenotrophomonas sp.*
ET662B	None	None
HP561A	None	None
HP561B	None	None
IS711A	None	None
IS711B	*Stenotrophomonas maltophilia*	*Stenotrophomonas sp.*
IS712A	None	*Klebsiella pneumoniae*
IS712B	None	*Staphylococcus aureus* plasmid pKH19 *Acinetobacter sp.* plasmid pRAY
JC272A	*Enterococcus faecium*	*Stenotrophomonas sp.*
JG351A	*Acinetobacter sp.* *Bacillus cereus*	None
JG352A	*Acinetobacter sp.*	None
	Alloicoccus otidis	None
JR281A	*Acinetobacter baumannii/ calcoaceticus* complex	None
JR342A	*Acinetobacter baumanii*	*Acinetobacter baumannii* ATCC 17978 pAB2 *Acinetobacter sp.* plasmid pRAY
JT691A	None	None
JT691B	*Staphyllococcus capitis*	*Acinetobacter SUN* plasmid pRAY
JT691C	None	*Stenotrophomonas sp.*
KS702A	None	None
KS702B	None	None
LM671A	None	None
LM671B	None	None
MW651A	None	None
MW651B	None	None
RH491A	None	None
TN631A	None	None
TN631B	*Enterococcus faecium*	None
ZB191B	*Enterobacter cloacae* *Acinetobacter baumannii*	*Enterobacter cloacae* plasmid pEC01

These initial results underscore the importance of a more comprehensive microbial detection strategy. As was noted, a number of the nosocomial pathogens detected by the LLMDA were not cultured via standard techniques. Additionally, a number of other microorganisms (Appendix A)

not previously implicated in wound or hospital-acquired infections were identified to be present. These organisms may very well play an as of yet unrecognized but important role in wound healing, which could be relevant to the course of treatment for a patient.

Summary of observed technical challenges and practiced mitigation

1. Initial detection methods did not yield the sensitivity required to detect bacterial organisms cultured from wounds by NMRC. We therefore modified our processing protocols by performing whole genome instead of whole transcriptome amplifications.

2. Using our revised protocols, several cultured organisms were still not detected using the LLMDA v4. The bioinformatics team at LLNL recently completed computational design of the updated LLMDA v5, which will include all newly sequenced bacterial, viral, fungal, and protozoan and archaea pathogens. More than 5700 microbial species were represented total including 3179 viral species, 2223 bacterial species, 124 archaea species, 94 protozoa and 136 fungi. This will provide a much more comprehensive panel for future testing of wound samples.

Aim 2: Identification of biomarkers for host responses in combat wounds (UC Davis, Naval Medical Research Center)

Methods: Optimization of 2-D difference gel electrophoresis

Biomarker discovery from combat wounds will be assessed using 2-D difference gel electrophoresis (2D DIGE). Using 2D DIGE, we will identify proteins observed at significantly different levels between normal and wound tissue. In order to optimize our methods, we performed studies using samples which were likely to be similar to those obtained from combat wounds. We conducted preliminary proteomics experiments by determining the optimal sample preparation method for proteomic analysis of burn wounds. Freshly obtained normal and burn skin was subjected to a single freeze/thaw cycle. Although differences were seen between frozen and fresh tissue, discrepancies were minor and, due to the likely difficulty in obtaining fresh tissue for this project, differences were deemed acceptable. Moreover, we recently examined paraffin-embedded fixed tissue as compared to tissue stored in RNA-Later® (Ambion). Initial data suggest RNA-Later® may be superior for preserving protein integrity relative to formalin fixation.

Results: Preliminary protein level observations

In our preliminary proteomic sample preparation studies, we detected a large number of protein spots and a fair number of differentially expressed spots when comparing healthy and burn wound tissue. We detected over 600 protein spots, and several were found to be differentially expressed between healthy and burn tissue, as well as different between burn tissue with and without collection of a fatty layer of tissue. Although differences between freeze/thaw and glass bead homogenization were not dramatic, both techniques improved upon the initial sample preparation of dounce homogenization in a different chemical lysis buffer. In addition, we analyzed the amount of tissue needed from each sample for this study, and found that 500 mg of tissue will be sufficient regardless of the sample preparation chosen. We also determined that the freeze/thaw protocol actually provided the highest amount of total protein as compared to other sample preparation methods.

Most importantly, we established an appropriate sample preparation method for obtaining high quality protein spot maps for healthy and burn wound human skin tissue. Several techniques were evaluated and compared including glass bead homogenization and chemical lysis using freeze/thaw cycles. Glass bead homogenization is most appropriate for the sample size and sample status of the tissue obtained from NMRC. In addition, we examined the proteomic differences between different skin regions on the same individual and healthy tissue between two individuals. As expected, there were no noticeable differences between skin regions on the same individual, and the differences between two individuals showed the most dramatic differences among the pilot studies so far. These results strengthen the need to examine multiple samples from the same individual (samples collected over time) to truly understand the patient-to-patient variability in wound samples. Combat wound samples were recently sent from NMRC to UC Davis, and will soon be processed using these empirically-determined optimized protocols.

Aim 3: Data analysis and clinical correlations (LLNL, UC Davis, and Naval Medical Research center)

Once Aims 1 and 2 are completed, LLNL will construct statistical models to predict clinical outcomes given (i) identified microbial flora and (ii) host response biomarkers. Standard multivariate regression methods will be used to predict categorical responses (wound closure vs. acute infection vs. chronic infection), and a Cox proportional hazard model will be applied to predict quantitative measures such as time to closure. LLNL and UC Davis have statisticians with the appropriate bioinformatics and clinical trials experience, respectively, for statistical analysis and computational modeling of the wound profiling data that will be generated in this project.

KEY RESEARCH ACCOMPLISHMENTS

Aim 1:

- Designed and optimized protocols for extraction of nucleic acid from wound tissue.
- All samples provided by NMRC extracted and hybridized to LLMDA v2 at LLNL. Determined that protocols did not provide optimal sensitivity for detection of bacterial species from tissue samples.
- Redesigned processing procedures and analysis thresholds. Reprocessed all samples using LLMDA v4.
- Established successful detection of nosocomial pathogens in nine out of 10 tissue samples from which bacteria were cultured, and achieved species-level identification in four of these samples.
- Detected nosocomial pathogens in four tissue samples from which organisms were not cultured, including several samples indicated to harbor coinfection with one or more species.

Aim 2:

- Determined ideal sample quantity and storage medium prior to analysis via 2D-DIGE.
- Optimized sample preparation methods and run parameters for 2D-DIGE.
- Determined that a large quantity of protein spots are obtained following analysis of preliminary burn wound samples.

- Identified several protein spots in preliminary samples whose presence was differentially observed between different sample groups, supporting the feasibility of this method for the discovery of possible biomarker candidates.

REPORTABLE OUTCOMES

Data submitted in part as an abstract, accepted to the 5th National Bio-Threat Conference:
Be NA, Gardner S, McLoughlin K, Thissen JB, Slezak T, Jaing C. A comprehensive microbial detection array applied to biodefense and public safety. In: 5th National Bio-Threat Conference; Denver, Colorado; 2012 March 27-29.

CONCLUSION

Our studies thus far, using the LLMDA, have identified numerous wound-relevant pathogens in tissue samples from wound biopsies. We identified cases in which the array-detected organism matched the cultured organism, as well as situations where nosocomial pathogens were detected in samples from which no microorganisms were successfully isolated. The latter circumstance is highly relevant toward the purpose of this project, to identify wound pathogen profiles which cannot be effectively obtained through standard microbiological techniques. It is likely that many varieties of wounds contain organisms which would not ordinarily be cultured, particularly those species involved in biofilm formation or which exhibit drug resistance. In combination with host biomarker discovery, the process for which has been prepared and optimized, these profile data will provide highly valuable information toward novel wound characterization.

Continuing work will address a number of project points. The LLMDA v5 probe design has been completed, and the array and analysis will be ready for operation in 1-2 months. Select previously run tissue samples, as well as all new samples, will be processed using the new array. Work is currently being performed to incorporate fungal detection into the computation for the new array. Once this is complete, we will also run these analyses, as fungal colonization is a significant clinical concern in wound infections. Further, extraction protocols will be tested which are specifically designed for genomic DNA purification from tissue. At present, our extraction protocols are geared toward whole nucleic acid and RNA purification due to previous experience with viral detection. Optimizing for genomic DNA may yield improved detection of bacterial and fungal species. Once additional samples are processed using these updated protocols, results will be correlated with pending 2D-DIGE proteomics data to construct deliverable profiles of wounds which are successfully resolved, versus those hampered by complications.

In both the military and clinical fields, treatment of wound infections still relies heavily on conventional microbiology, which is not powered to reliably assess the full spectrum of factors involved in patient recovery. Our technical resources allow us to assess an abundantly comprehensive range of infectious organisms, constructing a truly complete pathogenic profile. Once combined with host biomarker data, we will acquire a much clearer picture of the complex host-pathogen interactions vital to the healing process. Such information is crucial to effective case management of service members and other patients undergoing wound treatment.

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APPENDIX A.

Full list of pathogens detected from wound samples using LLMDA.

Key:

Cultured, detected
Cultured, not detected
Nosocomial pathogen not cultured, but detected

Sample	NMRC Cultured	LLMDA Detected	Notes
JR342A	Acinetobacter baumanii	Acinetobacter baumannii ATCC 17978 pAB2 Acinetobacter sp. Plasmid pRAY Streptomyces roseosporus GR feline sarcoma virus Clostridium asparagiforme HERV K115	
IS711A	None	Croceibacter atlanticus Streptomyces roseosporus Streptomyces sp. SPB74 Tupaiid herpesvirus 1 Bovine herpesvirus 5 Endoriftia persephone	
IS711B	Stenotrophomonas maltophilia	Stenotrophomonas sp. Streptomyces sp. C Bovine herpesvirus 5 Escherichia coli B7A Clostridium asparagiforme HERV K115	
TN631A	None	Clostridium asparagiforme Tupaiid herpesvirus 1 Endoriftia persephone Streptomyces sp.	
TN631B	Enterococcus faecium	None	No probes on array specific for E. faecium
EB471A	Acinetobacter baumannii	Acinetobacter SUN plasmid pRAY Stenotrophomonas sp. Escherichia coli B7A Streptomyces roseosporus HERV K113	
ZB191B	Enterobacter cloacae Acinetobacter baumannii	Enterobacter cloacae plasmid pEC01 Escherichia coli plasmid pIGRW12 Escherichia coli B7A Clostridium asparagiforme Streptomyces roseosporus	Plasmid pIGRW12 encodes E. cloacae MobC homolog

		Coxiella burnetti HERV K115	
JC272A	Enterococcus faecium	Stenotrophomonas sp. Streptomyces albus Escherichia coli B7A Propionibacterium phage Faba bean necrotic yellows virus HERV K115	No probes on array specific for E. faecium
JR281A	Acinetobacter baumannii/calcoaceticus complex	Escherichia coli B7A Streptomyces albus Streptomyces hygroscopicus Streptomyces roseosporus HERV K115	
DM231B	None	Escherichia coli B7A Streptomyces albus Ostreococcus virus Thiocystis sp.	
RH491A	None	Escherichia coli B7A Coxiella burnetti Endoriftia persephone Tupaiid herpesvirus 1 Herpes simplex virus 2 HERV K115	
CG531A	None	Acinetobacter sp. Plasmid pRAY Klebsiella pneumoniae plasmid pKlebB Escherichia coli B7A Streptomyces roseosporus Tupaiid herpesvirus 1 HERV K115	
HP561A	None	Azotobacter vinelandii Ostreococcus virus Streptomyces roseosporus Faba bean necrotic yellows virus Coxiella burnetti HERV K115	
HP561B	None	Escherichia coli B7A Streptomyces roseosporus Coxiella burnetti Clostridium asparagiforme Tupaiid herpesvirus 1 HERV K113	
MW651A	None	Azotobacter vinelandii GR feline sarcoma virus Uncultured Chromatiaceae bacterium Streptomyces roseosporus	

		HERV K115	
MW651B	None	Escherichia coli B7A Streptomyces albus Pennisetum mosaic virus Uncultured Chromatiaceae bacterium	
JG351A	Acinetobacter sp. Bacillus cereus	Beggiatoa sp. Escherichia coli B7A Streptomyces albus Streptomyces hygroscopicus GR feline sarcoma virus HERV K115	
JG352A	Acinetobacter sp. Alloicoccus otidis	Escherichia coli B7A Magnetospirillum gryphiswaldense GR feline sarcoma virus HERV K115	No probes specific for A. otidis
LM671A	None	Azotobacter vinelandii Streptomyces albus Pennisetum mosaic virus C Escherichia coli B7A Propionibacterium phage Clostridium asparagiforme HERV K115	
LM671B	None	Escherichia coli B7A Pennisetum mosaic virus C Stretomyces albus GR feline sarcoma virus Magnetospirillum gryphiswaldense Tupaiid herpesvirus 1	
JT691A	None	Streptomyces lividans Clostridium asparagiforme Tupaiid herpesvirus 1 Herpes simplex virus 2 Endoriftia persephone	
JT691B	Staphyllococcus capitis	Acinetobacter SUN pRAY Escherichia coli B7A Escherichia sp. Pennisetum mosaic virus C Streptomyces albus HERV K115	No probes on array specific for S. capitis
JT691C	None	Stenotrophomonas sp. Streptomyces albus Streptomyces lividans Clostridium asparagiforme GR feline sarcoma virus Azotobacter vinelandii HERV K115	

KS702A	None	Shigella boydii Ralstonia pickettii plasmid Pennisetum mosaic virus C HERV K115 Steptomyces roseosporus	
KS702B	None	Shigella boydii Streptomyces lividans HERV K115	
IS712A	None	Klebsiella pneumoniae Escherichia coli B7A Micrococcus luteus GR feline sarcoma virus Streptomyces lividans HERV K115	
IS712B	None	Staphylococcus aureus plasmid pKH19 Acinetobacter sp. Plasmid pRAY Escherichia coli B7A Streptomyces roseosporus GR feline sarcoma virus Staphylococcus sciuri HERV K115	
ET662B	None	Streptomyces lividans Clostridium asparagiforme Tupaiid herpesvirus 1 Bovine herpesvirus 5 Herpes simplex virus 2 Endoriftia persephone	