PANTON-VALENTINE LEUKOCIDIN EXPRESSION IN COMMUNITY ASSOCIATED METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS ISOLATES FROM RURAL ALASKA

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ABSTRACT AND SPECIFIC AIMS

Incidence of infection by Methicillin resistant *Staphylococcus aureus* (MRSA) has historically been attributed to nosocomial sources\(^1\), but there is currently an increasing trend towards community acquired infection not associated with hospital settings. Community-associated MRSA (CA-MRSA) is most often associated with skin and soft tissue infections rather than invasive disease.\(^\text{ii,iii,iv,xiv}\) The majority of isolates causing skin infections in healthy people carry a gene for a toxin called Panton-Valentine leukocidin (PVL).\(^\text{v}\) Presence of the *PVL* gene in the majority of community-associated human skin infections has led to general acceptance for PVL as an important virulence factor. One recent study, however, has suggested that presence of the *PVL* gene has no effect on MRSA virulence \(^\text{vi}\) and so this has become a point of contention in the understanding of the pathogenesis of these infections. The objective of this experiment is to verify if PVL is indeed a virulence factor in community associated-MRSA (CA-MRSA) by studying isolates collected from a skin infection outbreak in rural communities in southwestern Alaska.

This experiment will detect and quantify the expression of PVL by studying messenger ribonucleic acid (mRNA). PVL mRNA levels will be measured using reverse transcriptase real-time polymerase chain reaction (RT-PCR). I am hoping to determine whether or not CA-MRSA strains that carry the *PVL* gene are actually producing the PVL toxin. Isolates that were found to cause infection (invasive strains) will be compared to strains that were not found causes infection (carrier strains). If findings show that the *PVL* gene expression is higher in invasive strain than in carrier strains, then it can be suggested that PVL plays a role in the pathogenicity of CA-MRSA skin infections. If *PVL* expression levels do not show a pattern between carrier and invasive isolates of the same outbreak, this might provide supporting evidence for an alternate mechanism of infection.
INTRODUCTION

*Staphylococcus aureus* are Gram-positive bacteria that exists as part of the normal flora on human skin. Approximately 20% of the human population has this bacteria dwelling in their nasal mucosa without ever getting sick. Staphylococcus aureus are opportunistically pathogenic, and infections due to this microbial organism have always been considered one of the most common causes of morbidity and mortality worldwide.

The development of antibiotic resistance has led to increasing pathogenicity of S. aureus. Methicillin-resistant *S. aureus* (MRSA) is one of the most prevalent forms of antimicrobial-resistant bacteria. Since its discovery in the 1960s, MRSA has been mostly confined to hospitals. Recently, however, it has been recognized as an emerging community-associated pathogen; healthy individuals who had no previous history of long term hospital care, antimicrobial therapy, or intravenous drug use have become infected with MRSA.

The majority of these strains of *S. aureus* causing illness in healthy young adults were found to carry the gene for Panton Valentine Leukocidin (PVL). One study found that 93% of the community-associated *S. aureus* causing skin infections such as furuncles, possessed the PVL genes. It has been proposed that MRSA strains that have the ability to cause severe skin and soft tissue infections, do so in a PVL-dependent manner. This suggestion is even more compelling when taking into account the fact that only a small percentage of *S. aureus* strains are known to carry the genes for PVL.

PVL is a bacterial toxin made up of two components called LukS-PV and LukF-PV. These components work synergistically to damage the membranes of macrophages and polymorphonuclear leukocytes, important components of the immune response. The high specificity against these defense cells increases the likelihood for PVL to play a role in MRSA skin infections in humans. It is therefore thought to be an important virulence factor for *S. aureus*. A paper was recently published in which direct testing of PVL-positive and PVL-negative strains in mice were found to exhibit comparable virulence, thereby disputing the role for PVL as a major virulence factor in CA-MRSA. This study was, however, limited to exclusive use of a mouse model, where as other studies have suggested that the mechanism of PVL is species-specific.

Indeed, many studies have been conducted that have associated CA-MRSA with the gene that encodes PVL. However, not many studies have verified the production of PVL nor quantify the levels of mRNA synthesized by CA-MRSA. Studying whether or not MRSA carries the *PVL* gene does not necessarily guarantee that gene expression occurs. This experiment will use reverse transcriptase real-time PCR to detect and measure mRNA levels for PVL. mRNA is genetic material transcribed from DNA that is ready for translation into a protein by ribosomes. Studying mRNA levels gives insight into the expression of the toxin, by not having to directly test for the protein itself.

The results from this study will determine if the PVL positive CA-MRSA specimens from the *S. aureus* skin infection outbreak in southwestern rural Alaska express PVL. The outbreak started in May of 1999 and was studied by investigators at the Arctic Investigations Program. It was found that MRSA skin infections had increased from 5 to 56 infections per month. Of the 168 infections from that outbreak, they discovered that 86% were MRSA.

Further investigation determined that 97% of those CA-MRSA isolates possessed the gene for PVL. The detection and quantification of mRNA levels of these isolates from rural Alaska will determine if a relationship exists in PVL expression. Researching PVL and its relationship to CA-MRSA allows for a better understanding of the pathogenicity of the
disease. By determining if PVL is a virulence factor, treatment methods could be developed to specifically target the expression of the PVL gene in strains of CA-MRSA.
METHODS

Reverse transcriptase real-time PCR was chosen for the determination and quantification of toxin expression. This method was chosen because previous studies have found that RNA analysis methods, particularly reverse transcription real-time PCR, are more sensitive than antibody-based detection methods. Furthermore, protein isolation techniques of PVL through column extraction in other studies have been used mostly for isolating the protein but not for quantification purposes.

Growing Bacteria
*S. aureus* strains are grown on blood agar plates at 37°C in 5% CO₂. The bacteria are cultured in broth. 100μl of bacteria are cultured in a 2-liter Erlenmeyer flask filled with 120 ml of yeast extract Casamino Acids-pyruvate medium at 37°C and shaken at 180 rpm.

mRNA extraction

From the bacteria culture, total RNA will be extracted using Ambion’s RiboPure-Bacteria RNA Isolation Kit. Protocols and recommendations of the manufacturer will be followed. To isolate and enrich mRNA from total RNA, Ambion’s MICROBExpress™ Bacterial mRNA Enrichment Kit will be used. Again, protocols and recommendations of the manufacturer will be followed. Quality and purity of total RNA will be determined from absorbance readings at the optical densities of 260 nm and 280 nm.

Reverse Transcription

Complementary DNA (cDNA) must be transcribed from mRNA in order to amplify mRNA using PCR methods. Stratagene’s AffinityScript Multiple Temperature cDNA synthesis Kit which includes oligonucleotides, primers, and reverse transcriptase enzyme will be used to transcribe the mRNA. Manufacturer’s protocols and recommendations will be followed.

Real-time PCR

The purpose of real-time PCR is to quantify the levels of PVL expression. In this step, mRNA that has been transcribed as cDNA will be amplified using the method of one enzyme/one tube TaqMan real-time PCR. The particular kit that will be used is Stratagene’s Quantitative PCR probe and Brilliant QPCR Master Mix. Target specific primers for cDNA reaction, will use the following sequence:

Luk-PV-1 5’-ATCATTAGGTAAAATGTCTGGAACATGATCCA-3’
Luk-PV-2 5’-GCATCAASTGTATTGGATAGCAAAAGC-3’

Quantitation

Quantitation of mRNA will be achieved using a method that compares crossing threshold (CT) values with one sample. Strains from American Type Culture College that are PVL positive and PVL negative will determine appropriate CT values for positive and negative results for PVL production. A molecular beacon probe that detects mRNA encoding ribosome will act be used as an exogenous control that will be co-amplified with samples to normalize the data.
**ANTICIPATED RESULTS**

In the case where the results show that *PVL* is not expressed is not detected, then this research will provide additional support for the hypothesis that *PVL* is not a significant virulence factor in CA-MRSA infections, and more attention should be given to other potential factors that may be more directly involved in the disease process. The prevalence of the *PVL* gene may have more to do with a gene encoding another important virulence factor transmitted to *S. aureus* via transduction.

If *PVL* expression is detected in all the CA-MRSA isolates, then the results will provide additional support for the hypothesis that *PVL* is an important virulence factor in CA-MRSA skin infections. The circumstantial evidence is too strong to suggest that the prevalence of *PVL* in CA-MRSA is completely coincidental or can be ignored, especially considering that one study had found that <5% of *S. aureus* possess the *PVL* gene. Moreover, the majority of CA-MRSA strains that cause skin infections possesses the gene to produce the toxin.

In addition to detecting the expression of *PVL*, if the quantified levels of mRNA expression occurs at consistent levels for tested isolates then some conclusions can be made about characteristic features of CA-MRSA strains that caused the skin infection outbreak in rural Alaska. On the other hand, if *PVL* expression tends to be inconsistent then no succinct conclusions can be made without further investigation. For further analysis, it would be of interest to compare mRNA production to the severity of the skin infection in the patient where the isolate was collected.

Although a recent study has effectively argued that *PVL*’s involvement directly in the pathogenesis is comparable to CA-MRSA strains that lacked the gene for *PVL*, perhaps the interpretation of how *PVL* is an important virulence factor in CA-MRSA must change. CA-MRSA strains that possess the *PVL* gene may be more opportunistic or fit than strains that lack the *PVL* gene. Furthermore, *PVL*-positive strains of MRSA may be a more competitive and effective pathogen than other common organisms that exist on the skin, given certain conditions. It is thought that the factors that facilitate the transmission of the infection include crowding, frequent contact, compromised skin, contaminated surfaces and shared items, poor hygiene, and antimicrobial use.

By understanding the role of *PVL* in CA-MRSA outbreaks, preventative measures can be taken to prevent future outbreaks. In communities where skin infections are found to be *PVL*-positive MRSA, concentrated efforts in educating the public concerning how to avoid the spread of the disease can be an important tool in preventing infection. In addition to preventative measures, treatment of CA-MRSA infections or development of vaccines that target *PVL* may someday be reasonable options.
PROJECT BUDGET

- Ambion RiboPure™ Bacteria Kit $292.00 each
  5 units = $1460.00
- Ambion Vortex Adapter $83.00 each
- Ambion RNAlater® 100 ml $85.00 each
  5 units = $425.00
- Qiagen QIAquick PCR Purification Kit $88.00
- StrataScript® One-Tube RT-PCR kit with Easy-A™ PCR Cloning enzyme
  $240.00
- Stratagene AffinityScript™ Multiple Temperature cDNA synthesis kit $240.00
  5 units = $1200.00
- Stratagene Brilliant® QPCR Master Mix $335.00
- Air travel to Chicago $700.00
- Hotel Stay for five nights in Chicago $350.00
- Shipping and handling costs $100.00

---------------------------- Total $4,981.00----------------------
PROJECT BUDGET JUSTIFICATION

- Ambion RiboPure™ Bacteria Kit is necessary to isolate RNA from the methicillin-resistant *Staphylococcus aureus*. There are 50 reactions for each kit. Five kits are requested because there are approximately 110 samples to test and a standard must be prepared from control bacteria. Furthermore, the kit will be required to do optimization runs that may require 100 reactions.
- The Ambion Vortex Adapter is required to run the samples using the Ambion RiboPure™ Bacteria Kit.
- Ambion RNAalater® 100 ml allows for the bacteria to be stored for a week before extraction while maintaining the integrity of the RNA. Five units are required, because approximately 100 ml will be used for every RNA isolation kit.
- StrataScript® One-Tube RT-PCR kit with Easy-A™ PCR Cloning enzyme is requested in order to amplify standard control’s cDNA for producing a standard curve.
- Qiagen QIAquick PCR Purification Kit is requested to purify control DNA amplified by PCR to make standard dilutions of known concentration.
- Strategene AffinityScript™ Multiple Temperature cDNA synthesis kit converts mRNA to cDNA, so that PCR methods can be used to amplify the sample. cDNA synthesis kit from Strategene is specifically requested, because the reagents have been specifically optimized for the instrument that will be used for this experiment. Five kits are requested, because there are 50 reactions for each kit and as previously stated over 200 reactions worth of reagent will be required for the samples, standards and optimization runs.
- Stratagene Brilliant® QPCR Master Mix allows for the quantification of mRNA using real time PCR. Stratagene’s kit has been requested, because the reagents have been specifically optimized for the instrument that will be used for this experiment.
- Air travel and hotel stay in Chicago are requested in order to visit the laboratory at the University of Chicago. The laboratory regularly handles and studies the PVL toxin. By observing the techniques and procedures used by the laboratory, I will have a better understanding of how to successfully carry out the work described in this proposal and what problems I may encounter. (Please refer to letter attached to appendix).
PROJECT TIMELINE

- Late July, 2007 – Complete background research
- August, 2007 – Visit University of Chicago’s Laboratory for a week
- August, 2007 – Purchase kits and reagents
- August-November, 2007 – Optimize procedure for sample testing
- December, 2007 – Test samples
- December-January, 2007 – Do data reduction and analysis of results
- January-March, 2007 – Write final report on results
- Mid-April, 2008 – Presentation at the Undergraduate Research Symposium
- May 31, 2008 – Expenditure deadline
- June 15, 2008 – Final written report deadline
PROJECT REFERENCES

1 Barrett FF, McGehee RF, Finland M. 1968. Methicillin-resistant *Staphylococcus aureus* at Boston City Hospital. N. Eng J Med. 96:526-32
17 Gorwitz RJ & Jernigan J. Epidemiology and Control of Methicillin-Resistant *Staphylococcus aureus* in the Community. Community MRSA Presentation Slides archived at Centers for Disease Control and Prevention’s National Laboratory Training Network.