Project Title: Genomic Sequencing of a Diversity of *Staphylococcus* Isolates to Determine the Genetic Basis for Drug Resistance in *S. aureus.*

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Executive Summary:

The prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) is of growing concern, particularly due to the more recent increased frequency of community acquired MRSA in the population. The prevalence of MRSA has placed additional urgency to understand the mechanisms of resistance to methicillin as well as second line antibiotics. To date, the sequencing of MRSA genomes has not yielded significant insights into the epidemiology of *S. aureus* MRSA infections. The scope of work described in this proposal takes a multi-pronged approach to enabling improved understanding of *S. aureus* drug resistance. First, we propose to sequence two methicillin sensitive (MSSA) isolates representing each of the 26 identified sequence types encompassing the full diversity of *S. aureus* species. This important framework will greatly enhance the power of comparative genomics for this important pathogen for the *S. aureus* research community. Second, we will sequence several isolates of the sibling species *S. epidermidis*, thought to be an important and prevalent reservoir of genes acquired by horizontal transmission in *S. aureus*. Third, we will sequence a diverse collection of thirty MRSA isolates collected in Army clinics over the past several years. These isolates will be directly compared to the appropriate reference genome sequence type to better understand the basis for the virulence properties associated with these MRSA. Last, we will sequence *S. aureus* genomes that have been subjected to antibiotic challenge in the laboratory to select for resistant organisms. The sequencing of these genomes will reveal specific point mutations that give rise to antibiotic

resistant phenotypes and will provide clinical and academic laboratories with improved diagnostic markers for the screening of existing strain collections and patient samples.

Project Descriptions and Background:

Project 1: Comprehensive Sequencing of MSSA to Provide a Comparative Genomic Framework for MRSA (Peterson, Archer, Kreiswirth). Emergence of broad-spectrum antibiotic resistant bacteria is a global phenomenon, which poses an imminent threat to established treatment protocols for infectious disease agents. The pathogenicity, limited treatment options, ease of transmission and widespread occurrence of MRSA (Klevens et al. 2007; CDC, 2007) challenge our ability to control and treat this infectious agent. One of the foremost experts in the field of antibiotic resistance frames the problem of antibiotic resistance research into three major priorities: 1.) A detailed understanding of the molecular/genetic basis, evolution and disemmination of resistance. 2.) Development of new compounds. 3.) Development of innovative strategies to extend the useful lifespan of existing antimicrobials (Wright, 2007). Our proposal seeks to address the first of these goals in an aggressive and comprehensive manner. Importantly, success in this goal will enable subsequent success in the second and third priorities. The development of drug resistance in response to antibiotic challenge occurs through a variety of mechanisms including: point mutation, gene acquisition, and expression modification.

Resistance to methicillin/oxacillin in *S. aureus* is based on the presence of the SCCmec transposable element (Kwon, et al., 2005). This element contains the *mec*A gene and when present gives rise to methicillin resistant *S. aureus* (MRSA). Likewise, the presence of *van*A leads to vancomycin resistant strains of *S. aureus* (VRSA). These genetic determinants of resistance account for most of the observed clinical, drug-resistant *S. aureus*. Additional antibiotic resistance phenotypes due to the presence of other resistance genes include resistance to macrolides, tetracycline, streptogramins, beta-lactams, clindamycin, sulfamethazole, trimethoprim and aminoglycosides.

Alternatively, resistance to other front-line antibiotics used to combat *S. aureus*, occurs through more subtle mechanisms involving SNPs and possible regulatory mutations. The genetic basis for such resistance is generally not understood, with the exception of mutations in RNA polymerase B subunit that confer resistance to rifampin. Similar mechanisms give rise to antibiotic resistant *S. aureus* for the following drugs: vancomycin (VISA) [vancomycin intermediate-resistance *S. aureus*], methicillin (non-*mec*A mediated), fluoroquinolones, daptomycin, linezolids and rifampin. The administration of antibiotics induces error prone repair systems that enhance mutation frequency and result in an increased incidence of mutations leading to antibiotic resistance (Cirz et al., 2005). This phenomenon, first described in *E. coli* has been shown to apply to *S. aureus* (Cirz, et al., 2007).

In trying to provide a sound comparative genomic framework for the scientific community, we have selected *S. aureus* isolates for genome sequencing to span the entirety of diversity within the species. **Figure 1** shows a phylogenetic tree of *S. aureus* (Cooper and Feil) and the distribution of Sequence

Types (STs) across the species. The 26 STs shown on the map represent the major Strain Types. We will identify at least two representative strains for each of the STs, for a total of 52 strains.

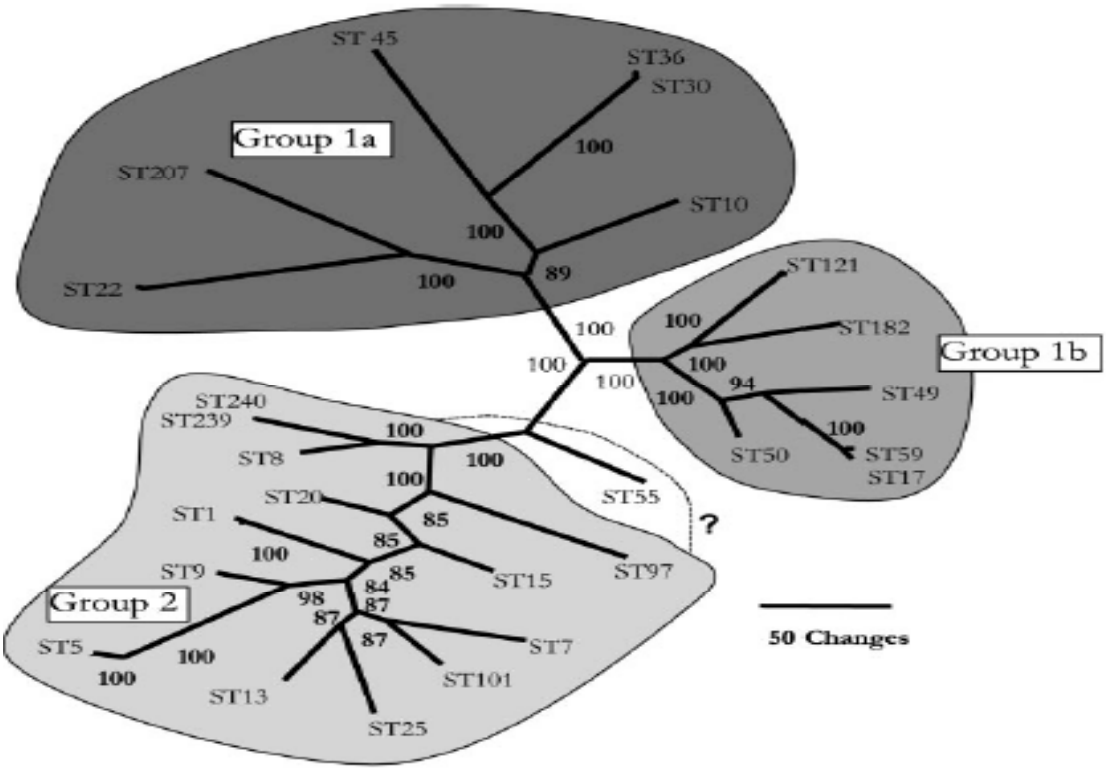

Figure 1. The *S. aureus* Clonal Complex based on MLST Sequence Data. Shown are the 26 STs comprising the *S. aureus* species.

Project 2: Sequencing Coagulase-Negative Staphylococci Genomes (Peterson, Archer).
Staphylococcus epidermidis is an important cause of infections of human indwelling foreign devices but it possibly has a more important role as a reservoir for genes that ultimately end up in *S. aureus*. Thus, we can learn a lot about what has happened and what may happen in *S. aureus* by sequencing the genomes of a collection of *S. epidermidis* strains. *S. epidermidis* is the major aerobic component of the skin microbiome and evolutionary data suggest that its genome is about four times more recombinogenic than that of *S. aureus*. In addition, *S. epidermidis* is carried in abundance on everyone's skin while *S. aureus* is present in only about 30% of individuals and usually only in the anterior nares. Thus, the role of *S. epidermidis* as a genetic reservoir is entirely plausible from an ecological standpoint.

The genetic reservoir is most obvious for antimicrobial resistance genes. Support for this comes from several observations. The timing of the occurrence of antimicrobial resistance in the two species as well as molecular evidence that many resistance genes were present in *S. epidermidis* before they were found in *S. aureus*. For example SCC*mec*, was found in 80% of nosocomial methicillin-resistant *S. epidermidis*

(MRSE) isolates in the 1970's at a time when fewer than 5% of *S. aureus* were methicillin resistance (MRSA). Furthermore, the SCC*mec* type that was not seen in *S. aureus* until the 1980's (SCCmec Type IV) was the most common type found in MRSE in the 1970's. Finally, an insertion event that inactivated a *mecA* regulator and became incorporated into *S. aureus* as part of Type I SCC*mec* had to have occurred in a coagulase-negative staphylococcus because the intact, inserting IS element is only found in coagulase-negative staphylococci, never in *S. aureus*. That insertion event only occurred once because the insertion junction is identical between that seen in the early isolates and in all of the evolved SCC*mec* elements (Types I and IV) that are seen currently. Additional antimicrobial resistance genes and mobile genetic elements such as conjugative and mobilizable plasmids appeared in *S. epidermidis* isolates years before they were described in *S. aureus*. The conjugative plasmids were able to transfer between *S. epidermidis* and *S. aureus* and were the type of plasmid on which the *vanA* gene from *E. faecalis* was first found.

Finally, there is a species of coagulase-negative staphylococcus, *Staphylococcus lugdunensis*, that has been found to have primary virulence as a specific cause of abscesses and wound infections. The virulence of this species has been confirmed in the *C. elegans* virulence model with many isolates killing worms as rapidly as *S. aureus*. There are no sequenced genomes of this species. Of equal importance will be the ability to find those genes that contribute to the appearance and evolution of antimicrobial resistance in staphylococci. If we systematically screen clinical isolates of coagulase-negative staphylococci for the emergence and evolution of resistance to current and future anti-infective agents, we can identify new genes or mutations associated with the phenotype by comparison with the large genome sequence bank generated by sequencing current and historic isolates. The new resistance genotype can then be monitored for its appearance in *S. aureus*. *S. epidermidis* will be the genomic canary in the coal mine, providing an early warning system for resistance before its appearance in the more virulent species.

We propose to sequence a collection of *S. epidermidis* held at VCU in Dr. Gordon Archer's laboratory that is selected for virulence (*S. epidermidis* native valve endocarditis [NVE; high virulence], *S. epidermidis* prosthetic valve endocarditis [PVE; low virulence], *S. lugdunensis* [high virulence]); sequence type (all *S. epidermidis* are typed by MLST and chosen to represent both common and unique types); time of isolation (*S. epidermidis* from the 1970's and 1995 – 2006); antimicrobial susceptibility (multiresistant and methicillin susceptible *S. epidermidis*; mostly susceptible *S. lugdunensis* with one methicillin-resistant); SCC*mec* type; and geography. In total we propose to sequence 19 strains of *S. epidermidis*. from NVE, 13 strains of *S. epidermidis* from PVE and finally 5 strains of *S. lugdunensis.*

Project 3: MRSA in the US Armed Forces (Peterson, Huang, McGann, Nikolich). MRSA has emerged as one of the most frequent pathogens responsible for nosocomial infections worldwide, particularly among intensive care units. A patient population of unique interest is wounded soldiers. Since the initiation of Operation Iraqi Freedom in 2003, the US Armed Forces have experienced a dramatic increase in the number of methicillin resistant *Staphylococcus aureus* (MRSA) infections and approximately half of all multiple drug resistant organisms isolated are MRSA. Because prognosis and

treatment are possibly strain dependent and because outbreaks can lead to increasing morbidity, characterization, tracking and treatment of MRSA is a very high priority.

Tracking MRSA requires epidemiological studies and molecular characterization. Multilocus Sequence Typing of non-military, clinical MRSA isolates revealed that most belong to one of five major clonal complexes (Karauzum et al, 2008). Though recent epidemiological studies suggest that these clonal groups have disseminated worldwide, the relevant studies have each been geographically limited, with reliable data available from only a small number of countries. In contrast, the majority of MRSA isolates from wounded US service members may be acquired during deployment to Iraq and represent a distinct reservoir of unique clonal groups (MEDCOM Pulse Field Gel Electrophoresis, or PFGE, data). PFGE characterization already encompasses over 150 isolates from the wounds and peripheral blood of wounded US service members and Iraqi civilians, allowing tracking of individual clonal outbreaks.

Patient management of MRSA infection also requires molecular characterization. Certain clonal groups of MRSA are significantly more virulent than others. Tracking virulent strains is important, but understanding their virulence and rationally designing strategies to frustrate it requires well characterized strains of varying virulence. Recently, hospitals in the United States have experienced the supplantation of less virulent strains of MRSA by a hypervirulent variant (USA300) in nosocomial infections (Como-Sabetti et al 2009). It would not be surprising if successive waves of infection in the military setting also had different degrees of virulence and required different treatment strategies. Therefore, emergent MRSA strains must be fully characterized and a framework of existing genomic data is required to make sense of data collected during an outbreak. While tracking can be accomplished by PFGE, rational assignment of therapies requires more thorough characterization.

A first step is collecting genomic data from military hospital MRSA. There are currently 22 MRSA genome sequences deposited at GenBank, but only 14 of these have been fully annotated and closed. The current genome data set for MRSA is >95% identical to some of the CDC USA collection (CDCUSA100 through CDCUSA1100, CDC, Atlanta, GA, personal communications). In contrast to this collection, PFGE analysis in the Nikolich laboratory at WRAIR has demonstrated that ~40% of our Iraqi isolates represent novel strains of MRSA that share only a distant relationship to any of the strains housed in the CDC collection. As a result, the existing data set sheds relatively little light on the strains that plague US soldiers and Iraqi civilians. To explore those strains will require additional genome sequences.

From the existing database, Baba and co-workers recently compared 12 genomes, uncovering significant inter-strain differences in gene content, particularly of virulence gene determinants, insertion sequences, transposons, prophage genes and other genomic islands (Baba et al 2008). These diverse genomes alone represent a significant asset to researchers in many areas. At the same time, sampling has not begun to saturate the discovery of strain variations. Because of phylogenetic distance between the sequenced strains and the military strains, gene discovery is unlikely to follow a smooth rarefaction curve. Instead, one would expect sequencing of the military-derived strains to be high-yield and to

reveal a large bounty of toxin, resistance, and virulence genes. New genomes will be made public to assist researchers in many important communities.

Thirty MRSA strains will be selected by scientists at WRAIR for genome sequencing based on PFGE, to avoid resequencing of clones. Strains that represent major PFGE clusters and isolates with unique PFGE patterns will be chosen for genome sequencing at the JCVI.

Project 4: Multiplex Microbial Genome Sequencing by 454 (Peterson, JCVI). A high prevalence of Single Nucleotide Polymorphisms (SNPs) and a wide diversity of genomic sequence variants within the *S. aureus* species make the identification of the genetic basis for drug resistance impossible by comparative genomic analysis. Identifying the precise SNPs contributing to antibiotic resistance is made difficult given they exist amidst a sea of other irrelevant SNPs that have become fixed through divergent evolution. By identifying SNPs and other genome sequence variations associated with antibiotic resistance in the laboratory, we will create a valuable resource for the scientific community. We propose to characterize antibiotic induction via the SOS-response pathway and the resulting genomic changes to develop a clear understanding of resistance-conferring mutations. The primary value of identifying these genomic alterations using the controlled *in vitro* selection approach is that we are able to compare any set of genome sequences to a sensitive parental genome sequence very precisely. In experiments performed to date, we typically see on the order of 1-20 base positions that differ in wild-type and resistant isolates making the validation of such candidates far more realizable.

We will generate several lineages representing low, moderate and high level resistance isolates *in vitro* for the antibiotics, vancomycin and daptomycin. These selections are performed serially since such low-level resistant parent strains give rise to moderate resistance progeny etc. This experimental design is beneficial since SNPs that are real are observed in specific genome sequences and also in all of their downstream progeny. Genomic DNAs will be isolated from individual colonies (~8/sequence run) and pooled together in equal concentrations for sequencing using the 454 platform. We have obtained 8-12-fold coverage of genomes using this pooling strategy that has allowed adequate depth of coverage and only rare instances of single read coverage across the genome. The resulting sequence reads will be compared to the reference genome sequence. The serial accumulation of mutations leading to high-level resistance to antibiotics is a hypothesis supported by growing number of studies, including our own (Figure 2).

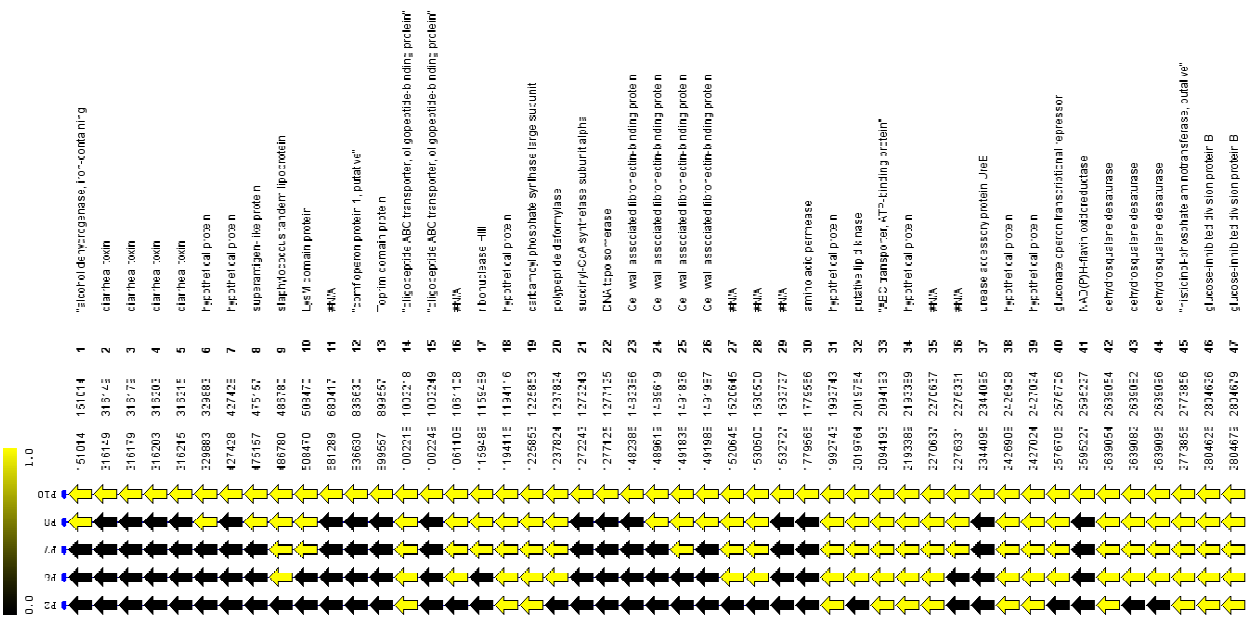

Figure 2: Stepwise selection of *S. aureus* isolates to increasing concentrations of an aminoglycoside drug. Black indicates wild-type bases and gold represents identified SNPs. Drug selection was performed at 0, 4, 8, 16 and 32 ug/mL of drug.

We have applied this approach in the model systems of *Mycobacterium tuberculosis*, *S. aureus* and *Pseudomonas aeruginosa* with excellent outcomes. In every instance we have been able to identify strong candidate SNPs to explain drug resistance. Another example from *S. aureus* involves a sensitive/resistant pair of isolates for the drug daptomycin. Only one SNP was identified as distinguishing these two genomes. That SNP mapped to a previously described gene related to resistance to vancomycin. In the data summary shown, we see the accumulation of SNPs in certain loci. These events are particularly informative since it is difficult to imagine how such mutations would accumulate at random if not under strong positive selection due to increasing drug concentration challenge. The strategy of comprehensive identification of antibiotic resistance conferring mutations *in vitro* represents a powerful approach. The establishment of specific mutations capable of conferring resistance to clinically relevant drugs is badly needed and currently represents a significant technology gap. Once defined, these SNPs can be translated into low-cost diagnostic assays for both clinical and academic research laboratories.

Strain Validation. All of our experiences to date using this genomic sequencing of *in vitro* generated drug resistant isolates have led to one or more strong candidate SNPs, likely to be directly responsible for the observed resistance. We will validate these SNPs by transferring mutant genes from drug resistant *S. aureus* strains into the sensitive parent strain. The mutant genes will be cloned into a *S. aureus* plasmid, pRN7240. This plasmid replicates autonomously in *S. aureus* under permissive conditions. However, by shifting the temperature to 37 ^{o}C, in the presence of selection for the plasmid marker, allows the selection of plasmid integration events into the chromosome. These events are either legitimate or illegitimate events. Only the legitimate events represent likely gene replacement events.

These colonies will be challenged with the antibiotic used for the original mutant gene selection to validate whether the presence of the mutant gene confers antibiotic resistance. The validation of the drug resistance SNPs or combination of SNPs defines the basis of one of two deliverables to the scientific community. The specific coordinates and base changes associated with *S. aureus* drug resistance will be made available to the scientific community via scientific publication, website presentation and annotation of genomic DNA sequences deposited into GenBank. The strains will also be deposited at NARSA.

Overlap with Current Sequencing Efforts. To the best of our knowledge, the Broad Institute is conducting a comprehensive evaluation of USA300 and USA400 strains in order to better understand the shift in MRSA that has been observed clinically and community acquired resistance. The extent of overlap is minimal with strains defined here. When appropriate, we will defer to existing sequence data from ST belonging to MRSA USA300 and USA400 isolates. The strains being sequenced from WRAIR appear to be unique variants based on the common USA typing scheme.

Scientific Community Depth of Interest. The proposal to sequence *S. aureus* and *S. epidermidis* isolates as a means of generating a functional framework through which causative genotypes can be related to antibiotic resistance was described to several members of the *S. aureus* community. The utility of creating a catalogue of mutations/strains that confer resistance as a means of enabling diagnostics was emphasized as the primary long-term goal of this effort. We received thoughtful replies from Robert Bonomo, Floyd Romesberg, Gordon Archer and Emmanuel Mongodin. There was universal enthusiasm for the approach described and great interest in obtaining a better set of diagnostic markers that could be used to evaluate the multitudes of strains held in their respective strain collections. In addition, the PFGRCs whitepaper efforts to sequence antibiotic resistant isolates of *S. aureus* in collaboration with Susan Boyle-Vavra and Robert Daum is a testament to the utility and interest held by those investigators. Some cautionary notes were also provided by David Alland who reminded us that some mutations may be difficult to identify *in vitro* as there selection may require other mutations that do not impact the MIC of the isolate, but potentiate the acquisition of resistance by second site mutation.

Availability of Strains for Proposed study. All *S. aureus* and *S. epidermidis* strains to be sequenced are available via Dr. Barry Kreiswirth and Dr. Gordon Archer's collections. Each of these isolates has been de-identified with respect to human subjects.

The Division of Bacterial and Rickettsial Diseases (DBRD) is uniquely positioned to conduct this research as we have conducted PFGE analysis of 150 MRSA isolates from wounded US soldiers and civilians in casualty care facilities throughout Iraq as part of a Department of Defense Global Emerging Infections System (DoD-GEIS) funded project. The DoD-GEIS project includes alternative genetic analysis including microarray approaches which can augment and enhance the value of genomic data. Genome sequencing is not funded by DoD-GEIS, but DBRD can select isolates, culture them, and produce the DNA for genome sequencing with current resources. To capture the diversity within the DBRD collection, we propose selecting up to three isolates from each PFGE cluster for sequencing, depending on the degree of pattern diversity within a cluster. Using this approach, and in consultation

with JCVI researchers, 30-40 strains will be selected for sequencing. Ultimately, this data will enhance the tracking and treatment of MRSA within the military health system.

Nature, Availability & Source of Reagents/Samples: WRAIR has a collection of over 150 MRSA isolates of which WRAIR will prepare purified genomic DNA from selected strains (30 strains, depending on agreed-to scope) based on technical specifications required and deliver to JCVI for sequencing.

Collaborator Role: At WRAIR, Xiao-zhe Huang and Mikeljon Nikolich will select strains for sequencing based on their PFGE database. Dr. Huang will extract gDNA from the selected strains for JCVI and work on data analysis. Dr. Patrick McGann of the WRAIR group will also work on the requested JCVI sequencing project on data analysis.
NIAID's Genomic Sequencing Center Reagent, Data & Software Release Policy:
http://www3.niaid.nih.gov/research/resources/mscs/data.htm
Accept; DNA samples to be stored at JCVI. JCVI sequence data to be deposited in GenBank.

Investigator Signature: ~/please accept as electronic signature/~

Investigator Name: Mikeljon P. Nikolich, Ph.D.

Scope of *S. aureus* DNA sequencing.

MSSA-2X26 sequence types- 56 genomes (UMDNJ, JCVI)
S. epidermidis- 37 genomes (VCU, JCVI)
MRSA- 40 genomes (WRAIR, JCVI)
In vitro selection antibiotic resistant strains- 60 genomes (JCVI)

193 Staphylococcal genomes:
454 runs= 25 runs, multiplexing of 8 genomes/run.

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