RESEARCH PLAN: SPECIFIC AIMS

The ongoing opioid and injection drug use crisis in the United States is driving an emerging epidemic of invasive *Staphylococcus aureus* infections among persons who inject drugs. Persons who inject drugs are estimated to be 16.3 times more likely to develop invasive *S. aureus* infections than their peers and twice as likely to be re-hospitalized with a recurrent *S. aureus* infection. Deaths from these invasive infections are also rising at an alarming rate, and deaths from injection drug use associated infective endocarditis are expected to result in over 7,260,000 years of potential life lost in the next 10 years.

Despite the overwhelming number of infections, little is known about their potential source and routes of transmission among persons who inject drugs. Our group recently published the first large scale study examining transmission dynamics of *S. aureus* bacteremia among persons who inject drugs and identified that 29% of cases could be linked to clonal transmission clusters within the injection drug use community. However it is unclear if these cases stem from 1) skin colonization of patients reflecting social interactions leading to expansion of colonizing strains, or if instead 2) the high number of transmission clusters reflects ongoing survival of *S. aureus* on drug use equipment which is shared among people who use drugs.

Our proposed Longer Life Foundation grant application is entitled "Pathogenesis of *S. aureus* bloodstream infections persons who inject drugs." We propose to recruit 50 patients hospitalized with *S. aureus* bacteremia related to injection drug use, and collect skin swabs, swabs of their drug preparation equipment in addition to their *S. aureus* bloodstream isolates. We will follow this with whole genome sequencing of all *S. aureus* strains to identify the source of individual patient infections (be this drug use equipment, skin colonization or none of the above). **We hypothesize** that *S. aureus* survives on drug use equipment and that drug use practices, including the sharing of drug preparation equipment, results in transmission of *S. aureus* strains and clonal expansion of *S. aureus* lineages among persons who inject drugs. Our specific aims are as follows:

Aim 1. Collect *S. aureus* bloodstream infection isolates, swabs to assess for colonization with *S. aureus,* and drug preparation equipment swabs from patients hospitalized with injection drug use associated blood-stream infections.

We will recruit 50 patients hospitalized with injection drug use associated bloodstream infections who are admitted to BJH. We will survey patients on their drug use practices and obtain culture swabs of the nares, axillae, and forearms of all participants to assess colonization with *S. aureus*. We will also collect *S. aureus* bloodstream isolates that are isolated in the microbiology lab from blood cultures. All participants will be provided with a swab kit for swabbing drug preparation equipment following discharge from the hospital.

Aim 2. Define transmission routes for *S. aureus* infections through epidemiologic and comparative genomic analysis of matched *S. aureus* strains.

We will perform whole genome sequencing (WGS) on all strains collected in Aim 1. We will use network analysis to identify the source of blood-stream infections, and risk factors for transmission of *S. aureus* within the injection drug use community.

BACKGROUND and SIGNFICANCE

Significance *1. Staphylococcus aureus* blood-stream infections are an emerging epidemic among persons who inject drugs. The syndemic of opioids, stimulants and other substances is driving an emerging epidemic of infectious diseases; chief among these maladies are invasive staphylococcal infections. People who inject drugs (PWID) are estimated to be 16.3 times more likely than peers to develop invasive staphylococcal infections, with one in every ten invasive staphylococcal infections in the US now related to IDU[1]. All regions of the United States have been affected in recent years with *S. aureus* infections due to injection drug use rising at an alarming rate[2]. Deaths from injection related infective endocarditis are estimated to result in over 7,260,000 years of potential life lost over the next 10 years[3]. Efforts against invasive staphylococcal infections in general are complicated by the existence of multiple disease subtypes, including central line-associated BSI (CLABSI), endocarditis, osteomyelitis, septic arthritis, and epidural abscess. Each disease manifestation necessitates preventative and therapeutic strategies tailored to characteristic epidemiology, pathobiology and host risk factors[4,5]. However, the epidemiology and transmission of IDU-associated *S. aureus* invasive infections is poorly defined, as few studies have investigated it as a separate disease entity.

Understanding injection drug use related bloodstream infection (IDU-BSI) as a unique entity is important because outcomes vary dramatically, with a lower mortality rate observed in patients with IDU-BSI compared with patients who have conventional *S. aureus* bloodstream infections (cBSI)[6]. By understanding how injection drug use associated infections occur, and if any differences in the *S. aureus* strains themselves are occurring between IDU-BSI and cBSI we may be able to develop effective strategies to both prevent recurrent infections and improve outcomes for both IDU-BSI and cBSI.

IDU-BSI has a unique pathogenesis and distinct clinical outcomes: Conventional *S. aureus* bloodstream infections (cBSI) require several key steps to develop an invasive staphylococcal infection, including breaches in skin barrier function or the presence of an underlying prosthetic device, which may serve as a nidus for biofilm formation for cBSI infections[7,8]. However, injection drug use related bloodstream infection (IDU-BSI) may skip several of these steps, potentially altering the evolutionary pressures on canonical virulence factors while also selecting for traits that allow for prolonged durations of bacteremia within the host. Clinical characteristics also support distinguishing IDU-BSI from cBSI. IDU-associated staphylococcal infections have been associated with prolonged duration of bacteremia and infectious sequelae such as endocarditis, possibly due to challenges faced by PWID in completing the standard-of-care, multi-week treatment regimens prescribed by guidelines[1]. Despite these poor prognostic indicators, emerging evidence suggests that, compared to cBSI, IDU-BSI exhibits comparable-to-lower mortality rates[9-14]. A mix of host and pathogen factors likely influence these discrepant observations. However, the paucity of studies comparing IDU-BSI to non-IDU staphylococcal BSI has resulted in

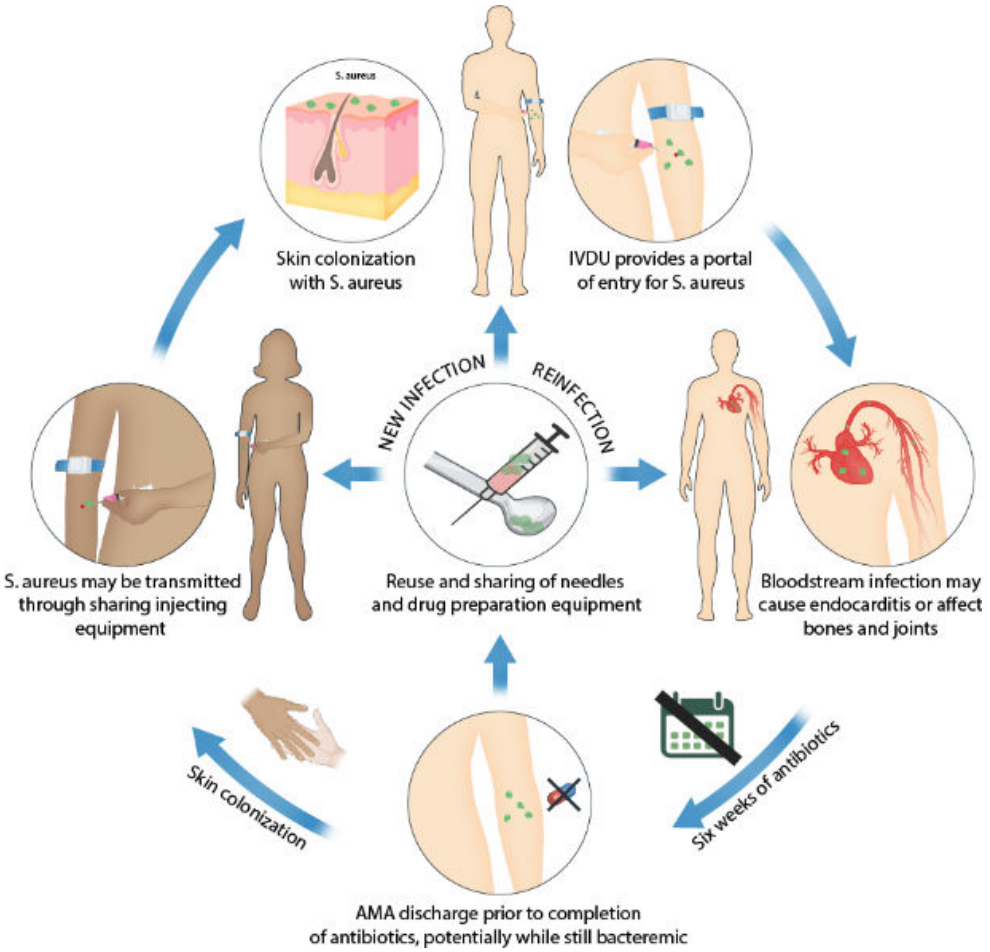

Figure 1. Proposed cycle of S. aureus infection and hypothesized transmission among PWID. AMA, against medical advice; IVDU intravenous drug use;

a critical knowledge gap in our understanding of unique factors governing IDU-associated infections.

Prior research has suggested clonal expansion of IDU-BSI isolates. Early reports on an IDU-associated outbreak in Detroit identified being unhoused and using shared injection equipment as risk factors[15], and phage typing and antibiotic susceptibility data identified common *S. aureus* lineages shared by many of these cases[16]. Subsequent reports further argued a link between IDU and transmission of methicillin-resistant *S. aureus* (MRSA) lineages among PWID[17-19]. However, these studies lacked comparator groups and/or employed low-resolution molecular methods and small cohort sizes, which limits the interpretation of their findings. Our recent publication in *Communications Medicine* used whole genome sequencing (WGS) to examine 254 *S. aureus* strains from a mix of IDU-BSI and cBSI cases in the St. Louis metropolitan area[6]. In this publication, we identified clonal expansion of IDU-BSI lineages which suggest clonal community transmission of *S. aureus* between PWID. However, to date there is no research demonstrating if IDU-BSI stems from expansion of colonization within the injection drug use community and within these social networks, or if it stems from shared use of contaminated drug preparation equipment. As community IDU-associated transmission represents an attractive target for BSI prevention (Figure 1), the route and impact of potential transmission and infection sources among PWID must be clearly established.

SCOPE OF WORK AND RELEVANCE TO LONGER LIFE FOUNDATION

S. aureus bacteremia carries a significant morbidity and mortality rate, with an estimated 30-day all-cause mortality rate of 20%, and high rates of treatment failures and recurrent infections[20]. This work will address the longer life foundations mission of studying factors that predict mortality and influence improvements in longevity, health and wellness, by contributing to a deeper understanding of where invasive *S. aureus* infections ultimately stem from among this population with a high risk for recurrent infections. We will do this by two important questions about *S. aureus* infections.

1) Does skin colonization or drug preparation equipment contamination act as a potential source of *S. aureus* bacteremia among PWID?
2) What is the role of colonization compared to contamination and sharing of drug preparation equipment in allowing for transmission of *S. aureus* infections within the injection drug use network?

To our knowledge, this study will represent the first molecular epidemiological analysis comparing both the potential source and transmission of *S. aureus* IDU-BSI and cBSI. Our case-control approach will allow us to identify characteristic features of IDU-BSI that are critical to consider in developing future investigations and interventions for this emerging disease. Application of WGS, and social network analysis can provide additional information that may facilitate rapid, directed public health action. This approach is powerful but is dependent on the availability of granular clinical data for the strains of interest. This is something our group is uniquely poised to provide with our dedicated multidisciplinary clinical program for the care of persons with IDU- related infections (Bridge to Health Program), which has high rates of patient participation in research studies.

Impact: Results from the work proposed in this application have the potential to directly impact patient care. Our research team hypothesizes that IDU-BSI represents a unique clinical entity distinct from cBSI with disparate clinical outcomes, transmission dynamics, and virulence characteristics. Furthermore, the confirmation of transmission networks within the PWID community, as well as the identification of the source of recurrent infections among PWID, would support the value of more targeted clinical trials which could evaluate if decolonization or harm reduction counseling can decrease the risk of *S. aureus* bacteremia, or recurrent infections in both IDU-BSI and cBSI participants alike. Previous decolonization trials have not evaluated recurrent *S. aureus* bacteremia infections in either patient population and have instead focused on populations at lower risk for recurrent infections. Methods to reduce the risk of recurrent *S. aureus* bacteremia infections are urgently needed to reduce morbidity and mortality related to *S. aureus* bacteremia. Conversely, the lack of any such transmission network would suggest *S. aureus* bacteremia among PWID is instead related to poor injection techniques and provide a focus for harm reduction education efforts.

RESEARCH DESIGN AND METHODS

Feasibility and approach: All preliminary research and proposed research plans have been approved by our local IRB (IRB #202110184). Biospecimen repository development has begun with strains from 24 patients collected to date, demonstrating the feasibility of this proposal.

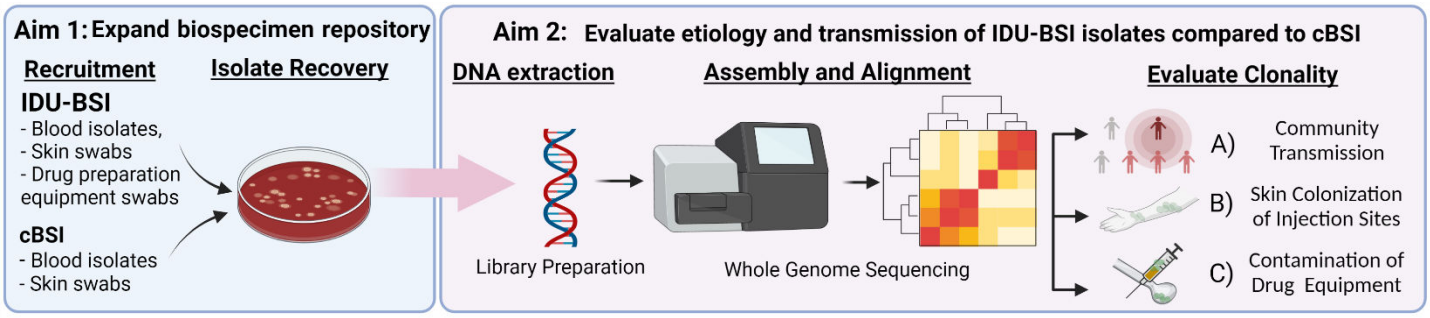

Figure 2. Proposed workflow for Aims 1 and 2. Participant recruitment and specimen collection will occur in Aim 1. Aim 2 will focus on distinguishing etiology and potential *S. aureus* routes of infection and transmission.

Aim 1. Expand an existing biospecimen repository with *S. aureus* BSI, skin, and drug preparation equipment isolates from IDU-BSI patients, and BSI and skin isolates from cBSI patients.

Preliminary data: IDU-BSI is characterized by clonal expansion of distinct lineages. My prior research has focused on identifying injection drug use related infections and defining the microbial epidemiology of these in Missouri[6,21-23]. As part of this research, I have recently published on the clinical characteristics and molecular epidemiology of *S. aureus* IDU-BSI and non-IDU cBSI which occurred at Barnes Jewish Hospital (BJH) from 2016 - 2019 [6]. This work identified an increasing proportion of IDU-BSI cases over that time-period (9.1% in 2016 to 13.4% in 2019) correlating with a surge in the opioid and stimulant crisis in the Midwest. We performed WGS on 254 *S. aureus* BSI isolates, and, using a case-control approach, identified over-representation of multiple distinct multi-locus sequence typing (MLST) among IDU-BSI when matched to cBSI on date and location of patient residence. In phylogenetic analyses, 29% of IDU-BSI strains grouped into unique clonal clusters which were geotemporally related[6]. These results demonstrate that pathogen community transmission distinctively spurs IDU-BSI. However, these findings are based on BSI isolates alone and cannot distinguish between expansion of skin colonizing strains, spread due to contaminated drug preparation equipment, or spread due to under-treated chronic infections among PWID (Figure 1). Collection of swabs and robust culturing of drug preparation equipment is of particular interest, as Kasper et al. previously found that 14% of cookers/filters used for injection of opioids were contaminated with *S. aureus*[24]. These *S. aureus* strains may have been injected during routine substance use. Differentiating between these potential sources of infection and transmission will require collection of additional blood isolates matched with sampling of skin and drug preparation equipment.

Research Strategy: We will recruit 100 IDU-BSI patients and 100 patients with no history of drug use (cBSI) who are admitted to BJH. *S. aureus* isolates from the blood will be collected from all participants. We will obtain culture swabs of the nares, axillae, and forearms of all participants. All participants will have blood collected for host immune response studies described in future directions. For IDU-BSI participants, skin swabs of drug preparation equipment will be collected.

Specimen processing. *S. aureus* isolates recovered from the bloodstream will be collected from the BJH clinical microbiology lab. Culture swab samples (BD ESwab) will be obtained at the enrollment visit to detect *S. aureus* colonization. Three swabs will be used for each participant to separately sample the anterior nares, axillae, and forearms. In addition, for IDU-BSI participants, any site(s) of injection drug use will be swabbed. Colonization swabs will be plated onto Mannitol Salt Agar (MSA) plates for selective identification of *S. aureus*. For sampling of drug preparation equipment, IDU-BSI participants will be provided with a culture collection kit (BD ESwab) along with return mailing envelope, and a flyer with sampling instructions for swabbing any drug preparation equipment they used prior to admission to the hospital. Swabs will be returned to the research team by participants using pre-addressed and pre-paid mailing envelopes. All culture swabs will be processed using Baird-Parker Agar contact plate and MSA plates for the isolation of *S. aureus* strains. For all isolates, we will perform broth enrichment followed by *S. aureus* identification confirmation by VITEK MS MALDI-TOF MS v2.3.3. according to established procedures[25,26]. All isolates will be stored in tryptic soy broth with glycerol at -80°C. To-date we have collected and processed isolates from 24 participants demonstrating feasibility of this proposal.

Clinical Data: Patients with an IDU-BSI will be instructed to complete a questionnaire on drug preparation practices and substance use history. The electronic medical record will be used to pull on patient demographics including zip code, comorbidities, *S. aureus* infection characteristics including duration of bacteremia, type of

clinical syndrome, number and type of metastatic sites of infection, vasopressor requirements, 1-year rates of recurrent *S. aureus* infections and mortality outcomes. We will use a combined endpoint of mortality and hospital readmission and will perform a competing-risk survival analysis. Covariates to be included in the analysis include measures of acute and chronic illness severity/burden.

Potential Pitfalls and alternative strategies**:** One potential pitfall, would be if rates of admissions for IDU-BSI decreased significantly over the study period, limiting enrollment of patients for collection of matched skin and drug use paraphernalia swabs as well as collection of further BSI isolates. Given the national increase in both overdose deaths and admissions for IDU-related infections this is considered unlikely. If patient enrollment is insufficient, we expansion collection efforts to other hospitals served by the BJH microbiology laboratory.

Aim 2. Define transmission mechanisms for *S. aureus* infections through epidemiologic and comparative genomic analysis of matched clinical data and *S. aureus* strains.

Hypothesis: Drug use practices (e.g., needle sharing) result in transmission of *S. aureus* strains and clonal expansion of *S. aureus* lineages among PWID.

Preliminary Data and rationale: The transmission of *S. aureus* within IDU networks had been previously proposed, but evidence was limited to studies with low-resolution molecular methods or small cohort sizes which lacked comparator groups, limiting interpretation of their findings[15-18]. We have recently published on the expansion of clonal *S. aureus* lineages within the injection drug use network[27]. This preliminary data was obtained from WGS of 254 index *S. aureus* isolates from BJH patients hospitalized between 1/2016 – 12/2019. We determined clonality of isolates according to pairwise SNP distance by analyzing the 1782 genes shared by all genomes in our cohort (i.e., the 'core genome'). By compiling the pairwise core genome SNP distances between index isolates from unrelated patients, we determined that 46 index isolates from unrelated patients are the result of from clonal transmission with core genomes that differ by <15 SNPs (Figure 3). In phylogenetic analyses, 45/154 and 1/91 contemporaneous IDU-BSI and non-IDU BSI staphylococcal isolates, respectively, grouped into multiple, unique clonal clusters, revealing that pathogen community transmission distinctively spurs IDU-BSI[27]. However, these findings do not distinguish between three potential hypotheses for transmission described in Figure 1:

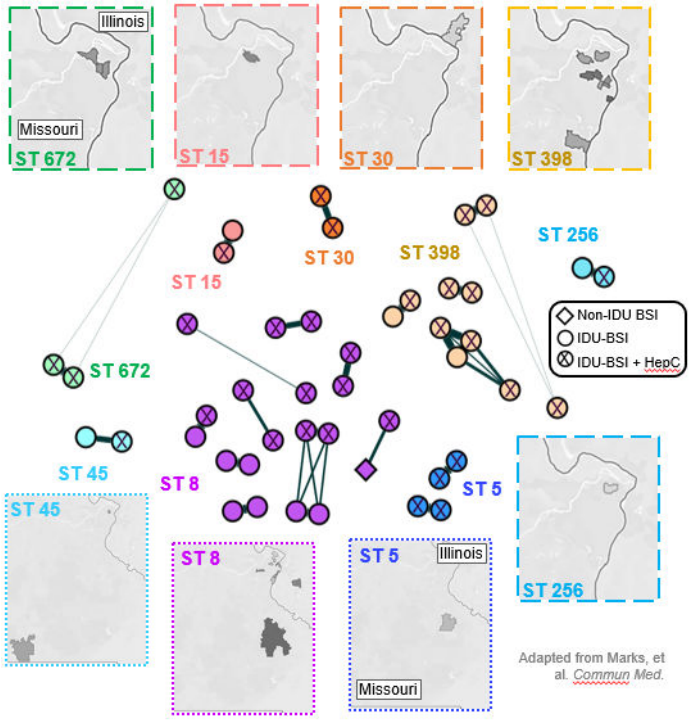

Figure 3. A) Networks of isolates with ≤15 core genome SNPs. Each node represents one of 46 index isolates, and are color coded by MLST, shaped according to whether associated with IDU-BSI (circle) or non-IDU BSI (diamond), and labeled according to HCV serostatus. Heat maps of BSI patients' zip code of residence for all isolates included in clonal clusters are displayed.

A) expansion of skin colonizing strains among PWID,
B) contamination and sharing of drug preparation
C) an independent pathway such as person-to-person transmission spurred by bacteremic patients with a delayed presentation to care.

As community IDU-associated transmission represents an attractive target for BSI prevention, the existence, impact, and potential routes of transmission of pathogenic strains among PWID must be clearly established. A WGS approach of our case-control strain library and strains collected in Aim 1 will be used, as it provides greater specificity compared to MLST which result in losses of phylogenic resolution in this genetically dynamic genus[27].

Aim 2a. Evaluate the presence of transmission clusters among *S. aureus* BSI isolates using social network analysis and whole genome sequencing.

Research Strategy: We will perform WGS on all additional *S. aureus* strains collected from Aim 1. Genomic DNA will be extracted from pure *S. aureus* cultures using Puregene Yeast/Bacteria Genome Purification Kit (Qiagen, Hilden, Germany). Sequencing will be performed on-site at the McDonnell Genome Institute (MDGI,

https://www.genome.wustl.edu/) using an Illumina platform. *In silico* sequence analysis will use the Washington University High Throughput Computing Facility (https://htcf.wustl.edu). Following de-multiplexing, reads will be processed with trimmomatic and deconseq to remove Illumina adapters and contaminating reads, respectively[28,29]. We will continue to assemble draft genomes using SPAdes[30]. Multilocus sequence typing will be performed with MLST-check[31], respectively. Prokka v1.13.7[32] will be run on scaffold files to identify open reading frames >500 bp in length. For phylogenetic analysis, the gff files produced by Prokka will be used to construct a core genome alignment with Roary v3.13[33]. The alignment will be used to generate a maximum likelihood tree with raxML v8.2.11[34], and visualized with iTOL[35]. For clonality analysis, Snp-sites v2.4.0[36] will be used to remove indels and create multiFASTA alignments containing the single nucleotide polymorphism (SNP) sites for each core genome. We will apply strict, empirically-derived clonality criteria[37], to identify transmission cluster isolates of fewer than 15 core genome SNP (cgSNP)).

A post-hoc social network analysis will be conducted on isolates meeting the criteria for transmission cluster isolates (<15 cgSNP). Each individual will be assigned to a community (a group of densely connected individuals) within the network, using the igraph algorithm in R (R version 3.2.0, R Foundation for Statistical Computing, Vienna, Austria). Clinical trait data will be merged with genomic data. Clinical trait data of cBSI or IDU origin, drug use habits (needle or equipment sharing, type of substance), and communicable diseases of HIV, HCV, or HBV, will be assessed as risk factors for risk of *S. aureus* transmission.

Aim 2b. Identify potential sources of *S. aureus* bacteremia among PWID.

Hypothesis: We hypothesize that cBSI participants will have higher degrees of relatedness between colonization and blood isolates compared to IDU-BSI participants. If transmission of IDU-BSI is mediated by contaminated drug preparation equipment as we hypothesize, we expect lower pairwise cgSNP distances between blood and drug preparation equipment isolates in IDU BSI compared to skin and blood isolates in IDU-BSI.

Research Strategy: We will directly compare clonality of *S. aureus* isolates between colonization sites and blood isolates, by comparing pairwise cgSNP distance between these skin and blood for IDU-BSI vs cBSI participants. For IDU BSI participants, we will further compare pairwise cgSNP distance between drug equipment and participant colonization and blood isolates.

Aim 2c. Determine the rate of strain replacement compared with reinfection among IDU-BSI and cBSI

Hypothesis: We hypothesize that cBSI are more likely to experience strain replacement as they interact with and acquire *S. aureus* strains from health-care facilities. Conversely, we hypothesize that IDU-BSI are more likely to experience re-infection with a related strain, due to an ongoing source in their environment; either re-use of contaminated drug equipment, or ongoing interaction with colonized or infected individuals in their community.

Research Strategy: We will collect isolates during any admissions patients experience for recurrent *S. aureus* bacteremia or other invasive *S. aureus* infections. We will compare the pairwise cgSNP distance between *S. aureus* bloodstream isolates collected during each infection episode to distinguish between strain replacement and re-infection with the initial strain.

Aim 2 (a-c) Potential Pitfalls and alternative strategies: Aim 2 is dependent in part on Aim 1, which is a consequence of the translational nature of this project. However, we have enrolled 24 patients to date and collected associated strains demonstrating feasibility so we do not foresee any significant difficulty in enrolling the remaining 76 patients.

FUTURE DIRECTIONS

A holistic analysis of the host response during IDU-BSI is outside of the scope of this proposal. However, recognizing that host immune response likely exerts a key role in differentiating outcomes among patients with IDU-BSI and cBSI we are collecting blood from all participants and performing isolation of peripheral blood mononuclear cells which will be saved and bio-banked for future research. These banked peripheral blood mononuclear cells will be used to evaluate markers of adaptive immunity by evaluating mass cytometry-based characterization of the CD4 T cell response to *S. aureus* using *in vitro* stimulation of PBMCs with heat killed *S. aureus* vs. tetanus toxoid, propidium monoazide, and ionomycin. To analyze the humoral immune response a multiplex-based evaluation of IgG response to *S. aureus* antigens will be performed. These studies will complement the proposed bacterial studies and provide an improved understanding of the host response during *Staphylococcus aureus* bacteremia, and how it may contribute to the lower mortality observed in patients with injection drug use associated bacteremia compared with patients who do not use drugs.

FUNDING PLAN AND TRANSITION OF PROPOSED RESEARCH TO HUMAN STUDIES/TREATMENT

FUNDING PLAN FOR PROPOSED RESEARCH AND EXTENSIONS STUDIES

Longer Life Foundation (LLF) support for this project in year 1 would allow us to complete specimen collection and sequencing of *S. aureus* isolates. If we are unable to secure LLF support for the proposed project, I plan to apply in the fall of 2019 for an Institute of Clinical and Translational Science – Clinical and Translational Research Funding Program (ICTS-CTRFP) award to support this project. With LLF or ICTS-CTRFP support, we will develop preliminary data to help identify etiology of recurrent *S. aureus* infections. In addition I am currently working on a resubmission of my K08 application which focuses on this same research question. Data developed in this LLF application would bolster a resubmitted application if I am not successful in the current funding cycle.

I then plan to use this data to support a summer 2024 R34 clinical trial planning grant application to support the development of either targeted harm reduction studies or decolonization studies to reduce the risk of recurrent *S. aureus* infections depending on the results of our basic science studies.

TRANSITION OF PROPOSED RESEARCH TO HUMAN STUDIES/TREATMENT WITH EXTENSION FUNDS

This proposal seeks to identify the etiology of recurrent *S. aureus* infections and whether these stem from colonization, or from contamination of drug preparation equipment. **There is a clear pathway to achieve clinical application as outlined in the following timeline**:

Year	Plan
1	Patient enrollment and isolate collection.
2	Completion of sequence assembly for all *S. aureus* strains and network anlaysis
3	Application for clinical trial planning grant to support clinical strategies of either harm reduction or decolonization to reduce rates of recurrent *S. aureus* bacteremia. Strategy to be based on the results from network analysis and colonization studies performed in year 2.

Impact: Identifying the source of *S. aureus* infections in persons who use drugs would allow us to develop clinical interventions specifically focused on mitigating both the initial infection and recurrent infections in this population. If *S. aureus* isolates on drug preparation equipment was found to match isolates identified from patient's bloodstream infections, then strategies should focus on harm reduction education including the use of sterile supplies, methods of sterilizing equipment and heating drug solutions to kill bacteria. If instead *S. aureus* infections were identified as being clonally related to skin isolates then interventions should focus on decolonization of persons who inject drugs with cost effective methods such as mupirocin, bleach baths, or cleaning injection sites with alcohol swabs. The data gained from this proposal would support an R21 application to test these hypotheses in a clinical trial.

The goals of our Development Research Award proposal support the Longer Life Foundation's stated mission specifically by providing basic science data to aid in developing interventions that prevent disease and promote longevity. The completion of this work will allow us to pursue additional funding to conduct clinical trials on interventions designed to directly impact the transmission route of invasive *S. aureus* infections in persons who inject drugs. In addition the studies proposed in this application will also expand our understanding of the pathogenesis of *S. aureus* bacteremia among persons who inject drugs. We hope that you will find our proposal interesting and worthy of your support.

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