Development of a Non-Human Primate Model for Staphylococcus aureus Nasal Carriage

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DEVELOPMENT OF A NON-HUMAN PRIMATE MODEL
FOR *STAPHYLOCOCCUS AUREUS* NASAL CARRIAGE

by

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ABSTRACT

Staphylococcus aureus nasal carriage (SANC) is largely asymptomatic, but presents a risk of autoinfection and dissemination to new immunocompromised hosts. SA disease states range from mild cutaneous infections to life-threatening bacteremia. Historically utilized rodent models do not naturally carry SA in the nose, are insufficient in longitudinal SANC experimentation, and lack immune factors that are vital in human clearance of SA. The nasal passages of non-human primates are similar anatomically and histologically, and reproductive mucosal studies have shown similar immune responses to pathogens and human-relevant microbial profiles. Seventeen captive pigtailed macaques (*Macaca nemestrina*) were found to naturally carry SA in the nose and pharynx, while topical mupirocin ointment effectively decolonized SA, similar to humans. Colonization was established with a human-relevant inoculum of 10^4 SA CFUs per nostril in four independent experiments, including with a human isolate (ST398). Autologous and non-autologous macaque strains were carried similarly in load and duration, each surviving over 40 days. Animals that cleared SA showed a rapid neutrophilic innate response, with up-regulation of IL-8, MCP-1, and IL-1β following inoculation, as observed in human hosts. Assessment of the nasal microbiome of pigtailed macaques and humans demonstrated similar relative abundance of the most prevalent genera: Staphylococcus, Corynebacterium, and Acinetobacter. Collectively, these multidimensional analyses provide evidence that the pigtailed macaque is a novel physiological model of human SANC that may be useful for testing novel SA decolonization strategies.

For my angel of a mother, Emily R. Lasseter.
Thank you for letting your chickadee find her wings.

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A quote displayed at my bench since the beginning of my stay in the Cole lab is as follows:
"Ability is what you're capable of doing. Motivation determines what you do. Attitude determines how well you do it." -Lou Holtz

…and a quote that I have lived by for many years:
"No one can make you feel inferior without your consent" -Eleanor Roosevelt

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CHAPTER 1: INTRODUCTION

Staphylococcus aureus Nasal Carriage and Population Dynamics

Staphylococcus aureus (SA) is a gram-positive, coagulase-positive, clustered cocci that is a part of normal skin flora of a wide range of mammalian hosts, including humans [1, 2]. SA transiently colonizes healthy human skin; however, a myriad of common SA disease states put pressure on our healthcare system, ranging from mild skin and soft tissue infections (SSTIs), like furuncles, boils, and impetigo, to life-threatening infections, like toxic shock and scalded skin syndromes, endocarditis, and bacteremia [3]. These are more likely to occur in those who have weakened immune systems, such as the elderly, newborns, those with preexisting immunocompromising conditions, and in close-contact populations with less than ideal sanitation, like prisons, military bases, nursing homes, hospitals, and schools [4, 5]. Since the widespread, and often unnecessary use of antibiotics began, SA has rapidly evolved resistance to the majority, like penicillin, methicillin, and vancomycin, as well as evolution of multi-drug resistant strains [6]. Antibiotic susceptible and resistant strains of SA are recognized as the most relevant nosocomial pathogen, easily spread through passive contact, often from healthcare worker to patient [7, 8]. This increases the duration of hospital stays, co-morbidities, and deaths [9, 10], driving up the cost of healthcare. *Staphylococcus aureus* alone accounts for approximately 25% of nosocomial infections, with a great majority of these post-operative infections [11], which places a heavy burden on the healthcare field, increased costs of medical treatment, and extended hospital stays.

The main ecological niche of SA in the human host is the moist mucosa of the anterior nares, surviving well in low-nutrient and increased salinity of nasal fluids. Introduction to the

nose is typically through fomite-hand-nose, due to the ability of SA to survive long-term on inanimate surfaces [12]. *Staphylococcus aureus* nasal carriage (SANC) is a largely asymptomatic state, even in many cases of methicillin-resistant strains, only creating a problem when the host becomes immunocompromised due to illness or defect. At any point in time, *Staphylococcus aureus* persistently colonizes the nasal passage of 25-30% of healthy adults on a world-wide scale [13, 14], and up to 80% intermittently [15]. Nasal carriage is associated with increased rates of carriage at extranasal sites, such as the pharynx, hands, perineum, etc. through passive contact of the hands with the face [8]. Also colonized are many companion animals like dogs [16], and large domesticated livestock such as cattle and pigs [2, 17], and humans can acquire the same strains through associating with animal carriers. SA nasal carriers have historically been categorized into one of three groups based on frequency of SA detection during longitudinal studies: persistent, intermittent, and non-carriers [18]. Categorization of carrier types is inconsistent between studies, especially from different countries, due to differences in protocols and in experimental limitations. Determined through longitudinal nostril swabs of human subjects, the nostrils of persistent carriers constantly contain detectable levels of SA when cultured, intermittent carriers will clear SA to undetectable levels and recover it, and rare non-carriers will not swab positive for SA at all [2]. It has been recently suggested that only two categories really exist, persistent and intermittent [2, 19], since it is expected that those that are classified as non-carriers are harboring SA below culturable detection limits. SA is ubiquitous, and humans are colonized on the skin at birth from the mother [20], so the likelihood for a person to never nasally carry SA is slim. Current knowledge has not fully explained why some healthy humans carry SA persistently, intermittently, or seemingly not at all, but what is known

is that SANC is influenced by highly complex interactions between SA genetics, host innate and adaptive immunity, environmental factors, and nasal microbiome biodiversity and interference.

Bacterial Determinants of *Staphylococcus aureus* Nasal Carriage

Staphylococcus aureus is very adept at persisting in the nostrils, regardless of its low iron and nutrient availability and high salt concentration, and despite the presence of antimicrobial peptides produced by nasal epithelial cells and commensal bacteria. The nasal passage is complex, with epithelium that transitions from the moist anterior portion characterized by keratinized stratified squamous cells, hair follicles and various glands, to pseudostratified columnar cells and a heavy mucous layer that is rapidly turned over by cilia and replaced [21]. Prior studies have determined that in human SA nasal carriers, the heaviest concentrations are localized in the distal portion of the anterior nares lacking cilia [21, 22]. Colonization of SA in the anterior nares relies upon very specific interactions between several SA surface proteins and protein components synthesized by keratinized cells [23]. Clumping factor B (ClfB) is a Staphylococcal surface adhesin that is mainly responsible for SA colonization, specifically binding to loricrin, a protein expressed by keratinocytes to protect the exterior-most barrier of the nasal mucosa [17]. Most disease states of SA involve these extracellular interactions with cells or biofilm formation, but evidence has accumulated that suggests that SA can evade the host immune system by inducing its own internalization by a zipper-type mechanism [24]. This internalization allows SA to persist and multiply, or induce host cell death through production of pore-forming toxins [24], releasing cellular contents and further increasing inflammation at the site of infection.

Staphylococcus aureus possesses 200-300 virulence factors, but few mechanisms are understood, especially when it comes to nasal carriage, as nasal carriage strains are rarely virulent. Once SA has adhered to the exterior surface of keratinized stratified squamous cells, it has to evade neutrophils recruited by the host's innate immunity as the first line of defense [25]. Staphylococcal protein A (SpA) is a multifunctional surface protein that binds Immunoglobulin G (IgG) across the bacterial surface in an incorrect orientation to prevent recognition as another method of evading neutrophil recognition, among other inflammatory functions [26]. Specific G protein-coupled receptors on the surface of neutrophils recognize concentration gradients of various small proteins that are released during SA opsonization or protein synthesis byproducts [27]. These concentration gradients in addition to pro-inflammatory proteins produced by host cells recruit resident neutrophils from surrounding tissues to the site of infection, however diapedesis can be inhibited at several points along the neutrophil's route by several virulence factors that block interactions between receptors on the neutrophil and endothelial cells [27]. Clearance of SA from the nasal mucosa relies on a rapid and robust neutrophilic response, and recent studies suggest that the nasal environment and host innate responses are primarily responsible for allowing nasal carriage rather than bacterial factors [28].

Host and Environmental Determinants of Staphylococcus aureus Nasal Carriage

Prior studies have suggested that nasal carriage is determined less by strain characteristics of the bacterium itself, but influenced heavily by host and environmental factors [28, 29]. SA is highly adept at rapidly adapting to fluctuations in the nasal environment due external influences such as change in climate, diet and health of the host, AMP concentration in the nasal fluid, oxygen availability, and more. SANC risk factors like HIV, Type II diabetes, cancer, prolonged

hospital stays, and many others alter the nasal mucosal environment and decrease the ability of the host's immune system to respond appropriately to SA, increasing the likelihood for SA to establish colonization in the nose and lengthen duration of carriage [5, 30].

Mucosal innate immunity is a group of processes involving receptors and effector proteins that within minutes to hours of a detected threat activates to protect the host from a potential infection. Keratinocytes on the epithelial surface of the anterior nares synthesize the keratinous protective layer in the skin and act as immunological surveyors, producing pro-inflammatory proteins such as cytokines and chemokines, and up-regulating their constitutive production of β-defensins when SA is sensed [31], representing the first line of defense against pathogen invasion [32]. Adherence to nasal epithelial cells allows detection of SA through pattern recognition receptors (PRRs) on the cell surface, principally Toll-like Receptor-2 (TLR-2) in conjunction with additional TLRs [33]. TLR-2 forms heterodimers with TLR-6, recognizing a conserved gram-positive bacterial cell wall component, lipoteichoic acid (LTA), triggering an intracellular cascade that results in mitogen-activated protein kinase and nuclear factor kappa-light-chain-enhancer of activated B cells (MAPK, NF-κB) mediated transcription of pro-inflammatory cytokines and co-stimulatory molecules to further activate innate and adaptive immunity [34, 35]. The release of these molecules leads to development of the characteristics of inflammation, such as increased fluid retention at the site of infection, promotion of blood-clotting, proliferation of host cells, and neutrophil influx from the bloodstream to tissue [36, 37].

An influencing factor of SA pathogenicity is its ability to invade host cells as a subversion tactic to avoid phagocytosis [36]. Once SA has gained endosomal entry into the host cell, it can take several paths, dependent upon toxin or enzymatic gene presence and may be phagosomally destroyed, proliferate within endosomes or in the cytoplasm following escape, or

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induce intrinsic or extrinsic apoptosis [24]. A cytoplasmic PRR, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is stimulated by a peptidoglycan monomer; muramyl dipeptide (MDP) to further activate NF-κB mediated transcription of cytokine and chemokines to promote neutrophil diapedesis from the bloodstream [38]. Extracellular and intracellular PRRs play a crucial role in the innate immune response, further demonstrated in TLR-2 and NOD2 gene knock-out mice, which cleared less SA and developed more serious skin lesions when compared to wild-type after subcutaneous injection with SA [39].

When stimulated with staphylococcal proteins, antimicrobial ability of nasal secretions varies among individuals from differences in regulation of gene expression of AMPs and pro-inflammatory proteins due to host genetics or environmental factors, however persistent human carriers generally have been found to have low antimicrobicidal activity in nasal fluid compared with non-carriers [21, 29, 40]. There are documented associations of polymorphisms in innate immune genes, defensins for example, resulting in a lowered constitutive threshold of well-functioning AMPs in the nasal epithelia, leaving them susceptible to various infections [29], including opportunistic SA strains. The immunocompromised are at especially high risk of infection, and with roughly a third of the human population asymptomatically colonized; there exists high risk of dissemination in healthcare facilities and within communities [41].

Microbiome Interference has an Antagonistic Effect on *Staphylococcus aureus* Colonization

Human host pro-inflammatory responses and SA genetics are not the only factors in the establishment of SA colonization, but the variety and abundance of non-SA microbiota has also been demonstrated to prevent or remove SA from the nares and is at least partially influenced by sex and host genetics [42]. The human nasal microbiome is under-explored, mainly due to low

comparative microbial population and few serious disease states associated with the upper respiratory system, while the majority of microbiome analyses have been focused on the gut due to serious illnesses such as Crohn's disease [43]. Limitations of microbial culturability and subsequent error in determining species-level identification of a total sample have proven difficult as nutrient-poor nasal passages contain folds less microbes as compared to the nutrient-rich gut.

There are many significant relationships that have been identified between SA and other nasal commensal species that have been determined to be an additional factor of carriage outside of the host immune response [44, 45]. Biological and niche interferences between SA and other coagulase-negative staphylococci, *Streptococcus pneumoniae*, and members of phylum Actinobacteria (*Corynebacterium, Propionibacterium*), have been analyzed and determined to have a role in SA carriage duration, however the complexities of the relationships have been limited by microbacterial culture techniques [44]. Interaction with *Corynebacterium* species in particular have been observed to cause a shift toward commensalism through inhibition of the accessory gene regulator (*agr*) system which is responsible for regulating quorum sensing, thus decreasing translation of virulence factors associated with that system [45]. Another common nasal inhabitant, *Staphylococcus lugdunensis,* is associated with inhibition of SA colonization through production of a novel antibiotic that has been commercialized since to decolonize SA and a wide range of other gram-positive microbes [46]. Diversity of species in the nares is also correlated with decreased ability for SA to establish persistent nasal carriage. Hospital patients persistently colonized with MRSA have been found to generally have less diversity of species in the nares than non-colonized controls, but the presence of *Streptococcus* and *Lactobacillus* species were found to have a protective effect [47], with *Streptococcus* also reported to compete

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with SA in neonatal units [47, 48]. This shift observed in SA from a pathogenic to a commensal phenotype may be explained by interference from other nasal commensals and would be important to further characterize in humans to understand the dynamics of nasal carriage and clearance in persistent, intermittent, and non-carriers. The pigtailed macaque nasal microbiome has not been characterized thus far, however benefit would be had in making comparisons in determining its use as a human-relevant model and in uncovering any relationships between SA and inhibitors that could lead to future development of novel antibiotics.

Rodent Models of *Staphylococcus aureus* Nasal Carriage Translate Poorly to the Human Condition

Historically, rodents have been extensively utilized in cancer, HIV, microbial, and many other areas if study because they are relatively economical, easily and inexpensively maintained, docile in handling, and can be genetically modified in ways to fit the study in question, allowing researchers great insight to mammalian biological mechanisms. Mice have been implemented in experimental models for SA induced cutaneous infections, sepsis, endocarditis, and others and have identified key SA virulence factors and mechanisms of adhesion [49]. These studies have greatly added to the scientific knowledge of SA, however failures in progression of therapies from rodents into human trials have led to the question as to whether mice are acceptable models for human responses to SA infection [49].

The nasal anatomical and histological differences between mice and humans are significant, since mice are obligate nose breathers [50]. The nares contain the same epithelial cell types, transitioning from stratified squamous in the anterior to pseudostratified columnar in the posterior, but in dissimilar ratios and with a five-fold increase in complex turbinate surface area in mice [51]. Rodents live with their faces about an inch off the ground, and the primary

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function of the murine nasal passage is olfaction, which is reflected in the differences in anatomy and cell-type striations as compared to humans where the function of the nasal passage is respiration [50, 52]. They are quadrupedal, and humans and non-human primates are mostly bipedal, therefore the head and nasal cavities evolved differently to reflect this, and rodents have a more pronounced backward tilt to the head that likely affects rate of sinus drainage. Another important factor, rodent nasal openings are devoid of hair follicles, a major niche occupied by SA and its competitors in the human nose, likely a main reason that SA is not naturally supported [22, 50]. When using mice for intranasal SA studies, longitudinal studies are impossible as their noses are too small to be swabbed. Monitoring for SA carriage leads to sacrifice of subjects at varying time points and surgical removal of noses for excision of nasal tissue and quantification of total nasal flora by plate counts [50, 52]. These aspects are less than ideal for tracking SA carriage rate and correlating individual innate responses with bacterial load over time as can be done with human studies.

Another undesirable aspect of utilizing mice as SANC models is that their host defense is dissimilar to humans in pronounced ways, therefore, rodents respond to the presence of SA differently and are resistant to nasal colonization [53]. In the human innate response, host nasal epithelial cells sense SA cell wall constituents and rapidly release chemokines to attract neutrophils to the site of infection [54]. Once neutrophils arrive and mature, they clear extracellular bacteria through release of oxidative bursts and phagosomal destruction of cells by the release of defensins, an important mechanism that is absent in mouse neutrophils [54]. Human blood contains approximately 70% circulating neutrophils, while mouse blood is more leukocyte-laden, with only about 20% neutrophils [52]. The innate response to nasal SA in rodents is primarily T helper cell mediated, with a corresponding up-regulation of IL-17 [55].

Further discrepancies include variances in B- and T-cell signaling pathways, cytokines and their receptors, expression of co-stimulatory molecules, PRRs, balance of leukocyte subsets, among many other inconsistencies [52].

Previous research has shown that most animals, including mice, implemented in studying SA pathogenesis are not natural nasal carriers of SA, a feature beneficial in mimicking human nasal carriage [56]. This contributes to establishing only transient SANC in the majority of study subjects for only a span of a few days, even when up to a billion SA CFUs are inoculated into each nostril and streptomycin antibiotic is provided in drinking water to reduce non-SA competition in the nose [1, 50, 57]. Human carriers readily support SA nasal inoculations over periods of weeks and months with no outside intervention to force nasal carriage [28, 58]. A new model is needed that also readily supports human physiologically relevant inoculations of SA without enormous inoculums and antibiotic knockdown of the non-SA commensal microbiome, as this is not true to the human condition.

The cotton rat (*Sigmodon hispidus*) model has been used for identifying bacterial determinants of SANC [10, 59], as its anatomy and histological striations are more comparable to human nasal passages than typical mouse models [60], however, in terms of the nasal microbiome, very different bacterial profiles are supported and longitudinal studies are still impossible in this model due to its small size. As discussed previously, the human nasal microbiome is dominated by Staphylococcal species and can be highly variable, while implementation of next-generation sequencing (NGS) showed that the cotton rat nasal microbiome is comprised of roughly half Campylobacter species, and the rest are potential gastrointestinal and oral pathogens, all not found in human noses [61]. These differences in supported species are likely due to the absence of hair follicles in the nostrils of the cotton rat,

which is an important ecological niche occupied by SA and its competitors in the human nose. The interactions between SA and the rodent nasal microbiome must therefore be dissimilar to those in the human nose and compounded with the immune response differences already discussed presents additional motivation for an improved model for human SANC.

Regardless of these points, implementation of rodent models for SANC studies is not ideal for a simple fact that longitudinal correlations between the presence or absence of SA with immune factors is impossible because rodent noses are too small to be swabbed, leading to the sacrifice of several animals every few days. Measuring the individual rodent's response to SA inoculation is impossible and knowing that the human response to SA is very personalized, a human-relevant model is needed that can be easily monitored over a long period of time to make similar distinctions as we can in humans to determine the mechanisms explaining why healthy people asymptomatically carry SA while others seemingly do not.

The Pigtailed Macaque *(Macaca nemestrina)* as a Potential Human-Relevant Model of SANC

Ideally, research on human SANC would be performed in the most accurate model, the human, however, in the US, there are strict ethical and procedural limitations to human experimentation. SANC is largely asymptomatic, so there is little pressure in recruitment of subjects from clinical settings since most nasal carriers are unaware that they are carrying, and retention of subjects is not always maintained due to the absence of symptoms and the presence of human free-will. For participation in SANC studies, subjects are required to apply antibiotic to their own nostrils to clear SA and complete the full regimen, and it is difficult to ensure completion, which can lead to skewed results. A study cannot inoculate human subjects with potentially virulent or antibiotic resistant strains, in fact, the only SA strains acceptable for

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human nasal inoculations are isolates from each subject's own nostril, and must be self-applied [28, 58]. This prevents the increase in knowledge of colonization mechanisms and the immune response to virulent strains, which would be beneficial in understanding progression of SA disease states. Human study subjects are typically required to longitudinally return to the laboratory for nasal swabs, nasal fluid collections, etc. and the free-will factor can cause procedural limitations when subjects fail to return. Removing the free-will element and eliminating as many factors as possible (environmental changes, diet, stress, illnesses) that have been seen in humans to impact SANC in creation of a reliable model that will be present for all follow-up visits will be useful in circumventing the ethical and procedural limitations in human research.

The pigtailed macaque (*Macaca nemestrina*) is a medium-sized monkey found in Indonesia, Malaysia, and southern Thailand, with a captive life-span of 27-35 years [62]. Females and males have very different temperaments; females are typically docile unless threatened and can be trained to easily cooperate in certain treatments or interactions, while males are aggressive and are maintained for breeding purposed in a research setting [63]. Sexual maturity for females is about 3 years of age, and their yearly hormone fluctuations and 28-day menstrual cycle resemble the human female condition, including the ability to breed year-round (unlike other monkey models) [64]. Hormonal fluctuations have been shown to influence the human female immune response [65]; therefore to use an animal model with very different hormonal cycles (such as rodents) is erroneous in translation to the human condition. Collaborators of this study at the Washington National Primate Research Center (Seattle, WA.) primarily house the pigtailed macaque due to their similarities to humans, and have implemented this model for years in research centered on reproductive and rectal mucosa and potential

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microbicides to treat or prevent sexually transmitted infections (STIs) like HIV and chlamydia [64-66]. Similarities in mechanisms and acquisition of human-relevant STI's, clinical presentation of infection, innate responses and microbiome in the reproductive mucosa, fetal development of the respiratory system, and more have been determined through increasing use of this model over the past 10 years [67-71].

There are many similarities between human and rodent systems, but it cannot be assumed that what is true in murine immunology is true in the human, as these differences lead to billions of dollars in wasted funding in failed clinical trials every year, driving up the cost of drug development. Rodent models have uncovered many SA adhesion and virulence factor mechanisms that were once a mystery, however colonization and clearance mechanisms of SANC are still not understood due to the differences in systems. Why do so many healthy humans persistently carry millions of SA cells in the nose without symptoms, while others do not seem to ever carry or only intermittently? Nasal carriers are not suffering from a neutrophilic disorder and have just as many circulating in the blood as non-carriers. The lack of a human-relevant model has essentially brought SANC studies to a stand-still, and development of a model in which many subjects can be easily and simultaneously decolonized and recolonized with a variety of SA strains would circumvent limitations in murine and human experimentation. The cost of non-human primate research is considerably higher than murine studies, however, further development of an improved model may yield results with improved translation to the human condition, prevent the loss of funds in the transition from pre-clinical to clinical human trials for new therapies, and balance out the cost of maintaining the model.

This study implements the pigtailed macaque (*Macaca nemestrina*) in a series of independent decolonization and recolonization SA inoculation experiments with a variety of SA

strains, including one human isolate. Development of this potential model for human SANC includes: observation of nasal and extranasal acquisition and carriage patterns following safe and appropriate nasal inoculations, innate responses to SA presence in the nasal mucosa, and comparisons between components of human and pigtailed macaque nasal microbiomes.

CHAPTER 2: LONGITUDINAL ASSESSMENT OF *STAPHYLOCOCCUS AUREUS* NASAL CARRIAGE DYNAMICS IN A HEALTHY COHORT OF PIGTAILED MACAQUES

Introduction

Staphylococcus aureus is a transient part of the normal flora of human skin, but preferentially occupies the moist nasal vestibule of approximately 30% on a world-wide scale. Longitudinal human studies have elucidated three accepted categories of nasal carriage: persistent (always SA-positive), intermittent (periodically clears SA from the nares), and non-carriers (always SA-negative by standard culture methods). Human SANC studies have also elucidated that nasal colonization is not solely reliant upon the bacterial strain but is also impacted by a myriad of other factors, such as host genetics, the speed and effectiveness of immune responses, and environmental factors. A study from the Netherlands, where fewer procedural limitations exist, discovered that intermittent and non-carriers share similar anti-staphylococcal antibody profiles, and that persistent carriers tend to preferentially select their autologous, or self-isolated strain from a mixed inoculum [2]. Procedural and ethical limitations in the US prevent human studies of this type since only autologous strains are allowed for inoculation studies, however following development of a human-relevant model this and other limitations could potentially be circumvented. The pigtailed macaque has been well-established as a suitable model for the female reproductive system and has many advantages over using common rodent and other small animal models. The tissue structure and immune responses in the reproductive mucosa of pigtailed macaques has proven similar to that of human females and support similar vaginal flora, and both have similar 28- to 30-day menstrual cycles and hormonal fluctuations [66, 72]. Implementation of the pigtailed macaque reproductive model has been

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useful in studying sexually transmitted infections like HIV, trichomoniasis, and chlamydia [64, 72], and several potential microbicide trials have resulted [66, 73].

An effective model for SANC would require the possibility for many subjects to be simultaneously decolonized of SA and recolonized again with a variety of strains to longitudinally follow carriage patterns and immune responses. This study over the course of 18-months validated six female pigtailed macaques as natural SA carriers, then follows their carriage patterns and their innate immune response to SA presence through a series of various autologous and non-autologous strain inoculations, one of which was a human nasal SA isolate.

Materials and Methods

Study Participants and Ethics.

This study was performed with pigtailed macaques (*Macaca nemestrina*) housed at the Washington National Primate Research Center (WaNPRC), in accordance with all institutional and federal guidelines for the care and use of laboratory animals. Housing and care conditions at the WaNPRC facilities meet AAALAC accreditation standards for non-human primates and follow NIH guidelines for ABSL-2 containment facilities. Ambient temperature in the animal rooms was between 22.2° to 25.6° C with relative humidity between 30-50%, and maintained on 12 hour light and dark cycles [74]. The study-specific protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Washington. On days where mupirocin ointment was applied only, animals received a light dose of 0.8-mg/kg ketamine, with antibiotic applied while still in their cages. For screening and sample collections (nasal and extranasal swabs, nasal fluid collection, and blood draws), animals were sedated with 1.2-mg/kg ketamine and moved from their cages to a procedure room. Six healthy, sexually

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mature female pigtailed macaques were selected from a colony of 17 healthy individuals whose nasal and pharyngeal SA carriage status was monitored for two months. Subjects were chosen based on ability to naturally acquire and carry SA at mid-high SA loads in the nasopharynx as well as availability for participation in the study for the approximate 18-month duration. There were no clinical adverse effects of nasal SA inoculation reported during the duration of the study. The study subjects were monitored for changes in temperature and body weight at each weekly sedation, with no changes in behavior or diet noted. Previous human nasal carriage studies performed by the Cole Laboratory that are referenced were previously described [58, 75] and were performed under guidelines of the Institutional Review Board of the University of Central Florida.

Animal Screening for SA Carriage and Sample Processing

To screen pigtailed macaques for SA carriage, the nostrils and pharynx were sampled using flocked Copan Diagnostics Eswab mini-tips and stored immediately in 1 ml of Eswab (liquid Amies) medium in transport tubes (Fisher Scientific #23600901). Samples were shipped overnight on ice packs to the Cole Laboratory and processed immediately upon arrival. Transport tubes were vortexed, and swabs were streaked onto BBL CHROMagar plates for colorimetric rapid validation of SA presence. Swab tips from each sample were reserved in a labeled cryotube and stored at -80°C for any further downstream use. Eswab media was diluted into Hank's Balanced Salt Solution (HBSS) at 1:10, 1:100, and an additional 1:1000 for pharynx and vaginal samples. These dilutions along with a straight (undiluted) sample were plated on Tryptic Soy Agar with 5% sheep's blood (TSA II, Fisher Scientific cat# B21261X) to quantify SA and non-SA colony-forming units (CFUs). Leftover Eswab sample was stored as

representative "early" glycerol stocks (500 µL sample + 500 µL 70% TSB/30% glycerol) at -80°C, and any leftover swab liquid (~100 µL) was cultured overnight (18 hrs/37°C/250rpm) in 2 mL TSB and stored at -80°C as a "late" glycerol stock (500 µL sample + 500 µL 70% TSB/30% glycerol). SA colonies were identified as phenotypic β-hemolytic colonies on TSA II and confirmed with Staphyloslide Latex Agglutination Test (Fisher Scientific cat# B4340953), which identifies presence of SA-specific protein A. Then the colony was plucked with a sterile pipette tip for overnight subculturing in TSB to reserve SA colony glycerol stocks for any future use. Following subject selection, this identical protocol was followed for all sample sites (left and right nostrils, pharynx, left and right axillae, left and right palms, and vagina) each week for ~18-months.

MLST and Spa Typing of SA Isolates

The nasal SA strains isolated from these animals were characterized using multilocus sequence typing (MLST), which exploits conserved genes that encode essential housekeeping enzymes within a species [76]. These are amplified with PCR, followed by analysis of the resulting DNA sequences of the MLST loci, then are assigned allele numbers, the combination of which identify the strain as a strain type (ST). USA300-0114 (NRS384) was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program, supported under NIAID, NIH Contract No. HHSN272200700055C and used as a positive control for PCR reactions. Human nasal isolates were collected as described previously [28], and processed identically to pigtailed macaque isolates described herein. SA colonies from each animal's nostril were plucked from TSA II plates used to enumerate SA and non-SA, and subcultured overnight in 5 mL TSB (18 hrs/37°C/250rpm). Genomic DNA was extracted from

these isolates using UltraClean Microbial DNA Isolation Kit (MO Bio Laboratories, West Carlsbad, California, USA) according to manufacturer's instructions, except instead of using 1.8 mL of sample, 800 µL were extracted unless bacterial pellet was insufficient. Resulting genomic DNA was placed in seven separate PCR conditions with the appropriate primers (**Table 1**) to amplify the seven MLST genes (arcC, aroE, glpF, gmk, pta, tpi, and yqiL) in adherence with the described protocol [77] with the remaining DNA numerically coded and reserved at -20° C until further use. PCR amplicon sizes were verified with negative (no DNA) and positive (USA300 amplicons) by gel electrophoresis, then sent to Eton Biosciences (Research Triangle Park, NC) for Sanger sequencing using the same primers as used in PCR. Mega7 was used to form a contiguous sequence using forward and reverse sequences. The *Staphylococcus aureus* MLST database (http://pubmlst.org/saureus/) was utilized to assign allele numbers and identify sequence types (STs) for each of the seven alleles and SA isolates respectively. SA genomic DNA was used in a similar manner for staphylococcal A protein (*spa)* typing according to previously described procedures [78]. Spa types were determined using Ridom StaphType software (http://www.spaserver.ridom.de/) after receiving *spa* sequence data from Eton Biosciences. Newly identified sequence types were synchronized with the RIDOM server, and were used throughout the study to validate nasal and extranasal SA detections as identical to the inoculated strain.

Comparative Genetic Analyses of Macaque and Human Nasal Isolates

Eight SA strains isolated from previous human study donors [75] were genetically compared to the three SA strains isolated from the six pigtailed macaques in this study. Multi-locus sequence typing (MLST) was performed initially for seven housekeeping genes (arc, aroE,

glpF, gmk, pta, tpi, and yqil) to characterize the isolates, and numerical identifications were obtained from the *Staphylococcus aureus* MLST database (http://pubmlst.org/saureus/). The seven MLST allele sequences were concatenated for the eight human and three macaque SA strains, aligned with each other, and analyzed using MrBayes v3.2 Bayesian analyses to form a phylogeny to view evolutionary relationships. Human SA isolate from donor (D) 579, which was previously identified as ST398 was found similar to macaque strains ST3814 and ST3815 while other human isolates were further diverged.

Verification of Methicillin-Susceptibility of Implemented SA Strains

It was important to ensure that the SA strains chosen for nasal inoculation studies in the chosen cohort of healthy pigtailed macaques were not methicillin resistant, as we wanted to avoid infecting the animals with difficult to eradicate virulent strains. The *mecA* gene encodes the penicillin binding protein, PBP2A, which is responsible for disrupting the lactam ring in several antibiotics and is present in methicillin resistant strains of SA. For validation of methicillin susceptibility, the PCR amplification of *mecA* was performed for each human and pigtailed macaque SA isolate using MR1 and MR2 primers (**Table 1**) under PCR conditions adopted from a previous study [79]. The absence of a corresponding 1.3 Kb band after gel electrophoresis was indicative of the lack of the *mecA* gene (as compared to USA300 MRSA strain as a positive control). These were further validated as *mecA* negative by plating corresponding bacterial samples on MRSASelect chromogenic agar plates (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. When plated, the USA300 MRSA strain control presented as pink colonies, indicative of methicillin resistance, while all other susceptible strains grew as white colonies.

<u>Verification of Mupirocin Susceptibility of Implemented SA Strains</u>

Macaque strain types 3813, 3814, 3815 and human strain D579/ST398 were verified susceptible to mupirocin antibiotic application through multiple liquid turbidity (growth) assays [80]. Snap frozen cultures were thawed on ice, vortexed vigorously, and 50 μL was proliferated in 5 mL TSB in a shaking incubator (2.5 hrs/37°C/250rpm) to log phase growth. Optical density (OD) of a 1-mL sample of each was measured at 550 nm to achieve a desired absorbance reading of 0.8, indicative of approximately 10^8 SA CFU/mL. Depending upon the OD measurement, approximately 100μL of each log phase SA culture was serially diluted in pre-warmed (37° C) Mueller-Hinton broth (MHB) (BD, cat# 275730) with 5% sucrose to achieve approximately 10^4 CFU in 90μL SA/well of a 96-well clear-bottom plate (TPP/ Midsci, cat# TP92096). Input CFUs were also verified through back calculating two serial dilutions of each strain on TSA II agar plates. A 1-mg/mL mupirocin (Sigma-Aldrich, cat# M7694) stock was prepared in dimethyl sulfoxide (DMSO), then diluted in HBSS to 80, 40, 20, and 10 μg/mL. These working solutions were diluted 1:10 in SA on a 96-well plate to achieve 1, 2, 4, and 8 μg/mL final concentrations of mupirocin. Controls for the assay included an aminoglycoside antibiotic, tobramycin, a known SA inhibitor; an antibiotic-free growth control with DMSO to ensure that the vehicle was not inhibitory to bacterial growth; and a negative control of MHB/5% sucrose to ensure sterile media. Once loaded, the plate was sealed with ThermoSeal A Film (Excel Scientific, Inc., cat# TSA-100) and placed into a pre-warmed (37°C) SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). Over the course of the 16-hour kinetic assay, turbidity as indication of bacterial growth at OD 550nm was measured every five minutes following brief 15-second agitations. OD 550nm measurements for all wells were exported and resulting growth curves were plotted against time (hourly time points) using GraphPad Prism 7

software. Following the 16-hour assay, 10 µL of each strain per treatment was plated on TSA II agar and β-hemolytic colonies were enumerated the next day following incubation. Each strain proliferated to approximately 10^8 CFU/90µL in antibiotic-free wells as back calculating CFU counts from serial dilutions on TSA II plates, and each strain was susceptible to mupirocin concentrations as low as 1 µg/mL.

Experimental Nasal SA Inoculation of Pigtailed Macaques.

Prior to experimental inoculations, the pigtailed macaques were decolonized of endogenous SA by the application of a pea-sized amount of 2% topical mupirocin ointment (Bactroban, GlaxoSmithKline, Philadelphia, PA) to each nostril followed by a brief massage of the exterior nostrils. This was performed under light anesthesia while remaining in their cage twice a day for five days to clear the body of SA. Following the fifth day of mupirocin application, the nasal microbiome was allowed to normalize over a four-week period prior to experimental SA inoculations. During this recovery period, the nostrils, pharynx, axillae, palms, and vagina were monitored for SA and to enumerate non-SA as an indication that the commensal population had replenished by inoculation day. During the fourth week of post-mupirocin commensal normalization, experimental strains were prepared and enumerated for inoculation as follows: SA colonies were proliferated in 50mL TSB (18 hrs/37°C/250rpm), after which 1-mL was subcultured in 50 mL TSB (2 hrs/37°C/250rpm). Liquid culture was added to a conical tube, centrifuged five minutes at 3000 xg, after which supernatant was removed and the bacterial pellet reserved. Pellets were resuspended in 35mL HBSS with 1% bovine serum albumin (BSA), spun down again, and the pellet resuspended in another 35mL HBSS/1% BSA. This suspension was well-mixed, transferred to 1.5mL microtubes and snap-frozen in liquid nitrogen for 3 hours,

then kept at -80°C until future application. The concentration of each SA strain (CFU/mL) was verified through dilution plating five aliquots of each snap culture on TSA II agar, enumerating SA colonies, and back calculating to determine the average original concentration of each strain. These back counts were used to design a dilution scheme per strain for the WaNPRC to deliver 10^3 or 10^4 CFUs in an inoculation of 20µL per nostril. Under ketamine anesthesia, a micropipette was used to administer the inoculum to both nostrils of the subjects, and the exterior of the nostrils were massaged for 30 seconds to distribute throughout the nasal passage. The first experimental inoculation implemented each individual pigtailed macaque's SA strain to determine an appropriate inoculum to successfully establish nasal and extranasal colonization with the least CFUs as possible in 20µL, beginning with 10^3 CFU/nostril. This inoculum was not successful in establishing carriage in most nostrils and extranasal sites as indicated through dilution plating methods, therefore two weeks following the first inoculation, a second inoculation was performed with 10^4 CFU SA in 20µL per nostril. This inoculum was successful in establishing carriage through day 70 post-inoculation, therefore 10^4 CFU per nostril was continued throughout the remaining three experimental inoculations of ST3813, ST3814, and human isolate ST398 applied to all subjects, with nasal carriage established in each inoculation for 63, 93, and 22 days respectively. Throughout each of the nasal microbiome normalization periods and four experimental SA inoculation studies, nasal and extranasal swabs, nasal fluids, and blood were collected at days 0 and 3 post-inoculation, then weekly until the end of follow-up.

All screening, turbidity assays, and carriage data were analyzed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Analyses of natural SA levels by body site (Fig. 3B) were log-transformed and compared using one-way ANOVA with Tukey's multiple comparisons test. Recovery of the non-SA nasal commensal population (**Fig. 4**) was determined by averaging the nostrils per individual, log-transforming them, and comparing the weekly results using paired t-tests. Autologous and non-autologous positive nostril SA CFU counts (**Fig. 7A**) were found not significantly different through unpaired t-test (two-tailed). Survival curves comparing autologous and non-autologous macaque inoculations (**Fig. 7B**) were found not significantly different using both the Log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test. Human and pigtailed macaque nasal SA isolates were genetically compared (**Fig. 8**) using Mr. Bayes v3.2 phylogenetic analyses.

Results

Pigtailed Macaques (*Macaca nemestrina*) are Natural Carriers of *S. aureus* (SA) Strains at Bacterial Loads Within Human Carriage Range

As previously mentioned, rodents are not natural carriers of SA in the nostrils and this presents issues in maintaining carrier status even when inoculated with a billion SA CFUs or more per nostril [1, 57, 59]. A natural nasal carrier of SA would be ideal for colonization studies; therefore, 17 pigtailed macaques were assessed for nasopharyngeal SA carriage by a two-month screening process. Each subject was swabbed in the nasal vestibule of both nostrils and the pharynx at two screening visits eight weeks apart. Thirteen animals were colonized in one or both nostrils at both visits, with all seventeen presenting positive for SA in either the nose

and/or pharynx in at least one screening (**Fig. 1**). Six healthy females of reproductive maturity were assigned to participate in a series of independent SA inoculation studies (red-boxed identifications in **Fig. 1**) based upon mid-to-high SA CFU carriage and availability to participate.
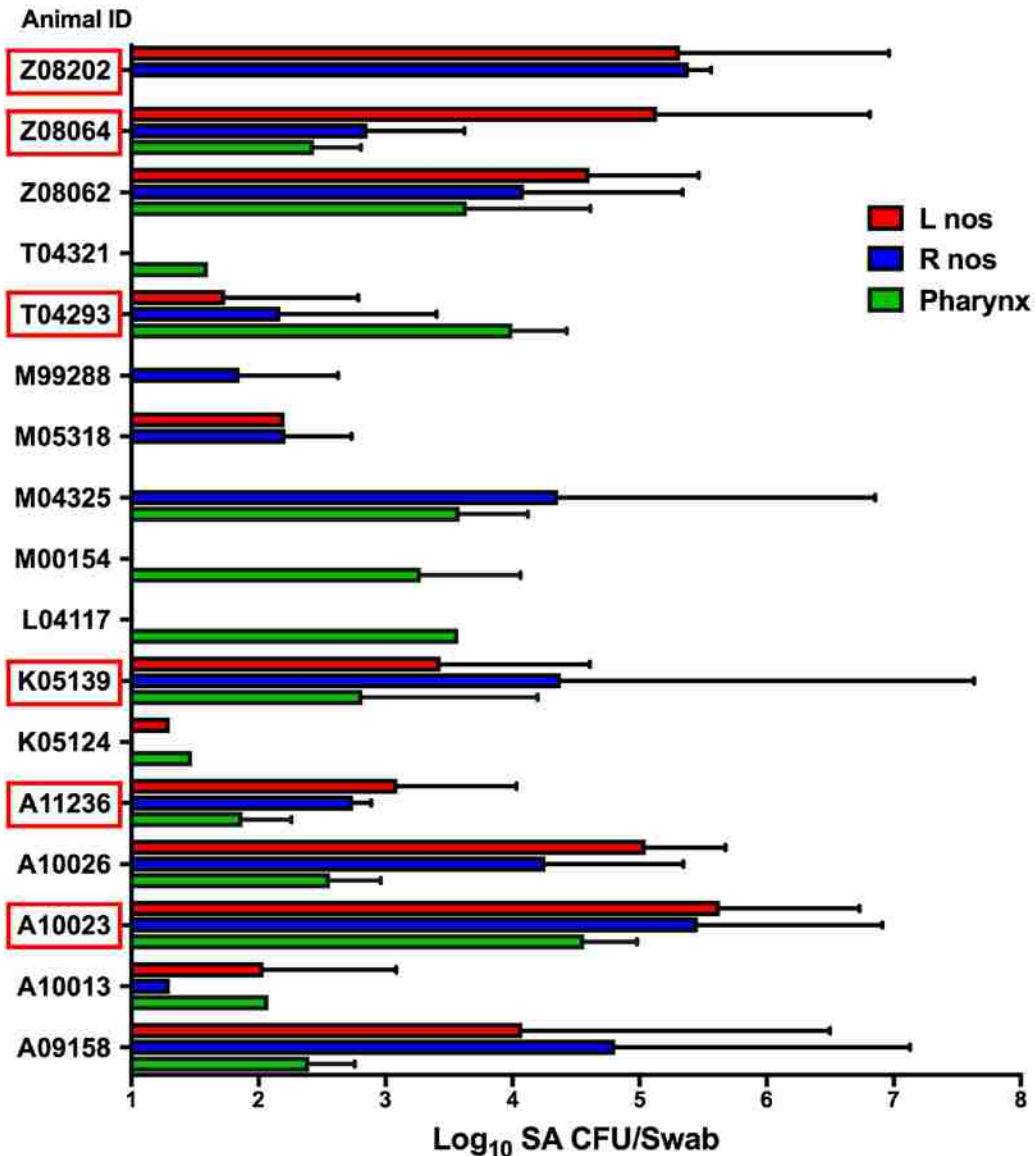
Figure 1: Pigtailed Macaques are Natural Carriers of SA in the Nose and Throat.

Seventeen pigtailed macaques at the WaNPRC were screened for SA in the nostrils and the pharynx twice, eight weeks apart and swabs were plated on TSA II to quantify SA. Boxed

animal identification codes (Animal ID) indicate the six animals chosen to participate in a series of inoculation studies. Error bars indicate mean±SD, the x-axis begins at the limit of SA detection as plated on TSA II (10 SA CFU/swab).

Once subjects were assigned to this study, animals were maintained in separate enclosures, and collected nasal isolates were genotyped by *spa* typing and multi-locus sequence typing (MLST) implementing primers described in **Table 1**. Characterization of the macaque strain genotypes was not only for the benefit of comparing the genetics to each other and to prior human SA isolates, but to also provide a method to longitudinally validate that any SA colonies detected nasally and at extranasal sites over the course of each individual experimental inoculation were genetically indistinct from the SA strain placed into the nostrils.

Table 1: Primers Implemented in this Study

Primers:	Primer Name	abbr	Forward (5' - 3')	Reverse (5' - 3')
SA MLST:	arcC	A	TTGATTCACCAGCGCGTATTGTC	AGGTATCTGCTTCAATCAGCG
	aroE	B	ATCGGAAATCCTATTTCACATTC	GGTGTTGTATTAATAACGATATC
	gLpF	C	CTAGGAACTGCAATCTTAATCC	TGGTAAAATCGCATGTCCAATTC
	gmk	D	ATCGTTTTATCGGGACCATC	TCATTAACTACAACGTAATCGTA
	pta	E	GTTAAAATCGTATTACCTGAAGG	GACCCTTTTGTTGAAAAGCTTAA
	tpi	F	TCGTTCATTCTGAACGTCGTGAA	TTTGCACCTTCTAACAATTGTAC
	yqiL	G	CAGCATACAGGACACCTATTGGC	CGTTGAGGAATCGATACTGGAAC
***spa* typing:**	1095F-1517R	spa	AGACGATCCTTCGGTGAGC	GCTTTTGCAATGTCATTTACTG
***MecA* (-/+):**	MR1/MR2 (F/R)	*mecA*	TAGAAATGACTGAACGTCCG	TTGCGATCAAATGTTACCGTAG
16S:	27F-1492R		AGAGTTTGATCMTGGCTCAG	GGTTACCTTGTTACGACTT
	341F-785R		CCTACGGGNGGCWGCAG	GACTACHVGGGTATCTAATCC

Sequence analyses showed that animals A10023, K05139, T04293, and Z08064 carried an indistinguishable sequence type (ST) 3813; A11236 carried ST3814; and Z08202 carried ST3815, each characterized as new SA strains (**Table 2**).

Table 2: Human and Macaque *Staphylococcus aureus* Isolate Characterizations

SA Strain ID	Origin	MLST	SpA Type	*mecA* (+/-)	mupR (+/-)
ST3813	Pigtailed macaque	3813	t15866	-	-
ST3814	Pigtailed macaque	3814	t15867	-	-
ST3815	Pigtailed macaque	3815	t15868	-	-
*USA300	Human wound	8	t008	+	-
D528	Human nose	8	t008	-	-
D547	Human nose	5	t688	-	-
D20	Human nose	30	t12255	-	-
D579	Human nose	398	t571	-	-
D502	Human nose	105	t056	-	-
D812	Human nose	716	t008	-	-
D566	Human nose	30	t037	-	-

SA: *Staphylococcus aureus*; MLST: multi-locus sequence type; SpA Type: Staphylococcal protein A gene Ridom type; *mecA*: *mecA* gene presence (+) or absence (-); mupR: mupirocin resistance presence (+) or absence (-). *USA300 strain isolated from wound, acquired from NARSA program. *USA300-0114 (NRS384) was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program: supported under NIAID, NIH contract No. HHSN272200700055D.

SA Strains Implemented in this Study are Mupirocin-susceptible

Each strain isolated from the pigtailed macaques lacked the *mecA* gene encoding methicillin resistance; however, it was desired to implement a topical antibiotic other than methicillin to prevent the rapid acquisition of methicillin resistance in the pigtailed macaque colony once the study ended. All three macaque SA isolates and the single human SA implemented in this study were validated as mupirocin sensitive with liquid turbidity assays prior

to use in the animals. This study implements 2% mupirocin antibiotic ointment, applied twice-a-day for five days to each nostril of the pigtailed macaques, which is the same concentration and protocol used to eradicate nasal SA prior to surgery and clear skin infections in humans. Upon challenging 10^4 CFUs of each strain with a low-level range of mupirocin concentrations, from 1-8 μg/mL, SA was inhibited with as low as 1 μg/mL as compared to the antibiotic-free growth curve (**Fig. 2A**). Each curve is the result of averaging hourly OD (550 nm) measurements (as indication of growth) of the three macaque SA isolates and the one human strain over the course of the 18-hour assay. For each strain per treatment, 10 μL from post-assay wells were plated on TSA II/5% sheep's blood and any resulting SA colonies were enumerated and compared to input and antibiotic-free SA CFU counts (**Fig. 2B**). Antibiotic-free/DMSO (vehicle) treatments of all four SA isolates proliferated to 10^8 CFUs from an approximate input of 10^4 CFUs (error bars indicate mean±SEM). Mupirocin concentrations of 1-8 μg/mL were inhibitory for all strains; post-assay SA colony growth from the 8 μg/mL treatments occurred only twice and only with macaque isolates ST3814. Post-assay growth for the 1 μg/mL occurred often and with all tested strains, suggesting that these low concentrations used in turbidity assays are bacteriostatic rather than bactericidal. *In vivo* application of mupirocin ointment delivers concentrations estimated between 5000-10,000 μg in a pea-sized amount (estimated as a fourth to a half of a gram) of 2% mupirocin ointment, twice-a-day, for five days and not only in one application as in the *in vitro* turbidity assay.
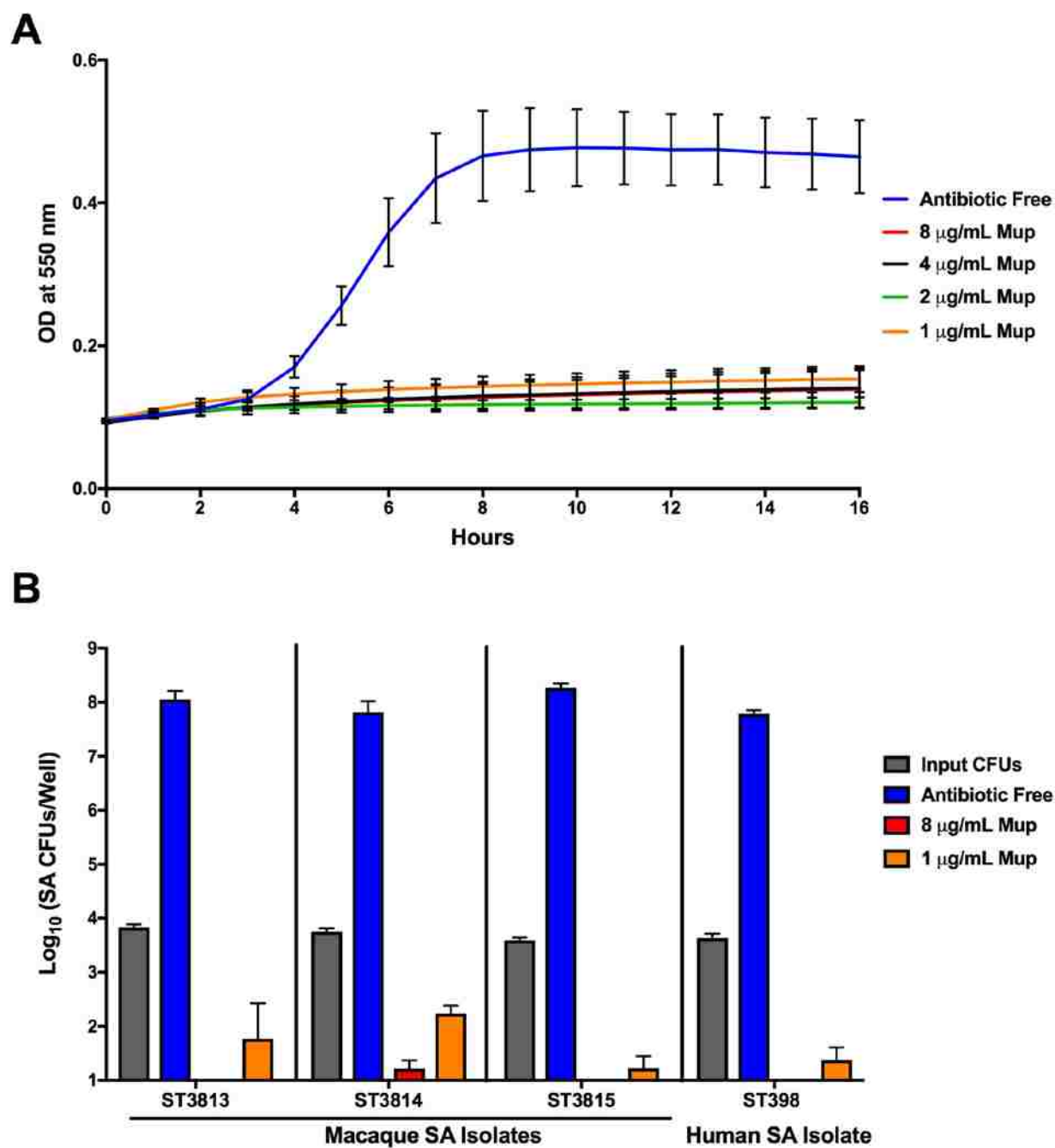
Figure 2: Nasal SA Isolates Implemented in Experimental Inoculations were Susceptible to Mupirocin Application

A) Liquid turbidity assays with low-level range of mupirocin concentrations (1-8 µg/mL) were applied to four SA isolates (three macaque, one human). Growth curves (OD plotted against time) were generated for each treatment versus an antibiotic-free growth curve (blue) for comparison. B) One well per each strain was plated on TSA II per treatment (including

30

antibiotic-free) following the 16-hour assay. Resulting SA colonies were counted and compared to input CFUs (indicated through pre-assay plate counts). n=15 (5 experiments, 3 replicates per strain), error bars indicate mean ± SEM.

The Nasal Vestibule is the Primary Reservoir of SA in the Pigtailed Macaque

Screening SA CFU counts prior to any manipulation revealed that pigtailed macaques naturally support SA in the nose and pharynx within range of human nasal carriers. To determine if the nose is also the reservoir for SA in the macaque, animals were swabbed in the left and right nostrils, pharynx, left and right axillae, left and right palms, and vagina to enumerate the comparative SA loads of these sites that are observed in humans to have increased SA during heavy nasal carriage. Immediately following, application of 2% topical mupirocin ointment to the nostrils was continued twice-a-day for five days, then these same sites were followed for four weeks. **Figure 3A** shows the average SA load at eight swab sites at "Pre-Mupirocin", and Weeks 1-4 "Post-Mupirocin" for 6 macaques during four independent experimental inoculations. Pre-mupirocin counts show that the nostrils contained significantly higher SA loads compared to all other extranasal sites, and the pharynx contained the most SA out of the six extranasal sites but less than the nostrils. Mupirocin ointment was only applied to the nasal vestibule, but by week one post-mupirocin, all sites were cleared. Out of 24 total weekly swabs per site over the post-mupirocin periods, ≥21 samples were negative for SA once the nostrils were cleared.
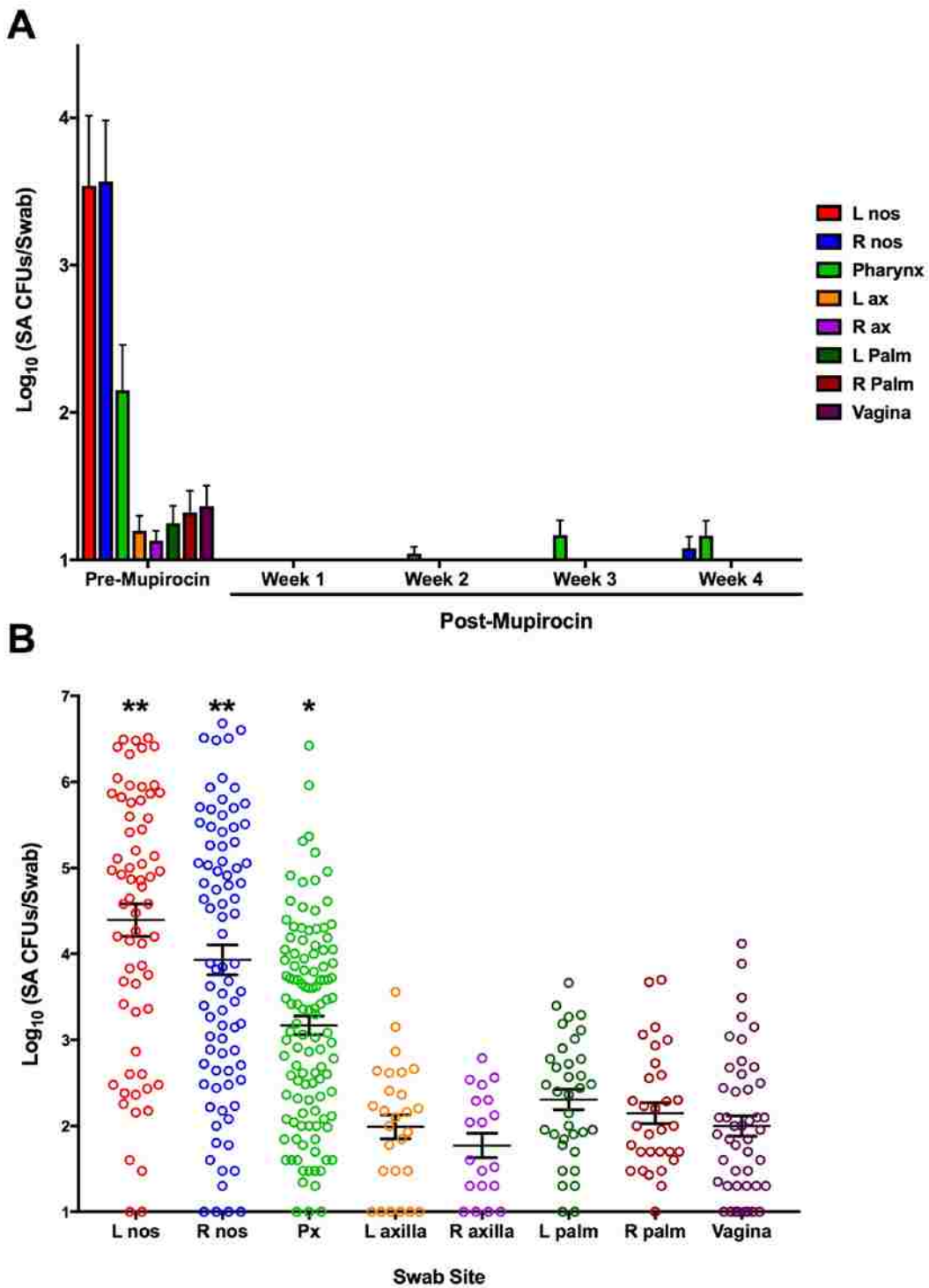
Figure 3: The Nasal Vestibule is the Predominant Reservoir of SA in the Pigtailed Macaque

A) The nostrils and each extranasal site were swabbed prior to antibiotic treatment, then weekly after the five-day course of 2% mupirocin ointment applied to the nostrils only. The nostrils and

rest of the body were effectively cleared of SA. Error bars indicate mean±SEM, n=24 (6 animals, 4 experiments). B) Detection of natural SA carriage (i.e. not due to experimental inoculation of the nostrils) collected over an 18-month period shows elevated SA load in the nares (double asterisk). Single asterisk indicates an elevated SA load when compared to extranasal swab sites. n= 19-115 swabs depending on anatomical site. Error bars indicate mean±SEM.

To further demonstrate that the nostrils are the primary reservoir, natural SA levels of the six extranasal sites were analyzed concurrently alongside the nostrils of the six animals: the pharynx, left and right axillae, left and right palms, and the vagina. **Figure 3B** displays natural SA positive swabs, excluding nasal SA CFU counts resulting directly from experimental inoculations of SA. Nostril swabs included in this figure are screening CFU counts, pre-inoculation samplings, or when clearance of experimentally inoculated SA was observed for two consecutive samplings before detection. All SA positive swabs were included for the six extranasal sites as these never received any experimental inoculations and naturally acquired SA from the individual's own nose. Natural SA carriage in the nostrils significantly outnumbered all other extranasal sites for frequency of positive swabs as well as SA load as indicated by double asterisks. Considering the extranasal sites only, the pharynx swabbed positive for SA at a higher frequency with a higher density of SA, however again, lower in positive swab frequency and SA load than the nostrils indicated by a single asterisk (**Fig.3B**). Taken together, these data suggest that like human SANC, the primary reservoir of SA in the pigtailed macaque is the nasal vestibule and is spread to extranasal sites through passive hand-face-body contact.

Nasal Non-SA Commensal Population Recovers Prior to Experimental Inoculation

To validate an animal model of SA nasal carriage, multiple animals need to be simultaneously cleared of SA, nasally inoculated with strains of choice, and followed up together easily. For each of the four individual experimental inoculations, all six subjects were cleared of

nasal SA successfully with mupirocin antibiotic ointment and swabbed for four weeks to validate

SA clearance and monitor the non-SA commensal population using dilution plating on TSA II

agar. Other gram positive non-SA commensals are susceptible to mupirocin, so it was expected

that the non-SA CFU counts would decrease at least temporarily, but recovery should occur by

inoculation day so that SA can be introduced to as close to a normal environment as possible for

each individual. **Figure 4** displays that the mupirocin regimen significantly decreases the non-

SA nasal population as demonstrated by week one TSA II plate counts.
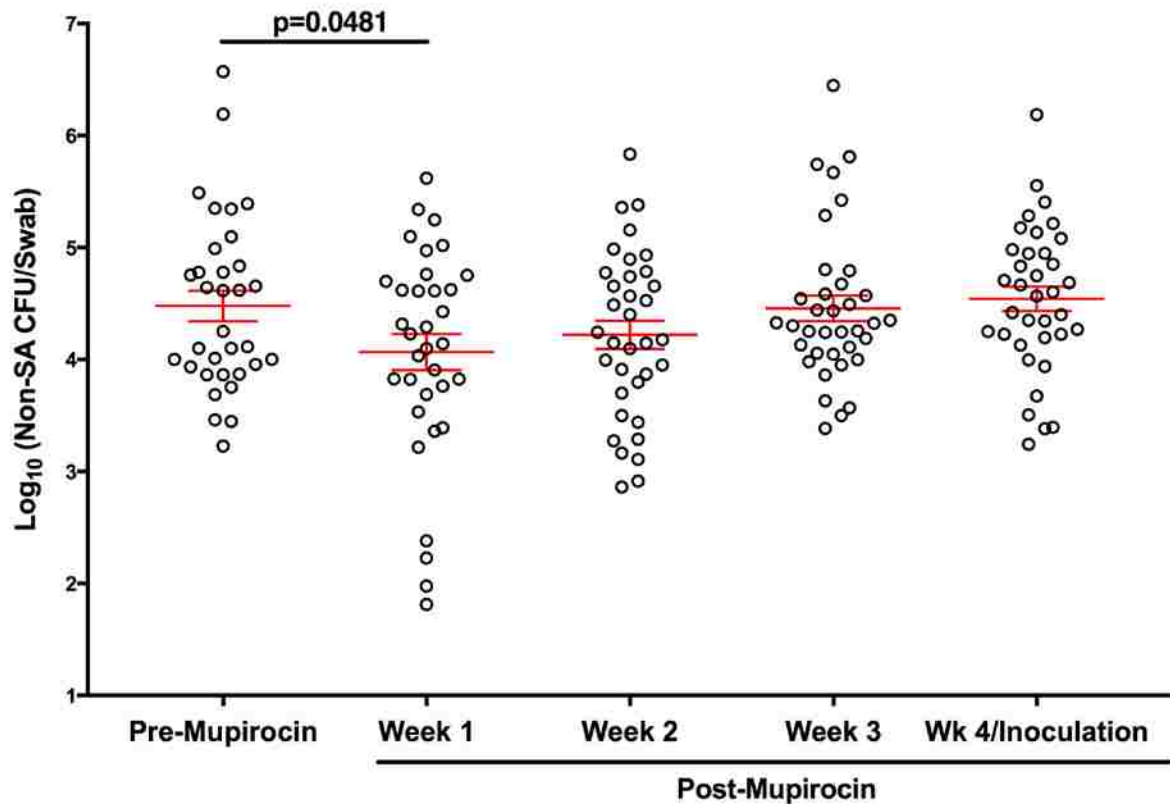
Figure 4: The Non-SA Nasal Commensal Population Recovers Prior to Experimental Nasal Inoculations

Macaque nostrils were swabbed prior to the first nasal mupirocin application, then weekly for four weeks afterwards, and the non-SA population was enumerated on TSA II blood agar plates. Paired non-SA CFU enumerations show the commensal population is significantly decreased one

week after mupirocin application (p=0.0481), however recovers by Week 3 Post-Mupirocin. Experimental inoculations occurred following the Week 4 swab collection. Error bars indicate mean±SEM, n=36 at each time point (6 animals, 2 nostrils each, 3 inoculation studies).

By Week 3 Post-Mupirocin, the nasal commensal population appeared to be fully restored, and the animals remained SA negative except for one individual (data shown **Fig 3A**). Experimental SA nasal inoculations took place immediately after the Week 4 Post-Mupirocin samples were collected.

A Human-relevant Inoculum of *Staphylococcus aureus* Establishes Asymptomatic Nasal and Extranasal Carriage in all Subjects

As this is the first study of this type, there is no prior knowledge on how much SA is required to establish carriage with a nasal inoculum of SA in the pigtailed macaque. The first inoculation was designed to identify the lowest nasal inoculum of SA to not only establish nasal and extranasal carriage in most subjects within the first two weeks of follow-up, but also prevent erroneously overloading the nasal mucosa and microbiome with SA. The first inoculation experiment involved clearing the nares and re-introducing the strain of SA isolated from each individual. **Figure 5** is a heat map generated from log-transformed carriage data to give a quick visual representation of relative SA density in the nose and extranasal sites over ten weeks of follow-up. Green is indicative of an SA count at or below the limit of detection based on dilution plating methods (LOD≤10), and ranges from yellow (low SA load, >10) to brick red (highest SA load, ≥10^6). For paired sites (nostrils, axillae, palms), SA detections are averaged. Six animals were first inoculated with 10^3 CFU/20 µL of their own individual isolate in each mupirocin-cleared nostril (Inoc. 1), and followed at day 2, then for two weeks by swabbing the nares and extranasal sites for detection of SA on TSA II agar. SA detection on day 2 post-

inoculation one showed that 5 of 6 subjects were positive for SA in at least one nostril, and few extranasal sites. Day 7 post-inoculation one SA quantification indicated that 3 of 6 subjects had cleared both nostrils, and only 6 of 36 extranasal sites were detectable for SA. After sample collection on day 14, each subject received another nasal inoculation (Inoc. 2) of approximately 10^4 SA CFU in each nostril (same self-strain). Sample dilution plating of day 14 samples revealed that 3 of 6 animals remained nasally colonized and only 5 of 36 extranasal sites were still detectable for SA. Following the second inoculation, day 21 of follow-up showed that SA established colonization in all nostrils, and then subjects were followed for an additional 8 weeks of nasal and extranasal swabs, nasal fluid, and serum collections for a total of 70 days. Most extranasal sites were eventually colonized during this time in subjects with high nasal SA loads, importantly Z08064 and Z08202, both of which reached levels from 10^5-10^6 CFUs/swab and tended to carry higher when positive for SA over the course of the study. One animal (T04293) cleared the nostrils of SA by day 56 and remained clear until the last sample before mupirocin clearance, important to note as this shows that the inoculum was adequate in establishing carriage but was not so high to prevent clearance.

Animal ID	Swab Site	Inoc. 1 (10³ CFU/nostril) 0	Post-Inoculation 1 2	7.0	14.0	Inoc. 2 (10⁴ CFU/nostril) 14.5	Post-Inoculation 2 21	28.0	35	42.0	49	56.0	63	70
A10023	Nostrils	3.0	3.8	3.9	1.0	4.1	5.1	2.6	4.5	4.2	5.6	4.0	4.7	6.2
	Pharynx		1.0	1.0	1.0		1.3	1.0	1.2	2.5	3.9	2.3	1.6	6.4
	Axillae		1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3
	Palms		1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	Vagina		1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
A11236	Nostrils	3.5	2.8	3.1	5.0	3.9	3.5	3.6	4.8	5.0	4.3	4.3	3.7	4.6
	Pharynx		1.0	1.6	1.7		2.1	1.0	4.0	3.4	1.0	3.5	1.0	2.9
	Axillae		1.0	1.1	1.0		1.0	1.0	1.0	1.3	1.9	1.0	1.0	1.0
	Palms		1.0	1.0	1.0		1.0	1.0	1.8	2.6	1.0	1.0	2.5	1.0
	Vagina		1.0	1.0	1.0		1.0	1.0	1.0	1.3	1.0	1.0	1.0	1.0
K05139	Nostrils	3.0	1.0	1.0	1.0	4.0	4.2	4.8	4.7	4.7	4.2	1.9	2.8	4.2
	Pharynx		1.0	1.0	1.0		1.0	1.0	3.7	1.0	1.0	1.0	1.0	1.0
	Axillae		1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	Palms		1.0	2.4	1.0		1.0	1.0	1.0	1.0	1.5	1.1	1.0	1.0
	Vagina		1.0	1.3	1.0		2.5	1.0	1.0	1.0	3.9	2.1	1.0	1.2
T04293	Nostrils	3.5	2.7	1.0	1.0	4.0	3.2	1.0	5.5	4.6	5.3	1.0	1.0	1.0
	Pharynx		3.0	1.6	3.4		2.3	3.3	2.8	4.5	4.3	3.1	4.4	1.0
	Axillae		1.0	1.0	1.0		1.0	1.0	1.0	1.2	1.5	1.0	1.0	1.0
	Palms		1.2	1.0	1.0		1.0	1.0	1.0	2.2	1.0	1.0	1.0	1.0
	Vagina		1.0	1.5	2.0		1.0	1.0	1.0	1.8	1.0	1.0	2.6	1.0
Z08064	Nostrils	3.0	1.1	1.0	5.4	3.9	6.1	6.7	5.5	4.9	5.4	5.1	5.7	5.0
	Pharynx		1.0	1.0	3.9		4.3	3.8	2.0	3.2	3.0	2.5	3.5	1.0
	Axillae		1.0	1.0	1.0		1.0	1.0	2.6	1.2	1.9	1.0	1.0	1.1
	Palms		1.0	1.0	1.0		1.0	1.2	2.8	2.2	1.7	3.1	1.0	1.0
	Vagina		1.0	1.0	1.0		1.3	1.0	1.7	1.0	1.0	1.0	1.0	1.0
Z08202	Nostrils	3.2	5.1	6.1	5.6	3.9	5.6	6.5	5.8	5.6	5.9	6.0	6.2	6.2
	Pharynx		1.2	1.0	3.6		3.7	2.9	4.0	1.0	1.0	2.1	2.0	1.0
	Axillae		1.0	1.0	1.0		1.0	1.2	1.0	1.0	1.8	1.0	2.4	1.9
	Palms		1.0	1.0	1.0		1.0	2.0	1.0	1.5	1.0	2.2	1.7	3.4
	Vagina		1.2	1.0	1.0		2.1	2.8	3.0	1.0	2.1	2.7	1.3	3.3

SA Carriage Timeline (Days)

Color Legend — SA CFU/swab:
- ≥ 10⁶
- ≥ 10⁵
- ≥ 10⁴
- ≥ 10³
- ≥ 10²
- > 10
- ≤ 10 (LOD)

Figure 5: Long-term Nasal and Extranasal SA Carriage is Established with a Human-relevant Nasal Inoculation of 10^4 SA CFUs/nostril

Animals were cleared with a regimen of mupirocin ointment and recolonized with their own SA isolate. After 4 weeks of microbiome normalization, 10^3 SA CFUs were delivered to both nostrils. CFU counts as detected on TSA II plates are shown as log-transformed, and paired sites are averaged and shaded according to SA load, legend on the right. By day 7, 3 of 6 subjects had cleared both nostrils, and only 6 of 36 extranasal sites contained detectable SA. Immediately following day 14 swabs, all nostrils received 10^4 CFUs of their own isolate again, which was sufficient in establishing carriage in all nostrils, and animals were followed for another 8 weeks. Most extranasal sites were eventually detectable for SA, especially in animals that achieved high SA load in the nose.

<u>Autologous and Non-autologous SA Strains Were Supported Similarly During Longitudinal</u>
<u>Pigtailed Macaque SANC</u>

Once an appropriate inoculum was determined with each of the subjects' own strains, two cycles were performed of mupirocin clearance of the nostrils, a 4-week normalization period, and inoculation of 10^4 SA CFUs into each of the pigtailed macaques' nostrils. Carriage of autologous versus acquisition of non-autologous SA strains has been implicated in influencing duration of human nasal carriage and bacterial load [2]; therefore it was desired to determine if the pigtailed macaques are similar. Two macaque SA isolates were implemented in separate inoculation studies, ST3813 and ST3814, applied to all animals' left and right nostrils, then followed for 63 and 93 days respectively. Inoculation of ST3813 to all six animals was autologous (indistinguishable from original nasal strain) to four, and non-autologous to two macaques. Inoculation of ST3814 was autologous to only one animal, non-autologous to the other five. These were administered to each animal regardless of whether it was autologous or not to the originally detected strain to determine if strains are carried differently if they were originally residing in that individual's nostril or completely new to the environment. Animals were swabbed in the nostrils and at each extranasal site (pharynx, axillae, palms, vagina), nasal fluids were collected, and blood drawn each week throughout. Nasal and extranasal SA and non-SA were enumerated weekly through dilution plating swab samples on TSA II plates. Each dot on **Figure 6A** represents one animal's log-transformed averaged nostril, separated by autologous (left) or non-autologous (right) positive SA loads. There was a total of 5 autologous and 7 non-autologous inoculations, with most of the non-autologous inoculations occurring in the independent study with the longer duration (93 versus 63 days). The supported SA load was not significantly different between autologous and non-autologous groups as determined through a

two-tailed unpaired t-test (p=0.1281). Importantly, the mean averaged nostril detection for both autologous and non-autologous macaque strains was approximately 10^4 SA CFUs/ swab, while the median SA load in human nasal carriage is 9.8 x 10^4 SA CFUs/swab [58]. Additionally, there was no significant difference in duration of survival between autologous and non-autologous SA strains. Survival curves were generated using the same longitudinal CFU counts from day 2 or 3 post-inoculations, then weekly over the duration of both inoculation studies (**Fig. 6B**). All nostrils received identical treatments; therefore, detectable SA in at least one nostril was considered survival in that animal. Autologous and non-autologous SA strains both persisted over 40 days, autologous with a median survival of 43 days and non-autologous with a median of more than 57 days, and the generated survival curves were statistically similar (p=0.3668).
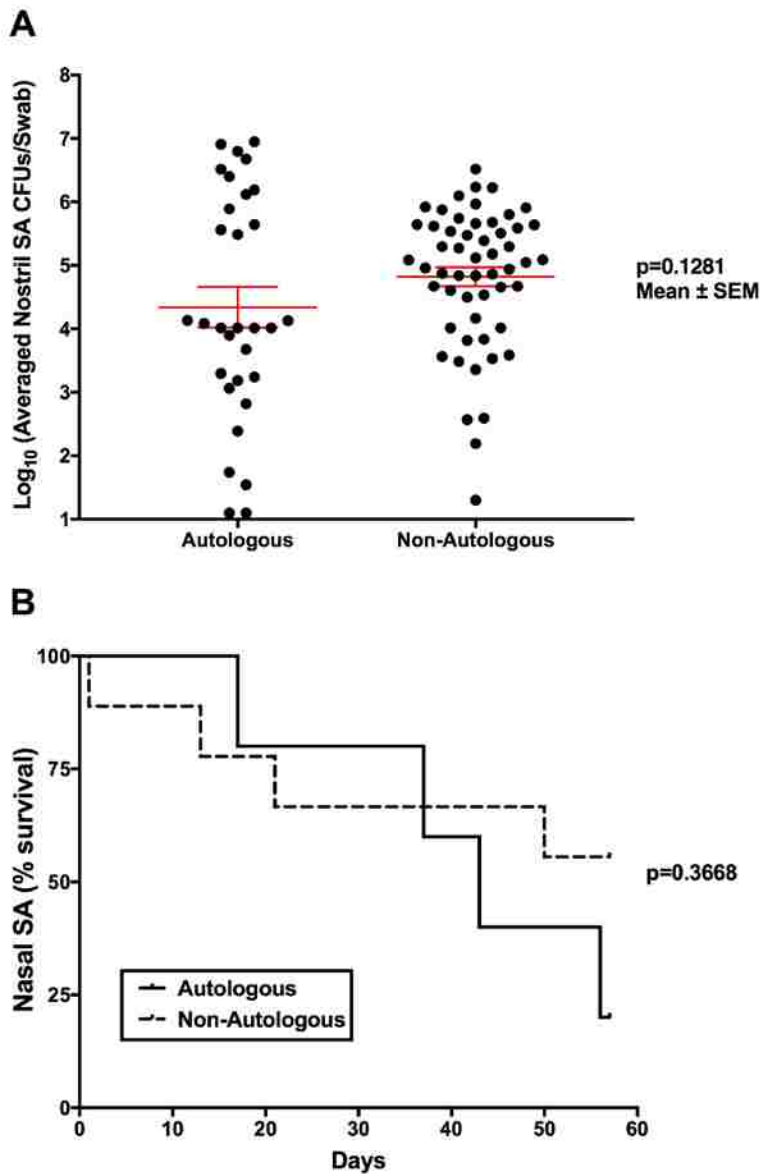
Figure 6: Autologous and Non-autologous Pigtailed Macaque Nasal SA Strains may be Implemented in Successful Nasal Carriage Studies

Animals' nostrils were inoculated with 10^4 CFUs of SA in two independent inoculations with either genetically distinct (non-autologous) or indistinct (autologous) from their screening isolate as determined through MLST and *spa* typing. Nasal swab samples were plated on TSA II weekly to quantitate SA CFUs longitudinally. A) Positive nasal SA counts were averaged per individual and separated based on if the SA present was autologous or non-autologous to that animal's original nasal strain, with statistically similar (p=0.1281) supported SA loads. Autologous n=31, non-autologous n=54, as most of the non-autologous data was collected during an extended follow-up duration, ~4 weeks longer than the inoculation including most of the autologous data collections. B) Percent survival was determined from these same

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longitudinal carriage data points, with SA detection in one nostril considered survival as all nostrils were treated with the same inoculum in the two independent studies. Autologous and non-autologous strains survived statistically similar (p=0.3668) for over 40 days.

Pigtailed Macaque SA Strains are Genetically Similar to Several Human Isolates

The final inoculation was a short pilot study in which it was desired to determine if the nostrils and immunity of the pigtailed macaques would allow the colonization of a human SA strain. The three pigtailed macaque SA strains isolated in this study were genetically compared to seven prior human study nasal isolates as well as prototypical human wound MRSA isolate, USA300 (wound isolate NRS384, ST8/t008) [75] to determine if the lab already possessed a suitable human strain as similar to the macaque's strains as possible to establish carriage. All nasal SA strains (**Table 2**), whether human or pigtailed macaque (other than USA300), were lacking the *mecA* gene that codes for methicillin resistance as determined through the absence of a band in a DNA gel following PCR amplification using *mecA* primers. At the beginning of this study, multi-locus sequence typing (MLST) was performed for seven housekeeping genes for every strain to identify each isolate's sequence type. These seven MLST DNA sequences were concatenated per strain, aligned against each other, and arranged into a phylogeny tree according to sequence similarity using MrBayes v3.2 phylogenetic analyses (**Fig. 7**). Human donor (D) 579 carried ST398 and was found to be genetically similar to macaque strains ST3814 and ST3815 (based solely on MLST gene sequences). It was desired for the last pilot study to implement a non-virulent, antibiotic susceptible human strain with similarity to the SA detected in this cohort to avoid harm to the animals and to yield as much information as possible. These comparisons heavily influenced the selection of which human nasal SA isolate was implemented in the pilot trial involving experimental inoculation of a human SA nasal isolate into the nostrils of the pigtailed macaques.
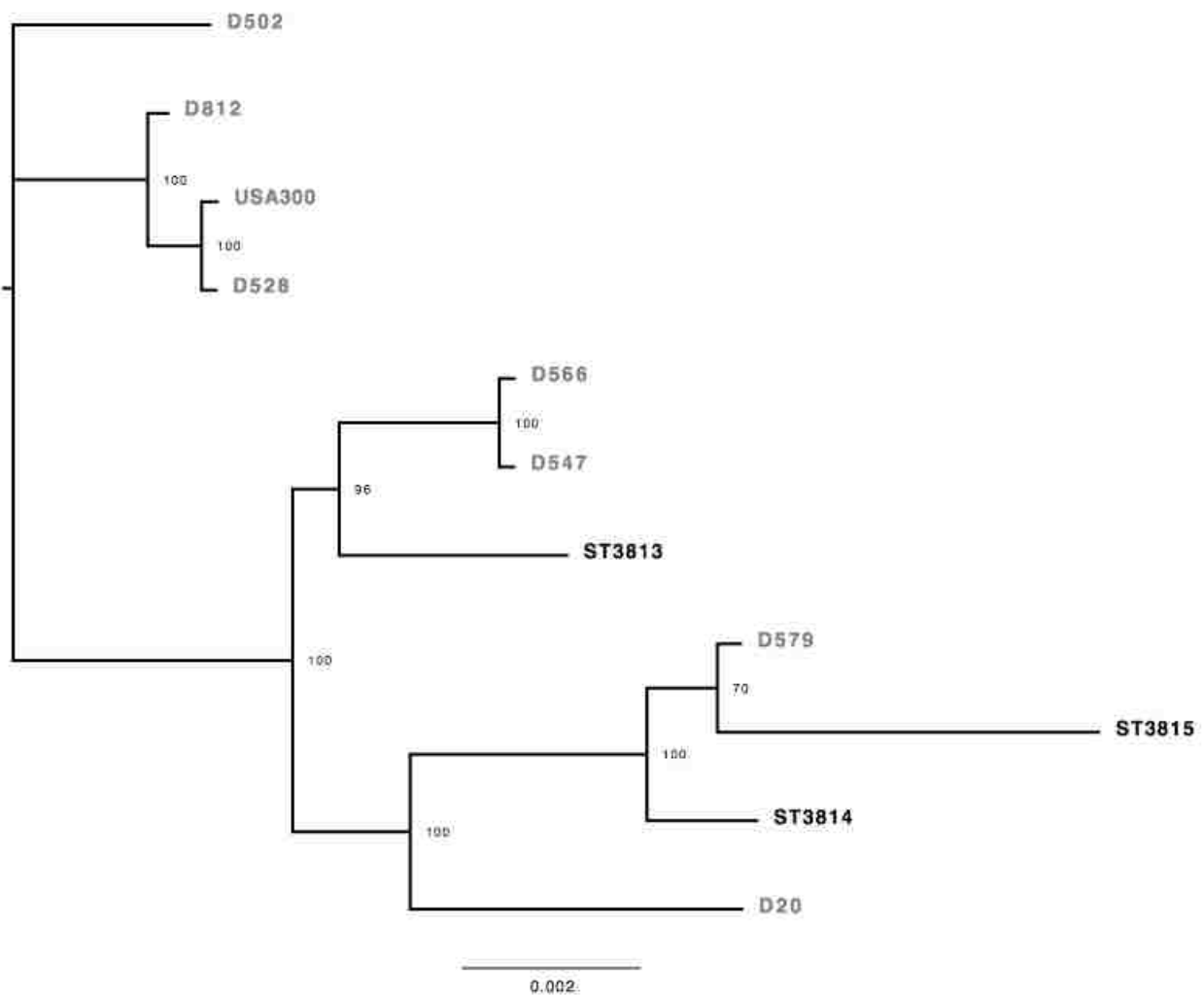
Figure 7: Nasal SA Isolates from Six Captive Pigtailed Macaques are Genetically Related to Human Nasal Carriage Strains

Evolutionary relationships between 8 human and 3 macaque nasal SA isolates in were analyzed using Bayesian analyses. Seven MLST loci per strain were concatenated, then aligned together and arranged into a phylogeny according to sequence similarity using MrBayes v3.2 phylogenetic analysis. Human isolate D579 (ST398) was chosen for experimental pilot inoculum based on genetic MLST similarity to macaque isolates ST3814 and ST3815. The average standard deviation of similarity between samples was 0.00157. Scale bar of 0.002 indicates number of substitutions per site. Numbers at each node are indicative of posterior probability support.

<u>Nasal Colonization of Human Isolate ST398 was Successful and Comparable to Human SANC</u>

Following the autologous and non-autologous inoculations of pigtailed macaque SA isolates ST3813 and ST3814, animals were cleared of SA with mupirocin and the nasal microbiome was allowed to normalize over four weeks. Sufficient funding remained for a 22-day pilot study implementing human SA isolate ST398 using identical protocols and inoculum size as previous macaque inoculations. **Figure 8A** displays that nasal colonization was successful in all six subjects, as indicated by log-transformed averages of each animals' left and right nostrils (animal ID in legend on the right) over the duration of the study period. Two animals (A10023 and A11236) naturally cleared the SA within 2 weeks, while the remaining four animals maintained carriage until force-cleared with mupirocin on day 22. Animals were returned to the colony after all sites were verified cleared on day 28 post-inoculation. This study was abbreviated and did not allow determination of the possible duration of carriage of the four that maintained carriage, or if the two that cleared would eventually swab positive again. The preceding macaque strain inoculations were each at least 63 days, and most extranasal sites eventually swabbed positive for SA within that time. **Figure 8B** shows the SA load days 2-22 for the nostrils and all extranasal sites, each dot representing a log-transformed CFU count for one animal (6 animals, 4 swab dates). As seen previously, the nostrils carried significantly higher than all other sites ($p<0.0001$), indicated by a double asterisk. The pharynx supported higher SA loads than the other extranasal sites ($p<0.005$) but not close to the nostrils, indicated by a single asterisk. This is indicative that the shortened study duration was not conducive to establishment of extranasal acquisition of the inoculum, however like previous inoculation studies, the pharynx acquired carriage of the same strain once the nostrils were detectable.
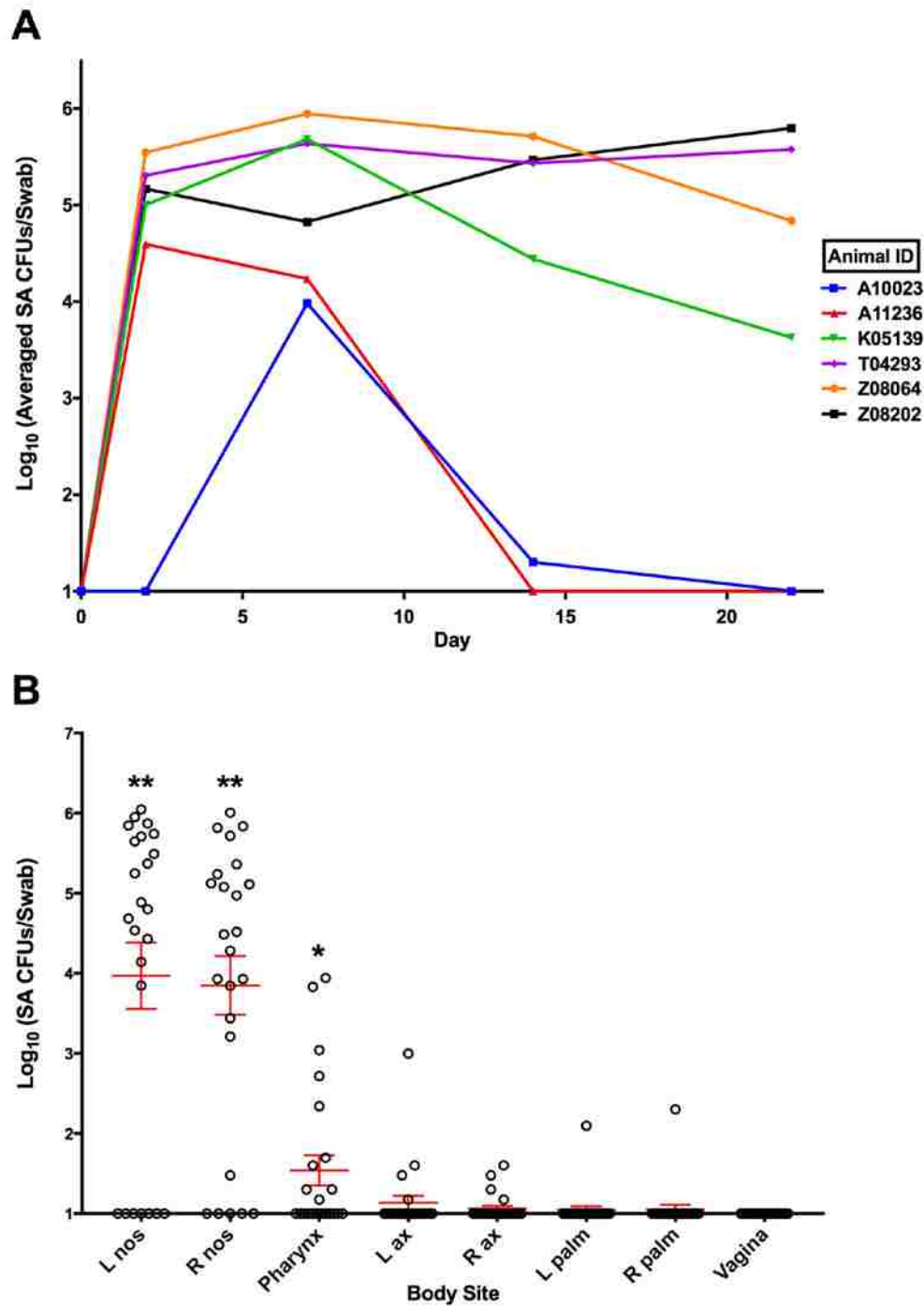
Figure 8: Experimental Inoculation of Human Nasal Isolate ST398 Established Nasal Carriage in all Subjects

Macaques were cleared of SA as described in Materials and Methods and reinoculated with 10^4 CFU/nostril of human isolate ST398. Nasal and extranasal swabs were collected weekly for SA quantification. A) Averaged nostril SA CFU/swab for each animal shows carriage was

established in each subject with two animals clearing within 22 days, while the remaining four subjects carried until cleared by mupirocin. Legend to the right indicates Animal IDs. B) SA load days 2-22 in the nostrils and extranasal sites. Double asterisks indicate elevated SA load compared to all other sites ($p<0.0001$). Single asterisk indicates elevated SA load compared to axillae, palms, and vagina ($p<0.005$). Error bars indicate mean±SEM, n= 24 (6 animals, 4 swab days). A, B) y-axis begins at the limit of SA detection (10 CFUs/swab).

Discussion

Prior to any experimental inoculations, the first step of this study was determining whether pigtailed macaques (*Macaca nemestrina*) at the Washington National Primate Research Center (WaNPRC) naturally carry SA in the nose, as this is a major drawback to attempting the use of rodent models for SANC. Thirteen of seventeen animals were found to carry SA in at least one nostril in the first screening visit, and fourteen were positive two months later (**Fig. 1**). Most animals were also positive in the pharynx at one or both visits. An important finding to note is that macaques carry nasal SA at a significantly higher rate than humans, with 14 of 17 (82%) screened pigtailed macaques in this study and 82% of 48 rhesus macaques in a longitudinal study [56], compared to an approximate third of healthy adults carrying worldwide at any given time [14, 15]. Previous human studies have attributed increased carriage rates in close quarter populations that may have less than ideal sanitation like prisons, retirement homes, daycares, and schools [4,5], and captive monkeys live in close contact and their hygiene is lacking. Access to wild pigtailed macaques is extremely outside of our scope, as they are native to Thailand, Malaysia, and Indonesia, therefore it cannot be said definitively that all pigtailed macaques are natural nasal carriers of SA. The likelihood, however, that nasal carriage in this population is a result of living in captivity and being cared for by human handlers is low, as the caregivers go to extreme measures to prevent the transmission of microbes by wearing personal

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protective equipment including: full-coverage garments, face masks, face guards, and double-glove their hands. Each strain isolated from the chosen subjects was identified as new, with new MLST and *spa* types (**Table 2**), which is suggestive that natural SA carriage of this cohort of macaques is not likely transmitted through human handling. Prior clinical evaluations have shown that most babies are colonized with SA within hours after birth, and usually identical to the mother's nasal strain [81, 82], but husbandry records at the WaNPRC validate that the four animals sharing an autologous strain are not related and are not all housed together, with SA negative animals co-inhabiting with SA positive animals according to animal housing charts (records not shown).

In humans, the anterior nares have been accepted as the site of predominant SA carriage since SA is extremely adaptive to that low nutrient, high saline environment. Prior to invasive surgical procedures, 2% mupirocin antibiotic ointment is applied to the nostrils to prevent autoinfection, and this is sufficient to also eradicate SA from the rest of the body, as the nose serves as a reservoir to spread SA through passive hand to face contact. This study implemented the same antibiotic ointment with an identical regimen, identical to what is applied to humans pre-surgery. The three pigtailed macaque isolates were susceptible to mupirocin *in vitro* (**Fig. 2**) and *in vivo* (**Fig. 3A**), and just like pre-surgical humans, mupirocin cleared not only the nostrils, but also the rest of the body as indicated through the observable absence of β-hemolytic colonies on blood agar plates. This suggests that in the pigtailed macaque, the nostrils also serve as a reservoir for SA, spread to the rest of the body through casual hand-to-face contact. It was not determined how long the subjects would retain SA negative status in the absence of experimental inoculation, but the nostrils, pharynx, axillae, palms, and vagina remained mostly clear of SA for four weeks prior to each separate inoculation. This demonstrates that a common human

antibiotic is effective in clearing the primary reservoir of SA in the pigtailed macaque, also clearing the body similar to humans.

Development of a protocol for SA eradication and recolonization like prior human studies was a main goal, with a giant step forward in the introduction of a variety of strains to the nostrils of the pigtailed macaque. Once subjects were cleared of SA, recolonization was required implementing an appropriate inoculum that would introduce just enough SA to establish nasal and extranasal carriage in most subjects, while also avoiding the abrupt introduction of an excessive inoculum that may erroneously overload the nasal mucosa. A human-relevant inoculation of 10^4 SA CFUs delivered in 20uL to both nostrils was sufficient in re-colonization of each subject after mupirocin clearance. (**Fig. 5**). This is an important finding, as a prior Cole laboratory survey of 30 healthy human carriers in 2016 determined a range of SANC from 10^1 to 10^6 CFUs per nostril, with a median of 9.8 x 10^4 SA CFU per nostril [58]. This same human-relevant inoculum was successful in establishing nasal and extranasal carriage in all subjects for a total of four independent experiments (**Figs. 5 & 8**), significantly not requiring millions of SA CFUs like rodent models.

A significant limitation to human SANC research in the U.S. is the inability to implement SA inoculations other than the subject's own nasal isolate, which prevents understanding adhesion and virulence mechanisms of potentially virulent or antibiotic resistant strains. The possibility of inoculation studies with a variety of strains would present a means to longitudinally observe colonization and pathogenesis of non-autologous strains in a human-relevant model. In two independent studies, 10^4 SA CFUs of a macaque isolate (3813 and 3814) was introduced to the nostrils of all animals regardless of whether it was autologous or not to the nostril and followed for several weeks. Autologous and non-autologous macaque strains were supported

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similarly, with both approximate means of 10^4 SA CFUs (**Fig. 6A**). Autologous and non-autologous strains also survived similarly in the macaque nostrils for over 40 days (**Fig. 6B**), determined from SA-positive status in at least one nostril (considered survival as all nostrils were treated with the same inoculum). This implicates the usefulness of the pigtailed macaque in a variety of SANC strain inoculation studies with a physiologically relevant inoculum.

A concluding three-week pilot study that was designed to determine if there was potential for a human SA isolate to establish carriage in the pigtailed macaque with the same physiologically relevant inoculum that was sufficient in the macaque strain inoculations. Human donor 579 (D579) carried ST398, a strain implicated as a host-species-adaptable methicillin-susceptible SA that can acquire resistance [84, 85]. ST398 is commonly associated with livestock (cattle, pigs) and meat-processing and is often transmitted to humans in these lines of work [86, 87], and was determined genetically similar to two macaque isolates through comparisons of concatenated MLST sequences (**Fig. 7**). Two animals naturally cleared the nostrils by day 22, while the remaining four subjects carried an average of 2.7×10^5 SA CFUs per swab until to mupirocin clearance. The 22-day duration of this pilot study was not sufficient for the majority of extranasal sites to acquire SA from the nose, but as seen in prior macaque inoculations, the nostrils were the primary reservoir with significantly higher SA loads, and the pharynx contained the highest of the extranasal sites (**Fig. 8B**). Prior inoculation durations were for at least eight weeks of follow-up, so given more time the extranasal sites would acquire increased SA loads. This data demonstrates that the pigtailed macaque can acquire and maintain carriage of clinically-relevant human strains in addition to non-autologous pigtailed macaque isolates, which has major future SANC research implications.

Throughout each of the four inoculation studies, MLST and *spa* typing for sequencing analysis was performed periodically to ensure that the SA colonies detected were identical to the inoculated strain and not a genetically distinct strain. At least two MLST loci and the *spa* type were required to match to be considered "genetically indistinct". These analyses were performed at times when i) an animal swabbed positive for several consecutive weeks, ii) times when the nostrils swabbed positive after extended periods of undetectable SA, and iii) the last positive swab on each extranasal site for each study. Detection of a strain genetically distinct from the inoculum was rare, however did occur in the first study in which the appropriate inoculum was determined. This was a rare occurrence, with extranasal SA matching the inoculum in 105 of 110 (95.5%) of the analyzed swab samples, and this did not occur in any of the other three independent inoculations. These animals were housed in separate cages, with only visual contact with each other, suggesting that this alternate SA strain may have been introduced in their food supply, as these animals were not housed together prior to this study.

Longitudinal analysis of SANC in *Macaca nemestrina* elucidated patterns of carriage observed in prior healthy human studies. Most human carriers are intermittent, with the ability to clear SA to undetectable levels, then swab positive again. Some human carriers are persistent and never appear to clear, and some appear to not carry at all, and the reasons and mechanisms for healthy carriers to support SA in the nares and the body asymptomatically are not fully understood. Similar carriage patterns were seen in the macaque subjects over several weeks of follow-up over the course of the 18-month study, therefore it will be highly beneficial to analyze the immune response during times of SA nasal carriage and clearance.

CHAPTER 3: ANALYSIS OF THE PIGTAILED MACAQUE NASAL RESPONSE TO SANC REVEALS SIMILARITIES TO THE HUMAN CONDITION

Introduction

Until now, the nasal innate immune response to SA has not been characterized for the pigtailed macaque. The majority of pigtailed macaque research has centered on potential microbicides, HIV, and other human sexually transmitted diseases, with tissue structure, clinical presentations, and immune responses similar to the human condition [66, 72]. Previous research implementing this model found similarities in fetal development of the respiratory system and immune function in pigtailed macaques [67, 68] therefore parallels are expected in innate responses in the nasal mucosa in the presence of SA. In humans, however, the pro-inflammatory response to SA in the nose has been well documented, although the mechanisms are not fully elucidated due to the complexities of the associated relationships. Dual PRRs, TLR-2 on the surface of phagocytic nasal mucosal cells and cytoplasmic NOD2 sense the presence of conserved gram-positive cell wall proteins either through direct contact or protein turnover during biosynthesis. This mounts an immune response that either effectively clears the invasion or further promotes inflammation that potentially damages the tissue [88]. Recognition of SA by nasal cell PRRs in the skin elicits redundant pathways to the nucleus to up-regulate expression of AMPs and contributes to rapid innate activation. Various pro-inflammatory cytokines and chemokines are up-regulated in response to SA presence; acting to recruit neutrophils through diapedesis to infected tissue and maturation once they arrive [89]. Once matured, neutrophils release oxidative bursts, sequester nutrients, produce more AMPs, and use phagocytosis to digest SA cells encountered in the tissue [25, 90, 91], thus, defects in pro-inflammatory proteins,

receptors, mechanisms, or decreased neutrophil volume can allow for SA persistence in the nares.

Previous studies in the Cole laboratory centered on host factors contributing to SANC have elucidated significant relationships between several key pro-inflammatory mediators and the introduction of SA to the nose. In humans, enhanced expression of IL-8, IL-1β, macrophage inflammatory protein (MIP-1β), monocyte chemoattractant protein-1 (MCP-1), granulocyte colony-stimulating factor (G-CSF), and a decreased IL-1 receptor antagonist to IL-1β (IL-1RA:IL-1β) ratio detected in nasal fluids at post-inoculation time points as compared to pre-inoculation were associated with SA clearance groups only [28, 58]. IL-1β is a significant inflammatory marker that first exists as a precursor that requires cleavage into mature IL-1β by caspase-1, induced through inflammasome activity [92] and heightens the inflammatory state once secreted into infected tissues. Inhibition of IL-1β signaling occurs through non-productive competition due to high levels of the IL-1β receptor antagonist binding at the cell surface IL-1 receptor [91]. Interestingly, dysregulation of IL-1β has been implicated as the primary cause of inflammation in pancreatic islet cells, leading to progression of Type II diabetes [93], which is known as a major risk factor of SANC. Rapid up-regulated transcription of the IL-8 gene results in chemotaxis of neutrophils to the site of infection and induces phagocytosis once they reach infected tissue, clearing SA more quickly than a delayed reaction [58]. MCP-1 and MIP-1β are monocyte and leukocyte chemoattractants produced by activated macrophages that further induce pro-inflammatory pathways in response to presence of SA [94, 95]. Examination of the pigtailed macaque innate response will focus on these pro-inflammatory mediators as well as a few others implicated as relevant to the SA response or lack there-of in other studies. Rodent SANC studies have shown that IL-17 deficiency is responsible for decreased AMP production

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and increased carriage duration of SA as compared to wild type animals [55]. Induction of pro-inflammatory cytokines IL-6 and TNF-α has been observed in humans to be elevated in obesity [96], which is an additional risk factor for SANC. Addition of SA peptidoglycan to keratinocytes *in vitro* triggers up-regulation of vascular endothelial growth factor (VEGF), which has been observed *in vivo* to be associated with SA-associated psoriasis [97]. Highly expressed in the nasal environment, VEGF increases permeability of capillaries, increasing fluid influx into inflamed tissues, which facilitates influx of white blood cells [98].

Previously shown, the pigtailed macaque is easily decolonized of SA in the nose and body with a common human antibiotic often applied to pre-operative humans, and they remained decolonized through four weeks of nasal microbiome equilibration. Re-colonization of several animals is easily established and reproducible with autologous and non-autologous strains using a human-relevant inoculum of 10^4 SA CFUs per nostril. Nasal carriage of SA inoculations in this cohort of six pigtailed macaques was followed longitudinally over 18 months, and this data will be implemented in comparing subsequent detection of the 10 pro-inflammatory mediators just discussed to the presence or absence of SA in the nasal mucosa. This will allow further comparison of this proposed model to the human condition based on importance of certain proteins in the innate response, as well as differences in expression in the nasal mucosa of animals that were successful in clearance of SA from the nares to those that maintained carriage.

Materials and Methods

Blood and Nasal Fluid Collection and Processing.

Each week, 3-5 mL of blood was collected into serum separating tubes to acquire 1-2 mL serum, stored at -80°C until use. Nasal fluid was also collected each week by inserting two

Whatman 540 low-ash filter paper strips (0.3 x 2.4 cm) into each nostril and massaging the exterior of the nose for 30 seconds. Paired left and right nostril strips were stored in separate tubes at -80°C. Frozen serums and nasal strips were batch-shipped at the end of each inoculation study on dry ice to the Cole laboratory for subsequent processing and analysis. Nasal fluid proteins were extracted from paper nasal strips by vigorously vortexing for 20 minutes in 900 μL of 10% glacial acetic acid (in molecular grade water), transferring the supernatant containing soluble proteins to a fresh tube (on ice), then an additional vortex of strips with 500 μL acetic acid for 10 minutes. The supernatants were combined per animal nostril, balanced in a SPD1010 Speedvac (ThermoFisher), and vacuum dried ≤100 μL using consistent 45° C heat. Two washes were performed with molecular grade water and re-evaporating to a ring of moisture. Dried samples were resuspended in nine parts Dulbecco's Modified Eagle Media (DMEM) with 1% BSA and one-part RIPA buffer (50mM Tris, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and then neutralized to approximately a pH of 7 using 1N or 10N sodium hydroxide as needed in 1μL increments. Samples were standardized to 130μL per extract, then insoluble debris was clarified by centrifugation (10,000 x g for 1 min) and stored in 65μL aliquots at -80°C until protein analysis. Total protein was quantified with an average of 9 mg/mL with consistency between subjects. Six of 188 extracts were eliminated from Luminex analysis due to unreadable protein quantifications, either due to insufficient collection or blood content.

Total Protein Quantification Assays.

Select nasal protein extracts were chosen to validate the acid extraction process and quantify total proteins by implementing the bicinchoninic acid assay (Micro BCA Protein Assay

Kit, Thermo Scientific, Rockford, IL Lot #PD199651). Standards were made by diluting 2mg/mL bovine serum albumin (BSA) stock with DMEM/BSA/RIPA diluent 1:10 for 200 μg/mL, which was then diluted 1:1 to create 100, 50, 25, 12.5, and 6.25 μg/mL BSA standards. BCA working reagent was mixed according to manufacturer's directions, and 150 μL of samples/standards was combined with 150 μL working reagent on a 96-well clear-bottom plate (TPP/ Midsci, cat# TP92096), with standards ran in duplicate. Controls included were molecular grade water and any buffers used to ensure no protein contamination. The plate was covered and incubated for 2 hours at 37°C, then removed from heat and allowed to cool to room temperature while setting up the SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). Protein content is directly proportional to the intensity of purple color created in a two-step reaction between copper and proteins in the solution, which is measured in absorbance. Absorbance was read at 562nm according to manufacturer's instructions, and protein content was determined by correlating absorbance of samples with absorbances of known concentrations of a duplicated standard curve.

Multi-plex Bead Immunodetection Assays.

Nasal protein extracts and serum aliquots corresponding to sample date were thawed on wet ice prior to multiplex bead assay. A custom ProcartaPlex non-human primate 10-plex cytokine assay (Life Technologies Corporation, Carlsbad, CA) was performed for detection of G-CSF, IL-1β, IL-1RA, IL-6, IL-8, IL-17α, MCP-1, MIP-1β, TNF-α, and VEGF. Manufacturer instructions were followed step-by-step for preparing standard curves, serum dilution, incubation times, and instrument settings. Left nostril extracts were analyzed for 3 of 4 inoculation experiments (n=6 each) and right extracts were used for the other to best utilize observed

transitional negative to positive SA detections. Nasal protein extracts (54μL) were mixed with 6μL of DMEM/5% BSA so both the standards and samples were diluted in a nearly equivalent mixture of DMEM, RIPA, and .5% BSA to create a protein background and to prevent aggregation of target proteins and non-specific adherence to the wells of the 96-well plate. A final volume of 50μL per well of prepared standards and samples was combined with antibody-conjugated beads and washed using a Bio-Rad Bio-Plex Pro II wash system. Completion of washes and samples readings were performed using a compatible high-throughput Bio-Plex 200 reader (Luminex xMap technology). All standard curves exhibited good fit down to 0.5-4 pg/mL with detection limits included in figures. Data is presented as log-transformed pg/mL for both nasal fluid extracts and serums as they were processed by equal volumes.

Statistical Analyses.

BCA and Luminex multi-plex bead protein detection assays were analyzed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Prior to analyzing all extracted nasal fluids, reproducibility of analyte detection was validated in nasal fluids by pooling four animals' nasal fluid extracts from one sample date and dividing into four wells for detection. Validation of low-level detection reproducibility in serum was determined through running one animal's serum from one sample date in triplicate for detection of analytes. Both were found to be highly accurate and reproducible. SA-negative to SA-positive transitional cytokine comparisons per individual were analyzed for significance using paired t-tests (two-tailed) (**Fig. 12**). Carriage durations for noses with different peaks of inflammatory proteins detected in nasal fluids were compared using ordinary one-way ANOVA with Tukey's multiple comparisons test (**Fig. 13**).

The Nasal Fluids of Humans and Pigtailed Macaques Contain Comparable Total Protein Levels

Six pigtailed macaques were sampled for nasal SA beginning with pre-mupirocin time points, for four weeks post-mupirocin, inoculation day zeros, day 2 or 3, then for several weeks following inoculation. After nasal swabbing with flocked swabs, nasal proteins were collected through wicking nasal fluid onto paper strips (Whatman 540) and blood was drawn to separate out approximately 1mL serum. Adsorbed nasal fluid proteins were extracted in 10% glacial acetic acid, concentrated with a total of three desiccations (with molecular water as a diluent in between), and neutralized in a pH buffer compatible with protein quantification assays and Luminex-based multi-plex bead assay of pro-inflammatory cytokines, chemokines, and growth factors. Human nasal secretions (14 humans, 1 collection each) were self-collected via suction catheter, and total protein as measured through bicinchoninic acid assay (BCA) averaged 5.5±2.5 mg/mL (mean±SEM). Pigtailed macaque nasal secretions (6 macaques, average of 14 collections each) were comparable to human nasal fluid protein content at an average of 9.9±3.1 mg/mL total protein (**Fig. 9**). For the macaque data, only the extractions of non-bloody are included, as bloody strips yielded 2 to 4-fold higher protein detections as detected through BCA. Nasal fluids collected from animal Z08202 had frequent visibly bloody strips, therefore had the fewest data points, but strips consistently contained higher amounts of protein than other animals.
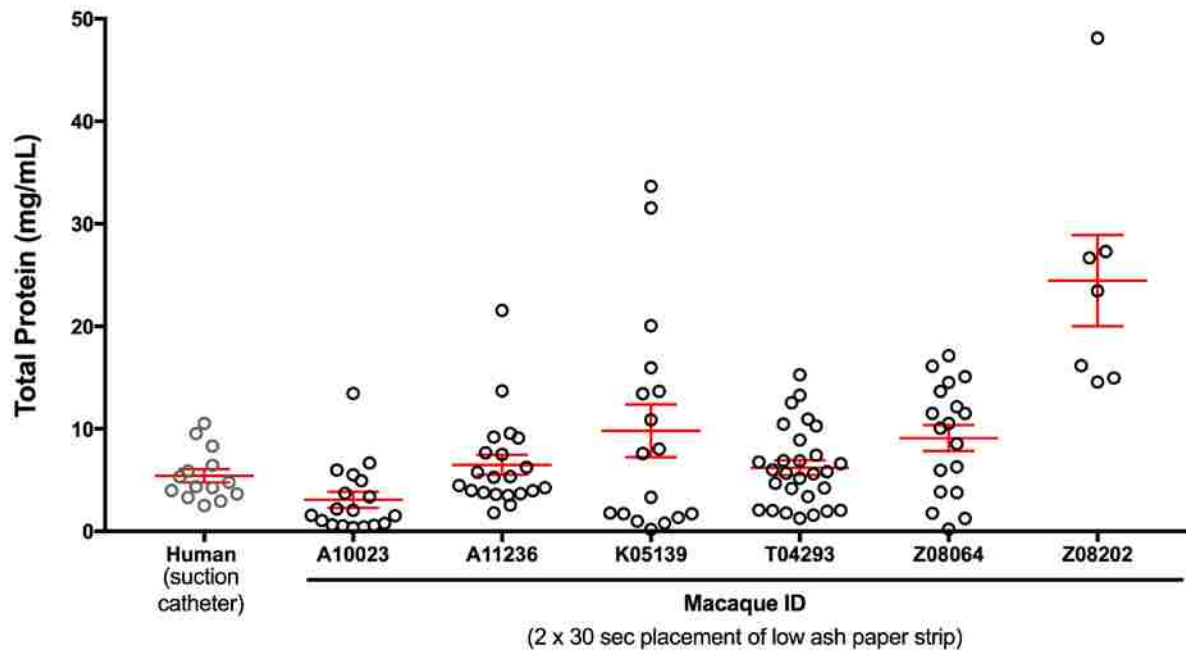
Figure 9: The Nasal Fluids of Humans and Pigtailed Macaques Contain Comparable Total Protein Levels

Following acid extraction of macaque nasal strips, soluble nasal fluid proteins were quantified and compared to human nasal fluid protein content in mg/mL (mean±SEM) collected through self-applied suction catheter. For the macaque data, these were the extractions marked "non-bloody", as bloody strips yielded 2-4-fold higher protein values as detected through BCA colorimetric assay, n=14 human nasal fluids, n=7-28 macaque nasal fluids per individual depending on blood content of strips.

SANC is Asymptomatic in Both Humans and the Pigtailed Macaque

In healthy human hosts, SANC is mostly asymptomatic, unless the host at some point becomes immunocompromised, which increases the risk for dangerous invasive skin infections leading to systemic infections like bacteremia. Systemic responses to an infection cause signs of illness such as fever, malaise, and behavioral changes like loss of appetite. Throughout this 18-month study, the cohort of six pigtailed macaque subjects were monitored daily for any adverse changes in condition or behavior. Body weight and temperature remained within a normal range as they were measured each week as the animals were sedated for mupirocin applications or

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sample collections. Appetites and thirst remained constant as indicated through daily protein biscuit counts reported by the animal husbandry staff. There were no discernible changes in behavior throughout the study, nor any reports of malaise or illness, suggesting that SANC in the pigtailed macaque is asymptomatic. Other than observable signs of illness caused by a systemic inflammatory response, increased expression of pro-inflammatory proteins would be detected in the blood following experimental inoculations. **Figure 10** displays detection of IL-8 and IL-1β in the nasal fluid compared to serum, as these were two key pro-inflammatory mediators in the human innate response to SA inoculation.
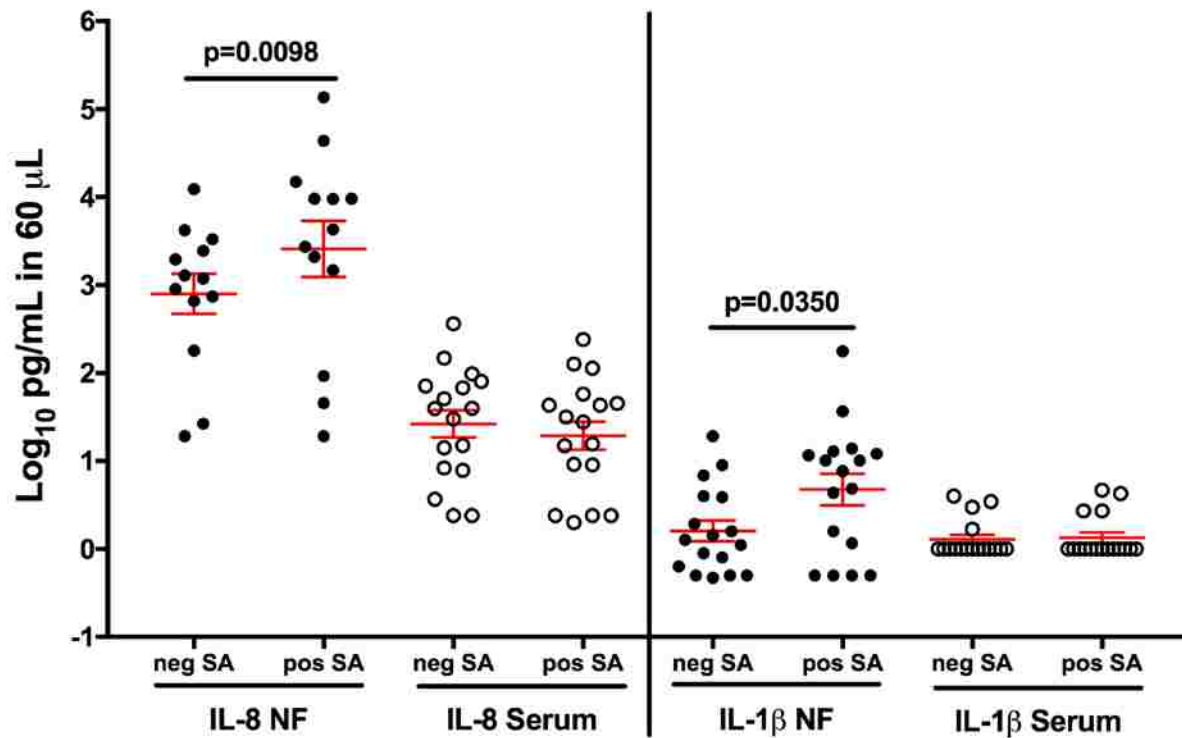
Figure 10: Asymptomatic SANC does not Elicit a Systemic Response in the Pigtailed Macaque

Pigtailed macaque nasal fluids and serums were collected on day 0 directly prior to inoculations, day 3, then weekly until the end of follow-up periods. Nasal fluids and serums for each of the four independent experiments were assayed for ten analytes using multi-plex bead assays and flow-based enumerations, with IL-8 and IL-1β detections shown in closed circles (nasal fluid

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detection) and open circles (serum detection). Values are shown for paired transitions from times at which SA was not detected on TSA II agar plates either prior to inoculation or if the animal naturally cleared the assayed nostril (neg SA), to the next sample corresponding to SA positivity on TSA II plates (pos SA). Error bars represent mean±SEM.

These are paired comparisons of expression from samples transitioning from SA-negativity to SA-positivity from days 0-14 of each study. Absence was either prior to experimental inoculation or if the animal had naturally cleared SA, and presence was the first sample at which SA was detected on TSA II agar. IL-8 and IL-1β were both significantly up-regulated in the nasal fluid when SA was present (p=0.0098 and p=0.0350 respectively), but not in the serum which indicated a local innate immune response rather than a systemic response. In fact, there was no up-regulation of any of the ten analytes in the serum (data not shown), and animals did not display any visible symptoms of illness over the course of the 18-month study. which strongly suggests that the response in the pigtailed macaque to SANC is local to the nasal mucosa only as it is in the human condition.

Clearance of Experimentally Introduced SA is Reliant on Appropriate Shifts in Nasal Mucosal Expression of Neutrophil-associated Inflammatory Mediators

Nasal fluids were assayed with a 10-plex non-human primate-specific antibody profile to simultaneously measure the presence of IL-8, IL-1β, IL-1RA, MCP, MIP-1β, IL-17A, G-CSF, VEGF, IL-6, and TNF-α. Previously discussed, human studies have elucidated relationships between the local innate response indicated by levels of detection of several key inflammatory mediators and the SA load present in the nostrils. When SA is present, more neutrophil-associated proteins are produced by the nasal mucosal cells than when SA is absent for the purpose of recruiting neutrophils to the site of infection to aid in clearance. For four total independent inoculation studies, pigtailed macaques were cleared of SA by a five-day mupirocin

regimen, the nasal microbiome was allowed four weeks to normalize, and on "Day 0" animals were swabbed, and nasal proteins were collected, followed immediately by inoculation of SA. All animals were confirmed clear of SA on inoculation day by the absence of both β-hemolytic colonies on TSA II and purple colonies on CHROMagar. **Figure 11** shows paired transitional detections of analytes in individual animals' nostrils as they transitioned from SA-negative (neg SA) to SA-positive (pos-SA). These data points were further striated depending on whether the animal cleared SA (open red circles) from the analyzed nostril or carried consistently (green open circles) until conclusion of the follow-up period. Like the human condition, up-regulation of IL-8, MCP-1, and IL-1β corresponded to the individuals that cleared SA from the analyzed nostril. In addition to the increase in IL-1β expression in SA clearers, expression of the IL-1 receptor antagonist (IL-1RA) did not significantly change, resulting in an approximate 10-fold decrease in the IL-1RA:IL-1β ratio in the clearance group **(Fig. 11C)**. IL-1β expression was a bit higher at baseline in the carriers than the clearance group, but SA carriers lacked the up-regulation of IL-1β; however nasal mucosal cells significantly up-regulated the IL-1RA, with no change in the IL-1RA:IL-1β ratio. Expression of MIP-1β increased when nostrils transitioned from SA-negative to positive regardless of whether that nostril cleared or not, and detection of both TNF-α and IL-17A remained low in most samples (<10pg/mL) (data not shown). IL-17A has been implicated as a key cytokine in murine SANC experiments, but it does not appear to be important in the human or pigtailed macaque response to SA inoculation in the nose. These data together are suggestive that like human SANC, dysregulation of neutrophil-associated pro-inflammatory protein expression and the IL-1 pathway in pigtailed macaque nasal mucosa lends to sustained carriage of SA in the nose, likely due to decreased neutrophil influx and decreased production of antimicrobial peptides.
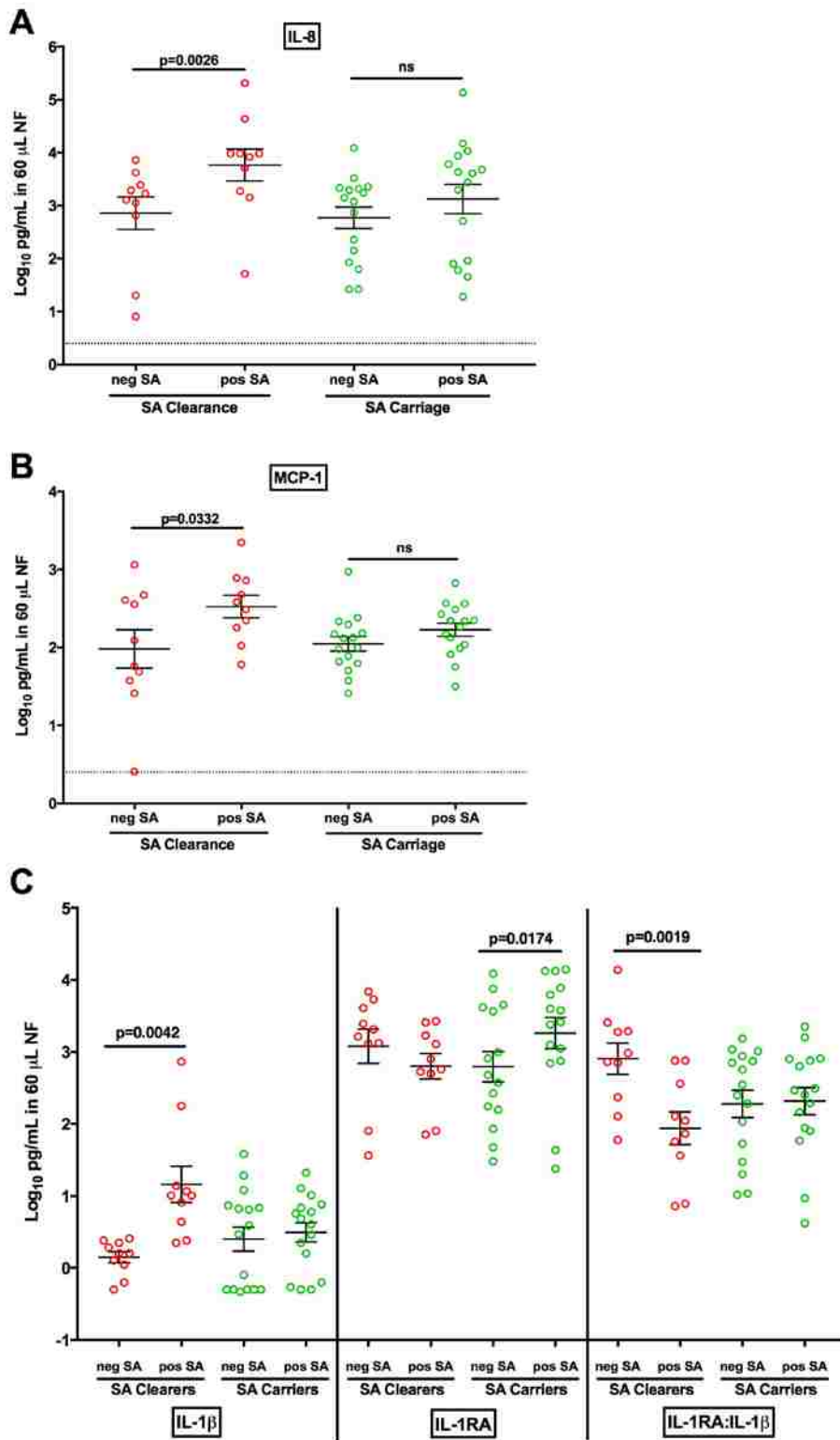
Figure 11: Clearance of Experimentally Inoculated SA from the Nares is Reliant on Appropriate Shifts in Nasal Mucosal Expression of Neutrophil-associated Inflammatory Mediators

Macaques were cleared of endogenous SA and reinoculated with various strains as described in Materials and Methods. Nasal swabs and nasal fluids were collected at days 0 and 3, then weekly following nasal inoculation with SA. Luminex multi-plex bead assays were used to detect pro-inflammatory mediators in nasal fluids. Cytokines A) IL-8, B) MCP-1, and C) relationships between IL-1β and its receptor antagonist, IL-1RA are shown as paired transitions from times at which animals were negative for SA (neg SA) to positive for SA (pos SA). Data was further striated into groups of those that naturally cleared the assessed nostril of SA (SA Clearers, red open circles) or maintained carriage for the duration of the study (SA Carriers, green open circles). Error bars represent mean±SEM.

Duration of SANC in Pigtailed Macaques Associates with Peaks in Expression of Inflammatory Markers

The Cole laboratory has previously conducted human SANC experiments in which 43 healthy adults self-applied mupirocin ointment to clear the nostrils of SA, then self-applied their own isolate again into each nostril [28, 58]. Subjects were swabbed in the nostrils, followed by nasal fluid collection via suction catheter on days 0 and 3, then weekly for a month of follow-up to compare SANC with nasal mucosal expression of pro-inflammatory proteins. Peak expression of IL-8 and IL-1β within 7 days correlated with shortened duration of SA nasal carriage, an average of 19±3 (mean±SEM) days, while a delayed response of ≥16 days correlated with longer carriage or no clearance at all [58]. Seen over the course of three independent inoculations, pigtailed macaques displaying peak expression within the first 7 days post-inoculation cleared SA within an average of 11±3 days, and the absence of peak expression or a delayed response of 16 days or more resulted in nasal carriage of at least 40 days (**Fig. 12**). Three animals with peak expression at 16 days post-inoculation or later maintained carriage until the final day of follow-up (red circles) and would have likely continued nasal carriage in the absence of antibiotic clearance. These points together suggest that IL-8 and IL-1β function similarly in humans and the pigtailed macaque, and appropriate up-regulations of these key inflammatory mediators in the nasal mucosa drive duration of *Staphylococcus aureus* nasal carriage.
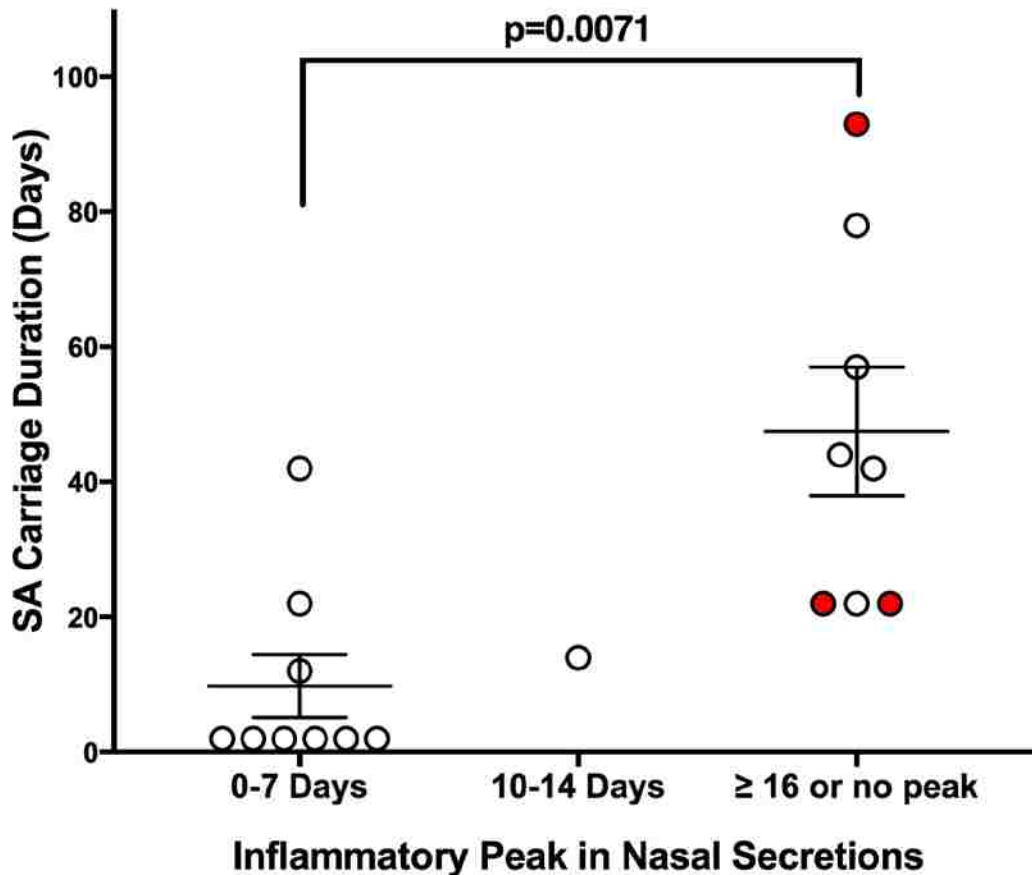
Figure 12: Duration of SANC in Pigtailed Macaques Associates with Peaks in Expression of Pro-inflammatory Mediators

For each of the 18 macaque inoculations, peak nasal expression of pro-inflammatory proteins IL-8 and IL-1β was assessed by Luminex multi-plex bead assays of extracted nasal fluids. Occurrence of peak detections (x-axis) per individual was plotted against duration of SANC in days (y-axis), error bars indicate mean±SEM. Red-filled circles indicate individuals that were still positive for SA in the assessed nostril on the final day of follow-up (thus SA carriage was likely longer).

<u>Discussion</u>

Previous human SANC studies have proven that human local responses to SA inoculation in the nose are neutrophilic in nature with observations of rapid and robust up-regulations of IL-8, IL-1β, and a decrease in the ratio between IL-1β and its competitive receptor antagonist (IL-

1RA) in human hosts with the ability to clear SA from the nose [28, 29, 58]. Human IL-8 is a chemokine that recruits neutrophils to the site of infection, and its increased expression is correlated with increased detection of SA CFUs [32], also observable in pigtailed macaque sample detections. The quick up-regulation of expression of the IL-8 gene is vital to human clearance of SA, and if it is not correctly regulated, SANC will likely persist. This aspect of human SANC is not modeled in rodents, not even in the more physiologically relevant cotton rat as the IL-8 gene is absent, however in the pigtailed macaque it is not only present, but functions similarly as it does in human SANC (**Fig. 11A**).

Staphylococcus aureus nasal carriage in humans is typically asymptomatic, even when SA is detected in the millions of CFUs per swab in healthy hosts [58]. Although the reasons and mechanisms behind this asymptomatic state is not well understood, the human innate response to SA presence is heavily neutrophilic, with local up-regulations of IL-8 and IL-1β in nasal fluid, among others [28, 29, 58]. Pigtailed macaque nasal mucosal cells also significantly up-regulate expression of IL-8 and IL-1β (p=0.0098 and 0.0350 respectively), with no systemic up-regulations detected in the serum (**Fig. 10**). Macaques also showed no changes in body temperature, appetite, body weight, behavior, and no other outward signs of illness were observed over the course of the 18-month study. Together, these data show that SANC is also an asymptomatic state in the pigtailed macaque.

Elucidations of the pigtailed macaque responses to nasal inoculations of SA were obtained through simultaneous detections of 10 pro-inflammatory mediators in the nasal fluid: IL-1β, IL-1RA, IL-6, IL-8, IL-17A, MIP-1β, MCP-1, G-CSF, VEGF, and TNF-α. Prior human SANC research showed that IL-1β, IL-8, MIP-1β, MCP-1 and G-CSF were significantly up-regulated in nasal fluids of the subjects that cleared SA from the nose [28, 58]. TNF-α and IL-6

are well-known pro-inflammatory cytokines; VEGF maintains homeostasis and remodeling of mucosal barriers damaged by inflammation [28, 98]; and IL-17A up-regulation is the primary response to nasal SA inoculation in rodents [15, 55]. For four independent inoculations, nasal fluids from one nostril per individual was analyzed as they transitioned from SA-negative status (day 0 or after natural clearance) to the next sample obtained when SA was detectable (SA-positive), then further striated based upon the individuals' ability to clear SA from the analyzed nostril or maintain carriage (**Fig 11**). These paired transitions elucidate that *Macaca nemestrina* accurately models the human neutrophilic response to SANC. The SA clearance group showed significant up-regulations of IL-8 (**Fig. 11A**), MCP-1 (**Fig. 11B**), and IL-1β with a 10-fold decrease in the IL-1RA:IL-1β ratio (**Fig. 11C**), indicating that like human SANC, dysregulation of expression of these neutrophil-associated pro-inflammatory mediators in the pigtailed macaque disrupts neutrophil diapedesis and SA clearance to allow persistence. IL-17A, VEGF, IL-6, G-CSF, and TNF-α expression were not significantly up-regulated when SA was present, and MIP-1β was up-regulated whether the animal cleared or carried SA (data not shown). This data aligns with previous studies implementing this species for human sexually transmitted infections and fetal respiratory development, each concluding that the pigtailed macaque is currently the most-human relevant model for mucosal interface structure and immune responses [64, 67, 99, 100].

Examining the detections of these pro-inflammatory mediators longitudinally over the course of each of the four independent SA inoculations revealed an important correlation between when expression of neutrophil-associated proteins and the resulting duration of SA carriage. A peak in IL-8 and IL-1β expression within the first 7 days corresponds to 9.8±4.6 days until SA clearance, significantly shorter than 47.5±9.5 days when the peak was at 16+ days

or not at all (p=0.0071) (**Fig. 12**). Three individuals indicated as red-filled circles were still positive for SA at the end of follow-up periods and would likely continue carriage in the absence of mupirocin application. IL-1β signaling is vital for induction of neutrophilic killing of SA by phagocytosis, oxidative bursts, and the release of neutrophil extracellular traps (NETs), and dysregulation of this cytokine and its non-productive receptor antagonist (IL-1RA) has been implicated to allow persistent carriage of SA [39]. The IL-1 axis is often dysregulated in diabetes and obesity; two risk factors for persistent SA nasal carriage that often lead to autoinfection [101] and nosocomial comorbidities. These significant observations in this model indicate that like human SANC, dysregulation of these pathways also leads to extended duration of SANC.

CHAPTER 4: THE NASAL MICROBIOME AS AN UNDEREXPLORED DETERMINANT OF *STAPHYLOCOCCUS AUREUS* NASAL CARRIAGE

Introduction

Nasal microbiota structure is temporally variable and is reactive to hygiene, stress, climate, environment, and interpersonal relationships [102], and not fully elucidated or understood. Displaying potentially extensive diversity, commensal microflora are an additional source of protection from pathogens through release of various protein products, and niche competition. Disturbances in a commensal population possibly contribute to inflammation, problematic in determining whether microbiome shifts act as the cause or the effect of SA infection, or both [103]. Numerous studies on human nasal commensal relationships have revealed specific species that at least partially inhibit persistent *SA* colonization through various mechanisms that alter the nasal environment [104]. An inverse relationship has been observed between SA and notable microbes like *Corynebacterium spp.* [45], *Propionibacterium spp.* [105], *Staphylococcus epidermidis*, [42, 102], and *S. lugdunensis* [46], likely through production of various bacteriocins [46, 102], niche competition, or competition for nutrients and surface area for attachment [48]. These associations affect SA colonization ability, presumed to be at least partially responsible for allowing persistent carriage in some individuals and not others in human studies, however most of these studies have used culturable methods. It has been shown recently through next-generation sequencing (NGS) methods that the most human-relevant rodent model for SANC, the cotton rat, do not support any of these important bacterial species that are found in human nostrils, but instead contain primarily Campylobacter species and potential human oral and gastrointestinal pathogens [61]. Prior research implementing this model did not consider the constituents of the nasal microbiome, however to validate the pigtailed macaque as a human-

relevant model of human SANC, these comparisons must be made. Prior studies have determined the pigtailed macaque as a physiologically relevant model for human reproductive and rectal mucosa, and through culturable methods determined that the mucosal normal flora endogenous to the vaginal tract is like humans in frequency of colonization with Lactobacilli [106, 107].

Analyses of the human microbiome composition has been limited because using Sanger sequencing requires streaking a series of different agar types, plucking colonies to incubate overnight, extracting DNA, running 16S PCR, sending samples for Sanger sequencing, and BLAST analysis of the results, which proves to be slow and expensive with low turnout [108]. Comparatively few species of bacteria will grow on plates, some are fastidious and require special additions, and some proliferate more rapidly than others, leading to overrepresentation of species with underrepresentation of others or missing them completely. Another aspect to consider is that culturable methods only allow detection of viable cells and it would be beneficial to determine if SA is present in the nostrils of those categorized as non-carriers either as free-DNA, dead cells, or viable but nonculturable cells (VBNC). SA frequently exists in a VBNC state in the low nutrient environment of the nasal mucosa [109] and is likely also present in non-carriers.

Significant advancements in NGS technologies and databases in recent years have improved acquisition of metagenomic information and have allowed this long and problematic process to be circumvented, and instead analyze all DNA present in a sample. Illumina MiSeq Sequencing System is a cost-effective, high-throughput system used for targeted gene sequencing, gene expression analyses, and metagenomics that has a maximum read length of 2x300 base pairs of the 16S rRNA gene [110]. The 16S gene is 1500 base pairs that encode the

constituent of the bacterial small ribosome that binds to the Shine-Dalgarno sequence when the ribosome is recruited to bacterial mRNA. The gene is made up of conserved and hypervariable regions, conserved regions being identical between bacterial species, and the hypervariable regions being species-specific, an aspect that is exploited in improvements in next-generation sequencing technologies [111]. The ability to identify down to genus or species level of a population sample relies on the hypervariable region(s) of the 16S gene chosen for amplification and the methods used, especially when several species are closely related, like those in genus Staphylococcus [109]. For this study, the V3-V4 hypervariable regions were chosen for amplification as it has been seen in prior research to provide the most accurate genus/species representation [111]. A positive feature of the advancements in sequencing is that upwards of 200 samples can be combined together into one library to analyze on the same microchip by adding a unique "barcode" not found in nature to each strand in an individual sample, so even after combination with many other samples, the microbial species detection data can be identified and separated apart [111].

Importantly, prior *in vitro* and *in vivo* methods in this study have shown the absence of both mupirocin and methicillin resistance in the three nasal macaque strains and the human isolate implemented, and all animals were successfully cleared of SA for several weeks while allowing the nasal commensal microbiome to normalize. The six healthy pigtailed macaques enrolled in this study are fed the same diet, treated identically, and live in climate and humidity controlled individual enclosures, which eliminates some of the free-will factors that contribute to fluctuations of the nasal microbiome in humans. It was evident through longitudinal plate counts on TSA II agar plates that mupirocin application temporarily decreased the non-SA commensal population in the nose, but by week three of the post-mupirocin normalization period that

population numbers were restored. Although population numbers were restored by week three, there was no way to know what commensal species were represented and what resulting environment was created by the post-mupirocin nasal microbiome. Since mupirocin inhibits a wide range of gram-positive bacteria, determination of the affected nasal commensal species other than *Staphylococcus aureus* and observation of any microbial shifts that occur following mupirocin application would be a beneficial addition to scientific knowledge and further understanding of the dynamics of SANC. This study seeks to i) compare culturable components of the human and pigtailed macaque nasal microbiomes, ii) determine culturable species affected in one pigtailed macaque's nose over three weeks and any microbial shifts once mupirocin opens the niche that SA occupies, iii) compare these culturable affected species to MiSeq detections in the same animal's nose, and iv) compare MiSeq detections of nasal commensals in select pigtailed macaque and human samples. This will broaden scientific knowledge as the pigtailed macaque nasal microbiome has not been described thus far, and the use of next-generation sequencing may provide insight to what culturable microbes are missed in plating methods. Comparisons between the relative abundance of human and pigtailed macaque nasal inhabitants will also provide another level of evaluation of the potential of this species as an improved model for human SANC.

Materials and Methods

Culturable Microbiome Analyses

The natural nasal microbiome of fourteen human and six pigtailed macaque nostrils were analyzed prior to any experimental manipulation. Representative aliquots over the course of both human [58] and macaque studies were reserved in sterile glycerol stock at -80°C until

further use. Selected samples were kept on dry ice to keep stock as a mostly frozen "slush", mixed with a sterile spatula, and approximately 100 μL of frozen slush removed and allowed to fully thaw. This was diluted 1:10 in HBSS, and plated on an array of six agars: nutrient agar (NA), plate count agar (PCA), CHROMagar, tryptic soy agar (TSA), tryptic soy agar/ 5% sheep's blood (TSA II), and mannitol salt agar (MSA). Each plate received 100 μL of sample and was incubated 24-48 hour. Colony morphology was recorded and plucked for overnight subculturing in 5 mL TSB (18 hrs/37°C/250rpm). Genomic DNA was extracted from these isolates using UltraClean Microbial DNA Isolation Kit (MO Bio Laboratories, West Carlsbad, California, USA) according to manufacturer's instructions, except instead of using 1.8 mL of sample, 800 μL were extracted unless bacterial pellet was insufficient. 16S PCR was performed, verified by gel electrophoresis, then sent for Sanger sequencing at Eton BioSciences (Research Triangle Park, NC). Sequencing files were analyzed using Mega7.0.20 software to align 16S sequences in preparation for BLAST analysis for prospective species identification. These identifications were analyzed using GraphPad Prism 7 software.

To analyze fluctuations in the culturable nasal microbiome due to 2% mupirocin application, the right nostril of animal Z08202 was chosen and three time points were chosen: i) the natural balance of the nasal microbiome at the first pre-mupirocin nasal swab (prior to any experimental manipulation), ii) week one post-mupirocin, and iii) week three post-mupirocin to determine the natural balance of species compared to the time point at which the population was decreased the most and when the population was restored. Early glycerol "slush" was removed from each sample, and the remainder was quickly returned to -80°C for future use. Fully thawed sample was serial diluted in HBSS at 1:10 and 1:100 and plated on Hoyle's agar, TSA II, plate count agar, and nutrient agar. These were incubated at 37°C for 24 hours (TSA II) and 48 hours

for growth. Plates were counted, twenty colonies per plate type were documented for colony growth morphology, plucked with a pipette tip, and each propagated in 5mL TSB overnight at 37°C in a shaking incubator. DNA extraction of each colony stock was performed and reserved at -20°C, with subsequent PCR to amplify the 16S rRNA gene. The presence of 16S rRNA amplification was verified through visualization of a distinct band corresponding to 1500 base pairs after gel electrophoresis. Successful amplifications were then sent for Sanger sequencing at Eton BioSciences (Research Triangle Park, NC). Sequencing files were analyzed using Mega7.0.20 software to align 16S sequences in preparation for BLAST analysis for prospective species identification. These identifications were analyzed using GraphPad Prism 7 software.

DNA Preparation for Comprehensive Microbiome Analyses

To analyze fluctuations in the comprehensive nasal microbiome due to mupirocin application, total DNA extractions were performed of representative "early" glycerol stocks of Z08202 left nostril corresponding to sample dates analyzed using culturable methods. These were fully thawed on wet ice, vortexed well, and 800 μL was transferred per sample into a 1.5mL microcentrifuge tube, reserving the remainder at -80°C for future applications. 800 μL of each sample was spun down at 14000 xg for 10 minutes at 4°C, then 500 μL of the supernatant was spun through a MWCO 3 Amicon Ultra filter for 20 minutes at 14000 xg to obtain a concentrated super for inclusion in the DNA extraction process. We modified the ZymoBIOMICS DNA Microprep kit (Zymo Research, Irvine, CA Lot #ZRC201522) protocol to include a heated enzymatic incubation step by resuspending each pellet in 41 μL Tris/EDTA (TE) buffer, 50 μL (10mg/mL) lysozyme, 3 μL (4KU/mL) lysostaphin, and 6 μL (25 KU/mL) mutanolysin and incubating at 37°C/1hr/350-400 rpm. This enzymatic incubation and additional

spin of supernatant was to promote lysis of any hardier gram-positive cell walls present and prevent exclusion of smaller microbes that may not have been pelleted. 200 µL of combined pellet and retentate was DNA extracted following instructions provided. Resulting DNA concentrations were determined using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, P11496) in a 96-well format with fluorescence read by SpectraMax i3X to ensure adequate DNA extractions of at least 5 ng/µL. Data was exported to Excel to determine dilution schemes to input 5ng/µL of each sample into DNA library preparation for Illumina MiSeq sequencing of the V3-V4 hypervariable region of the 16S rRNA gene.

Using longitudinal carriage data, six human and six pigtailed macaque samples were extracted implementing this same protocol for inclusion in this preliminary MiSeq run to determine similarities and differences in comparative carrier and non-carrier/clearer nasal microbiomes. Macaque samples were analyzed at days 0 and 3, weekly until day 22, then at time points when individual animals naturally cleared SA, swabbed positive again, and either cleared again naturally or at the last day of follow-up. Human persistent, intermittent, and non-carrier samples were collected at less consistent time points than the macaques, but samples with at least a 2-week period in between collections were chosen, if possible.

Illumina MiSeq Library Preparation

For this project, the taxonomic compositions of 15 samples were analyzed to determine comparative microbiomes of healthy pigtailed macaque and human SA nasal carriers versus non-carriers/clearers. 5 ng/µL of total microbial DNA extractions from representative nasal swab glycerol stocks were each individually entered into a first stage PCR to amplify the V3-V4 hypervariable region of the 16S gene out of genomic DNA following protocols and using

primers and kits provided by Illumina MiSeq. This was done using previously described 341F, 785R primers [112] with universal overhang adapters upstream and downstream of the V3-V4 region. Quality control of PCR products were run on a TapeStation system to verify the approximate 550 bp size of the amplicon plus the two ~50 bp adapters. A cleanup step followed using beads graciously provided by Dr. Anna Forsman at the University of Central Florida to eliminate free primers, primer dimers, and any amplicons that were not the approximate 550 bp size. These were then entered into a second round of PCR to add unique indices to each amplicon in each individual sample, followed by an additional beaded cleanup to purify the final library prior to another quantification by PicoGreen assay, and finally pooling samples into an equimolar library. This resulting library made up of approximately 255 individually barcoded samples in equimolar representations was sent to the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR), then processed using MiSeq v3 reagent kit for optimal kinetics. Prior to cluster generation and sequencing, a denaturation process using buffers and heat must be done, then the library is diluted, transferred to the flow cell, and run on the MiSeq system.

Taxonomic Analyses

Percent representations of Sanger sequencing detections of human and macaque nasal microbiome species/genera were analyzed using GraphPad Prism 7 and calculated as part of a whole (**Figs. 13-15A**). Dr. Shibu Yooseph of the University of Central Florida kindly analyzed resulting Illumina MiSeq sequence data using the USEARCH/UPARSE program suite algorithms to rapidly search global databases and cluster sequences with a reported ≤1% incorrect base error rate [113, 114] (**Figs. 15B-17**). Taxonomy was assigned to sequences

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implementing a naïve Bayesian classifier in mothur [115, 116] to define the relative abundance of taxa present in each analyzed nostril, mostly represented at the genus level.

<div align="center">Results</div>

Humans and Pigtailed Macaques Support Similar Culturable Nasal Commensals

Fourteen human and six pigtailed macaque swab fluids were diluted in HBSS and plated on an array of agars to analyze comparative culturable natural nasal microbiomes. A total of 83 colonies were plucked from human plated samples, and a total of 182 colonies were plucked from macaque sample plates. The relative abundance of species detected through plucking colonies and Sanger sequencing of the full-length 16S rRNA gene revealed a majority of species in common between humans and macaques (**Fig. 13**).
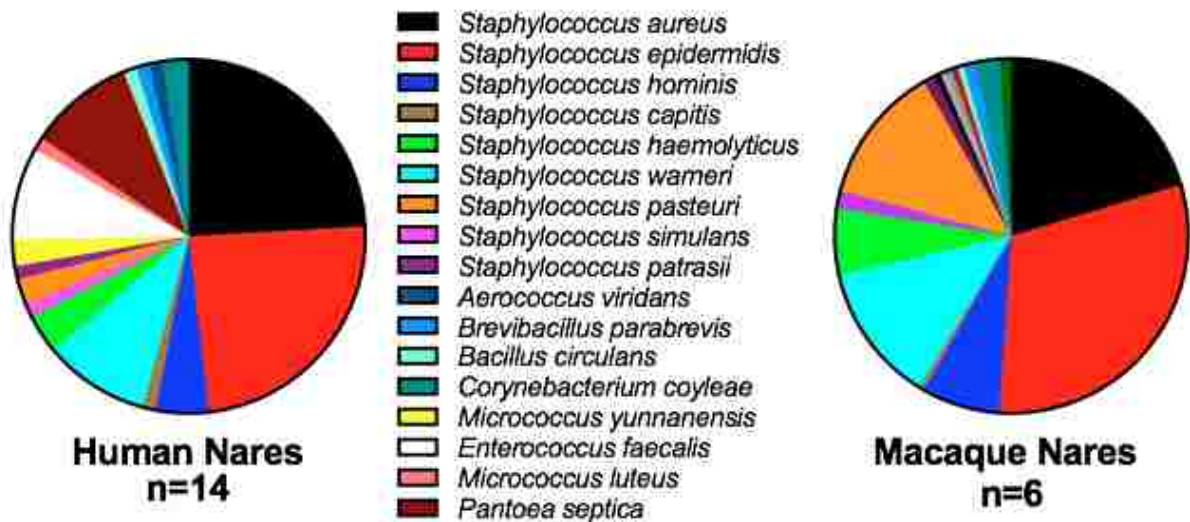

Figure 13: Humans and Pigtailed Macaques Support Similar Culturable Nasal Commensals

Representative glycerol stocks from 14 human and 6 macaque nostrils were dilution plated on an array of agars and incubated 24-48 hours, then colonies were described and proliferated overnight in TSB for DNA extraction. 16S rRNA gene was amplified for each colony and sent for Sanger sequencing to identify each. These preliminary investigations into the natural nasal inhabitants of human and captive pigtailed macaques show a predominance of Staphylococcal species in the culturable population, mainly *S. aureus* and *S. epidermidis,* as well as many additional microbial species in common. n= 83 human and 182 macaque colony identifications.

Staphylococcal species represented the majority of 16S species identifications, with humans supporting 72.3%, and pigtailed macaques supporting 92.9% including: *S. aureus*, *S. epidermidis*, *S. hominis, S. capitis, S. haemolyticus, S. warneri, S. pasteuri, S. simulans, and S. patrasii*. In terms of *Staphylococcus aureus* representation specifically, pigtailed macaque nostrils contained 20.3%, while humans supported 24.1%. Roughly 100 more colonies grew on macaque dilution plates with the same dilution scheme as the human samples, indicating that as expected, these animals support more bacteria as they lack the basic hygiene of humans. Two major Staphylococcal species comprised roughly half of both human and pigtailed macaque total species identifications, *S. aureus* and *S. epidermidis,* however further analyses should be explored to determine if this is true due to competition of these common skin flora for the same niche, or due to the bias created by using culturable methods as Staphylococcal species are hardy and proliferate well on a wide variety of media.

Mupirocin Significantly Reduces the Nasal Staphylococcal Population Causing a Microbial Shift

To determine the effect of nasal application of mupirocin ointment to the microbiome, one animal's natural microbial balance in one nostril prior to any experimental procedures was compared to weeks one and three post-mupirocin application in the same nostril (**Fig. 14**). These representative glycerol stock samples were dilution plated on Hoyle's, nutrient, plate count, and TSA II agars, with 20 colonies per plate type (if present) identified by Sanger sequencing of the

16S gene. Supporting prior observations of the original weekly longitudinal carriage counts, week one post-mupirocin dilution plates showed that along with SA eradication, there was also a significant decrease in non-SA population CFU counts that recovered by week 3 (pre-mupirocin n=83 colony identifications, week 1 post-mupirocin n=57, and week 3 post-mupirocin n=73). **Figure 14** shows the colony species identifications that are shared between the three analyzed time points with any identifications not shared compiled into an "other" category for clarity.
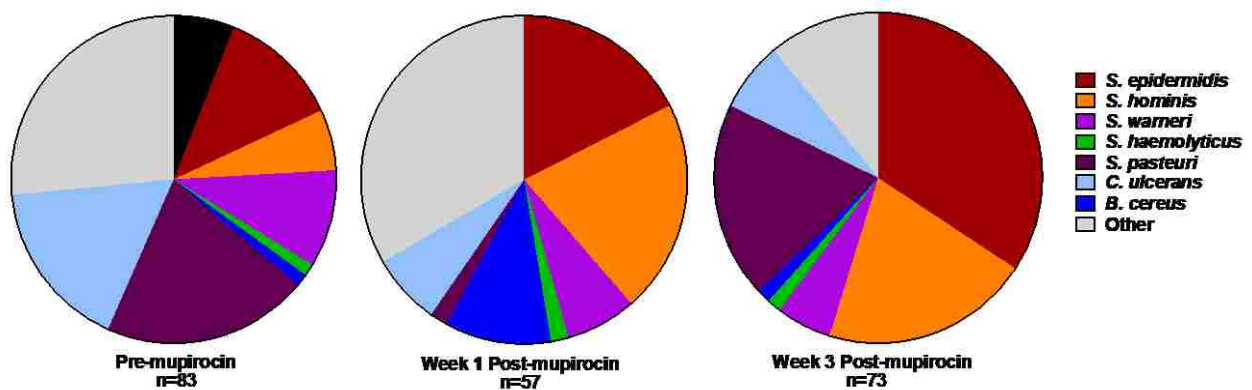
Figure 14: Application of 2% Mupirocin to the Nares Causes a Microbial Shift

Representative glycerol stocks from one individual at pre-mupirocin and weeks one and three post-mupirocin were each individually plated on an array of agars and incubated 24-48 hours, then colonies were described and proliferated overnight in TSB for DNA extraction. The 16S rRNA gene was amplified for each colony and sent for Sanger sequencing to identify each at the species level. Eradication of SA and significant inhibition of *S. pasteuri* provides a niche for other less inhibited species to shift into.

In this individual's right nostril, the natural culturable microbiome contained 6% SA (in black), 12% *S. epidermidis*, 6% *S. hominis*, 20.4% *S. pasteuri*, 17% *Corynebacterium ulcerans*, and 26.5% other species prior to any manipulation. By week one post-mupirocin, SA was eradicated, *S. pasteuri* decreased by 19%, and *C. ulcerans* decreased by 10%, allowing a 5% increase of *S. epidermidis*, a 15% increase in *S. hominis*, and detection of "other" species increased by 7%.

This indicates that eradication of SA and significant inhibition of *S. pasteuri* and *C. ulcerans* by mupirocin application, allows other species, including Streptococcus, Bacillus, Psychrobacter, and other Staphylococcal species to proliferate into the unoccupied niche. By week three post-mupirocin, *S. epidermidis* and *S. hominis* made up more than half of the species identifications, *S. pasteuri* increased by 17% and the "other" species decreased by 22%, indicating a decrease in biodiversity as Staphylococcal species flourish and outcompete other less-represented nasal commensals. Again, these associations were made through implementation of culturable methods and as such are likely biased toward Staphylococcal species, however are still useful in comparisons NGS detections.

Comparison of Nasal Microbiome Determined from Culturable Bacteria Versus a DNA-based MiSeq Approach

The Illumina MiSeq platform was used to sequence the V3/V4 hypervariable regions of the 16S gene of total microbial DNA extracted from Z08202's representative glycerol stocks of the left nostril that correspond to the same days as prior culturable analyses of the right nostril in which the 16S gene in its entirety was sequenced from colonies grown on an array of agar plates. **Figure 15** shows genera detections through both Sanger sequencing of the full 16S gene (**Fig. 15A**) and MiSeq sequencing of V3/V4 (**Fig. 15B**). In comparing the resulting detections of individual strands of DNA present in representative samples, it is evident that Staphylococcal species are over-represented when using culturable methods. Corynebacterium species are under-represented through culturable methods, especially seen in weeks one and three post-mupirocin where culturable methods only allowed detection of 22.8% and 6.8% respectively, while in the relative abundance detected through MiSeq sequencing were approximately 55% and 72% respectively. Week one detections using MiSeq showed very low Staphylococcal

species representation, and approximately 50% Corynebacterium, which is very different than culturable detections. MiSeq allowed detection of Dolosigranulum, a genus that only has one known species, *D. pigrum,* which was not detected on any agar plate at any time.
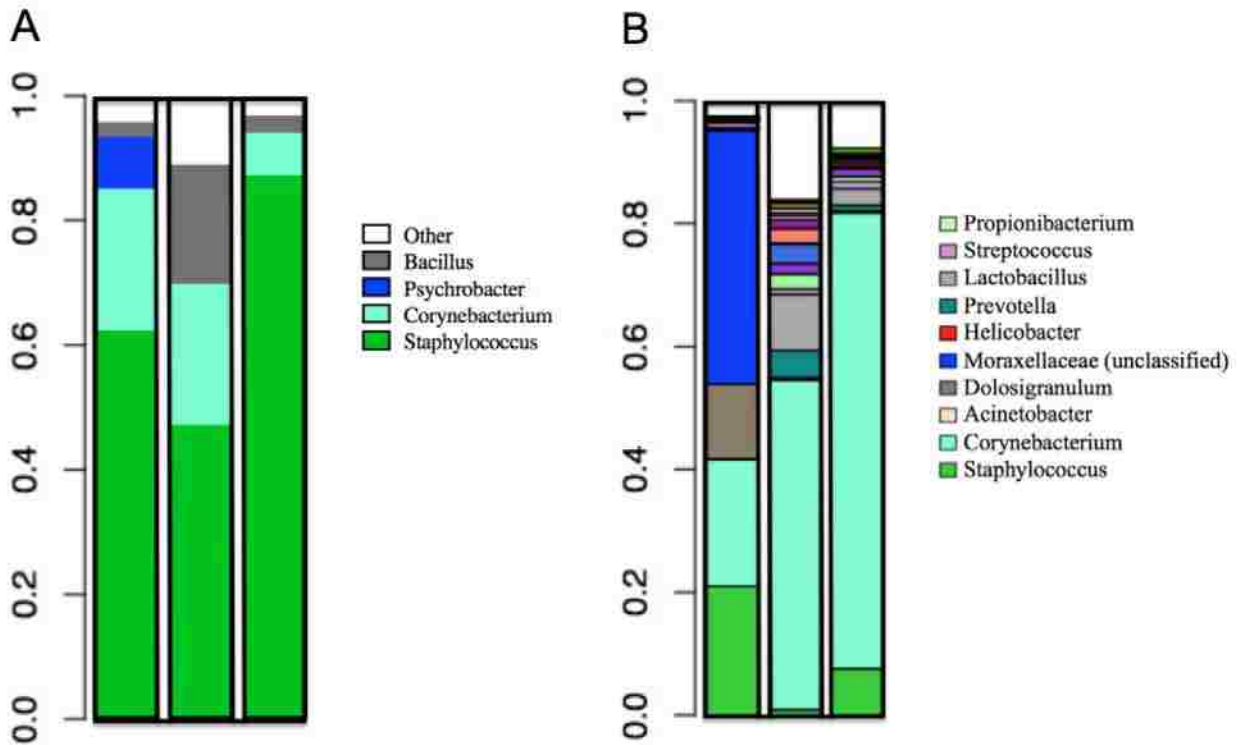
Figure 15: Comparison of Nasal Microbiome Determined from Culturable Bacteria Identification Versus a DNA-based MiSeq Approach

Comparisons of relative abundance of genera present in the nose of one individual macaque detected through: A) Sanger sequencing of the full-length 16S rRNA gene of plated colonies (right nostril), and B) Illumina MiSeq detection of the V3-V4 hypervariable region of all microbial DNA present in representative samples (left nostril). The three bars in both A and B from left to right represent pre-mupirocin, week 1, and week 3 genera detections respectively.

MiSeq also detected an unclassified Moraxellaceae family member at about 40% relative abundance prior to mupirocin treatment, which was eradicated with mupirocin treatment. A member of this family was identified in the pre-mupirocin culturable Sanger identifications,

Psychrobacter pulmonis, so this is likely at least part of the unclassified Moraxellaceae detected along with another member, genus Acinetobacter. The genus Acinetobacter was not specifically detected in this animal's nostrils but was detected with MiSeq in other pigtailed macaques at many times throughout the study as well as many human samples. Comparisons between Sanger and MiSeq showed that mupirocin treatment increased biodiversity at least temporarily as indicated by increased detection of "other" species, indicated more clearly in **Fig. 15B**, as well as the limitation of culturability that creates bias when plating samples.

MiSeq Detected Similar Nasal Microbiome Profiles in Pigtailed Macaque and Human Nasal Samples

Illumina MiSeq sequencing of V3/V4 hypervariable regions of the 16S gene was used to analyze the comprehensive nasal microbiome following extraction of total DNA from representative glycerol stocks reserved over the course of a prior human study [28] and one independent SA inoculation in the nostrils of the six pigtailed macaques. Samples were analyzed at days 0 and 3, weekly until day 22, then at time points when individual animals naturally cleared SA, swabbed positive again, and either cleared again naturally or at the last day of follow-up. Human persistent, intermittent, and non-carriers were analyzed for future determinations of differences in nasal microbiome components, if any. Comparisons of the relative abundance of genus-level identifications were made between three human and three macaque samples from individuals that maintained persistent nasal carriage of SA (**Fig. 16**). Staphylococcus and Corynebacterium represented more than 75% of the genus make-up of the nasal microbiome of SA carriers, with few other genera represented in the remaining 10-20% detections. Dolosigranulum, Acinetobacter, Streptococcus, and others were represented in much smaller abundance in both human and pigtailed macaque samples.
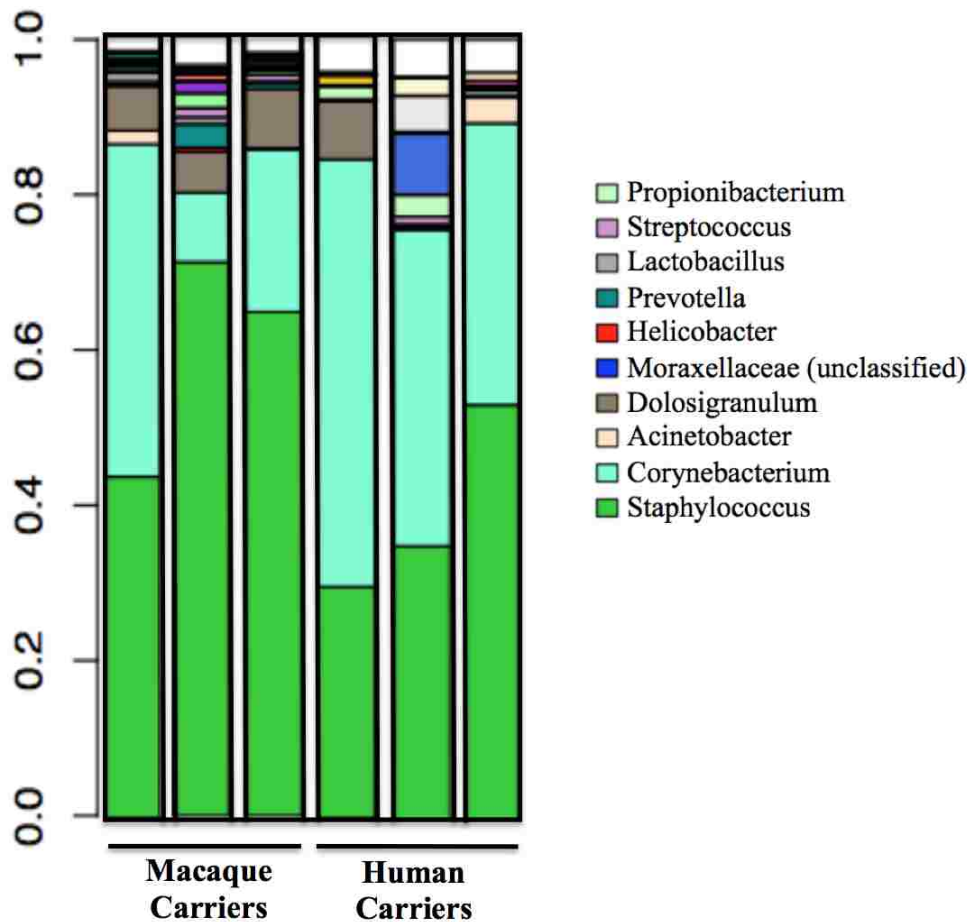
Figure 16: Pigtailed Macaque and Human Persistent SA Carriers Support Similar Genera in Nasal Microbiome as Detected Through Illumina MiSeq

V3-V4 amplicons of the 16S rRNA gene of extracted DNA from representative sample glycerol stocks from three human and macaque persistent carriers were sequenced implementing the Illumina MiSeq platform. Comparative relative abundances (y-axis) of the ten most represented genera (legend on the right) are shown, with Staphylococcal and Corynebacterium species predominating at an approximate 80% of the detections in both human and macaque carrier groups.

Comparisons of the relative abundance of genus-level identifications were made between three human non-carriers and three macaques that quickly cleared SA from the nose using longitudinal carriage data in selection of subjects to include in comparisons (**Fig. 17**). These samples contained considerably less Staphylococcal, Corynebacterium, and Dolosigranulum genera than

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the relative abundances found in nasal SA carriers (**Fig. 16**), but interestingly had noticeably increased representation of Acinetobacter in both human and macaque samples at about 30%, also with more biodiversity in most samples.
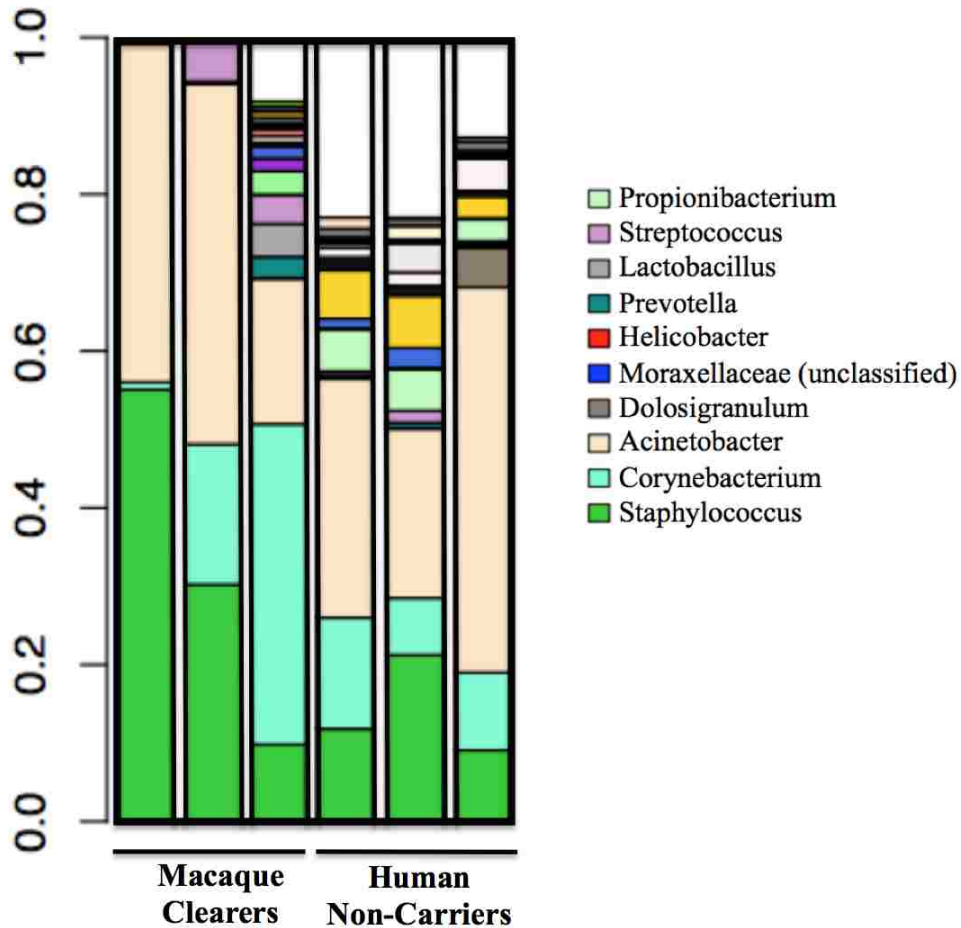
Figure 17: The Nasal Microbiomes of Pigtailed Macaques that Clear SA from the Nose Compared with Human Non-carriers Contain a Significant Percentage of Acinetobacter

V3-V4 amplicons of the 16S rRNA gene of extracted DNA from representative sample glycerol stocks from three human non-carriers and three macaque clearers were sequenced implementing the Illumina MiSeq platform. Comparative relative abundances of the ten most represented genera (legend on the right) are shown, with significant detections of Acinetobacter in each, which was not observed in persistent carriers.

<center>Discussion</center>

The components of the pigtailed macaque nasal microbiome have not been characterized prior to this investigation into the suitability of this proposed model for human *Staphylococcus aureus* nasal carriage, however previous studies have shown that the vaginal mucosal microbiome is similar to human females [66, 71, 99, 107]. The human nasal microbiome is underexplored due to low pressure from disease states, but it is known to vary between individuals with many factors contributing to the species supported in the nose as well as SANC. Initial preliminary investigations were limited by culturability on an array of agars, but did allow determination that both macaque and human nares support *Staphylococcus aureus*, a feature lacking in historic rodent models. Culturable methods also showed that representative swab fluids contained mostly Staphylococcal species in both humans and macaques, with SA, *S. epidermidis*, *S. hominis, S. warneri, S. capitis*, and *S. haemolyticus* in similar proportions in both (**Fig. 13**). Application of mupirocin antibiotic ointment in the nose significantly affects the culturable nasal microbiome, as many other gram-positive nasal commensals are inhibited along with SA eradication. *S. pasteuri* and *C. ulcerans* were significantly decreased by week one post-mupirocin, allowing *S. epidermidis*, *S. hominis*, and several "other" genera like Bacillus and Streptococcus to temporarily flourish and occupy the niche left open in an increase in biodiversity (**Fig. 14**). By week three post-mupirocin, the population counts had restored, and *S. epidermidis*, *S. hominis*, and *S. pasteuri* overtook most of the culturable nasal microbiome, decreasing biodiversity by displacing the "other" species by a 20% decrease in detection.

Realizing that culturability of microbes is a limiting factor for microbial identification, the Illumina MiSeq sequencing platform was implemented to detect each piece of bacterial DNA in representative samples frozen in glycerol rather than only what will grow on an array of

<center>83</center>

plates. Comprehensive detections of representative samples using NGS methods are reliant on the chosen method, as well as several other factors. The nasal mucosa has a relatively low bacterial biomass as compared to the lower respiratory system and the gut, and is made up mostly of difficult to lyse gram positive microbes. Without an enzymatic step, many cell walls will fail to lyse, and misrepresentation of inhabitants is likely. All analyzed samples were treated with identical enzymatic treatments and library preparation steps to maintain consistency and prevent wasting irreplaceable representative samples.

Comparing the percentages of genera detected on plates against what was detected through MiSeq when analyzing the effect of mupirocin on the same animal's nose, it is evident that Staphylococcal species are highly overrepresented using culturable methods, by 40% or more between each of the three days analyzed (**Fig. 15**). The MiSeq platform allowed for a wider range of microbial identifications that were not detected on agar plates, however culturable methods allowed identification of Psychrobacter species, while MiSeq detected the family it belongs to, Moraxcellaceae. MiSeq data represents a roughly even relative abundance of the natural balance of Staphylococcal and Corynebacterium species detected in the pre-mupirocin sample (**Fig. 15B**), as well as detection of Dolosigranulum, which was never detected on agars. Week one post-mupirocin showed an increase in biodiversity, which was also reflected in culturable identifications, but week 3 post-mupirocin MiSeq detections show that Corynebacterium overtook the rebounded population, conflicting with culturable methods, which showed primarily Staphylococcal species (**Fig 15**). Using these sequencing methods in conjunction with each other is beneficial to acquire additional information than either one singly can provide.

Preliminary comparisons between the nasal microbiomes of human and pigtailed macaques were made implementing the MiSeq platform to determine if the nasal environment is similar in terms of commensal factors in addition to the similarities in carriage patterns and resulting pro-inflammatory protein expression that we have already observed. Microbial profiling of human and macaque persistent carrier samples revealed that the nasal microbiome is made up of approximately 75% Staphylococcal and Corynebacterium genera (**Fig. 16**), which aligns with prior findings [118, 119]. In addition, we observed a trend of similar profiles with significant representations of Acinetobacter and decreased Corynebacterium and Staphylococcal identifications in samples collected from pigtailed macaque clearers compared with human non- and intermittent carriers (**Fig. 17**). Interestingly, gram-negative *Acinetobacter baumannii* has previously been implicated as a major human pathogen with the propensity to acquire drug-resistance and maintain long-term concomitant persistent nasal carriage with MRSA in healthcare settings that can become invasive and life threatening [120, 121]. There are at least 46 species within the Acinetobacter genus, and since the MiSeq did not resolve down to the species level, we cannot assume that this detection is *A. baumannii*, but this genus was detected in similar relative abundances in both human and macaque non-carrier nasal microbiomes. This data suggests that whichever Acinetobacter species detected in these samples competes for niche occupancy with Staphylococcal and Corynebacterium species as these were found in less relative abundance in noses containing increased abundance of Acinetobacter. With respect to bacterial diversity in general, it appears that the clearers/non-carriers support a more diverse microbiome in most samples than persistent carriers in both the pigtailed macaque and human samples (**Fig. 16 and 17**). As previously discussed, the nasal microbiome of the most relevant rodent model for SANC has been previously characterized as primarily Campylobacter species [61] through

next-generation sequencing methods. Importantly, similar methods implemented in this study did not detect any of the species identified in the cotton rat model in either human or pigtailed macaque samples.

Prior human studies have suggested that Corynebacterium is a major inhibitor of SA colonization in the nares [48]; however, Corynebacterium was found in all nostrils whether positive or negative for SA, and was detected in a greater relative abundance in persistent nasal carriers. These findings likely support other groups that have suggested that interactions with Corynebacterium species causes a shift in SA fitness that promotes commensalism over virulence [45], allowing prolonged persistence in the nares with decreased immune detection.

This nasal microbiome data, paired with prior research that identified similarity in the reproductive system microbiome, supports the pigtailed macaque as a better model than rodents for the dynamic interplay between SA and the non-SA nasal commensal population during *Staphylococcus aureus* inoculation experiments. Comparisons between human and macaque nasal microbiomes have never been made, and elucidations of the similarities between taxonomic compositions of carriage groups will prove valuable in future applications of this model. Illumina MiSeq detections of V3-V4 amplicons of the 16S rRNA gene only allowed for mostly genus-level identifications, therefore future inquiries into the pigtailed macaque and human nasal microbiomes should implement a more sensitive method that can acquire species-level identifications.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Staphylococcus aureus nasal carriage is prevalent in at least 30% of the healthy world population and presents a risk of autoinfection to the host and dissemination to new immunocompromised hosts in nosocomial and at-risk communities. SANC is a dynamic state, and is influenced by SA strain genetics, host immune responses, and the nasal mucosal environment, which is impacted by factors like climate, health of the host, and non-SA commensals. The mechanisms that impact SANC are not fully understood as it results from complex interactions, and the lack of a human-relevant model prevents further study due to the ethical and procedural limitations set for human research. Historically implemented rodent models for SANC do not translate well to the human condition as they resist enormous nasal inoculations, resulting in only transient carriage. Murine immune responses lack key components that play primary roles in the human's heavily neutrophilic response to SA inoculations, and instead clear the nostrils through T cell mediation. Longitudinal studies are impossible with rodent models due to their small size, however SANC is very individualized, and longitudinal sampling is necessary to understand immune and nasal commensal responses to inoculations. Transitions of potential therapeutics to clinical trials from rodent models often fail, therefore development of an improved model is necessary for future research and clinical trials of novel antibiotics to combat the rapid evolution of SA antibiotic resistance.

Unlike rodents, pigtailed macaques naturally carry SA in the nose and throat and are easily decolonized of SA with 2% mupirocin topical antibiotic following an identical protocol used to clear the nostrils and subsequently the body of pre-surgical humans. Nasal and extranasal carriage was established in all six animals with a human-relevant inoculation of 10^4

CFUs per nostril of a variety of strains, with the nasal environment serving as a suitable reservoir for passive transmission of the same strain to the rest of the body. This important aspect provides a route around a significant limitation to human SANC studies, as in human SANC studies, only self-strains are ethically permitted for inoculation and must be self-applied by the subject. Acquisition and retention of human hosts for SANC studies has proven difficult as humans have free-will and are unpredictable, and in the absence of symptoms, the return to a laboratory for nasal swabs and nasal fluid collections becomes a burden and subjects are frequently lost from studies. This cohort of captive pigtailed macaques are maintained in individual enclosures with climate and humidity control, and are treated and fed the same, which eliminates the free-will aspect of study subjects as well as many environmental factors that can impact SANC. Additionally, this sufficient low inoculum size of 10^4 CFUs per nostril is a significant finding because in rodent models, only transient carriage of only a few days is established with enormous inoculations in a much smaller nose, even when antibiotics are added to drinking water to inhibit competition from nasal microbiome inhabitants. Further modeling the human condition of SANC, pigtailed macaque nostrils maintain the highest density of SA, which is an aspect not observed in rodent models of carriage. A three-week pilot study found that a human SA isolate shared genetic similarity to macaque isolates and nasal colonization was just as easily accomplished with the same inoculum size in this model as inoculations with various macaque SA strains. Once established, SANC in the pigtailed macaque is easily followed longitudinally with a simple nasal swab, a convenience that is lacking in rodent models, which results in the sacrifice of a large number of rodents, often without any benefit as many will clear SA rapidly from the nose. Carriage patterns in healthy pigtailed macaques model the

human well, and present as mostly intermittent with some individuals able to clear SA from the nostrils naturally while others maintain persistent carriage.

Macaques that were able to clear the nostrils of SA displayed up-regulations of key pro-inflammatory mediators once SA was detected as compared to its absence. These proteins are also up-regulated in the human response to SA and are associated with neutrophil recruitment and maturation as well as exacerbation of the inflammatory response in the nasal mucosa. Key neutrophil-associated pro-inflammatory proteins implicated in prior human research to mediate clearance of SA from the nasal mucosa were found to play important roles in the pigtailed macaque as well, also not modeled in even the most relevant rodent model. In macaques that were able to clear SA from the nose, IL-8, IL-1β, and MCP-1 were significantly up-regulated in the nasal mucosa as detected through multi-plex bead assays of nasal fluids, as well as a 10-fold decrease in the IL-1RA: IL-1β ratio. These up-regulations were not detected in the serum, and when paired with husbandry charts indicate that SANC in the pigtailed macaque is asymptomatic as it in the human condition. Animals that maintained persistent carriage failed to establish this required shift in pro-inflammatory expression similar to human persistent carriers. Carriage duration correlated with temporal peaks in IL-8 and IL-1β expression, the earlier the peak the more quickly clearance occurred. Animals that failed to accomplish a peak in expression or the peak occurred at 16 days or later maintained carriage for significantly longer or were unsuccessful in clearance of SA. This data suggested that as in humans, dysregulation of factors involved in neutrophil recruitment and function in the pigtailed macaque nasal mucosa leads to prolonged duration of carriage and/or inability to clear SA from the nose.

Pigtailed macaques and humans host similar microbes in the nose as determined through both culturable methods and MiSeq analysis of representative swabs samples collected

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longitudinally, with similar relative abundance of taxa, namely Staphylococcal, Corynebacterium, Dolosigranulum, and Acinetobacter. Species in the Acinetobacter genus may correlate to a shortened duration of SANC rather than previously implicated Corynebacterium, which was present in all samples. Like humans, pigtailed macaques support diverse genera in the nose, and the nasal microbiome appears to act as an additional major factor of SANC observed by notable differences in abundances observed between carrier and non-carrier groups.

This is the first study of its type to implement the pigtailed macaque in SA nasal inoculation studies, and these significant findings reveal great future potential applications for this model. Since these animals naturally carry SA in the nose and throat, they would be useful as a decolonization model for newly developed antimicrobials against SA that would not necessarily require inoculations of SA, but a simple screening process to determine carriage status. Through a series of directed inoculation studies, it was found that a variety of SA strains could establish carriage and persist in the nostrils, including a human nasal isolate. This indicates the likelihood for a wide variety of SA strains to successfully establish carriage, including potentially antibiotic resistant and virulent strains, providing a potential way to circumvent this limitation placed on human SANC studies. Longitudinal follow-up of SANC would be useful in conjunction with pro-inflammatory protein detections of corresponding days to observe human-relevant immune responses to virulent SA. In addition to SANC, considering the MiSeq analysis of the nasal microbiome revealed similarities in bacterial species and their relative abundances, proposed inhibitors of SA like Corynebacterium and Acinetobacter species could be introduced into the nostrils to correlate not only to duration of carriage, but also changes in SA gene expression in a living model as these kinds of studies have mostly taken place *in vitro*.

In terms of possibilities for future application of this project specifically, the second aliquot of nasal fluids analyzed through multi-plex bead assays are reserved, with potential for future detections of additional factors of SANC, such as defensins that could be correlated to the data already discussed herein. The acid extracted nasal fluids of only one nostril were assayed for ten potential mediators in the inflammatory response, therefore the protein laden nasal strips corresponding to the opposite nostril are still reserved at -80°C and could be extracted and assayed for the analytes found significant in this study in addition to others. Also remaining at -80°C are representative glycerol stocks of the swab samples collected from the nostrils not implemented in the Illumina MiSeq microbiome analyses that could be analyzed using more sensitive metagenomic detections to identify down to the species level. With this model, knock out studies of adhesion proteins, virulence factors, and more could be performed, and elucidate mechanisms that have not been available for *in vivo* study thus far. Future directions of this laboratory in implementation of the pigtailed macaque as a human-relevant model for SANC aim to specifically focus on longitudinal analyses including: internalization of SA in nasal mucosal cells, SA genome evolution, and alterations in SA protein expression as it progresses from establishing adhesion and colonization to once prolonged nasal carriage is established. These analyses will increase understanding of other factors that may allow SA to persist through multiple methods of evading the immune system.

Together, these multi-dimensional analyses of the potential for the pigtailed macaque, *Macaca nemestrina,* as a model for human SANC demonstrate that they are much improved as compared to historic rodent models. As *Staphylococcus aureus* continues its rapid evolution of antibiotic resistance to every medication used to combat infections, a new model is crucial for discovery and development of novel therapies with the hopes of increased potential for success

in clinical trials. SANC is a highly dynamic and individualized state impacted by a multitude of factors that are not fully understood yet. In terms of human studies, the science has progressed as far as it can to elucidate dynamics between SA and the host immune system. Further understanding of the relationship between SA and the resident nasal microbiome, elucidating methods of immune evasion, and identifying shifts in SA protein expression is necessary for progressive intervention strategies. Circumvention of experimental and ethical limitations placed on human SANC research implementing the pigtailed macaque would better scientific knowledge on this multifactorial state and ease the burden on healthcare with improved decolonization strategies in nosocomial and at-risk community settings.

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