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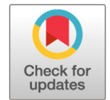


# Evaluation of the Pig-Tailed Macaque (*Macaca nemestrina*) as a Model of Human *Staphylococcus aureus* Nasal Carriage

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**ABSTRACT** *Staphylococcus aureus* nasal carriage is a common condition affecting both healthy and immunocompromised populations and provides a reservoir for dissemination of potentially infectious strains by casual contact. The factors regulating the onset and duration of nasal *S. aureus* colonization are mostly unknown, and a human-relevant animal model is needed. Here, we screened 17 pig-tailed macaques (*Macaca nemestrina*) for *S. aureus* carriage, and 14 of 17 animals tested positive in the nose at one or both screening sessions (8 weeks apart), while the other 3 animals were negative in the nose but positive in the pharynx at least once. As in humans, *S. aureus* colonization was densest in the nose, and treatment of the nostrils with mupirocin ointment effectively cleared the nostrils and 6 extranasal body sites. Experimental nasal *S. aureus* colonization was established with $10^4$ CFU/nostril, and both autologous and nonautologous strains survived over 40 days without any apparent adverse effects. A human nasal *S. aureus* isolate (strain D579, sequence type 398) was carried in 4 of 6 animals for over 3 weeks. Nostrils that did eradicate experimentally applied *S. aureus* exhibited neutrophilic innate immunity marked by elevated nasal interleukin-1$\beta$ (IL-1$\beta$), IL-8, and monocyte chemotactic protein 1 levels and a 10-fold decreased IL-1 receptor antagonist/IL-1$\beta$ ratio within 7 days postinoculation, analogous to the human condition. Taken together, pig-tailed macaques represent a physiological model of human *S. aureus* nasal carriage that may be utilized for testing natural colonization and decolonization mechanisms as well as novel classes of anti-*S. aureus* therapeutics.

**KEYWORDS** *Staphylococcus aureus*, human, mucosa, nasal carriage, nonhuman primate, pig-tailed macaque

*Staphylococcus aureus* colonizes the moist mucosa of the anterior nares of 20 to 30% of healthy adults on any given day, and most people and large domesticated mammals carry *S. aureus* transiently throughout their lifetimes (1, 2). Prevalence is not consistent between all demographics and is impacted by age, obesity, living in close quarters, HIV infection and type 2 diabetes, and lengthy hospital stays (3–5). *S. aureus* nasal carriage (SANC) is typically asymptomatic but presents a risk for autoinfection and dissemination of both drug-susceptible and multidrug-resistant *S. aureus* strains by common, nonintimate contact (6–8). *S. aureus* skin, soft tissue, and surgical site infections in humans place an enormous burden on health care. *S. aureus* infection of cows, pigs, and industrial farm workers is costly and problematic for agriculture and food processing.

Combinations of *in vitro* and *in vivo* approaches have been implemented to define host and bacterial determinants of human SANC; however, many limitations, both ethical and procedural, exist in human experimentation. As human SANC is nearly always symptomless and thus does not require clinic visits, recruitment and retention

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are difficult and thwart large longitudinal studies of natural carriage and clearance. Some European studies which used experimental inoculation of human noses with diverse *S. aureus* strains were conducted (2, 9, 10). However, institutional review board approval for experimental inoculation of human subjects in the United States has been limited to strains isolated from the subject's own nostrils (11, 12), without the possibility of testing potentially virulent or antibiotic-resistant strains. Rodent models have been useful for studying *S. aureus*-induced cutaneous infections, lung injury, sepsis, endocarditis, and more (13), and the cotton rat (*Sigmodon hispidus*) model was used for identifying bacterial determinants of SANC (14–16). However, murine and cotton rat noses do not appear to be naturally colonized with *S. aureus*, and inoculation with upwards of $10^7$ to $10^9$ CFU/nostril does not result in sustained carriage, even when providing streptomycin-containing drinking water to reduce the natural nasal flora (14, 15, 17). To monitor *S. aureus* carriage in these models, animals must be sacrificed so that the entire noses may be processed, precluding longitudinal analyses of host responses and *S. aureus* evolution within the nasal environment. Furthermore, mice are obligate nose breathers whose nasal mucosa has anatomical and resident cell type differences from the human nasal mucosa (18, 19). Whereas clearance of *S. aureus* from the human nose and skin is heavily reliant on a robust neutrophilic response (20), murine neutrophils play a different if not less dominant role in host immunity: murine neutrophils lack defensins and make up a much smaller percentage of circulating leukocytes than human neutrophils (19, 21). Humans and mice also differ in expression of cell differentiation markers, immunoglobulin classes, Toll-like receptors, and T cell subsets in the skin and mucosa (19); thus, translation of research findings from murine-*S. aureus* studies to the human SANC condition might be difficult.

Considering the experimental limitations that exist in studying SANC in humans, paired with the lack of a reproducible human-relevant model, we sought to determine whether pig-tailed macaques (*Macaca nemestrina*) are natural carriers of *S. aureus*, whether they may be experimentally colonized with *S. aureus* strains relevant to human *S. aureus* carriage, and whether their innate response to *S. aureus* models the human response. *Macaca nemestrina* pig-tailed macaques have previously been shown to be able to be used as a model of the human condition for airway development, immune function, hormonal regulation, and microbiome complexity (22–26). Here, we screened 17 pig-tailed macaques living in the Washington National Primate Research Center (WaNPRC) for nasal or pharyngeal *S. aureus*, and 14 were nasal *S. aureus* positive at one or both screening sessions (8 weeks apart). Similar to the human condition (27, 28), treatment of macaque nostrils with topical mupirocin ointment effectively cleared the nostrils and 6 extranasal sites (pharynx, left and right axillae and hands, and vagina), demonstrating that the nasal vestibule is the likely reservoir for colonizing *S. aureus*. We experimentally colonized macaque noses with both autologous and nonautologous *S. aureus* strains for over 40 days each, utilizing a physiologically relevant inoculum of $10^4$ CFU/nostril. Experimentally inoculated animals exhibited an *S. aureus*-associated host defense that was similar to the human nasal defense, in that the clearance of nostril *S. aureus* was associated with rapid elevation of interleukin-1$\beta$ (IL-1$\beta$), IL-8, and monocyte chemotactic protein 1 (MCP-1) levels and a decreased IL-1 receptor antagonist (IL-1RA)/IL-1$\beta$ ratio in nasal secretions. Collectively, pig-tailed macaques represent a physiologically relevant and reproducible model of human *S. aureus* nasal carriage.

## RESULTS

**Pig-tailed macaques (*Macaca nemestrina*) are natural nasal carriers of *S. aureus* strains.** Seventeen pig-tailed macaques were surveyed for *S. aureus* carriage by swabbing the nasal vestibule (both nostrils) and pharynx at two study visits that occurred 8 weeks apart. Thirteen macaques were colonized with *S. aureus* in at least one nostril at both visits, and all 17 animals were *S. aureus* positive in either the nose or the pharynx at least once (Fig. 1). Six macaques were assigned to participate in nasal inoculation studies (the animals whose identification codes are boxed in Fig. 1), and the nasal isolates collected from these animals were genotyped by multilocus sequence type

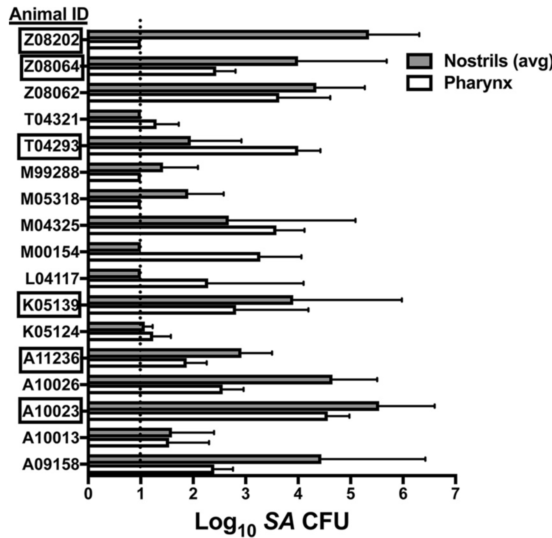



**FIG 1** Pig-tailed macaques are natural carriers of *S. aureus* (*SA*). Seventeen macaques at WaNPRC were screened for *S. aureus* in the nostrils and pharynx at two visits that occurred 8 weeks apart. The mean and SD are shown for each animal. The dotted line indicates the limit of detection (10 CFU/swab). Boxed animal identification (ID) codes indicate animals that were utilized for inoculation studies.

(MLST) analysis and *spa* typing. Animals A10023, K05139, T04293, and Z08064 all carried sequence type 3813 (ST3813), and animals A11236 and Z08202 hosted distinct STs, ST3814 and ST3815, respectively (Table 1). Like the majority of human nasal strains (29, 30), the macaque nasal *S. aureus* strains were devoid of the *mecA* gene, which confers methicillin resistance, and were mupirocin sensitive (Table 1).

**Clearance and recolonization of pig-tailed macaque noses with diverse *S. aureus* strains.** To create a model in which several animals could be nasally inoculated with *S. aureus* and followed up together, a nasal *S. aureus* decolonization protocol was established with topical nasal mupirocin, and the necessary post-mupirocin-treatment recovery period for restoration of non-*S. aureus* commensal microflora was evaluated. Following a 5-day, twice-daily nasal mupirocin treatment course, swabs were collected once per week for the next 4 weeks. In four independent experiments, each using 6 animals, nasal *S. aureus* was eradicated and the extranasal swab sites were cleared of *S. aureus* in 21 or more of the 24 sample collections (Fig. 2A). Commensal bacterial levels, as determined by enumerating all colonies cultured on blood agar, were fully restored by week 3 (Fig. 2B), and subsequent experimental *S. aureus* inoculations were administered during the 4th week following the completion of mupirocin treatment. Nasal inoculations with each macaque's own (autologous) *S. aureus* isolate were performed to determine an inoculum that would colonize the majority of animals during the first few weeks of follow-up. Animals were first inoculated with $10^3$ *S. aureus* CFU, placed into each nostril, and followed for 2 weeks by swabbing nostrils and

**TABLE 1** Nasal *S. aureus* strains utilized in experimental inoculation of *Macaca nemestrina* pig-tailed macaques[c]

| *S. aureus* strain designation | Host | MLST | *spa* type |
|---|---|---|---|
| ST3813 | Pig-tailed macaque | 3813[a] | t15866[b] |
| ST3814 | Pig-tailed macaque | 3814[a] | t15867[b] |
| ST3815 | Pig-tailed macaque | 3815[a] | t15868[b] |
| D579 | Human | 398 | t571 |

[a]Newly assigned MLST.
[b]Newly assigned *spa* type.
[c]MLST, multilocus sequence type; *spa* type, staphylococcal protein A gene Ridom type. The *mecA* gene and mupirocin resistance were absent in all strains.

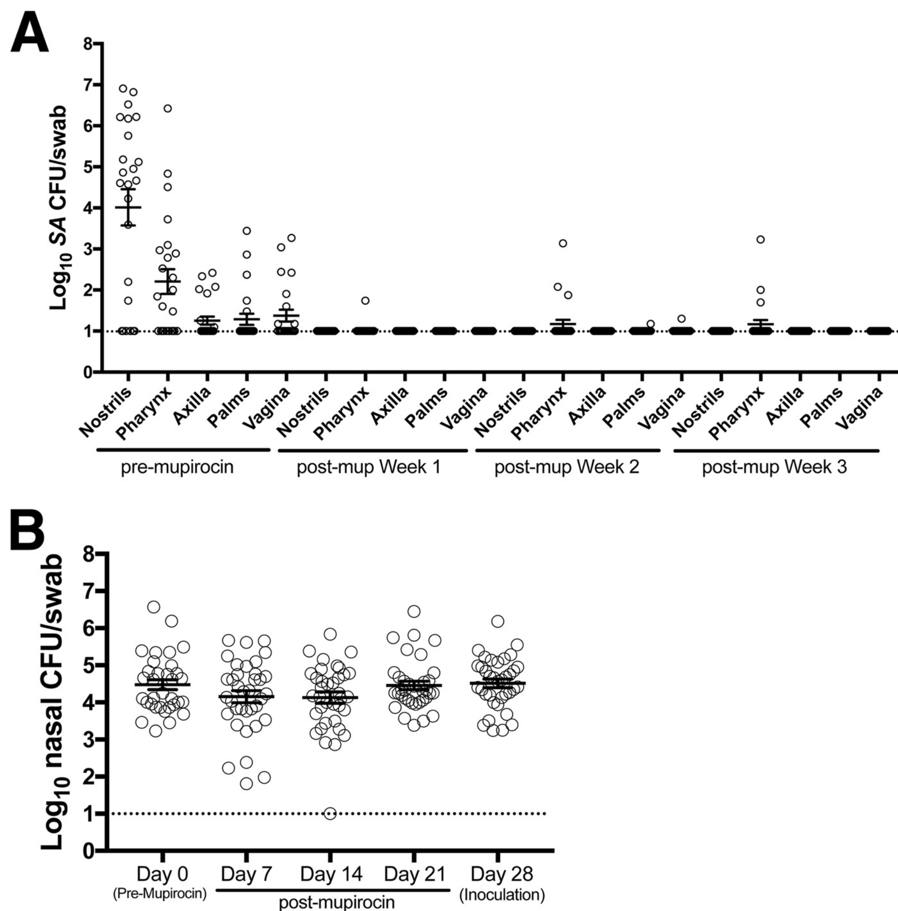
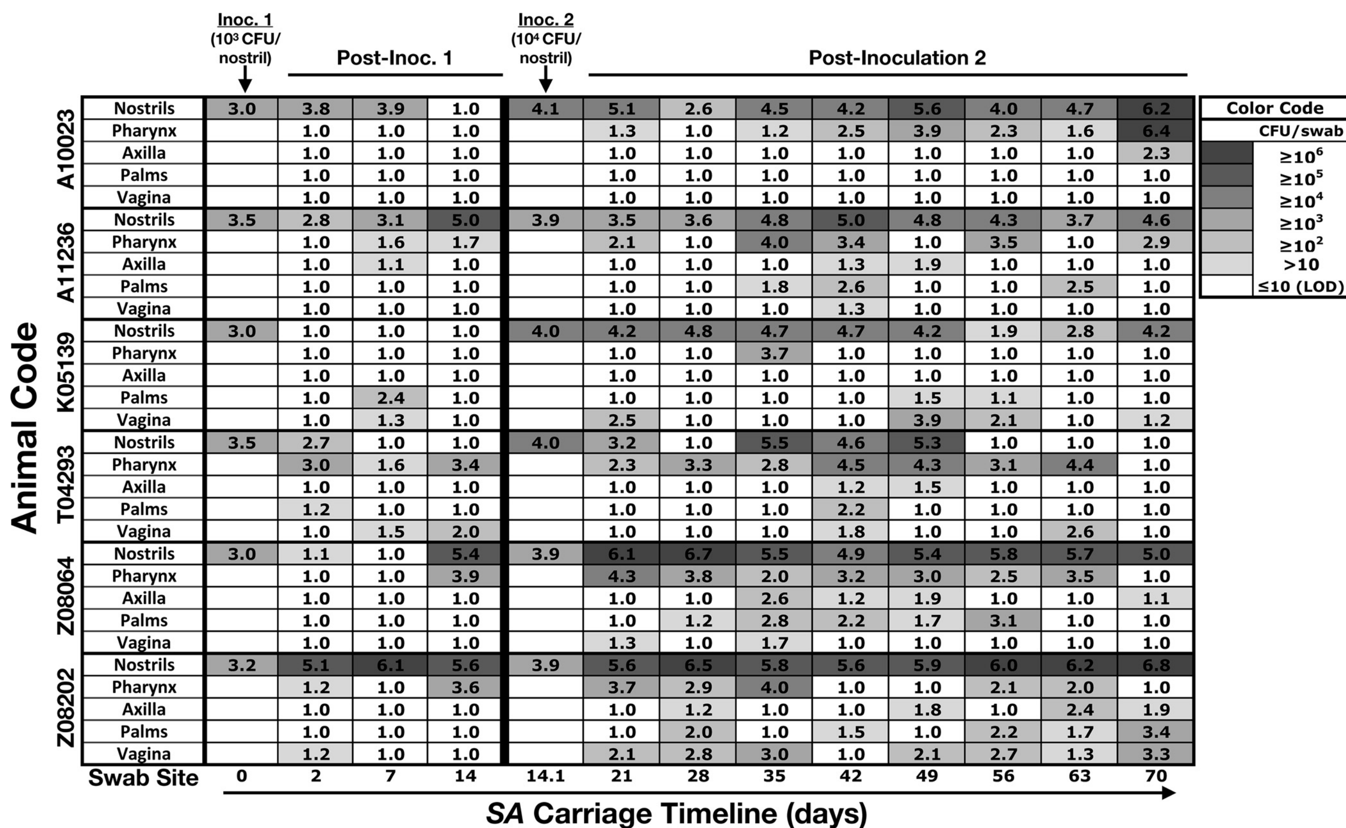



**FIG 2** Nasal mupirocin treatment effectively clears pig-tailed macaque nostrils and external sites. (A) The *S. aureus* load before and after nasal mupirocin (mup) application for each body site (average for the left and right for nostrils, axilla, and palms). Error bars indicate the mean and SEM ($n = 24$; 6 animals, 4 experiments each). (B) The animals' nostrils were swabbed prior to the first topical application of mupirocin and then weekly for 4 weeks, and the number of non-*S. aureus* CFU was enumerated. Error bars indicate the mean and SEM. The dotted horizontal lines indicate the limit of *S. aureus* detection (10 CFU/swab).

extranasal sites (pharynx, left and right axillae and palms, and vagina). At day 2 postinoculation, 5 out of the 6 animals' noses were *S. aureus* positive in at least one nostril, but by day 14, only 3 of 6 animals were colonized nasally and only 9 of 48 total body sites presented detectable *S. aureus* (Fig. 3). After sample collection on day 14, animals were nasally inoculated with $10^4$ CFU/nostril, and animals were followed up for another 8 weeks (through day 70 overall) before mupirocin treatment to clear *S. aureus*. Colonization was established in the noses of all 6 animals and in the extremities of animals with high nasal *S. aureus* densities (most notably, animals Z08064 and Z08202; Fig. 3).

Three subsequent decolonization/recovery/experimental inoculation cycles (6 animals treated identically, 18 total inoculations) were carried out using $10^4$ CFU/nostril. Two macaque *S. aureus* isolates (ST3813 and ST3814) were administered, such that in total, there were 5 nasal inoculations with autologous MLST and *spa* types and 7 inoculations with nonautologous strains. Swab samples were collected at day 2 or 3 postinoculation and weekly thereafter, and *S. aureus* survival curves were generated. Detectable *S. aureus* in at least one nostril was considered survival. Both autologous and nonautologous *S. aureus* strains survived over 40 days (median survival times, 43 days for autologous *S. aureus* strains and over 57 days for nonautologous *S. aureus* strains; Fig. 4), and the survival curves were statistically similar ($P = 0.37$). A human *S. aureus* isolate (D579, whose characteristics are shown in Table 1) was carried in the nose




| Animal Code | Swab Site | 0 | 2 | 7 | 14 | 14.1 | 21 | 28 | 35 | 42 | 49 | 56 | 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 |
| | Axilla | | 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 | 4.8 | 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 |
| | Axilla | | 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 |
| | Axilla | | 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 |
| | Axilla | | 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 | 5.8 | 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 |
| | Axilla | | 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 | 6.8 |
| | Pharynx | | 1.2 | 1.0 | 3.6 | | 3.7 | 2.9 | 4.0 | 1.0 | 1.0 | 2.1 | 2.0 | 1.0 |
| | Axilla | | 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 |

**FIG 3** Nasal inoculation with $10^4$ CFU/nostril establishes long-term *S. aureus* carriage at nasal and extranasal body sites. Six pig-tailed macaques (the animal identification is shown at the left) were cleared of nasal *S. aureus* with mupirocin (5 days, twice daily) and recolonized with $10^3$ CFU/nostril (inoculation 1 [Inoc. 1] was on day 0) of their own *S. aureus* strain 4 weeks later. *S. aureus* values are shown as the number of $\log_{10}$ CFU per swab and color coded in the style of a heatmap according to the *S. aureus* load (see the key at the upper right). On day 14 after inoculation 1, when it was evident that *S. aureus* carriage had not been established in the majority of animals, $10^4$ CFU/nostril (inoculation 2 [Inoc. 2]) was administered after swab samples and nasal secretions were collected. The average for the left and right swab sites is displayed for the nostrils, axilla, and palms. Experimental inoculations 1 and 2 were designed to administer $10^3$ and $10^4$ *S. aureus* CFU/nostril, respectively, and actual values ($\log_{10}$) are shown. The limit of detection (LOD) was 10 *S. aureus* CFU/swab.

(range, $8.50 \times 10^3$ to $1.02 \times 10^6$ CFU/swab) and some extranasal sites (range, $5.20 \times 10^2$ to $8.75 \times 10^3$ CFU/swab) for at least 22 days in 4 of 6 animals. Mupirocin treatment was initiated at day 22 to clear the animals of *S. aureus* prior to return to the colony, and all sites were clear when checked at day 28 postinoculation.

To confirm that the detected *S. aureus* strain matched the inoculated strain, MLST and *spa* typing were performed on *S. aureus* colonies collected from (i) extranasal swab

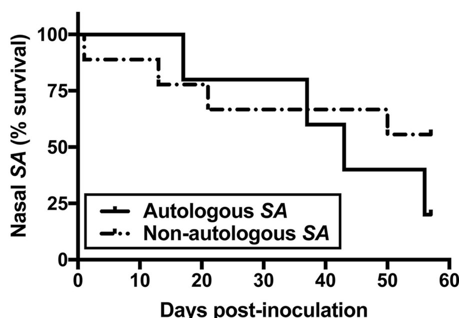



**FIG 4** Macaque noses may be experimentally colonized with autologous and nonautologous *S. aureus* strains. Each animal's nostrils were experimentally inoculated with $10^4$ CFU of an *S. aureus* strain either genetically indistinct from that animal's screening isolate (autologous *S. aureus*; $n = 5$ noses) or a nonautologous *S. aureus* isolate ($n = 7$ noses). Collection of nostril swab specimens was performed at day 2 or 3 and then weekly postinoculation through day 56. Detectable *S. aureus* in at least one nostril was considered survival.

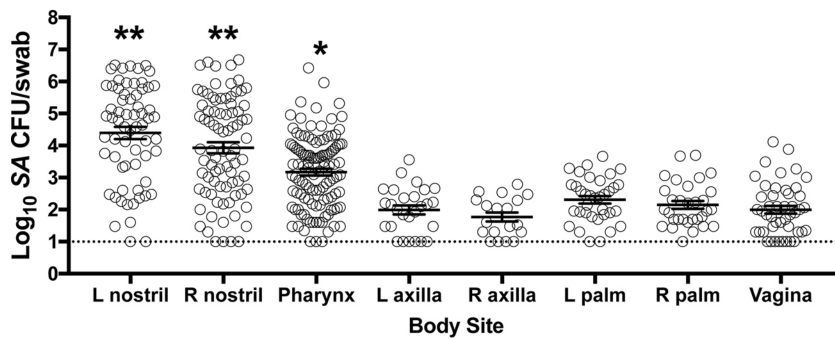



**FIG 5** The nasal vestibule represents the predominant reservoir of *S. aureus* during colonization. The results for naturally *S. aureus*-positive macaque body sites (i.e., sites positive not due to experimental inoculation of the nostrils) collected over an 18-month period are shown. **, elevated *S. aureus* load compared to all other sites ($P < 0.0001$); *, *S. aureus* load greater than that in the axilla, hands, and vagina ($P < 0.005$). Error bars indicate the mean and SEM. The dotted horizontal line indicates the limit of *S. aureus* detection (10 CFU/swab). L, left; R, right.

sites, (ii) nostrils that carried *S. aureus* continuously for several weeks, and (iii) nostrils that had apparently cleared *S. aureus* and then exhibited *S. aureus* positivity again at a subsequent visit. Acquisition of an *S. aureus* strain genetically distinct from the inoculated strain was rare but did occur for animal T04293 in the nose at day 35 and for animal K05139 in the nose at day 63 of the pilot $10^4$-CFU/nostril inoculation study (Fig. 3). The *S. aureus* strains from the extranasal sites matched the inoculated nasal *S. aureus* strains in 105 out of 110 (95.5%) swab samples that were evaluated. Taken together, the presented protocol for clearing and recolonizing pig-tailed macaques with *S. aureus* appears to be feasible and reproducible and pig-tailed macaque SANC appears to be analogous to human SANC (2, 11, 31, 32).

**Evaluation of natural *S. aureus* load by anatomical site.** To further determine the suitability of the pig-tailed macaque as a model of human SANC, we evaluated the natural *S. aureus* load at eight anatomical sites: left and right nostrils, pharynx, left and right axillae, left and right palms, and vagina. Figure 5 presents the *S. aureus*-positive body sites, excluding nostrils positive directly due to experimental inoculation (i.e., nostrils were considered positive only from screening or preinoculation samplings or when clearance of experimentally inoculated *S. aureus* was evident at two consecutive prior visits). *S. aureus*-positive nasal swabs far outnumbered *S. aureus*-positive axilla, palm, and vagina swabs; and nasal swabs contained the highest density of *S. aureus* compared to all other sites (Fig. 5). Pharyngeal swab specimens contained more *S. aureus* bacteria than axilla, palm, and vagina swab specimens but a lower *S. aureus* load than nostril swab specimens (Fig. 5). Taken together with the results in Fig. 2A, which showed nasal mupirocin's effectiveness at clearing nasal and extranasal sites of *S. aureus*, these data suggest that the *S. aureus* reservoir in pig-tailed macaques is the nasal vestibule.

**The pig-tailed macaque nasal mucosal response to *S. aureus* inoculation models the human nasal host defense against *S. aureus*.** Following swab sample collections during each of the pre- and postinoculation follow-up periods, blood was collected for serum storage and nasal fluid was collected from each nostril by twice placing Whatman 540 low-ash filter paper strips into the lumen and massaging the nose for 30 s. Adsorbed proteins were extracted, concentrated, and reconstituted in neutral-pH buffer suitable for total protein quantification and Luminex-based detection of cytokines, chemokines, and growth factors. Macaque nasal secretions averaged 9.9 ± 3.1 mg/ml total protein (mean ± standard error of the mean [SEM]), whereas human nasal fluid self-collected by suction catheter averaged 5.5 ± 2.5 mg/ml (6 macaques [an average of 14 collections from each macaque] versus 14 humans [1 nasal fluid sample from each person]). Macaque nasal fluid collected pre- and postexperimental nasal inoculation with *S. aureus* was assayed for IL-1$\beta$, IL-1RA, IL-8, macrophage

inflammatory protein 1α (MIP-1β), MCP-1, granulocyte colony-stimulating factor (G-CSF), IL-6, tumor necrosis factor alpha (TNF-α), IL-17A, and vascular endothelial growth factor (VEGF) using nonhuman primate-specific antibody sets. These analytes were chosen on the basis of the findings of previous experiments in human subjects that showed associations between increased IL-1β, IL-8, MIP-1β, MCP-1, and G-CSF expression and nasal *S. aureus* clearance (11, 12). IL-6 and TNF-α are hallmark inflammatory cytokines, IL-17A is associated with *S. aureus* host defense in mice (33, 34), and VEGF is important for mucosal barrier homeostasis and remodeling and is highly expressed in mucosal fluids (11, 35, 36). We assessed cytokine levels as the nostrils transitioned from *S. aureus* negativity to *S. aureus* positivity (paired comparisons for each subject, with one nostril analyzed per animal) and further stratified the data according to whether the nostril eventually cleared *S. aureus* or exhibited extended carriage. Similar to the findings for human subjects (11), *S. aureus*-associated elevation of nasal IL-1β, IL-8, and MCP-1 corresponded with *S. aureus* clearance (Fig. 6). Furthermore, macaque nostrils that eventually cleared *S. aureus* demonstrated a 10-fold decrease in the IL-1RA/IL-1β ratio postinoculation (Fig. 6C), while nostrils that carried *S. aureus* exhibited elevated IL-1RA and no change in the IL-1RA/IL-1β ratio (Fig. 6B and C). Increased expression of MIP-1β was observed in *S. aureus*-positive nostrils compared to negative nostrils, regardless of whether clearance was achieved (data not shown). The levels of TNF-α and IL-17A were low (<10 pg/ml) in most samples and near the lower limit of detection in the utilized assay, similar to other observations in human subjects (11, 12, 37). The serum levels of all 10 analytes were unchanged by experimental nasal *S. aureus* inoculation (data not shown). The animals' body weight and temperature were measured and documented each time that the animals were sedated for sample collections. Protein biscuits provided to the animals were counted and reported by the husbandry staff daily. No discernible changes in appetite, weight, or behavior of the animals were noted throughout the course of the experiments. Body temperatures remained within the normal range. Collectively, these observations suggest that the pig-tailed macaque inflammatory response to nasal *S. aureus* was not systemic in nature.

Our laboratory (A. M. Cole) has conducted 43 experimental nasal *S. aureus* inoculations in which healthy human subjects self-applied their previously isolated *S. aureus* strains and then returned for swab and nasal fluid collections for a month of follow-up to evaluate *S. aureus* carriage duration and mucosal host defense characteristics (11, 12). Both human subjects and macaques presented a trend that a rapid innate nasal inflammatory response, typified by peak IL-1β and IL-8 expression within 7 days of *S. aureus* inoculation, was associated with shortened carriage duration (Fig. 7). For macaques, nostrils that demonstrated peak nasal host defense by day 7 averaged 11 ± 5 days (mean ± SEM) of *S. aureus* carriage, while nostrils that exhibited a late peak (on or after day 16) or no peak averaged over 40 days of *S. aureus* carriage (Fig. 7A). Similarly, human noses that presented peak IL-1β and IL-8 expression by day 7 cleared *S. aureus* within 19 ± 3 days, while later responses to *S. aureus* inoculation resulted in carriage that extended beyond the month of follow-up for the majority of subjects (Fig. 7B). Taken together, these data suggest that the host effector molecules that mediate nasal *S. aureus* clearance in humans are present and function similarly in the pig-tailed macaque.

## DISCUSSION

Complications from *S. aureus*-induced infections burden most, if not all, medical specialties. Patients with HIV infection, with diabetes, and on dialysis, postoperative patients, and populations living in close quarters with less than optimal sanitary conditions (populations living on military bases or in prisons or nursing homes or attending day care centers) demonstrate enhanced susceptibility (1, 5). Although the reservoir for human-hosted *S. aureus*, the moist squamous epithelium of the nasal mucosa, has been appreciated for over 2 decades, the research community lacks an appropriate animal model for studying early nasal colonization events, asymptomatic

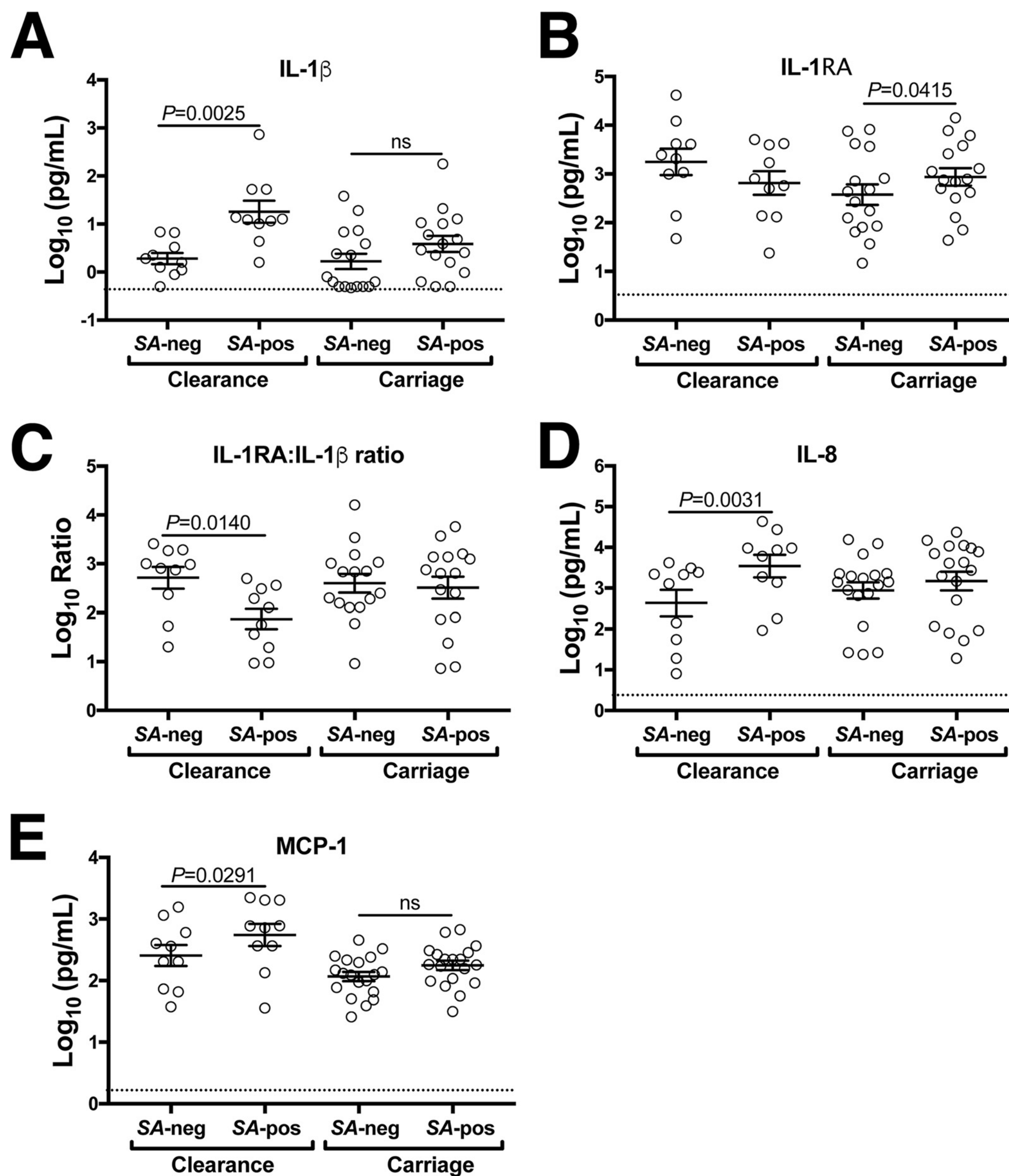



**FIG 6** Clearance of experimentally inoculated nasal *S. aureus* associates with augmented innate nasal mucosal defense. Six macaques participated in four *S. aureus* decolonization/experimental *S. aureus* recolonization protocols and had their noses sampled as described in Materials and Methods. Based on nasal swab testing results that indicated each nostril's *S. aureus* carriage pattern, either left or right nostril secretions (the same for all macaques in each experiment) were assessed for cytokines using a Luminex assay. The levels of IL-1$\beta$ (A) and IL-1RA (B), the IL-1RA/Il-1$\beta$ ratio (C), and the levels of IL-8 (D) and MCP-1 (E) in nasal secretions are shown for each nostril that transitioned from *S. aureus* negativity (*SA*-neg) to *S. aureus* positivity (*SA*-pos) either upon experimental inoculation or after testing negative for *S. aureus* and then positive again during the follow-up period. Cytokine data were further stratified according to whether the nostrils eventually cleared inoculated *S. aureus* (Clearance) or stayed colonized with inoculated *S. aureus* (Carriage). Error bars represent the mean and SEM. Dotted horizontal lines indicate the limit of detection.

carriage/immune tolerance, and clearance mechanisms. This presents a challenge against efforts to develop novel decolonization strategies and antibiotics.

We evaluated whether the pig-tailed macaque (*Macaca nemestrina*) is a natural host of nasal *S. aureus*. We determined that 13 of 17 animals carried *S. aureus* in at least one nostril at the first screening visit, and 14 of 17 animals were nasal *S. aureus* positive 8 weeks later (Fig. 1). Three animals (L04117, M00154, T04321) screened negative for nasal *S. aureus* at both sampling times but had detectable *S. aureus* in one or both pharyngeal swab specimens. Since we did not have access to any pig-tailed macaques

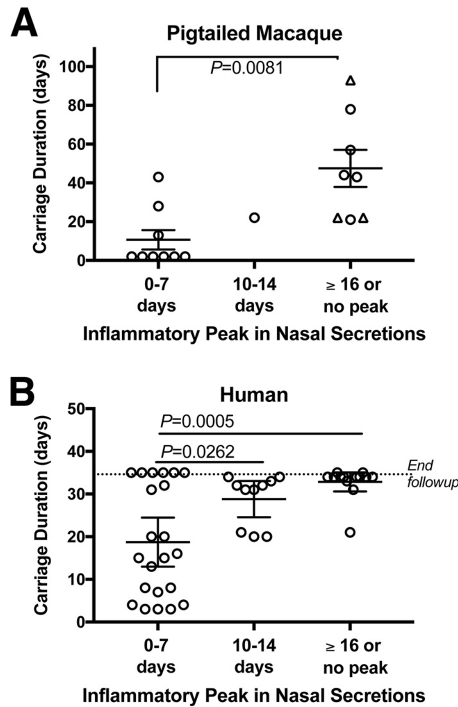



**FIG 7** Similar association between the nasal inflammatory peak and the *S. aureus* carriage duration in pig-tailed macaques and humans. The peak nasal inflammatory response to experimentally inoculated *S. aureus* was assessed by Luminex measurements of IL-1$\beta$ and IL-8 in collected nasal secretions. For each of 18 macaque inoculations (A) and 43 human inoculations (B), the occurrence of the peak inflammatory response postinoculation (*x* axis) was plotted according to the duration of *S. aureus* carriage (*y* axis). Error bars indicate the mean and SEM. The triangles in panel A represent animals that were carrying *S. aureus* nasally on the last day of follow-up (thus, the carriage duration was actually longer).

living in the wild, it cannot be ruled out that macaque *S. aureus* carriage at the WaNPRC is the result of captivity and human handlers. However, caregiver personal protective equipment (PPE) entails full coverage during animal handling, and review of the animal housing map indicated that the three nasal *S. aureus*-negative animals cohabited with the nasal *S. aureus*-positive animals. Animal Z08202 carried $>10^4$ *S. aureus* CFU/nasal swab at both screening visits and was housed in the same room as animal T04321, while animal Z08064 (*S. aureus* positive at both screening visits) was caged immediately adjacent to animal L04117 in a separate room. Thus, these animals' negative nasal *S. aureus* carriage status was not likely a result of different environmental or animal caregiver factors. Moreover, new MLST and *spa* types were assigned to the macaque strains (Table 1), suggesting that carriage owed only to human intervention is unlikely. Rhesus macaques from the Biomedical Primate Research Center in The Netherlands were found to host and easily exchange *S. aureus* within the colony (38). Although our study was limited to one primate research center, it appears that research *Macaca nemestrina* pig-tailed macaques carry *S. aureus* at least intermittently, with the nose containing the highest *S. aureus* density for nearly all animals (Fig. 1 and 5). Importantly, this corresponds with the prevailing paradigm that human SANC involves persistent carriers and others who host *S. aureus* transiently throughout life (2).

The high rate of carriage in macaques (14 of 17 animals in our study and 82% of 48 rhesus macaques sampled longitudinally [38]) exceeds that in humans and warrants further study. Cattle and pig farms report nasal *S. aureus* carriage rates in the 50 to 90% range (39, 40), suggesting that living in close quarters and hygiene differences support *S. aureus* colonization. The finding that individuals living in prisons and on military bases present elevated carriage rates (41, 42) lends support to this reasoning. Given the obvious hygiene differences between macaques and humans, it will still be important to determine potential host immune differences that might permit an elevated yet

asymptomatic *S. aureus* load in the nasal mucosa. Future studies that should be feasible in pig-tailed macaques include the determination of anti-*S. aureus* antibody profiles, intracellular *S. aureus* levels in the nasal epidermis of macaque noses (43), and identification of infiltrating immune cell populations during *S. aureus* carriage and upon its resolution.

An important finding of this study is that all macaques were successfully decolonized using a 5-day course of topical nasal mupirocin. Consistent with the human SANC condition (2, 11, 12, 31, 32), nasal mupirocin application eradicated *S. aureus* not only in the nostrils but also in the axillae, hands, and vagina, with pharyngeal *S. aureus* being cleared in 21 of 24 tests (Fig. 2A). This suggests that the anterior nasal vestibule of pig-tailed macaques is the reservoir for colonizing *S. aureus* and might be as attractive an environment to *S. aureus* as the human anterior nares. It should be noted that we did not test how long animals stayed clear of *S. aureus* following mupirocin treatment, though we did observe that all 6 animals generally stayed clear of *S. aureus* at all sites for at least 4 weeks. Decolonization of humans with mupirocin nasal ointment is considered beneficial for preventing postoperative infection (27, 44), but *S. aureus* carriage is recurrent in many subjects (45). More studies that investigate the impact of mupirocin treatment on microbial community dynamics as well as new agents to be used for *S. aureus* decolonization are needed, and the pig-tailed macaque might be useful in this regard. Our main objective was to develop a decolonization and inoculation protocol similar to what we previously reported for human subjects (11, 12). Importantly, recolonization of the macaques was established with a physiologically relevant inoculum of $10^4$ *S. aureus* CFU/nostril. Nasal swab collections indicated that macaque nostrils averaged approximately $10^4$ *S. aureus* CFU/nostril swab naturally (e.g., independent of experimental nasal colonization; Fig. 5), consistent with the findings for human noses, which carry *S. aureus* in the range of $10^1$ to $10^6$ CFU/nostril swab (median, $9.8 \times 10^3$ *S. aureus* CFU/swab for 30 healthy human subjects surveyed by our group in 2016 [12]).

A second indication that *Macaca nemestrina* pig-tailed macaques present a physiologically relevant model of human SANC is that both autologous and nonautologous strains colonized the macaque noses (at least one nostril) for over 40 days (Fig. 4), and 5 out of 6 macaques were nasally colonized with a human nasal *S. aureus* isolate for at least 14 days. One animal swabbed *S. aureus* negative at all body sites on day 14 postinoculation with human ST398 strain D579, while another was clear at all sites on day 22. Due to time and funding constraints, follow-up was restricted to 22 days following experimental inoculation with the human donor isolate, but 4 of the 6 inoculated animals carried an average of $2.7 \times 10^5$ *S. aureus* CFU per nostril swab on day 22 prior to mupirocin treatment to successfully clear the animals for return to the colony. Keeping in mind that the inoculum was only $10^4$ *S. aureus* CFU per nostril, this indicates that, like the human nasal mucosa, the macaque nasal mucosa provides favorable conditions for *S. aureus* survival. Colonization of the majority of animals with this common lineage, known to affect humans, cows, and pigs and to have the ability to acquire methicillin resistance (46–49), suggests that other clinically relevant *S. aureus* strains will be testable in pig-tailed macaques.

Consistent with human SANC (11, 12, 50), innate host defense against nasal *S. aureus* in macaque noses appears to be predominantly neutrophilic in nature, with the observed induction of IL-1$\beta$ and IL-8 and a 10-fold decreased IL-1RA/IL-1$\beta$ ratio in nostrils that eventually cleared *S. aureus* (Fig. 6). Modeling of the neutrophilic response of humans to nasal *S. aureus* with *Macaca nemestrina* macaques will undoubtedly prove useful in elucidating the specific cellular mechanisms that result in clearance in certain hosts but sustained asymptomatic carriage in others. Notably, this aspect of nasal host defense cannot be adequately modeled in mice, since mice do not make IL-8 and murine neutrophils lack defensins and thus have different means of degranulating and damaging *S. aureus* and forming extracellular traps. While we have not yet identified which immune cells populate the macaque nasal subepithelium, previous reports showed that the pig-tailed macaque perhaps represents the best-known model for the

human host defense at mucosal surfaces. It has been used to model human rectal and reproductive mucosae in studies of sexually transmitted pathogens, as well as in studies of infection-induced preterm birth and impaired fetal lung development (23, 24, 51–53). Furthermore, *Macaca nemestrina* macaques are colonized with a mucosal flora similar to that in humans, including with endogenous lactobacilli in the reproductive tract (54, 55). We have not yet fully evaluated the nasal microbiome of macaques at the DNA/metagenomic level, but the species in both human and macaque nares mainly comprised staphylococcal species in our initial evaluation of culturable microbes from swab fluid specimens. Importantly, *S. epidermidis*, *S. aureus*, *S. hominis*, *S. capitis*, *S. haemolyticus*, and *S. warneri* all had similar relative abundances in each host (unpublished observations). These preliminary data, combined with the known similarity between the human and pig-tailed macaque vaginal microbiome (54, 55), support the notion that the macaque nasal mucosa is preferable to rodents for studying microbial community dynamics with respect to *S. aureus* carriage.

In summary, the presented study demonstrates that *Macaca nemestrina* macaques are natural hosts of *S. aureus*, with the nasal mucosa containing the highest *S. aureus* density. This is similar to the human condition and not observed in current rodent models. Macaques were successfully cleared of *S. aureus* with the same topical mupirocin treatment used by humans, and 4 of 6 macaques carried an experimentally inoculated human nasal *S. aureus* isolate for over 3 weeks. Given that both humans and *Macaca nemestrin*a macaques exhibit a neutrophilic response to inoculated *S. aureus* in the nostrils that successfully clears the bacterium, there is the potential for studying host-*S. aureus* interactions and new decolonization strategies with this model of human *S. aureus* nasal carriage.

## MATERIALS AND METHODS

**Study participants, animal care, and compliance.** This study was performed with pig-tailed 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 nonhuman primates and follow NIH guidelines for animal biosafety level 2 containment facilities. The study-specific protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Washington. On sample collection days (collection of swab, nasal secretion, and blood samples), animals were sedated with 1.2 mg/kg of body weight ketamine and moved to a procedure room. On days in which the only intervention was to apply mupirocin nasal ointment, the animals were sedated with a light dose of ketamine (0.8 mg/kg) and treated while remaining in their cages. In all, six healthy sexually mature female pig-tailed macaques were selected from a cohort of 17 healthy individuals for monitoring of nasal and pharyngeal *S. aureus* carriage status for 2 months. Their ages ranged from 9 to 13 years, and they were born to different sets of parents and thus considered unique in terms of host genetics. The subjects were chosen on the basis of the individual's ability to naturally acquire and carry *S. aureus* as well as their availability for participation for the entire study duration (~18 months). There were no clinical adverse effects of nasal *S. aureus* inoculation reported during the duration of the study. The human nasal carrier studies referred to here were previously described (11, 12) and were performed under the guidelines of the Institutional Review Board of the University of Central Florida.

**Animal screening and sample collection.** To screen pig-tailed macaques (*Macaca nemestrina*) for *S. aureus* carriage, the left and right nostrils and pharynx of each animal were sampled using a flocked Copan Diagnostics Eswab minitip and immediately stored in 1 ml of Eswab (liquid Amies) medium in transport tubes (catalog number 23600901; Fisher Scientific). Samples were shipped overnight on ice packs to the A. M. Cole laboratory. Upon arrival, the transport tubes were vortexed vigorously and the swabs were swirled in transport medium and then streaked onto CHROMagar plates for colorimetric identification of *S. aureus*. The swab/transport liquid was dilution plated onto tryptic soy agar II (TSAII) containing 5% sheep blood (catalog number B21261X; Fisher Scientific) for enumeration of the *S. aureus* and non-*S. aureus* CFU. *S. aureus* colonies were confirmed using a BD Staphyloslide latex test (catalog number B4340953; Fisher Scientific) and subcultured overnight to make *S. aureus* colony glycerol stocks. The remaining swab fluid was prepared as an early glycerol stock (0.5 ml sample plus 0.5 ml Bacto tryptic soy broth [TSB], 30% glycerol, stored at −80°C) and also cultured overnight (250 rpm, 37°C, 18 h) in a total volume of 2 ml TSB prior for preparation of a cultured late glycerol stock.

**Experimental nasal inoculation of pig-tailed macaques with *S. aureus*.** Animals were decolonized of endogenous *S. aureus* by applying mupirocin nasal ointment (Bactroban; GlaxoSmithKline, Philadelphia, PA) twice daily for 5 days. For 4 weeks following the last mupirocin application, nostrils and extranasal sites (left and right axillae, left and right hands, pharynx, vagina) were swabbed and samples were shipped and processed as described above to confirm the clearance of *S. aureus* and recovery of the commensal nasal microflora. *S. aureus* strains were prepared for experimental inoculation during the 4th week after mupirocin treatment as follows: *S. aureus* colonies were grown in 50 ml of TSB for 18 h

(250 rpm, 37°C), and then 1 ml of culture was added to 50 ml TSB and cultured for another 2 h. The liquid culture was transferred to a conical tube and centrifuged at 3,000 × *g* for 5 min, and the supernatant was discarded. The bacterial pellet was suspended in 35 ml of Hanks' balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA), and then centrifugation and suspension in HBSS–1% BSA were repeated. The resulting liquid was portioned to microtubes and snap-frozen by placing the tubes in liquid nitrogen for 3 h, followed by transfer to a −80°C freezer. The concentration (the number of CFU per milliliter) for each strain's snap-frozen stock was calculated by dilution plating each of 5 aliquots on TSAII–5% blood agar. This information was used to determine the dilution scheme and final volume (≤20 μl) needed to achieve an inoculum of either $10^3$ or $10^4$ CFU/nostril. Inocula were administered using a micropipette and gently pinching/massaging the outside of the nose for 30 s. The first experimental inoculation tested whether $10^3$ CFU/nostril would colonize the majority of animals (it did not), and then $10^4$ CFU/nostril was tested and the animals were followed up at day 2 postinoculation, followed by weekly swab, nasal secretion, and blood collections until day 70. In the next 3 studies, the animals were decolonized with mupirocin as described above and then inoculated with either macaque nasal isolate ST3813 or ST3814 or human ST398 nasal isolate D579. The follow-up periods (day 2 or 3 and then weekly swab, nasal secretion, and blood collections) were 63, 93, and 22 days, respectively.

***S. aureus* genotyping and assessment of mupirocin sensitivity.** Strain USA300-0114 (NRS384) was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program. Human nasal *S. aureus* isolates were collected as described previously (11), and macaque *S. aureus* isolates were collected as described above. Genomic *S. aureus* DNA was extracted using an UltraClean microbial DNA isolation kit (Mo Bio Laboratories, West Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was placed under PCR conditions with primers to amplify the seven MLST genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*), *spa*, and *mecA* as described previously (56–58). PCR amplicons were sequenced by Eton Bioscience, Inc., and the Mega (version 7) program was used for analysis. The *S. aureus* MLST database (http://pubmlst.org/saureus/) was utilized to assign allele numbers and sequence types (STs) for each *S. aureus* isolate's seven alleles. Staphylococcal protein A (*spa*) gene typing was performed as previously described (57, 59) using Ridom StaphType software (http://www.spaserver.ridom.de/). Newly discovered *spa* types were synchronized with the Ridom server. For antibiotic resistance assessment, the presence of a 1,339-bp product or no amplicon indicated, respectively, the presence or absence of the *mecA* gene, which encodes the penicillin-binding protein (PBP2A) that confers resistance to β-lactam antibiotics. The results were confirmed by spreading bacterial cultures (the same ones used for DNA extraction) onto MRSASelect agar plates (Bio-Rad, Hercules, CA, USA).

Donor *S. aureus* strains were also tested for functional mupirocin resistance by performing turbidity (growth) assays (60). *S. aureus* isolates were grown to log phase in TSB, diluted to $10^4$ CFU/90 μl in Mueller-Hinton broth (Sigma-Aldrich, St. Louis, MO) containing 5% sucrose, and loaded to a 96W culture plate. Ten microliters of 2-fold serial dilutions of either mupirocin (catalog number M-7694; Sigma-Aldrich) or vehicle (volume-matched dimethyl sulfoxide) was added to the diluted *S. aureus* culture so that the final concentration of mupirocin ranged from 1 to 8 μg/ml. The culture plate was covered with ThermoSeal A film (catalog number TSA-100; Excel Scientific, Inc.) and placed into a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) programmed for an 18-h kinetic assay at 37°C. Turbidity measurements (optical density at 550 nm [$OD_{550}$]) were taken every 5 min following 15 s of agitation. Growth curves ($OD_{550}$ plotted against time) were generated for all wells, and both the input and 18-h incubations were plated on TSAII–5% sheep blood agar for enumeration. All strains grew to ∼$10^8$ CFU/0.1 ml in antibiotic-free medium. All strains were killed in the presence of ≤1 μg/ml mupirocin.

**Blood and nasal secretion collection and processing.** Each week, blood (3 to 5 ml) was collected into serum-separating tubes (SST) to separate 1 to 2 ml serum, which was stored at −80°C until use. Nasal secretions were collected by twice inserting Whatman 540 low-ash filter paper strips (0.3 by 2.5 cm) into each nostril and massaging the nose for approximately 30 s. The strips used to sample the left and right nostrils were stored in separate tubes at −80°C. Frozen serum samples and nasal strips were batch shipped on dry ice to the A. L. Cole lab for processing and analysis. Nasal fluid proteins were extracted by vortexing for 20 min in 900 μl of 10% glacial acetic acid (in molecular-grade water), transferring the supernatant containing soluble proteins to a fresh tube (on ice), and then vortexing the strips with an additional 500 μl of acetic acid solution for 10 min. The supernatants were combined (per animal nostril), balanced in a SPD1010 SpeedVac apparatus (Thermo Fisher), and vacuum dried to ≤100 μl. Two washes were performed by adding molecular-grade water up to 1 ml and reevaporating the sample to near dryness. Samples were resuspended in nine parts Dulbecco's modified Eagle medium (DMEM) and one part radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and then neutralized (if necessary) with a scant volume of 1 N or 10 N sodium hydroxide. Sample final volumes were standardized to 130 μl per nasal strip extract (left nostril or right nostril), and the extracts were clarified of insoluble debris by centrifugation (10,000 × *g*, 1 min) and stored in aliquots at −80°C until analysis. Total protein was quantified, and macaque nasal secretion extracts averaged 9 mg/ml, consistent between animals. Six extracts out of 188 were eliminated from Luminex analysis due to low protein levels.

**Detection of pig-tailed macaque cytokines.** Nostril secretion protein extracts and the corresponding serum aliquots (collected on the same day) were thawed on wet ice prior to multiplex bead assay. A ProcartaPlex nonhuman primate custom 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. Company instructions were followed step by step for standard curve generation, serum sample preparation, and instrument settings. Left nostril secretion extracts were analyzed for 3 of the 4

inoculation experiments (with 6 animals in each experiment), and right nostril extracts were run for the other inoculation experiment, as these were the nostrils in which the most transitions from *S. aureus* negative to *S. aureus* positive were observed. Nasal fluid extracts (54 $\mu$l) were mixed with 6 $\mu$l of DMEM containing 5% BSA, such that both standards and samples were diluted in a nearly equivalent mixture of DMEM, RIPA buffer, and 0.5% BSA, which acted as a background protein to help prevent target protein aggregation and nonspecific adherence to labware. A volume of 50 $\mu$l/well of the prepared standards and samples was mixed with antibody-conjugated beads to begin the assay. A Bio-Rad Bio-Plex Pro II wash system and Bio-Plex 200 reader with high-throughput fluidics (Luminex xMAP technology) were used to complete the wash steps and sample readings. Each standard curve demonstrated a good fit down to 0.5 to 4 pg/ml, and these detection limits are shown in the figures where appropriate. Data are presented as the number of picograms per milliliter or the log-transformed number of picograms per milliliter for each serum or nasal fluid extract, which was processed by equal volume throughout the work flow.

**Statistical analysis.** Data were analyzed using GraphPad Prism (version 7) software (GraphPad Software, La Jolla, CA). Survival curves for autologous and nonautologous *S. aureus* strains in macaque noses (Fig. 4) were compared and determined to be not significantly different by both the log-rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon test. Body site *S. aureus* levels (Fig. 5) were log transformed and compared using ordinary one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test. Cytokine comparisons (an *S. aureus*-negative to *S. aureus*-positive transition for individual animal nostrils; Fig. 6) were made using paired *t* tests (two-tailed). Carriage durations for noses with different inflammatory peak patterns (Fig. 7) were compared using ordinary one-way ANOVA with Tukey's multiple-comparison test.

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We declare no conflicts of interest.

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