Surface Proteins and Exotoxins are Required for the Pathogenesis of *Staphylococcus aureus* Pneumonia

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A model of *S. aureus* induced pneumonia in adult, immunocompetent C57BL/6J mice is described. This model system closely mimics the clinical and pathologic features of pneumonia in human patients. Using this system, we define a role for *S. aureus* strain Newman surface proteins and secreted exotoxins in pneumonia-related mortality.
Staphylococcus aureus is an important bacterial pathogen causing pneumonia in both adult and pediatric populations. Recent reports have described a growing incidence of severe S. aureus pneumonia in otherwise healthy individuals, often caused by multi-drug resistant strains (8, 9). In addition, S. aureus remains one of the most common causes of ventilator-associated pneumonia, contributing to significant morbidity and mortality (18). At present, little is known about the S. aureus virulence factors that play a role in lower respiratory tract disease. The development of an adult, immunocompetent animal model system recapitulating S. aureus pneumonia would provide a useful tool to allow for such investigations.

To date, small animal models of S. aureus pneumonia have relied on the use of surgical inoculation methods or the infection of immunocompromised animals (6, 17). While these models highlight the inflammatory response to intrapulmonary S. aureus, a detailed characterization of S. aureus-encoded virulence factors has not been possible as the organisms are rapidly cleared from the lungs. A murine model of pulmonary infection with agar-embedded S. aureus has defined a role for coagulase in hematogenous infection (34), while a neonatal mouse model of S. aureus pneumonia revealed the importance of the accessory gene regulator A (agrA), sarA, and staphylococcal protein A (spa) in the development of disease (10, 13). Together, these data are suggestive that multiple S. aureus virulence factors contribute to the pathogenesis of pneumonia.

We sought to develop a transnasal murine model of S. aureus pneumonia in adult, immunocompetent animals to permit the investigation of virulence
factors. To define infection parameters leading to evidence of pneumonia in 7 week old C57BL/6J mice (Jackson Laboratories), groups of 20 animals were inoculated via an intranasal route with either phosphate buffered saline (PBS) or one of three doses of S. aureus Newman, a human clinical isolate (7). Following dilution of an overnight culture 1:100 into fresh tryptic soy broth, staphylococci were grown with shaking at 37°C to an OD$_{660}$ of 0.5. Culture aliquots (50 mL) were sedimented by centrifugation, staphylococci washed, and suspended in 750 µl PBS. Animals were anesthetized with ketamine and xylazine as previously described (21). When appropriate anesthesia was documented, 30 µl of bacterial slurry were inoculated into the left nare and animals were held upright for 1 minute post-inoculation. All animals were provided with food and water ad libitum and continually observed over 72 hours. Immediately following inoculation, all animals displayed labored breathing marked by rapid respiratory rate and exaggerated chest wall excursion. This initial physiologic change resolved within six hours, such that all live animals at this initial time point were ambulatory and well-appearing. A small percentage of animals routinely succumbed within the first six hours following inoculation, likely from the combined effects of aspiration and anesthesia. These animals were not included in subsequent analyses. Inoculation with $4 \times 10^8$ colony forming units (CFU) of S. aureus Newman resulted in a mortality of approximately 50% at 24 hours, with an additional 20% of animals succumbing to infection within 48 hours following inoculation (Fig. 1A). Importantly, all infected animals appeared ill, demonstrating increased respiratory rate, hunched posture, and decreased
mobility at the 24 hour time point. Reduction of the bacterial inoculum to $8 \times 10^7$ CFU yielded no mortality, albeit that infected animals appeared ill. This group of animals demonstrated marked improvement in their condition by the 48 hour time point, resembling uninfected animals. Similar results were obtained with an inoculum of $1.3 \times 10^8$ CFU S. aureus Newman (data not shown). Inoculation with $8 \times 10^8$ CFU S. aureus Newman resulted in nearly 90% mortality by 24 hours, which was significantly greater than the mortality observed with an inoculum of $4 \times 10^8$ CFU at the same time point (p=0.02); surviving animals remained ill in appearance until 72 hours post infection.

To assess the kinetics of bacterial growth and clearance in the lung, animals were infected with $3-4 \times 10^8$ CFU wild-type S. aureus Newman. At indicated time points post-infection, animals were killed by forced CO$_2$ inhalation, in compliance with the University of Chicago Institute of Animal Care and Use Committee guidelines. The right lung was excised using aseptic techniques, suspended in 1 ml of PBS and tissue homogenized. Serial dilutions and plating were performed to enumerate staphylococcal burden in lung tissue. Immediately following infection, approximately one-third of the inoculum could be recovered from the lungs (Fig. 1B); this recovery was not significantly different from that observed at 6 hours post-infection. Interestingly, by 24 hours, most animals demonstrated significant increases of staphylococci in lung tissues (p=0.05), indicating that S. aureus Newman proliferated following infection. A decrease in the recovery of S. aureus was observed in the 48-72 hour time frame, corresponding to clinical improvement in the animals.
To discern whether pulmonary infection with *S. aureus* in this murine model was capable of causing pathologic lesions appreciated in human patients, we examined the lungs of infected animals for gross pathologic changes as well as histopathologic evidence of infection. The lung tissue of infected animals was red in appearance and displayed a firm texture (Fig. 2A). In contrast, the lungs of uninfected animals remained light pink and spongy. Inspection of the dissected left lung from a representative infected animal further revealed a heterogeneous red coloration, consistent with marked congestion (Fig. 2B, right panel).

For histopathologic analysis, the left lung was dissected and placed in 1% formalin. Formalin-fixed tissues were processed, stained with hematoxylin and eosin and visualized by light microscopy. Histopathologic examination revealed the consequences of *S. aureus* infection on lung parenchyma. As a control, normal alveolar architecture observed in uninfected animals, with thin-walled air spaces defined by a single layer of pneumocytes (Fig. 3, panel A). As early as 6 hours following inoculation with *S. aureus*, aggregates of dark purple staining immune cells are visualized in the lungs of infected animals (panel B; arrow). The overall lung architecture remains preserved at this time point, and no bacteria are evident in tissues. In contrast, by 24 hours, significant alveolar destruction has occurred with infiltration of large numbers of immune cells (panel C). Interestingly, large foci of staphylococci are noted in lung tissues at this time point (arrow), consistent with bacterial proliferation. Dense, eosinophilic staining consistent with proteinaceous edema is observed to fill the alveolar space in infected animals (panel D). By 48 hours, these bacterial foci are reduced in size.
or absent, and the re-emergence of air-filled spaces becomes evident (panel E). At the 72 hour time point, significant air space is restored, however the alveolar walls remain thickened (panel F). Together, these data establish a murine model of *S. aureus* pneumonia that closely mimics the clinical and histopathologic findings evident in human patients. It is likely that both the size of the inoculum and the mouse strain utilized in this system contribute to the development of pneumonia in this animal model. This combination had not been examined in previous studies. The large inoculum required to cause pneumonia in these animals speaks to the remarkable ability of the murine immune system to eliminate this pathogen from the lung, raising the possibility that an extension of this model system to other strains of immunocompetent mice may enhance our understanding of pulmonary immunity against *S. aureus*.

To define *S. aureus* virulence factors critical for infection of the lower respiratory tract, mortality following pulmonary infection of mice with wild-type *S. aureus* Newman or its isogenic mutants was assessed. *S. aureus* Newman strains carrying a deletion in *srtA* and *srtB* were described previously (24, 27). *agrA, spa, hla, and icaA* mutants harboring *bursa aurealis* insertions were transduced into wild-type *S. aureus* Newman using isolates of the Phoenix transposon library (2). All mutant strains were cultured in TSB supplemented with erythromycin (10\(\mu\)g/ml). When inoculated with wild-type Newman, slightly more than 70% of infected animals succumbed over a 72 hour period (Fig. 4A). Sortase A mutants (*srtA*) of *S. aureus* strain Newman are unable to anchor surface proteins with LPXTG sorting signals to the staphylococcal cell wall.
envelope; *srtA* mutations effectively disrupt the surface display of 17 polypeptides (Spa, FnBPA, FnBPB, ClfA, ClfB, SdrC, SdrD, SdrE, IsdA (SasE), IsdB (SasJ), IsdH (SasI), SasA, SasB, SasC, SasD, SasF, and SasH) involved in staphylococcal adherence to host tissues or immune evasive strategies (24, 25, 27). When compared to animals challenged with an equal dose of wild-type staphylococci, a significant reduction in mortality of animals infected with sortase A mutants was observed (p=0.001). Protein A, a surface protein with five immunoglobulin-binding modules, captures antibodies via their Fc portion (5, 14). *S. aureus* Newman insertion mutants in *spa*, with defects in protein A synthesis and in staphylococcal binding to immunoglobulin, also displayed a significant defect in *S. aureus* induced mortality. These data corroborate earlier observations on the requirement of protein A for the pathogenesis of staphylococcal pneumonia in newborn mice (10). Sortase B (*SrtB*) anchors IsdC, a heme-binding protein, to the cell wall envelope and mutants with a deletion in *srtB* display defects in staphylococcal heme-iron scavenging (22, 26). Deletion of *srtB* in *S. aureus* strain Newman caused only a small reduction in mortality, suggesting that heme-iron scavenging may not be essential for the pathogenesis of staphylococcal pneumonia. The exopolysaccharide poly-*N*-acetylglucosamine (PNAG) is synthesized by *icaABC* products (12, 29). PNAG conjugates may function as a vaccine as immunization of mice with this compound can protect animals against invasive staphylococcal disease (20, 29). Further, *icaABC* mutations cause a reduction in virulence in a mouse model of abscess formation in kidney tissues (19). However, *icaA* mutants displayed no defect in virulence,
suggesting that PNAG exopolysaccharide is not required for the pathogenesis of staphylococcal pneumonia in mice.

Previous work reported that tracheal instillation of anesthetized Sprague-Dawley rats with *S. aureus* strain 8325-4 into the lung causes damage to alveolar epithelia and erythrocytes in a manner requiring *hla*, which encodes staphylococcal α-toxin, the secreted hemolysin expressed by virtually all *S. aureus* strains (16, 28). After binding to receptor sites on cell surfaces, α-toxin assumes a heptameric assembly and funnel shaped pore that perforates host cell membranes (3, 35). *S. aureus* mutants lacking *hla* display reduced virulence in invasive diseases models as larger numbers of staphylococci are required to kill mice following either intra-peritoneal or intra-mammary infection (4, 33). These observations prompted us to examine the virulence of *S. aureus* Newman *hla* mutants in murine pneumonia. Interestingly, animals infected with the *hla* mutant strain appeared moderately ill within the 24 hours post-inoculation, however only a small number of these animals succumbed to infection (Fig. 4B). The death of these animals was delayed, occurring more than 48 hours post-inoculation. Expression of many staphylococcal genes is regulated by *agr*, the accessory gene regulatory locus. This locus provides for both quorum sensing and regulatory control of virulence (31). Briefly, AgrA and AgrC, a response regulator and sensory kinase, perceive environmental abundance of autoinducer peptide (AIP) to activate expression of an array of genes, including *hla* and other exotoxin genes, at threshold level (15). AIP, synthesized from AgrD proinducer, is processed and secreted by AgrB (23). Mutations in *agrA* are known to
abrogate quorum sensing (32). *S. aureus* Newman variants carrying a bursa aurealis insertion in *agrA* are avirulent in the murine pneumonia model, as none of the experimental animals succumbed to infection (Fig. 4). These findings can be explained by the regulatory defect of *agrA* mutations, which abrogate expression of many virulence genes including α-hemolysin, β-hemolysin, γ-hemolysin, δ-hemolysin as well as leukocidins (31). *S. aureus* Newman cannot express β-hemolysin, as the strain carries a phage insertion in the *hlb* gene (1). However, three secreted γ-hemolysins (HlgA, HlgB and HlgC) assemble into heterooligomeric toxins with structure and function similar to that reported for α-hemolysin (11). Thus, the observed virulence defect of *agrA* mutants in the murine pneumonia model is likely due to the aggregate loss of all secreted hemolysins and toxins (31).

The inability of *agrA* and *hla* mutant strains to contribute to lethality in experimental animals raises the interesting possibility that *S. aureus* exotoxins may play a pivotal role in lung parenchymal injury. It is readily appreciated that insults to the alveolar epithelium contribute to impaired gas exchange. Further, there are detrimental systemic effects of pulmonary inflammation, as patients with acute lung injury are susceptible to multiple organ dysfunction and increased mortality. These systemic effects are likely mediated by the combined effects of inflammatory cytokines, such as IL-1 and IL-8, along with the products of arachadonic acid metabolism including thromboxane A2 and prostaglandins. Our observation that *agrA* and α-hemolysin mutants fail to induce mortality may provide insight into the specific mechanism whereby *S. aureus*-induced lung
injury contributes to the significant morbidity and mortality associated with severe 
*S. aureus* pneumonia. Together with the observation that protein A is required 
for inflammatory responses in the lung following *S. aureus* infection, our data 
suggest that one of the principal functions of *S. aureus* virulence factors may be 
to cause lung parenchymal insult, facilitating bacterial survival and evasion of 
host defenses.

Multiple recent studies have highlighted the association of the Panton-
Valentine leukocidin (PVL) with *S. aureus* strains isolated from patients with 
severe necrotizing pneumonia (8, 30). Like the α-toxin and other hemolysins, 
PVL is a pore-forming toxin whose expression is regulated by *agr*. The precise 
role of PVL in pulmonary infection has not yet been elucidated. Considering the 
data presented here, it seems plausible for us to speculate that *S. aureus* α-toxin 
and PVL may both share the unique ability to induce pulmonary inflammation, 
resulting in systemic manifestations of disease and concomitant mortality. The 
murine model system presented herein will allow for a more rigorous assessment 
of the role these cytotoxins and other staphylococcal virulence factors in the 
pathogenesis of pulmonary infection.

ACKNOWLEDGEMENTS

We thank the Department of Pathology at The University of Chicago for 
preparation of histology samples. J.B.W. is an NICHD Fellow of the Pediatric 
Scientist Development Program (NICHD Grant Award K12-HD00850). Work on
the role of surface proteins and sortases in the pathogenesis of *S. aureus* infections is supported by United States Public Health Service Grants AI38897 and AI52474 from the National Institute of Allergy and Infectious Diseases, Division of Microbiology and Infectious Diseases to O.S.

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FIG. 1. Inoculum-based mortality and proliferation of *S. aureus* Newman in murine lung tissue. (A) C57Bl/6J animals were inoculated with either PBS or various doses of live *S. aureus* Newman via an intranasal route. Percent survival was recorded at 24, 48, and 72 hours post-infection. Animals appearing moribund were killed and counted as dead in the appropriate time frame. Results were analyzed for statistical significance using Fisher’s exact test (*p* ≤ 0.02). (B) Animals were inoculated with 3-4 × 10^8^ colony forming units (CFU) *S. aureus* Newman and bacterial CFU in both lungs (5 min) or the right lung (6, 24, 48, and 72 hr) were enumerated at indicated time points post-infection. Data were analyzed for significance using the Student’s t-test.

FIG. 2. Gross pathology of animals infected with *S. aureus* via intranasal route. Representative infected animals were compared to uninfected animals in order to reveal the gross pathologic findings of lungs *in-situ* (A) or following dissection of the left lung (B).

FIG. 3. Histopathologic findings following intranasal inoculation of *S. aureus*. Lung tissue harvested from animals infected with *S. aureus* Newman was prepared and visualized by hematoxylin and eosin staining. Representative histology is presented for an uninfected control demonstrating normal lung parenchyma and a series of images obtained from animals examined at the time
points indicated. Aggregates of purple-staining immune cells are seen as early as 6 hours post-infection (arrow), with dense accumulation of bacteria evident in tissues at 24 hours post-infection (arrow).

FIG. 4. *S. aureus* mutants lacking protein A (*spa*) or all surface proteins (*srtA*) or exoproteins (*agrA* and *hla*) are defective in the ability to cause pneumonia-related mortality. Animals infected with 3-4 x 10⁸ wild-type *S. aureus* Newman or isogenic mutant strains were scored for acute lethal disease, demonstrating a significant reduction in mortality in animals infected with both the *srtA* and *spa* strains (A). Analysis of mutants with *bursa aurealis* insertions in *agrA* and *hla* (α-toxin) likewise demonstrated marked reduction in the ability to cause acute lethal disease (B). Statistical significance was evaluated by the Fisher's exact test (*p=0.001, **p<0.002*).
A

![Graph](http://iai.asm.org/)  

**A**  

| CFU S. aureus Newman, intranasal | percent mortality |
|---------------------------------|------------------|
| -                              | 0                |
| 8×10⁷                           | 50               |
| 4×10⁸                           | 60               |
| 8×10⁸                           | 80               |

* * indicates a statistically significant difference.

B

![Graph](http://iai.asm.org/)  

**B**  

S. aureus Newman CFU  

dose: 3-4×10⁸ CFU S. aureus Newman

| time post-infection | 5 min | 6 hr | 24 hr | 48 hr | 72 hr |
|---------------------|-------|------|-------|-------|-------|
| S. aureus Newman CFU|       |      |       |       |       |
A

uninfected S. aureus Newman

B

uninfected S. aureus Newman
