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Permalink

https://escholarship.org/uc/item/94v4d884

Journal

The Pediatric Infectious Disease Journal, 38(7)

ISSN

0891-3668

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Publication Date

2019-07-01

DOI

10.1097/inf.0000000000002285

Peer reviewed

Bacterial Colonization of the Hospitalized Newborn: Competition Between *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Abstract

Background—In adults, *Staphylococcus epidermidis* and *Staphylococcus aureus* compete for colonization of the nasal mucosa and *S. epidermidis* strains that produce the Esp serine protease eradicate *S. aureus* nasal colonization. Whether similar phenomena are seen in newborn infants is unknown.

Method—Nasal swabs were obtained on admission and discharge from newborn infants (n = 90 and 83, respectively) in the neonatal intensive care unit at UC Davis Children's Hospital. Swabs were cultured for *S. aureus* and *S. epidermidis. S. epidermidis* isolates were tested for Esp expression, overall secreted protease activity, and biofilm inhibition.

Results—No infant had *S. aureus* on admission. *S. epidermidis* colonization was rare on admission in inborn infants (2.5%), but common in infants transferred from referring hospitals (50%). At discharge, most infants (96%) were colonized by staphylococci. *S. aureus* colonization was less common in infants with *S. epidermidis* colonization (9%), and more common in infants without *S. epidermidis* (77%) (relative risk of *S. aureus* colonization in infants colonized with *S. epidermidis* 0.18, 95% CI 0.089–0.34, p<0.0001). Compared with *S. epidermidis* strains from infants without *S. aureus, S. epidermidis* from infants co-colonized with *S. aureus* had lower total proteolytic enzyme activity and decreased biofilm inhibition capacity, but did not have lower frequency of Esp positivity.

Conclusion—In hospitalized neonates, *S. epidermidis* colonization has a protective effect against *S. aureus* colonization. Secretion of proteases by *S. epidermidis* is a possible mechanism of

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inhibition of *S. aureus* colonization; however, in this cohort of neonates, the source of major protease activity is likely other than Esp.

Keywords

Staphylococcus aureus; *Staphylococcus epidermidis*; neonate; serine protease; biofilm

INTRODUCTION

Fetal skin quickly becomes colonized at birth. The skin of newborns in the neonatal intensive care unit (NICU) often becomes colonized with hospital-derived strains of staphylococci.[1] Coagulase negative staphylococci (CoNS) are typically low virulence organisms that commonly colonize the skin and mucous membranes. *Staphylococcus epidermidis* is the most commonly identified of the CoNS and rarely causes infection in children or adults, but can be a cause of sepsis in premature infants.[2] *Staphylococcus aureus* is also a common skin colonizer, but is potentially a much more virulent bacterium associated with local and systemic infections in all ages. The emergence of methicillin-resistant strains of *S. aureus* (MRSA) is an especially daunting public health issue. The prevalence of such strains is increasing both in hospitals and in the community.[3] Mortality rates for adults with community-acquired MRSA infections and infections caused by methicillin-susceptible strains of *S. aureus* are similar;[4] however, the costs of treating MRSA infection are significantly higher.[5,6] The relationship between *S. aureus* and *S. epidermidis* colonization is of long-standing scientific interest and has been studied in man, in animals, and *in vitro* with a review of these studies published more than 40 years ago.[7] A gap in knowledge exists regarding the dynamics of staphylococcal colonization of hospitalized newborn infants.

In humans, the anterior nares are a prominent ecological niche that commonly harbors a large reservoir of *S. aureus*.[8,9] In a healthy population of adults, approximately 20% will be stably colonized with *S. aureus*, though prevalence varies in different groups. *S. aureus* can spread from skin reservoirs to the bloodstream to cause disease in multiple organs. The relative contribution of *S. aureus* genetics, host factors and environmental factors, including competitive interactions with co-colonizing bacteria, remain areas of active investigation.[10–13]

CoNS, including *S. epidermidis*, compete with *S. aureus* for stable colonization of human skin reservoirs by production of antimicrobial factors and perhaps by nutrient limitation.[14–16] The recent characterization of a strain of *S. epidermidis* that secretes a serine protease named Esp may have the potential to reverse the disturbing trend caused by MRSA. Esp secreting *S. epidermidis* (ESP-SE) can inhibit biofilm formation, destroy biofilms produced by *S. aureus*,[17,18] and inhibit nasal colonization by *S. aureus* in adults and in mice.[19,20] *S. epidermidis* also secretes other proteases including cysteine proteases and metalloproteinases; however, inhibition experiments suggest that serine proteases are the primary mode of inhibition of *S. aureus* biofilm production.[21]

The aims of this study were to characterize the pattern of colonization of the nares of infants in the NICU, to determine whether colonization with *S. epidermidis* is associated with

decreased colonization with *S. aureus* in this population and to analyze the prevalence and possible role of Esp in any observed protection.

METHODS

A prospective cohort study of 90 infants admitted to the NICU at UC Davis Children's Hospital (Sacramento, CA) between February and September 2012 included both term and preterm infants if the anticipated hospitalization was at least 7 days. Infants with congenital anomalies of the skin were excluded. The study subjects included inborn infants, transfers from other facilities, and admissions through our emergency department. This study was approved by the UC Davis Institutional Review Board. Informed consent was obtained from the parents of all enrolled infants.

Nasal swabs were obtained 12–24 hours after admission to the NICU on all 90 infants and within 24 hours of discharge for 83 of the infants. The swabs were cultured on selective media for staphylococci (mannitol salt agar and Spectra MRSA, Remel). Cultures were obtained from infants in the cohort with clinical signs suggestive of sepsis. Among the cohort, 10 infants had blood cultures positive for *S. epidermidis*, 5 infants had blood cultures positive for *S. aureus*. All *S. aureus* and morphologically distinct colonies of CoNS were preliminarily identified by phenotypic tests and confirmed by *S. epidermidis*-specific 16S rDNA PCR as described below. *S. aureus* strains were classified as methicillin–susceptible or resistant based on growth on screening agar, susceptibility and PBP2a latex agglutination testing. A total of 520 *S. epidermidis* isolates were obtained (median - 6 isolates per colonized infant, 25th centile 4 isolates and 75th% centile 7 isolates). DNA was extracted from bacterial cell pellets for each isolate with a standardized procedure (Qiagen, Germantown, MD) and tested for the *Esp* gene by PCR and sequence analysis as described below. *S. epidermidis* isolates (n=214) were randomly selected from the infants who were colonized (median - 2 isolates per colonized infant, 25th centile 2 isolates and 75th% centile 3.25 isolates), sub-cultured, and then analyzed. These 214 clones were analyzed by immunoblotting for expression of Esp and azocasein protease activity as described below. From the 214 clones, 33 isolates from 11 infants were analyzed for *S. aureus* biofilm inhibition as described below.

PCR analysis of isolated colonies

PCR primers were selected using MacVector software (Apex, NC) and purchased from BioSource International (Camarillo, CA). The following oligonucleotide primers were used in the study: 1) for taxonomic identification using bacterial DNA as template: Bac16s27F (5'- AGAGTTTGATCCTGGCTCAG) and Bac16s1391R (5'- GACGGGCRGTGWGTRCA), SE16s21F (5'- ATCAAAAAGTTGGCGAACCTTTTCA) and SE16s145R (5'- CAAAAGAGCGTGGAGAAAAGTATCA); 2) for *Esp* gene detection using *S. epidermidis* DNA as template: Esp10F (5'- TTACCTAATAATAATAGACATCAAATTTTT) and Esp654R (5'- TTACTGAATATTTATATCAGGTATATTGTTTCTT); Esp560F (5'- TGGCTAATGGTTTGTCACCA) and Esp1971R (5'- CAGCTAGCCCAGTTATTGG); Esp990F (5'- CCGTTCCTTTATTGGAGTG) and Esp1674R (5'-

CACCTACAACAACACCTG); Esp1001F (5'- TGGAGTGTCGGTTCGAT) and Esp1663R (5'- ACACCTGAACCACTCAT); Esp802F 5'- CAAATCCTATCTTCCGCTCCAATT) and Esp2136R (5'- ATACTTAATAGGGATAATATTTAG) and from the literature[19,20], EspF (5'- TTTGATGTCTATTATTATTAG), and EspR (5'- GAAACAATATACCTGATATAAATATTCAG). The Esp-targeted primer pairs were designed to span overlapping segments of the entire *Esp* gene to ascertain if a simple deletion or other obvious polymorphism could account for the cases of an apparent discrepancy between *Esp* gene detection, but no measurable protein expression.

Briefly, 100 ng of DNA was amplified with indicated oligonucleotide primer pairs using Roche FastStart Taq DNA Polymerase (Roche Applied Science, Indianapolis, IN). Unless otherwise noted, the specific conditions were: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 7 min. These conditions were modified for the following reactions: Esp10F/Esp654R (annealing at 50°). The PCR products were analyzed by 1–2% (w/v) agarose gel electrophoresis, and by Sanger DNA sequencing for taxonomic analysis and for *Esp* gene analysis from representative samples.

Immunoblot analysis of Esp protein

Bacterial protein extracts were prepared using a method described previously.[2] Aliquots were spotted onto an Immobilon-P membrane for dot-blot assay. To block non-specific interactions, the membrane was soaked in 5% skim milk (W/V in PBS) for 2 hours at room temperature. The membrane was then washed 3 times with PBS-0.01% tween-20 (PBS-T). The membrane was then incubated with anti-Esp primary antibody diluted at 1:1000 in 0.3% skim milk (W/V) overnight at 4°C (the anti-Esp polyclonal antibody was provided by coauthor TI). The membrane was then washed 3 times with PBS-T, followed by incubation with goat-anti rabbit IgG-HRP (Bio-Rad) at a dilution of 1:5000 in 0.3% milk (W/V) for 2 hours at room temperature. The membrane was then washed 3 times with PBS-T and immunoreactivity was detected using Immobilon western chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA). The chemiluminescent signal was detected with a Biospectrum AC Imaging System (UVP, Upland, CA).

Azocasein proteolysis assay

Proteolytic activity in *S. epidermidis* bacterial supernatants was assessed using reported methodology.[17,22] Briefly, individual *S. epidermidis* colonies were grown aerobically in trypticase soy broth (TSB) media, 50 ml for 18 hours at 37°C in a shaking incubator (200 rpm). The bacterial supernatants were clarified by centrifugation at $17,000 \times g$ for 10 mins, and then passed through 0.22 μm filter. An aliquot of each supernatant was diluted with assay buffer to 1%, 5%, 10% and 20% (V/V) and then incubated with 1% azocasein (w/v, Sigma-Aldrich, Saint Louis, MO) in 100 mM Tris-HCl (pH 8.0) at 37°C for 16 h (total volume: 200 μl). As a negative control, buffer was added instead of culture supernatant. The reaction was quenched by the addition of 5% trichloroacetic acid (200 μl) to precipitate uncleaved protein substrate. After centrifugation at $13,000 \times g$ for 10 min the clarified supernatant was assayed for absorbance at 450 nm using 96-well microplate reader. Absorbance values for the negative control reactions were then subtracted from each

experimental specimen and adjusted for the dilution factor to yield an estimate of total proteolytic activity reported as the A_{450} for a 20% v/v dilution.

Quantification of *S. aureus* biofilm inhibition by *S. epidermidis*

Assessment of *S. epidermidis*-mediated inhibition of *S. aureus* biofilm formation was based on methods of Sugimoto et al.[17,19,22] Briefly, for static *S. aureus* biofilm formation from growing bacterial cultures, sterile 96-well flat-bottomed plastic tissue culture plates were used. *S. aureus* was grown aerobically overnight at 37°C in TSB media with 0.05% (w/v) glucose in a shaking incubator (200 rpm). Culture supernatants were then diluted with TSB to an optical density of 0.05 (measured at 600 nm). Aliquots (200 μl) were then transferred to wells of the 96-well plate, and biofilms were allowed to form as the plates were incubated for 20 hours at 37°C aerobically with 5% CO_2. The supernatants were then removed and the wells were washed 3 times with 300 μl of sterile PBS. The residual attached biofilm was fixed with 100% ethanol (200 μl per well) for 2 minutes. The ethanol was removed and the wells were left to air dry. The biofilm was stained with crystal violet (100 μl per well) for 2 min. To remove excess stain, wells were washed 5 times with sterile PBS (300 μl each). The stained biofilm was then dispersed by incubation at room temperature for 10 min with 95% ethanol. An estimate of biofilm mass was then made by measuring absorbance of each well at 570 nm using a microplate reader.

To assess *S. epidermidis*-mediated inhibition of this static *S. aureus* biofilm formation, aliquots of *S. epidermidis* culture supernatant were included during biofilm formation. Thus, each Esp+ strain of *S. epidermidis* was grown aerobically overnight at 37°C in TSB media with shaking (at 200 rpm). The *S. epidermidis* culture supernatant was passed through 0.22 μm filter. Aliquots of the filtered supernatants were then added to *S. aureus* cultures just prior to transfer to 96-well plates for static biofilm formation. The supernatants were added to various proportions (1%, 5%, 10% and 20% V/V) of the *S. aureus* culture (200 μl final volume per well). Inhibition of biofilm formation was measured by comparison of the amount of biofilm made in the absence of *S. epidermidis* supernatant (control) to the amount of biofilm made in the presence of *S. epidermidis* supernatant and reported as the percentage at A_{570} in a 20% V/V dilution.

Statistics

Based on a literature search,[23–30] we anticipated a prevalence of nasal carriage at discharge of about 25% for *S. aureus*, 5% for MRSA, and 25% for *S. epidermidis*. The prevalence of nasal carriage of ESP-SE has not been explored in neonates. We hypothesized that neonates colonized with ESP-SE are significantly less likely to be colonized with *S. aureus* in general and MRSA in particular. To determine the prevalence of *S. aureus*, MRSA, *S. epidermidis* and ESP-SE, a sample size of 90 would allow us to estimate the prevalence with margins of error (95% confidence interval halfwidths) of 10 percentage points wide (or less).

To assess co-colonization of ESP-SE with *S. aureus*, associations were estimated with relative risks for 2×2 tables and exact 95% confidence intervals. Sample size calculations indicated that these analyses would have 80% power to detect large decreases in *S. aureus* colonization proportions that are associated with co-colonization by *S. epidermidis*. For

example, if *S. epidermidis* colonization occurred in 25% of patients and if the incidence of *S. aureus* colonization was reduced to 2% in this group, compared to 25% among patients who are *S. epidermidis* negative, the sample size of 90 patients provided 75% power, under two-sided exact testing with alpha 5% to detect a significant association.

Relative risks were calculated with MedCalc (https://www.medcalc.org/calc/relative_risk.php), and generation of normal quantile and box plots, regression analysis and Mann-Whitney tests were performed with Stata (version 12.1, College Station, TX). As the data for the three analyses of the *S. epidermidis* isolates were not normally distributed, the non-parametric Mann-Whitney test was used to compare isolates from infants not colonized with *S. aureus* to isolates from infants colonized with *S. aureus*.

RESULTS

Demographic and clinical data for the 90 infants enrolled in the study are presented in Table 1. Thirty-nine of the infants enrolled were female. At admission, none of the infants were colonized with *S. aureus*, while 2 of 80 inborn infants and 5 of 10 infants transferred from a referring hospital or admitted through the emergency department were colonized with *S. epidermidis*. We were able to analyze swabs from 83 of the infants at discharge; 76% of infants swabbed were positive for *S. epidermidis* alone, 12% were positive for *S. aureus* alone, 8% were positive for both *S. epidermidis* and *S. aureus*, and 4% grew neither bacterium (see figure, Supplemental Digital Content 1). Only one discharge swab grew MRSA. Based on these data, the nasal swabs that were positive for *S. epidermidis* were less likely to grow *S. aureus* (RR 0.13, 95% CI 0.061–0.28, p=<0.0001) (Table 2). Simple regression analysis showed no association between *S. aureus* colonization at discharge and gestational age, birth weight, days of antibiotics, or days intubated. Correlations with days of feeding tube and length of stay explained only a small percentage of the variance (see table, Supplemental Digital Content 2).

We tested 520 isolates of *S. epidermidis* for the presence of the *Esp* gene by PCR analysis. Ninety-six percent of the isolates tested harbored the *Esp* gene, but it remains uncertain if Esp is universally secreted by *S. epidermidis* in this population, or if Esp is only expressed by a subset of the isolated *S. epidermidis*. A dot-blot assay on culture supernatants using an antibody to Esp demonstrated 188 of 214 randomly selected isolates were positive for Esp (88%), while 26 were negative for Esp. Fifty-five infants had only Esp positive *S. epidermidis* isolates, 5 had only Esp negative isolates and 12 had a mixture of Esp positive and negative colonies. Based on published data from adults and mice [19,20], we hypothesized that ESP-SE colonies would be less likely to be present in infants co-colonized with *S. aureus* than *S. epidermidis* strains that do not express Esp. Figure 1A and Supplemental Digital Content 3 (table) summarize the co-colonization data and show no significant protective benefit of ESP-SE strains against colonization with *S. aureus* (RR 1.5, 95% CI 0.66–3.4, p=0.34). This was also true for the three subgroups of infants with either just ESP-SE, no ESP-SE and those with mixed colonies (Figure 1B).

We next hypothesized that *S. epidermidis* colonies expressing bacterial proteases would be less likely to be present in nares of infants co-colonized with *S. aureus*, as reported for

endotracheal tube colonization.[21] To test this hypothesis, we utilized an azocasein cleavage assay [17,22] as a measure of total protease activity for a given colony of *S. epidermidis* (rather than solely protease activity of Esp). Figure 2A summarizes protease activity for *S. epidermidis* strains that either co-colonized with *S. aureus* or did not. These data support that *S. epidermidis* isolates that co-colonized with *S. aureus* had lower protease activity than isolates from infants not colonized with *S. aureus* (Mann-Whitney p< 0.05). This was also true in the subset of isolates that came from infants colonized by just ESP-SE strains (Mann-Whitney p<0.01), but not for the subsets of isolates from infants colonized just with non Esp producing strains or infants with mixed colonization (though this is likely the result of low numbers of isolates analyzed, Figure 2B).

To further explore the capacity of isolated *S. epidermidis* strains to prevent colonization with *S. aureus*, we utilized an established biofilm inhibition assay.[17,19,22] We hypothesized that *S. epidermidis* strains capable of biofilm inhibition would be protective against co-colonization with *S. aureus* compared to *S. epidermidis* strains incapable of biofilm inhibition. The data in Figure 3A support this hypothesis (Mann-Whitney p<0.01). This difference was statistically significant in the *S. epidermidis* isolates from infants colonized with just ESP-SE strains (Mann-Whitney p<0.01) and those from infants with mixed ESP-SE and Esp negative strains (Mann-Whitney p<0.05) though the small numbers of isolates tested and infants analyzed suggest caution in interpreting these data.

DISCUSSION

Colonization of the nose with staphylococci begins within days after birth. In healthy term infants who are not hospitalized, colonization with *S. aureus* peaks at one month of age and then decreases and is influenced by day care attendance and colonization with respiratory pathogens.[31] In the cohort reported here, by the time of discharge from the NICU, 96% of infants were colonized with staphylococci. Our data support that strains of *S. epidermidis* with high protease activity and/or high biofilm inhibitive capacity were protective against co-colonization with *S. aureus*. Previous studies of nasal colonization with CoNS in the NICU demonstrated that both *S. epidermidis* and *S. haemolyticus* are common in the nasal mucosa of NICU infants.[1] We chose to focus on *S. epidermidis* to determine whether a mechanism demonstrated in adults was applicable to neonates. Our data may have particular clinical relevance in two areas.

First, the observation that both ESP-SE organisms and purified Esp introduced into the noses of healthy adults decreased colonization with MRSA and methicillin-susceptible *S. aureus* suggests that it is possible to alter the nasal microbiota without antibiotic therapy.[19,20] Our study suggests that competition between *S. aureus* and *S. epidermidis* may utilize different mechanisms in the infant than in the adult or that the current study population had a different epidemiologic mix of *S. epidermidis* strains than the previous studies. While 96% of the *S. epidermidis* isolates we analyzed contained the *Esp* gene, expression of the Esp enzyme was seen in 88% with moderate variability in Esp intensity among these isolates. Previous studies found that some *S. epidermidis* strains that expressed Esp showed little protease activity using an azocasein protease assay ([19,20] plus unpublished observations). The authors suggested that *S. epidermidis* can be divided into 3 groups: strains that do not express Esp,

and strains that express Esp either with or without azocasein protease activity. Although mechanistic details are required to more clearly understand this phenomenon, the determinants might result from multifactorial complex interactions. In the cohort reported here, ESP-SE colonies did not correlate with prevention of co-colonization with *S. aureus*, similar to what was reported in analysis of an adolescent population.[32] Rather, *S. epidermidis* colonies positive for azocasein protease activity and biofilm inhibition assays both correlated with protection from *S. aureus* co-colonization suggesting that one or more proteases other than Esp may be an important mechanism by which *S. epidermidis* outcompetes *S. aureus* in the NICU patient.

Second, methicillin resistance is common in CoNS organisms and appears to increase in the NICU population with length of hospital stay.[1] CoNS organisms may represent a reservoir of resistant organisms and transfer of methicillin resistance from CoNS to *S. aureus* through the staphylococcal cassette chromosome mec has been proposed as a source for novel strains of MRSA.[33] This would suggest that strains of *S. epidermidis* that are not able to suppress growth of or biofilm formation by *S. aureus* may be more likely to transmit methicillin resistance. Similarly, transfer of other virulence-related genes (e.g. those associated with proteases, biofilm formation and resistance to other antibiotics),[34,35] from *S. epidermidis* to *S. aureus* could conceivably influence competition between these two species.

Given the high cost, morbidity and mortality of staphylococcal infections, further investigations of interactions between *S. aureus* and CoNS microbes are indicated, including exploration of non-ESP proteases in the neonatal population. Non-antibiotic approaches to decreasing nasal carriage of MRSA are appealing but not yet justified in the immunocompromised or ICU patient. Animal models may have particular value in determining mechanisms of competition or resistance transfer.[36]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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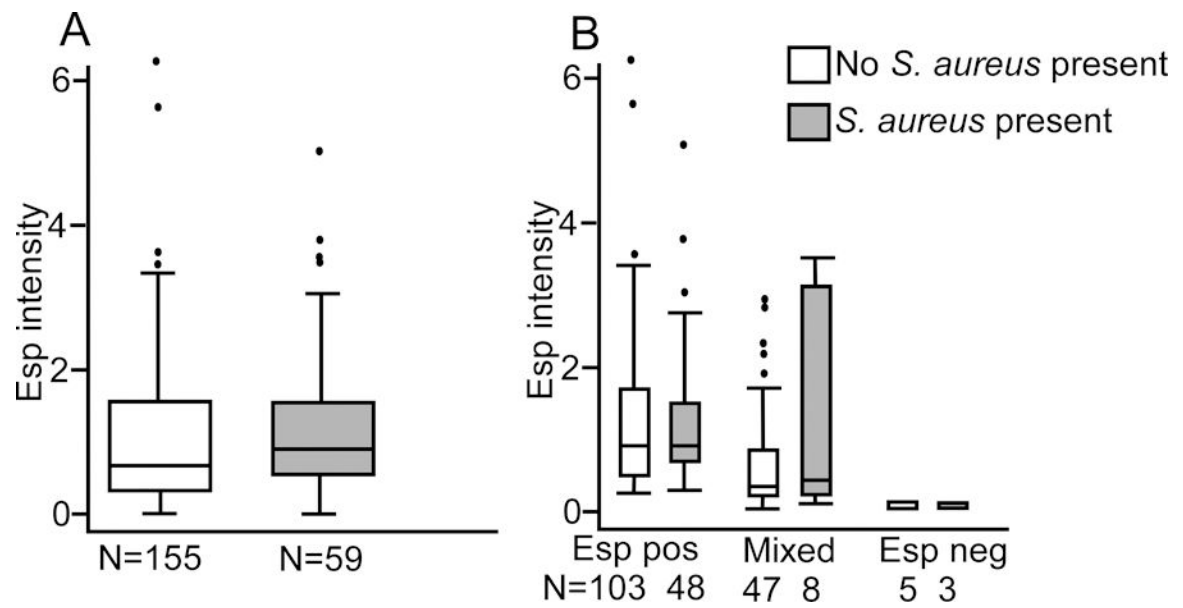
Figure 1:
(A) *S. epidermidis* isolates (N=214) tested for Esp activity by dot-blot assay and divided based on whether the isolate came from an infant that was not colonized with *S. aureus* (open boxes) or was colonized with *S. aureus* (shaded boxes). (B) The same data subdivided based on whether all isolates tested from the infant were positive for Esp, mixed, or negative for Esp. Mann-Whitney p values > 0.05 for all comparisons.
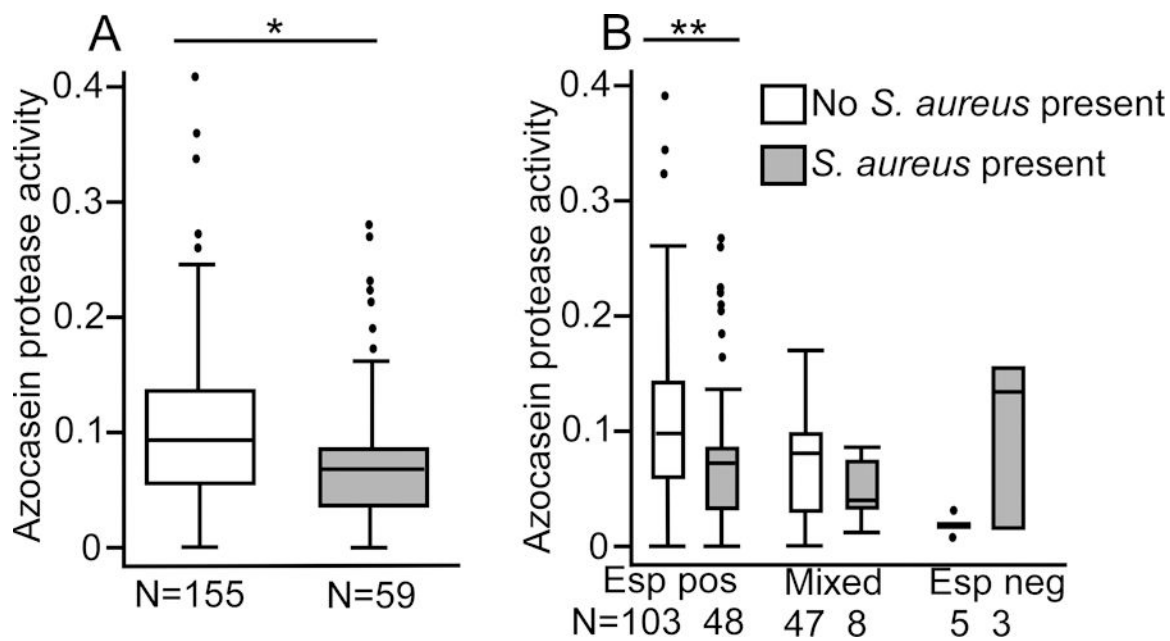
Figure 2:
(A) *S. epidermidis* isolates (N=214) tested for protease activity and divided based on whether the isolate came from an infant that was not colonized with *S. aureus* (open boxes) or was colonized with *S. aureus* (shaded boxes). (B) The same data subdivided based on whether all isolates tested from the infant were positive for Esp, mixed, or negative for Esp. Mann-Whitney *p<0.05, **p<0.01.
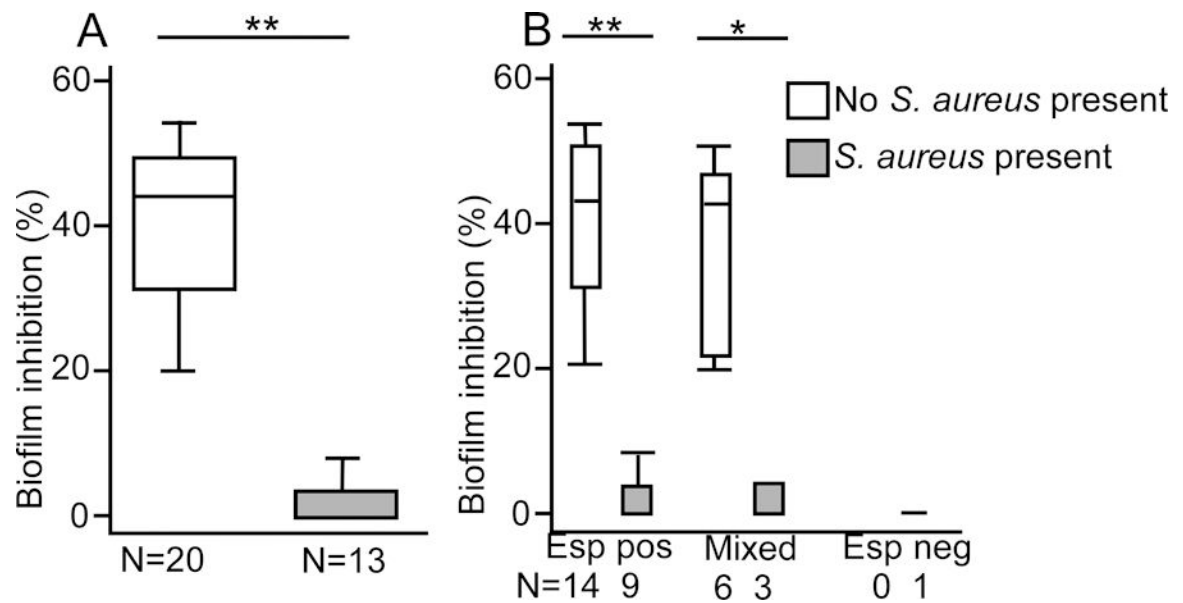
Figure 3:
(A) *S. epidermidis* isolates (N=33) tested for *S. aureus* biofilm inhibition divided based on whether the isolate came from an infant that was not colonized with *S. aureus* (open boxes) or was colonized with *S. aureus* (shaded boxes). (B) The same data subdivided based on whether all isolates tested from the infant were positive for Esp, mixed, or negative for Esp. Mann-Whitney *p<0.05, **p<0.01.

Table 1:

Demographic and clinical data for the full cohort (N = 90 infants)

	Mean	Range
GA (Weeks)	33	24–40
BW (Grams)	2248	610–5585
Days of Hospitalization	24	1–132
Days of Antibiotics	3	0–36
Days of Intubation	3	0–50
Days with a feeding tube	13	0–123

Table 2:

Infants colonized with *S. epidermidis* and/or *S. aureus* at discharge (N = 83 infants, p < 0.001 Fisher exact test)

	S. aureus **positive**	*S. aureus* **negative**
S. epidermidis positive	7	63
S. epidermidis negative	10	3