Published in final edited form as:
J Infect Dis. 2008 July 15; 198(2): 258–261. doi:10.1086/589307.

Development of real-time in vivo imaging of *Staphylococcus epidermidis* device-related infection in mice: influence of animal immune status

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Abstract

SUMMARY—We constructed a bioluminescent strain of *Staphylococcus epidermidis* and developed a catheter-related murine infection model for real-time monitoring of biofilm-associated infection in this leading nosocomial pathogen. Additionally, we compared immune-compromised versus immune-competent mice, demonstrating a substantial effect of animal immune status on susceptibility to experimental *S. epidermidis* infection.

Advanced modern detection techniques such as bioluminescence imaging (BLI) utilize a high sensitive optical biophotonic camera and subsequent digital quantification to measure *in vivo* processes. Bioluminescence results from the emission of visible light by metabolically active organisms through luciferase-catalyzed oxidation of the substrate luciferin. The bioactive process requires FMN_{H2}, ATP, and oxygen. Microorganisms can be engineered to produce bioluminescence by insertion of the *lux* genes into the bacterial genome. Thus, BLI permits visualization, quantification, and monitoring of "tagged" microorganism within intact animals (3,4,8,21).

Staphylococcus epidermidis is the leading pathogen in nosocomial infections associated with biofilm formation on medical devices (12,27). Despite the high prevalence of *S. epidermidis* infections in hospitals, not much is known about how *S. epidermidis* biofilms develop in the living host. Almost all animal infection studies require euthanasia at a final time point for the isolation of bacteria from implanted devices and tissues (18). However, samples obtained from these studies represent only a *status quo* of biofilm infection. In contrast, although biofilm-associated factors of *S. epidermidis* (e.g. *ica*, *atlE*, *agr*) have been investigated in several animals (18-20,26), *in vivo* dynamics of *S. epidermidis* biofilm-associated infection have not been monitored.

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Generation of SE Xen43 and BLI of S. *epidermidis* biofilm in mice

To set up a real-time monitoring model of *S. epidermidis* biofilm-associated infection using BLI, we first genetically engineered a bioluminescent *S. epidermidis* (SE) strain, SE Xen43, by insertion of the *luxABCDE* genes into the genome of the biofilm-positive clinical isolate SE 1457 (13) as reported previously (9,10). In this strain, the *lux* genes had inserted within the intergenic region between the open reading frames SE2196 and SE2197 (31)as determined by inverted PCR sequencing. To ascertain that the bioluminescent strain was phenotypically equal to the parental strain except for light emission, SE Xen43 and SE 1457 were tested *in vitro* for biofilm formation, extracellular polysaccharide production, growth, biochemical profile (API 20 STAPH System and DNAse activity), and hemolysis. No significant differences were found between both strains (data not shown). To compare *in vivo* biofilm capacity and virulence of the strains, we used a murine model of subcutaneous device-related infection with exponentially or stationary grown inocula. The two different inocula were selected to evaluate a potential influence of inoculum growth phase on disease progression. The experiment was performed as described previously using Balb/C mice (8 mice, female, 18-25 grams, Charles River, Wilmington, MA, per *S. epidermidis* strain, initial inoculation: 2×10^4 cells/catheter). Mice infected with SE Xen43 were anesthetized daily with gaseous isoflurane for BLI (9,10) with an IVIS 100 imaging system and quantitation using Living Image 2.11 software (Xenogen Corporation, Alameda, CA). Exposure was for a maximum of 5 min. Bioluminescence from predefined regions of interest (ROI) was expressed using a pseudocolor scale with red representing the most intensive and blue the least intensive BLS (9,10). The study protocol was approved by the "Animal Care and Use Committee" of the Rocky Mountain Laboratories.

We monitored progression of biofilm-associated disease caused by SE Xen43 using BLI within intact mice for 7 days (data not shown). Additionally, samples from catheters and surrounding tissues were collected at the final experiment day (day 7) for enumeration of bacterial colony forming units (CFUs) as published (11,25,26). We did not detect significant differences in infectivity between the strains or inocula from different growth phases (Fig. 1A). These results demonstrate that the *luxABCDE* genes insertion in SE Xen43 does not alter the capacity to establish biofilms on plastic polymer material *in vivo* or any other tested *in vitro* characteristics. Hence, SE Xen43 is a suitable strain for *in vivo* research related to *S. epidermidis* infections.

Immune deficiency impacts host susceptibility to biofilm-associated *S. epidermidis* infection

Defects in the host immune system commonly promote microbial infection. Neonatal, immune-compromised or granulocytopenia patients are examples of groups with a particularly high medical risk for *S. epidermidis* infection (17,24,28). Most animal studies of *S. epidermidis* biofilm-associated infection have been performed with immune-competent animals. Therefore, here we investigated how host immune competency impacts *S. epidermidis* biofilm-associated infection. We selected two severe combined immunodeficiency (SCID) mouse strains, Nu/Nu (female, 18-25 gram, Charles River, Wilmington, MA) and CBSCBG-MM (female, 18-25 gram, Taconic Laboratories, Hudson, NY), and compared their susceptibility towards biofilm-associated infection with the immune-competent Balb/C mouse strain. Nu/Nu mice are athymic and T-cell deficient but B-cells and natural killer cells positive (2,15,22,23). CBSCBG-MM mice carry SCID mutation (lack of both T and B lymphocytes), cytotoxic T cells and macrophage defects, and are impaired in natural killer cell function (1,14,16,29). The animal experiment was performed as described above (8 mice per strain) with stationary growth phase cultures of SE Xen43, because (i) we found no significant differences in infection between exponential and stationary growth phase inocula, and (ii) the physiological status of *S. epidermidis* colonizing its natural habitat is presumably more similar to stationary growth phase. Five days before the start of the experiment, administration of antibiotics to the SCID

mice was terminated not to kill *S. epidermidis* cells in the inocula. Biofilms were monitored daily by BLI of SE Xen43 in mice (Fig. 2A). Bioluminescence by SE Xen43 in almost all mice reached a maximum already at 24 h and decreased slowly afterwards (Fig. 2B), most likely due to an activated host immune system. We cannot exclude the possibility that bioluminescence intensity was affected by changes in the metabolic status of the bacteria, which is however a general limitation of BLI in all organisms. A slight increase in bioluminescence was observed in most mice after day 6 indicative of a regeneration of *S. epidermidis* infection or changes in cell metabolism. Notably, CFU determination indicated significantly higher differences in infectivity compared to data obtained from BLI at day 7 (Fig. 1B,Fig. 2B). Importantly, BLI and viable counts (CFUs) data showed that both immune-compromised mice strains were more susceptible towards *S. epidermidis* biofilm infections with Nu/Nu being the most susceptible of all tested strains. Our data clearly support the clinical evidence (5,6,28) that patients with immune deficiency are more susceptible to *S. epidermidis* biofilm-associated infection.

Concluding comments

Our study indicates that SE Xen43 is a suitable tool to study *S. epidermidis* virulence *in vivo* and demonstrates that mice that lack a functional immune system are much more susceptible towards *S. epidermidis* device-related infection compared to healthy mice. The limitation of BLI for *S. epidermidis* is the low intensity in bioluminescence signaling compared to many other bacteria (7,9,30). However, we believe that our results will initiate further investigation on *S. epidermidis* infections, especially to elucidate the specific mechanisms by which *S. epidermidis* evades host defenses *in vivo*.

ACKNOWLEDGEMENT

We would like to thank Ralph Larson, Donald Gardner, and Meghan Kahnle for technical supports with the animal studies.

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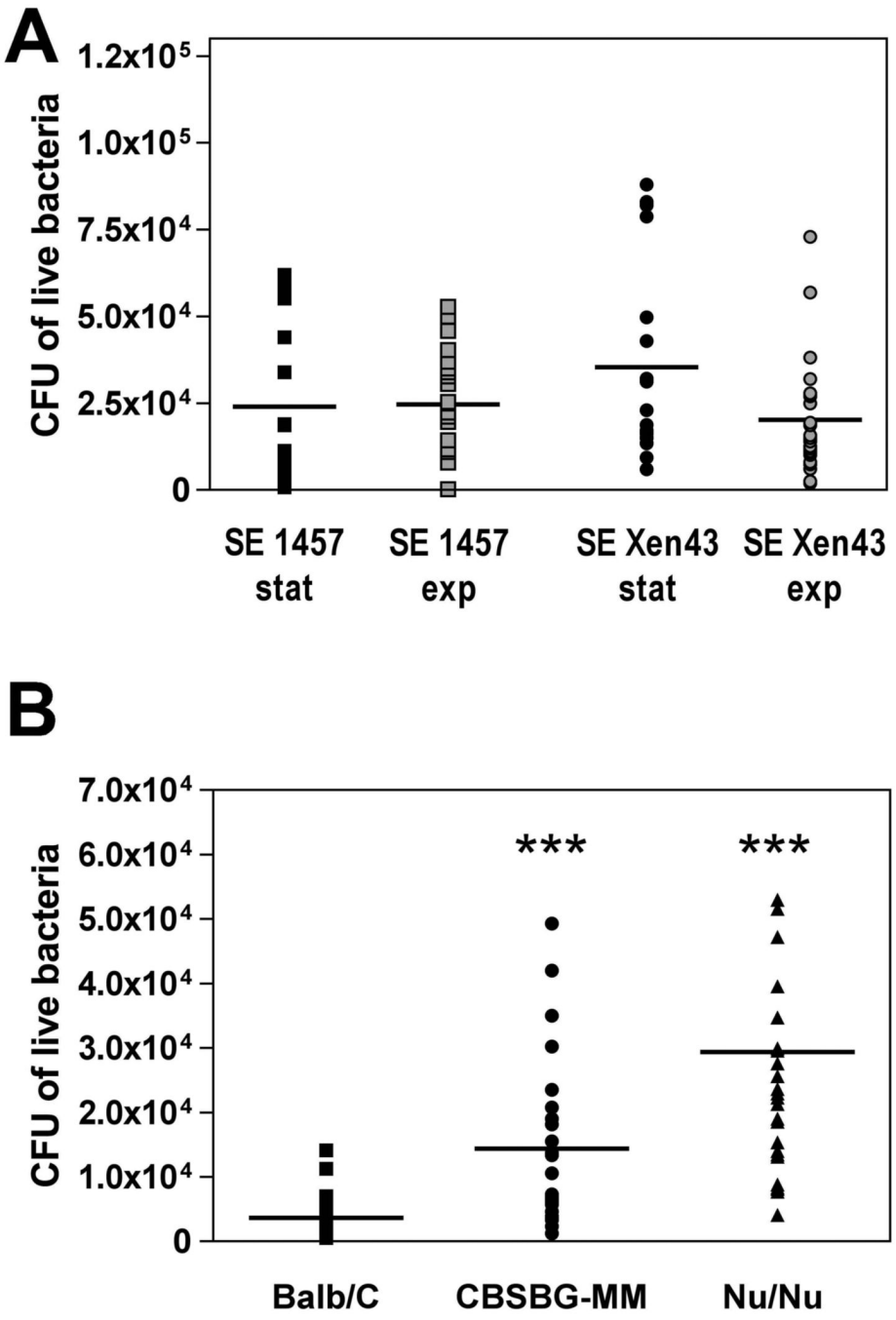

Fig. 1. Mouse biofilm-associated infection model, data from endpoint CFU determination

(A) Influence of inoculum growth phase and comparison of constructed bioluminescent strain to parental strain. A murine subcutaneous model of device-related infection was utilized to compare infectivity of the parental wild-type strain SE 1457 and its bioluminescent derivative SE Xen43. Exponential and stationary growth phase inocula of both strains were used to determine whether growth phase conditions impact biofilm virulence. After 7 days of infection, mice were killed by isoflurane overdose. Catheters and surrounding tissues were carefully removed from mouse bodies. Bacterial biofilms attached to catheter surfaces were isolated by sonication. Tissues samples were disrupted with a motorized homogenizer. Samples were plated on TSB agar plates, incubated at 37°C for 24 h and bacteria were determined as CFU.

CFU isolated from catheters and surrounding tissues were summarized in the graph. The mean is shown for each group. No statistically significant differences between the groups were detected. **(B)** Comparison of immune-competent with immune-deficient mouse strains. Immune-competent wild-type Balb/C mice, severe combined immunodeficiency mice (SCID) CBSCBG-MM mice, and SCID Nu/Nu mice were tested for their susceptibility towards biofilm-associated infection caused by bioluminescence SE Xen43. CFU counts from catheters and tissue samples were combined. The mean is shown for each group. ***, $p<0.001$ versus Balb/C group.

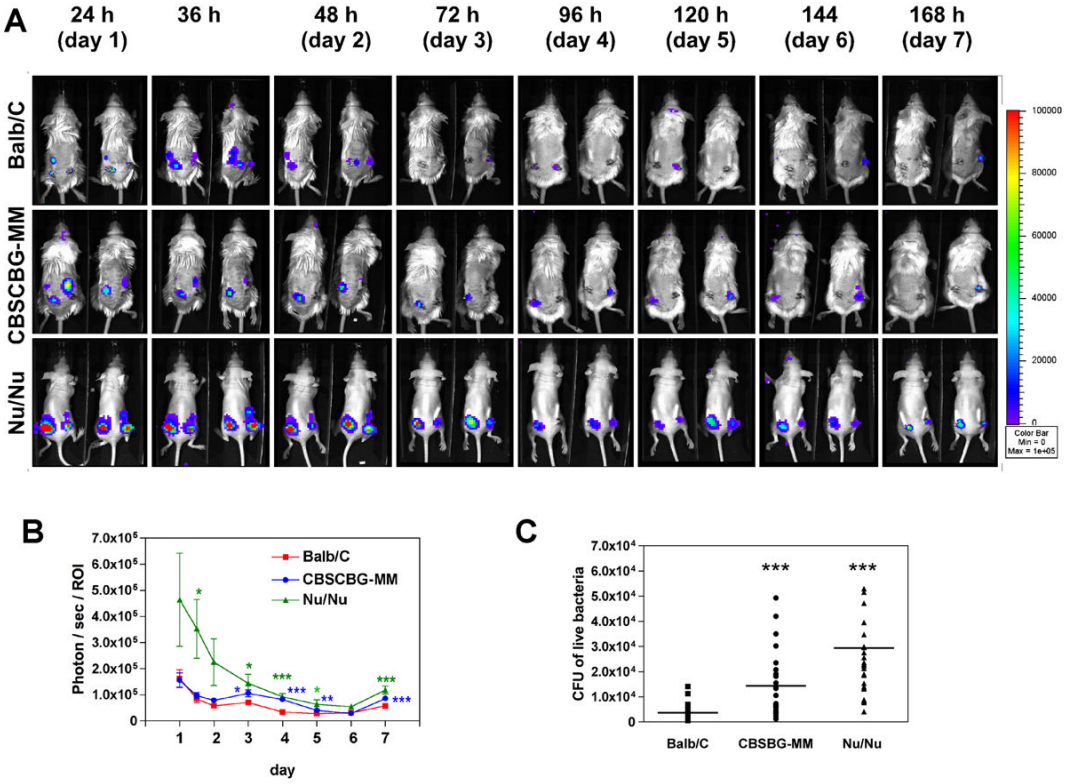

Fig 2. Mouse biofilm-associated infection model, data from real-time monitoring with BLI

(A) Biofilm-associated infection caused by SE Xen43 in intact Balb/C, CBSCBG-MM, and Nu/Nu mice was monitored in real time for 168 h (7 days). Images were taken every 12h for the first 36 h, and every 24 h afterwards. Mice were anesthetized continuously with isoflurane during imaging procedures. Photon/sec/ROI were presented as the cumulative photon counts collected within each measured ROI and quantified with Living Image 2.11 software. **(B)** Quantification of time course dependent bioluminescence of SE Xen43 in Balb/C, CBSCBG-MM, and Nu/Nu mice.