B-048

51st ICAAC Meeting
Chicago, IL
September 17-20, 2011

Alpha-Toxin Neutralization Significantly Impacts *Staphylococcus aureus* Biofilm Formation

M. E. Pulse, P. Nguyen, K. Peterson, J. Pierce, P. J. Renick, J. W. Simecka, D. Valtierra, W. J. Weiss

UNT Health Science Center-Preclinical Services, Fort Worth, TX

* Contact Information:
UNT Health Science Center
3500 Camp Bowie Blvd.
Fort Worth, TX 76107
mark.pulse@unthsc.edu
www.hsc.unt.edu/preclinical

Abstract

Background: Previous results generated by our lab have demonstrated that alpha-toxin (Atox), a hemolytic exotoxin, influences *S. aureus* biofilm formation on plastics. With these results in mind, the following study was designed to test whether neutralizing Atox decreases *S. aureus* biofilm formation and associated disease in a rat endocarditis infection (REI) model.

Methods: Hemolytic titers of the *S. aureus* endocarditis isolate (NRS234) used in the animal model were determined by incubating spent media harvested from 18-hour cultures with 1% rabbit blood cells. The spent media was also pre-treated with 0.1 mg/mL of polyclonal anti-Atox antibody (anti-Atox). In vitro biofilm assays were performed with a MBEC assay™ device that was inoculated with NRS234 and treated with 0.1 mg/mL of anti-Atox or serum control. After 24 hours at 37°C, well-associated biofilms were stained with crystal violet and pin-associated cells were recovered and plated for CFU counts. The REI model involved animals that were intravenously challenged with NRS234 that had been pre-treated with anti-Atox (10 mg/mL) or the serum control, and hearts were removed and processed for CFU recovery 4 days after infection.

Results: The mean hemolytic titer of NRS234 18-hour cultures was 37.3, while pre-treatment with anti-Atox resulted in a mean hemolytic titer of 2.3. Crystal violet staining of well-associated biofilms generated significantly different (P value <0.0001) mean absorbance (450 nm) values for NRS234 treated with anti-Atox (1.2) as compared to the serum control (0.12), while the mean CFU associated with the pegs were the same for both treatments (6.5 versus 6.2) after 24 hours. In the REI model, anti-Atox and serum control pre-treatment resulted in mean $\log_{10}$ heart-associated CFU of 9.5 and 9.7, respectively.

Conclusions: Neutralizing Atox significantly decreased in vitro biofilm formation of NRS234, which suggests that it may be a treatment option for *S. aureus* biofilm-associated infections. Even though differences were not observed in the REI model, Atox neutralization should be further explored for the treatment of *S. aureus* infections.

Introduction

Staphylococcus aureus has the ability to produce a number of virulence factors (toxins) that are thought to be important during the infection process and resulting disease state in the host. A well described staphylococcal virulence factor is alpha-toxin, which is a secreted cytotoxin that is known to lyse a number of host cells, including red blood cells. Caiazza et. al in 2003 published results that suggested alpha-toxin may be playing a role in *S. aureus in vitro* biofilm formation. Staphylococcal biofilms are bacterial communities that are formed on surfaces and contain metabolically slowed cells embedded in an exopolysaccharide matrix. Biofilms increase the persistence of *S. aureus* infections, boost associated antimicrobial resistance, and form on medical devices including intravascular catheters and pacemakers. Therefore, if a therapy could diminish *S. aureus* biofilm formation, then persistence and associated antibiotic resistance could potentially decrease, resulting in improved treatment outcomes for staphylococcal biofilm infections.

Previous work in our lab with *S. aureus* RN6390 and an alpha-toxin mutant of RN6390 revealed that the alpha-toxin mutant had an impaired ability to generate biofilms both *in vitro* and *in vivo* (Poster B-207, ASM 2007). With these results in mind, we hypothesized that if alpha-toxin production could be therapeutically neutralized, then *S. aureus* biofilm formation would be diminished and associated antibiotic resistance would be decreased. Here, we describe the results of several studies that were aimed at addressing this hypothesis.

Methods and Materials

Bacterial strains. *S. aureus* NRS234 strain was obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program supported under NIAID/NIH Contract #HHSN272200700055C, and the RN6390 strain was kindly provided by Dr. Mark Hart (NCTR, Jefferson, AR).

Culture conditions and inoculum preparation. For the *in vitro* biofilm assay and the mouse SC catheter model (MBF), *S. aureus* strains were grown in tryptic soy broth (TSB) supplemented with 0.9% NaCl and 0.25% glucose, and 0.1 mg/mL of serum control or polyclonal anti-alpha toxin (ATOX) antibody (Sigma Aldrich) was added to the inocula before being added to the wells of the MBEC™ Assay device or to tubes containing 1-cm length catheters (14 G, Teflon). For the rat endocarditis model (REI), plate cultures of *S. aureus* NRS234 were suspended in 0.9% NaCl saline, and 10 mg/mL of serum control or polyclonal anti-ATOX antibody was added to the inoculum before infection.

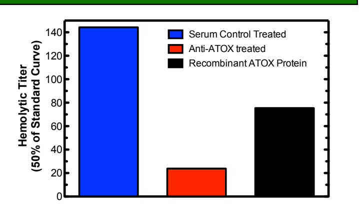
Hemolytic assay. The spent media from 18- hour cultures were harvested and sterilized through 0.1 µm filters. Spent media was 2-fold serial diluted in 96-well plates, and 0.1 mg/mL of the serum control or the anti-ATOX was added to designated wells before adding 1% rabbit blood cells. Hemolytic titer values were equal to 50% of the standard curve as determined by A_{450}.

***In vitro* biofilm and susceptibility assay.** *S. aureus* NRS234 inoculum was added to the wells of a MBEC™ Assay device and cultured for 24 hours. Well-associated biofilms were stained with crystal violet, and pins were sonicated and processed for CFU enumeration. Stain was removed with 33% acetic acid and stain intensity was measured as the absorbance value at 630 nm. For the susceptibility assay, 24-hour biofilms were challenged with 2-fold titers of vancomycin, and biofilms were disrupted with 0.05% Tween and plated for break point determination.

Mouse subcutaneous biofilm model (MBF). *S. aureus* infected catheters were surgically placed under the skin of female BALB/c mice. Catheters were removed +24 and +48 hours after implantation and processed for CFU recovery.

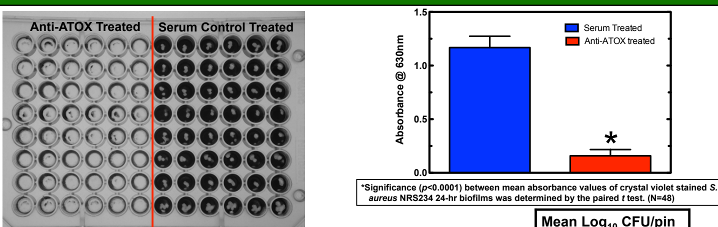
Rat endocarditis model (REI). Male Sprague-Dawley rats were anesthetized and a 4 – 5 cm segment of PE10 tubing was implanted into the heart through the right carotid artery. *S. aureus* NRS234 prepared inoculum was IV injected +48 hours after surgery, and vancomycin was dosed +6, +24, +48 hours after infection. Hearts were removed from euthanized animals at designated time points post-infection for CFU recovery and enumeration.

Panel 1: Anti-Alpha Toxin (ATOX) Treatment Reduces Hemolytic Activity of 18-hr *S. aureus* NRS234 Cultures



•Filter-sterilized spent media from 18-hour cultures of *S. aureus* NRS234 was pre-treated with the serum control OR with the anti-ATOX antibody (0.1 mg/mL) before incubation with 1% rabbit blood cells.
•Recombinant ATOX protein was 2-fold diluted from 1,000 units/mL and served as the positive control.

Panel 2: *S. aureus* NRS234 *In Vitro* Biofilm Density is Significantly Reduced with Anti-Alpha Toxin (ATOX) Treatment



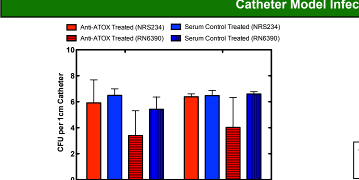
•Image of 96-well plate AFTER crystal violet staining of *S. aureus* NRS234 24-hr biofilms.
•Black smears at the bottom of each well represent the visual density of each biofilm.

*Significance (p<0.0001) between mean absorbance values of crystal violet stained *S. aureus* NRS234 24-hr biofilms was determined by the paired *t* test. (N=48)

	Mean $\log_{10}$ CFU/pin
Serum Treated	6.2
Anti-ATOX Treated	6.5

•*S. aureus* NRS234 CFUs were collected from the pins of the MBEC Assay™ after 24 hrs of growth.

Panel 3: Anti-Alpha Toxin (ATOX) Treatment Decreases Early *In Vivo* Biofilm Formation in a Mouse Subcutaneous Catheter Model Infected with *S. aureus* RN6390



Hours (After Implant)	$\log_{10}$ CFU of NRS234 per Catheter: Anti-ATOX Treated	$\log_{10}$ CFU of NRS234 per Catheter: Serum Control Treated	$\log_{10}$ CFU of RN6390 per Catheter: Anti-ATOX Treated	$\log_{10}$ CFU of RN6390 per Catheter: Serum Control Treated
24	5.9	6.5	3.4	5.4
48	6.4	6.5	4.0	6.6

•Catheter segments (14 gauge, 1 cm) were infected with 5.0 $\log_{10}$ CFU of *S. aureus* NRS234 or RN6390 that were pre-treated with serum control or anti-ATOX antibody (0.1 mg/mL).

Panel 4: Minimum Inhibitory Concentration (MIC) of Vancomycin against *S. aureus* NRS234

	NRS234	ATCC 29213*
Vancomycin (µg/mL)	0.5	2.0

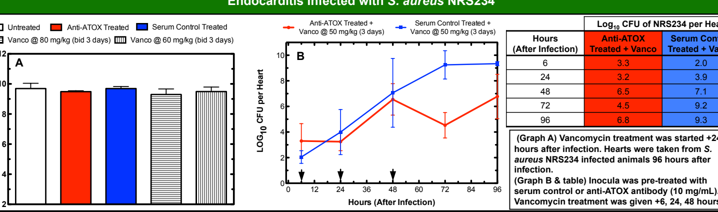
**S. aureus* reference strain included as a quality control. The microdilution method was used to determine the MIC value for vancomycin as outlined in CLSI guidelines.

Panel 5: Anti-Alpha Toxin (ATOX) Treatment Increases *In Vitro* Vancomycin Sensitivity of *S. aureus* NRS234 24-hr Biofilms

	NRS234: Serum Control	NRS234: Anti-ATOX
Vancomycin (mg/mL)	65.54	8.19

•*S. aureus* NRS234 inoculum was pre-treated with 0.1 mg/mL of the serum control OR the anti-ATOX antibody before inoculating 96-well plates.
•24-hr biofilms were challenged with titers of vancomycin, disrupted with 0.05% Tween, & plated for break point determination.

Panel 6: Anti-Alpha Toxin (ATOX) Treatment Enhances *In Vivo* Vancomycin Efficacy a in Rat Model of Experimental Endocarditis Infected with *S. aureus* NRS234



Hours (After Infection)	$\log_{10}$ CFU of NRS234 per Heart: Anti-ATOX Treated + Vanco	$\log_{10}$ CFU of NRS234 per Heart: Serum Control Treated + Vanco
6	3.3	2.0
24	3.2	3.9
48	6.5	7.1
72	4.5	9.2
96	6.8	9.3

(Graph A) Vancomycin treatment was started +24 hours after infection. Hearts were taken from *S. aureus* NRS234 infected animals 96 hours after infection.
(Graph B & table) Inocula was pre-treated with serum control or anti-ATOX antibody (10 mg/mL). Vancomycin treatment was given +6, 24, 48 hours.

Summary and Conclusions

•When compared to the hemolytic titer of serum control treated spent media, the titer value of *S. aureus* NRS234 cultures was decreased by ~7-fold after treatment with the anti-alpha toxin antibody (Panel 1).

•Treatment with the anti-alpha toxin antibody decreased the density of *S. aureus* NRS234 *in vitro* biofilms by 10-fold as compared to serum control treated biofilms. With CFU counts being approximately equal for each treatment, the reduction in biofilm density after anti-alpha toxin antibody treatment could not be attributed to decreased cell viability (Panel 2).

•Treating the *S. aureus* NRS234 inoculum with anti-alpha toxin antibody did not produce a pronounced decrease in CFUs associated with SC implanted catheters. However, when compared to the *S. aureus* RN6390 inoculum treated with the serum control, catheters infected with the anti-alpha toxin antibody treated inoculum had lowered CFUs 24 and 48 hours after surgical implantation (Panel 3).

•When compared to the serum control treated *S. aureus* NRS234 inoculum, inoculum treated with the anti-alpha toxin antibody increased vancomycin susceptibility by ~8-fold in 24-hour *in vitro* biofilms of *S. aureus* NRS234 (Panel 5).

•No difference in CFU counts was observed in hearts infected with *S. aureus* NRS234 inocula treated with either the serum control or the anti-alpha toxin antibody. However, vancomycin efficacy was enhanced in animals infected with the inoculum treated with the anti-alpha toxin antibody as compared to animals infected with the serum control treated inoculum (Panel 6).

•Treatment of *S. aureus* inocula with the anti-alpha toxin antibody reduced *in vitro* biofilm density and enhanced antibiotic efficacy in a device-induced model of experimental endocarditis. These results suggest that neutralizing alpha toxin during a *S. aureus* infection could reduce biofilm formation and potentially improve the clinical treatment for this type of staphylococcal infection.

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Acknowledgments

The mouse biofilm and rat endocarditis models were done in accordance with the protocols approved by the UNTHSC-Institutional Animal Care and Use Committee.

S. aureus NRS234 (endocarditis isolate) strain was obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program supported under NIAID/NIH Contract #HHSN272200700055C.