Salar Sanjari

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EDUCATION

Expected: May 2014 **Doctor of Dental Surgery / Masters of Clinical Research**
University of Maryland, Baltimore College of Dental Surgery; Baltimore, MD

2008 - 2009 **Masters of Molecular Biology**
University of Maryland Baltimore County; Baltimore, MD

2005 - 2008 **Bachelor of Science, Biology**
University of Maryland Baltimore County; Baltimore, MD

PROFESSIONAL EXPERIENCE

June 2013- Current **Advanced Education in General Dentistry**
University of Maryland, Baltimore college of Dental Surgery
Selected to join UMB's Advanced Education in General Dentistry residency for the entire senior year of dental school.

July 2013 **Externship**
Center Park Endodontics (Dr. S Craig Schneider)
Observed and assisted with variety of endodontic procedures in a private practice setting.

July 2012- June 2013 **Pediatric Clerkship**
University of Maryland, Baltimore College of Dental Surgery
Selected to further study pediatric dentistry in classroom and clinical setting. Attended weekly lectures and journal clubs and treated more complex clinical cases.

WORK AND RESEARCH ACTIVITIES

May 2011- Current **Master's Thesis: Development and evaluation of a Biofilm as well as Planktonic Specific Vaccine Against *Staphylococcus aureus* in a Murine Model**
University of Maryland, Baltimore College of Dental Surgery; Department of Microbial Pathogenesis, Dr. Mark E. Shirtliff's Laboratory

May - Aug 2010 **University of Maryland, Baltimore College of Dental Surgery; Department of Endodontics, Prosthodontics, and Operative Dentistry**

FAp-forming CPC and EndoSequence Compared to MTA: A Polymicrobial, dual-chamber Leakage Study

Sep 08- May 09 **Master's Thesis: Physiology of *Streptococcus mutans***
University of Maryland Baltimore County; Department of Biology

Jan 09- Jun 09 **Research Assistant: Studying cell migration using *Drosophila melanogaster* embryos.**
University of Maryland Baltimore County; Department of Biology

June 07- Aug **Summer Intern: Genomic and Proteomic Analyses of *Torpedo californica* as a Model of Mammalian Neuromuscular Junctions**
Children's National Medical Center, Center for Genetic medicine

Jan 07- May 07 **Undergraduate Teacher Assistant for Bio100**
University of Maryland, Baltimore County; Department of Biology

Sep 06- Dec 06 **University of Maryland Baltimore County, Shriver Center: Vertically Integrated Partnerships K-16 Project**
Spending 8 hours per week in Sherwood High School; teaching biology, helping high school students and assisting the teacher

Feb 06- Feb 07 **University of Maryland Baltimore County; Department of Biology and Education**
Research assistant: studying class activities and in class time allocation to different activities and their coloration with students' performances.

PRESENTATIONS, PUBLICATIONS AND CONFERENCES

Mar 2012 **Poster Presentation: "Evaluation of a Pentavalent Vaccine Against *Staphylococcus aureus* in a Murine Model of Osteomyelitic Implant Infection"**
IADR/AADR Annual Meeting, Tampa FL: NIDCR Trainee Session

Mar 2011 **3rd Author: "Evolution and comparative genomics of subcellular specializations: EST sequencing of Torpedo electric organ"**
Marine Genomics

Sep 09	**Chesapeake Dental Conference, Ocean City MD**
May 09	**Midatlantic Regional Meeting of the Society for Developmental Biology, College Park MD**
Jul 06	**Abstract publication and poster presentation: "A Method for Analysis of ClassTime Allocation in Higher Education"** University of Maryland Baltimore County: Undergraduate Research Festival

AWARDS AND LEADERSHIP

Fall 2013	**MD Senatorial Scholarship**
June 2013	**AEGD Diamond Scholar** Selected to join UMB's Advanced Education in General Dentistry residency for the entire senior year of dental school.
Apr 2013	**AADR/ADEA Annual Advocacy Day, Washington D.C.**
Sep 2012- Current	**University of Maryland, Baltimore College of Dental Surgery** Admissions Committee, Student Interviewer and Host
May 2012- Current	**University of Maryland, Baltimore College of Dental Surgery** Student Research Group, Program Director
Mar 2011	**American Student Dental Association, Annual Lobby Day, Washington D.C.** Attended a Three Day Event Focusing on Existing and Emerging Issues Affecting Dentistry and Met with Maryland Representatives
Mar 2011	**American Student Dental Association, Annual Meeting, Anaheim CA** 2nd Delegate Representing BCDS
Feb 2011	**University of Maryland, Dental School Advocacy Day, Annapolis MD**
Oct 2010	**American Student Dental Association, Eastern Regional Meeting, Chicago IL.** 2nd Delegate Representing BCDS

May 2010-2011	**University of Maryland, Baltimore College of Dental Surgery** American Student Dental Association, 2nd Delegate/VP
Mar 2010	**American Student Dental Association, Annual Meeting, Baltimore MD** Alternate Delegate Representing BCDS
Sep 09- May 2010	**University of Maryland, Baltimore College of Dental Surgery** American Student Dental Association, Alternate Delegate
Jan 09- May 09	**University of Maryland Baltimore County; Department of Biology** Faculty Search Committee, Graduate Student Member
May 08	**University of Maryland Baltimore County; Department of Biology** Dean's List
Sep 07- May08	**University of Maryland Baltimore County** Pre-Dental Society, President
Sep 06- May 07	**University of Maryland Baltimore County** Persian Student Association, Vice President

PROFESSIONAL AFFILIATIONS

Academy of General Dentistry
American Association of Dental Research
American Association of Endodontists
American Dental Association
American Student Dental Association

Reference

- Available upon request

Thesis Title: Evaluation of a Pentavalent Vaccine Against *Staphylococcus aureus* in a Murine Model of Chronic Peritoneal Abscess

Salar Sanjari, Master of Science, 2014

Thesis Directed by: Mark E. Shirtliff, Ph.D. Associate Professor, Department of Microbial Pathogenesis, School of Dentistry

Abstract

The majority (65%) of human bacterial infections involve biofilms, a complex heterogeneous community of microorganisms. *Staphylococcus aureus* causes a broad range of local and systemic infectious diseases including chronic biofilm-associated infections. The high morbidity, mortality and the ever-increasing associated healthcare costs have persuaded scientists to search for a silver bullet. Furthermore, emergence of antibiotic resistance and associated financial justifications have convinced many researchers and pharmaceutical companies that a vaccination approach can be a far more effective preventive approach against staphylococcal infections. As such, many researchers have focused their efforts to develop a vaccine against the free-floating, planktonic, *S. aureus*. In a new approach, we evaluated the efficacy of a pentavalent vaccine with four biofilm and one planktonic specific recombinant antigen against *S. aureus* in a murine model of chronic peritoneal abscess. Intraperitoneal challenge with $3x10^8$ CFUs of *S. aureus* (MRSA M2) in BALB/c mice resulted in 17% (n=2) mortality over 21 days in the vaccinated group and 92% (n=11) in the control group (Fig. 1) ($p< 0.001$). 80% (n=8) of the survived animals in the vaccinated group cleared the infection. Obtained results confirm the effectiveness of our pentavalent vaccine that targets both planktonic and biofilm-associated proteins in this specific model.

Evaluation of a Pentavalent Vaccine Against *Staphylococcus aureus* in a Murine Model of Chronic Peritoneal Abscess

By
Salar Sanjari

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2014

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Introduction

The Problem: The majority (65%) of human bacterial infections involve biofilms, a complex heterogeneous community of microorganisms. One of the most common and costly problems for the U.S. healthcare system is nosocomial infections (1), with *Staphylococcus aureus* being the second-leading cause of such infections (2). Methicillin-resistant *S. aureus* (MRSA) is responsible for 40-60% of all nosocomially-acquired *S. aureus* infections, and these resistant strains are now considered to be endemic in the hospital setting (3). Community-associated *S. aureus* strains may also acquire methicillin-resistance (CA-MRSA) and the modern emergence of such strains is of great concern (4-6). Recent studies indicate that *S. aureus* is also the major mediator of prosthetic implant infection (7, 8). Furthermore, due to development of antibiotic resistance, many members of medical field are hesitant to readily prescribe long term antibiotics, which makes the development of new antibiotics not financially feasible in many cases. On the other hand, if antibiotics are prescribed commonly, the chance of a rapid development of antibiotics resistance will increase in which case the specific drug will no longer be used. Consequently, in many cases it is financially unfavorable for the pharmaceutical companies to spend years to develop a drug, which may or may not stay on the market long enough to become profitable. The increasing involvement of *S. aureus* in foreign body-related infections, the rapid development of resistance to multiple antibiotics by these organisms, and the propensity of these infections to change from an acute infection to one that is persistent, chronic and recurrent have led to this organism once again being at the center of attention of the scientific community in search of silver bullet.

S. aureus Colonization and Infection: *Staphylococcus aureus* is a Gram positive bacterium that persistently colonizes the skin and nares of approximately 20-40% of healthy individuals (9, 10). *S. aureus* is associated with a wide range of acute and chronic diseases such as bacteremia, sepsis, skin- and soft tissue infections, pneumonia, endocarditis, and osteomyelitis (11), and has a high rate of mortality, estimated at 20-30% in bacteremia patients (12). The vast diversity in *S. aureus*-mediated disease is a consequence of the differential expression of >70 virulence factors that initiate colonization and growth, mediate damage to the host, and promote immunoavoidance (11) in response to the host environment. One crucial virulence factor associated with chronic infection is the biofilm phenotype, where sessile bacteria encapsulate their expanding population in a protective, extracellular polymeric matrix (13). In chronic diseases, this biofilm phenotype promotes persistence and complicates resolution of the infection because microbes in the biofilm are tolerant to antimicrobial agents (14) and the host immune response (15). In the clinical setting, *S. aureus* biofilms pose a significant complication to treatment of post-surgical infections on a growing patient population (16) with a dramatic economic burden (17). Treatment of *S. aureus* infections is further complicated by the increased incidence of antibiotic-resistant strains (18, 19). Together these *S. aureus* phenotypes limit the therapeutic options available to eradicate different type of infections. Therefore, researchers have sought to develop an effective vaccine to prevent acute and chronic infections.

Vaccines, Past, Present and Future: To date, previous vaccine studies have failed to identify antigen(s) with protective efficacy in clinical trials required to gain final

approval for human application (20-23). These include live attenuated or killed *S. aureus* (24), purified polysaccharide including capsular polysaccharide types 5 and 8 (25, 26) or the polysaccharide intracellular adhesin (PIA) composed of poly-N-acetylglucosamine (PNAG) (27, 28), isolated toxins such as Enterotoxin B (SEB) (29) or alpha toxin (30, 31), and recombinant subunit vaccines for microbial-surface-component-recognizing adhesive matrix molecules (MSCRAMM) including clumping factor A (ClfA) (32), clumping factor B (ClfB) (33), iron-regulated surface determinant B (IsdB) (34, 35), fibronectin-binding protein (FnBP) (33, 36), and protein A (SpA) (37). These studies focused on protecting against *S. aureus* in the planktonic-mediated infection but protective efficacy testing was not evaluated in biofilm-mediated infection challenge models. Protein expression differs significantly in planktonic and biofilm *S. aureus* (38-40). Despite the observed recalcitrance of the bacterial biofilm to the host immune response, proteins restricted to the biofilm growth phenotype are recognized by the immune system and elicit a humoral response (41). Based on those findings, a vaccine strategy was devised to boost and direct the humoral response against biofilm-specific antigens with sustained expression throughout the infection process in an effort to target and eradicate *S. aureus* throughout all stages of biofilm maturation. Unlike the multivalent vaccine approach by others that focused on antigen selection based on putative surface-exposure of the protein(s) (42), Brady et al. choose immunogenic proteins upregulated during *in vitro* and *in vivo* biofilm growth (39). Brady et al. found a quadrivalent vaccine composed of biofilm-specific antigens that provided protection against *S. aureus* biofilm populations in a model of osteomyelitis infection (43). However, protective efficacy was enhanced by administering antibiotics as an adjunctive

therapy to vaccinated animals in order to eliminate planktonic populations (43). In the present study, we proposed that the incorporation of a planktonic antigen into the previously tested quadrivalent biofilm-specific vaccine would eliminate the necessity for adjunctive antibiotic therapy to eradicate planktonic bacteria. This would be the first vaccine that would promote complete clearance of *S. aureus*. We evaluated the pentavalent vaccine for its ability to reduce mortality following challenge and to prevent the establishment of a chronic biofilm infection at 21 days post-challenge in a murine peritoneal abscess model that contains both biofilm and planktonic modes of growth (44). We verified that this multicomponent vaccine targeting both *S. aureus* planktonic and biofilm populations, thereby reducing mortality and providing for host clearance of *S. aureus.*

Materials and Methods

Bacterial Strains

The methicillin resistant *S. aureus* (MRSA) M2 strain (45) that was used for all challenge experiments was isolated from a patient with osteomyelitis at the University of Texas Medical Branch. The strain is characterized as sequence typeST30, spa type T019, and an agr III phenotype. *Escherichia coli* TOP10 and BL21 were used for recombinant protein expression.

Reagents

Unless otherwise specified, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cloning and Expression of 5 Antigens

Candidate antigens were previously identified by Brady et al. (39) (I&I 74:3415). The MRSA M2 gene sequences homologous to SACOL0486, SACOL0688, and the glucoaminidase fragment of SACOL1062 were subsequently cloned into pASK-IBA14 (IBA, Gottingen, Germany) and the MRSA M2 gene sequence homologous to SACOL0037 was cloned into pBAD-Thio/TOPO (Invitrogen Life Technologies, Grand Island, NY) by Brady et al. (43)(I&I 79:1797). For this study, we amplified the MRSA M2 gene sequence homologous to SACOL0119 using specific primers forward: 5’ CATGCCATGGACACGACTTCAATGAATG 3’; reverse: 5’AGCTTTGTTTAAACTCAATGATGATGATGATGATGAACTTTTTTGTTACTTT

GGTTC 3') and cloned the PCR product into pBAD-Thio/TOPO. The plasmid was sequenced to confirm the insertion. Verified expression vectors were transformed into *E. coli* BL21 or TOP10. Recombinant 0468, 0688, and glucoaminidase proteins were expressed by anhydrotetracycline induction (200 ng/ml final concentration for 3 hrs), and recombinant 0037 and 0119 proteins were expressed by arabinose induction (0.2% final concentration for 3 hrs)

Purification

Glucoaminidase (GLUC), 0486, 0688: Recombinant proteins were purified using StrepTactin Superflow columns (IBA) as instructed by the manufacturer's protocol with the exception of the lysis step. Here, we suspended the bacterial pellets in buffer P (100 mM TrisHCL, 500 mM sucrose, 1 mM EDTA) and lysed by sonication. This process was performed to enhance isolation of periplasmic proteins, but sonication also releases the cytoplasmic, unprocessed isoform of the recombinant proteins.

0119: Recombinant 0119 was purified under native conditions using the HisPur™ Ni-NTA resin (Thermo Scientific, Waltham, MA) as instructed by the manufacturer's procedure for purification by batch method with minor modifications. The 0019 protein extract was prepared by lysing the bacterial pellets in native binding buffer (250 mM NaH_2PO_4 pH 8.0, 2.5M NaCl, 3M imidazole) and 8mg lysozyme using sonication. Lysates were cleared by centrifugation and transferred to the HisPur Ni-NTA resin column. Diverting from the manufacturer's protocol, the protein extract was incubated with the resin by end-over-end rotation for 90 min at room temperature or overnight at 4°C. Subsequent wash and elution steps followed the manufacturer's protocol.

0037: Recombinant 0037 was purified under denaturing conditions using the HisPur Cobalt resin (Thermo Scientific) as instructed by the manufacturer's procedure for purification by batch method with minor modifications. The 0037 protein extract was prepared by lysing in a native buffer (50 mM Tris-HCl, 200mM NaCl pH 7.0) supplemented with 100 mM phenylmethylsulfonyl fluoride (PMSF) using sonication. Cell debris and insoluble proteins were isolated by centrifugation then resuspended in an inclusion body wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 1 mM EDTA; pH 7.4) and centrifuged again. After three washes with inclusion body wash buffer were performed, the pellet was resuspended in denaturing equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 6 M guanidine HCl, 10 mM imidazole; pH 7.4) with 1% Triton X-100. The insoluble proteins were extracted from the inclusion bodies by incubating the solution by end-over-end rotation for 90 min at room temperature or overnight at 4°C. Following this incubation, the denatured protein extract was cleared by centrifugation and transferred to the HisPur Cobalt resin column (Thermo Scientific). After this mixture was incubated by end-over-end rotation for 90 min at room temperature, the remaining purification steps were performed as instructed by the manufacturer.

Concentrating Proteins/Buffer Exchange

Recombinant 0486, 0688, and glucoaminidase were concentrated and exchanged into phosphate-buffered saline (PBS) (Sigma) using Amicon® Ultra 10,000 MWCO centrifugal filter units (EMD Millipore, Billerica, MA). Recombinant 0119 and 0037 were precipitated by trichloroacetic acid (TCA) precipitation from native or denaturing

elution buffers, respectively, and reconstituted in PBS. In short, TCA in a 1:10 dilution was added to the recombinant proteins, incubated overnight at -20°C., washed twice with cold acetone and dried pellet in a 95°C heat block for 5-10 minutes to drive off acetone. Antigens concentrations were quantified using the Advanced Protein Assay reagent (Cytoskeleton, Inc, Denver, CO) as directed by the manufacturer. The proteins' concentration and purity were confirmed by SDS-PAGE analysis using Tris-HCl precast gels (BioRad, Hercules, CA).

Immunization

Purified, recombinant antigens were combined together using a target protein concentration for glucoaminidase, 0486, 0688, and 0019 of 12 µg of each antigen per mouse, and 20 µg antigen per mouse for 0037. The volume of the antigen mixture was adjusted to 100 µl with PBS, and emulsified at a 1:1 ratio with Imject® Alum (Pierce Biotechnology, Rockford, IL; a subsidiary of Thermo Scientific) Thermo Scientific) for 30 minutes at room temperature. On day 0, six to eight week old, female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were immunized with the pentavalent vaccine or adjuvant alone by intraperitoneal injection (On day 14, experimental mice received a booster vaccine with the same concentration of each antigen but without the adjuvant. Control mice received sterile PBS.

Intraperitoneal Challenge for Murine Abscess Model

Three weeks following booster vaccination (Day 35), mice were challenged by intraperitoneal injection with 3×10^8 CFU of *S. aureus* MRSA M2. The inoculum was

prepared from an exponential phase M2 culture that was generated by diluting an overnight culture at 1:100 into fresh Tryptic Soy Broth (Fluka analytical, subsidiary of Sigma) and growing at 37°C with shaking. Bacteria were harvested after 3 to 3.5 hrs. The cells were pelleted by centrifugation and resuspended in PBS to obtain a concentration of 3-5 x 10^9 CFU/ml. The inoculum concentration was checked with using a Petroff-Hausser counting chamber. The concentration in the sample was verified by serial diluting and plating on tryptic Soy Agar (TSA) (Fluka). CFUs enumeration was completed following overnight incubation at 37°C. Infected mice were monitored for morbidity, specifically significant weight loss >15%, or recovery over a period of 21 days. Three weeks post-challenge (Day 56), the mice were euthanized by exsanguination to harvest blood, and both kidneys and all visible abscesses in the peritoneal cavity were harvested. These samples were sectioned in half, homogenized and serially diluted in PBS, and plated on TSA and *S. aureus* CHROMagar (CHROMagar, Paris, France), which is a selective media for *S. aureus*. Bacterial counts were enumerated from the serial dilution plates, and reported as colony-forming units (CFUs) per gram kidney or per gram abscess. Unused kidney sections were stored in paraformaldehyde for future *in vitro* applications. Serum was isolated from the blood samples for future use.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were performed to quantify the antibody isotype titers (IgG1, IgG2a, and IgG2b) elicited against each vaccine antigen in the surviving, vaccinated mice. Maxisorp ELISA plates (ThermoFisher) were coated with 0.5 µg of antigen per well (from a 5 µg/ml antigen stock) and incubated overnight at 4°C. The next day, the plates were washed

three times with PBS plus 0.05% Tween-20 (Sigma, ST. Louis) then blocked with 1% bovine serum albumin (BSA) (American Bioanalytical, Natick, MA) for one hr. at 37°C. For the primary step, each serum sample was serially diluted 10-fold in the ELISA plate starting from a 1:500 dilution. Duplicate dilutions of each serum sample were performed for each antibody isotype tested. Naïve serum was used as a negative control, and treated in an identical manner. Primary incubation was completed for one to two hours at 37°C. A 1:1000 dilution of horseradish peroxidase-labeled conjugates of IgG1 (Invitrogen), IgG2a (Santa Cruz Technology), or IgG2b (Invitrogen) were then added and incubated for one hr at 37°C. For both steps, the serum or secondary antibodies were diluted in PBS with 1% BSA, and the plates were washed after incubation completion with PBS with 0.05% Tween-20. Enzymatic activity was initiated by adding BD OptEIA TMB Substrate (BD Biosciences, San Jose, CA)) and stopped after 10 minutes by adding TMB® Stop Solution (KPL Inc., Gaithersburg, MD). Optical density readings at 450 nm (OD_{450}) were obtained using the DTX880 Multimode Detector (Beckman Coulter, Brea, CA). Data were plotted of OD (Y axis) versus logarithm of the reciprocal serum dilution (X axis). Titers are approximated based on identifying the effective dilution that is equivalent to 50% of the maximal absorbance in each sample.

Ethical Use of Animals

All animal procedures were approved and performed under the supervision of the University of Maryland Animal Care and Use Committee).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Mouse survival was analyzed for significant differences between vaccinated versus non-vaccinated mice using the two-tailed log-rank test. CFU levels were analyzed with the Mann-Whitney test for statistical significance (untailed Student's *t* test). Serum antibody titer levels that were established by ELISA were reported as the mean ±SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by a Tuke's *post hoc* test. A p-value < 0.05 was considered statistically significant.

Results

Mortality after challenge of mice by intraperitoneal injection of *S. aureus* M2. Vaccinated (n=12) and non-vaccinated (n=12) cohorts of BALB/c mice were infected via intraperitoneal injection with $3x10^8$ cfu of *S. aureus* MRSA M2, which resulted in a statistically significant reduction in mortality of vaccinated versus non-vaccinated mice (17% vs 92%; $p < 0.001$) over 21 days (Fig. 1). Weight of survived 11 animals initially dropped down but recovered after 11 days (Fig. 2).

Clearance of infection. Kidneys and obvious abscesses in the peritoneal cavity were harvested and examined for chronic infection. We found a non-renal abscess in the peritoneal cavity in 5 of the 10 (50%) surviving, vaccinated mice. Overall, eight of the 10 (80%) surviving animals in the vaccinated group cleared the infection in the kidneys and other abscesses in the peritoneal cavity (Fig.3). The remaining two animals with bacterial burdens present in peritoneal abscesses had concomitant renal infections.

ELISA testing showed in general low titer against 0037 and 0119 and lower IgG1 titers against 0119 antibody titers in 2 mice with persistent kidney infection. We tested titer levels of IgG1, IgG2a and IgG2b antibodies generated against the five recombinant antigens in the vaccine. In general, IgG subtype levels elicited against SA0688 were higher than the titers elicited against the other antigens. Overall, the mean values for the anti-0688 IgG subtype titers were significant higher than those for the anti-0037 and anti-0119 IgG subtype titers (Fig. 4).

Despite having a small number of mice for statistical evaluation, we found significantly lower IgG1 titers against 0119 in the two surviving, vaccinated mice with bacterial burdens compared to the remaining vaccinated mice that cleared the infection (IgG1 with 1:500 dilution, $p = 0.03$, 1:5000 dilution, $p = 0.003$). Antibody titers elicited against the other 4 recombinant antigens (0037, glucoaminidase, 0486, and 0688) were not significantly different between infected and non-infected vaccinated mice (figure not showed). Since we draw blood for antibody testing after challenging at time of tissue harvesting, we couldn't compare the antibody difference between killed and survived mice.

Discussion

S. aureus is a leading etiological agent of nosocomial infections (11) with high rates of morbidity and mortality (12). An effective vaccine against *S. aureus* would have a great impact in medicine due to the increased incidence of antibiotic resistant strains and the limited therapeutic options for treating biofilm-associated infections. Most of the previous vaccine studies have primarily focused on preventing acute infections such as bacteremia and sepsis or pneumonia (20-23). But so far, attempts to develop a vaccine that prevents *S. aureus* infection have failed (22, 23, 46, 47) due to the complexity of *S. aureus* pathogenesis. Mechanisms contributing to this complexity include the functional redundancy among some virulence factors, differential expression of virulence factors during different stages of growth (exponential versus stationary phase) or infection phenotype (planktonic versus biofilm-mediated), heterogeneity in protein expression throughout the bacterial biofilm, and lack of genetic conservation of some virulence factors amongst different strains (42, 46). In addition, *S. aureus* evades elimination by neutralizing the products of lysosomal enzymes after phagocytosis and persists in the compartment remaining inaccessible to the immune response (48). These features complicate the generation of an effective humoral response to protect against *S. aureus* when only a single virulence factor is targeted, which has led to a recent shift in vaccine design towards using a multicomponent strategy to target *S. aureus (42)*. Stranger-Jones used a multivalent vaccine approach that focused on antigens selection based on putative surface-exposure of the protein (IsdA, IsdB, SdrD, SdrE). But some antigens are not genetically conserved which is prerequisite for a successful vaccine. In addition, their chosen antigens were planktonic specific and had variable expression throughout the

infectious process. Our research group proposes that a multicomponent vaccine developed for acute and chronic biofilm-associated infections will be effective if it takes into account the planktonic and biofilm phenotypes, sustained *in vivo* expression, and genomic conservation. This concept emerged from our findings with a quadrivalent vaccine composed of biofilm-specific antigens, which were identified from immunoproteomic and transcriptomic studies (39, 41). Immunization of New Zealand white rabbits with the biofilm antigens (listed in Table 1) provided partial protection against *S. aureus* challenge. However, although the animals had reduced clinical and radiographic signs of osteomyelitis, bacterial burden was still observed (43). However, since the vaccine was biofilm-specific, it was hypothesized that the planktonic phenotype contributed to persistence. In a subsequent study, 87.5% of the vaccinated rabbits receiving an adjunctive antibiotic therapy cleared the infection, which supports the hypothesis that the antibiotic sensitive planktonic population shed from the biofilm mediated the persistence observed in the vaccinated animals. This study provided a promising lead towards developing complete protection against *S. aureus* and suggested the right direction for vaccine design as a multicomponent vaccine with both planktonic and biofilm-specific antigens.

In this murine model, we have established that use of one planktonic and four biofilm antigens generates a humoral response that targets planktonic cells to minimize initiation of colonization and early biofilm communities to prevent replication and maturation. Overall, this vaccine strategy eradicates the pathogen before a chronic biofilm is

established that is recalcitrant to the immune effectors. This study is a breakthrough in the development of a vaccine to prevent chronic infections mediated by *S. aureus.*

For this vaccine study, we choose to test our multicomponent vaccine against *S. aureus* in a peritoneal abscess model with an infection period of three weeks, which provides adequate time to establish a chronic biofilm infection. Recently, Rauch et al. used the same model to test the protective efficacy of the vaccine candidate alpha-hemolysin against *S. aureus* (30). In humans, peritoneal abscesses are mainly caused by infected implants, such as chronic peritoneal dialysis (CPD) catheters, ventriculo-peritoneal shunts, and vascular grafts, or as secondary sites of infection after appendicitis, pelvic infections, or infected gallstones (49) resulting in high morbidity and mortality (50). All of these diseases are difficult to treat without surgical intervention because of the bacterial biofilm and its recalcitrance to immune effectors and antibiotics. Despite the lack of an implant in this peritoneal abscess model, *S. aureus* forms abscesses after the intraperitoneal challenge providing a good model to examine vaccine efficacy against a chronic biofilm infection (30).

Our multicomponent vaccine design focuses on proteins differentially up-regulated during either different stages of biofilm maturation or planktonic growth. We incorporated a planktonic upregulated lipoprotein (0119) into our multivalent biofilm-specific vaccine, which is composed of glucoaminidase, a hypothetical lipoprotein (0486), an iron-regulated ABC transporter lipoprotein (0688), and a conserved hypothetical protein (0037). This lipoprotein (0119) was identified in the same

transcriptomic study that found the biofilm upregulated antigens (39). Detailed information about protein function, the experimental analysis used to identify each protein, and the experimental studies that were performed with, or examined the role of, each protein are summarized in Table 1. In this study, we showed that our multicomponent vaccine protects 83% of the vaccinated mice from mortality and completely eliminates *S. aureus* in 80% of the surviving, vaccinated animals in a chronic peritoneal abscess model. The remaining two vaccinated mice had intraperitoneal abscesses with high bacterial burdens and corresponding bacterial persistence in the kidney tissue. Overall, we demonstrated that this combination of the planktonic and biofilm antigens is effective at reducing mortality and promoting *S. aureus* clearance in a murine model of chronic infection.

We found that the highest IgG subtype titers in response to the vaccine were elicited against the ABC transporter lipoprotein 0688, which indicates that this antigen is highly immunogenic and might be an important component of our vaccine. An elevated humoral response to 0688 was also observed in other studies. In 2005, Dryla et al. screened 15 surface proteins and found that SACOL0688 was one of the 6 proteins that elicited higher IgG levels in infected patients (wound infections, bacteremia and sepsis, pneumonia, arthritis, urinary tract infections, catheter-related blood-stream infections, and peritonitis) compared to healthy controls (51). Likewise, Den Reijer et al. studied the humoral response against 56 staphylococcal antigens in bacteremia patients and found that the putative iron-regulated ABC transporter 0688 elicited the highest IgG titer amongst those proteins (52). Based on the results of these two studies, it seems that 0688 plays an

important role in both acute (planktonic) and chronic (biofilm) infections. Although 0688 is recognized by the immune system during both types of infection and is highly immunogenic, the humoral response to 0688 promotes limited clearance of *S. aureus.* This limited protective potential was evident within our original quadrivalent vaccine study completed in rabbits, where 0688 was among the four biofilm-specific antigens. Despite the evidence that suggests 0688 is expressed in both planktonic and biofilm phenotypes, an adjunctive antibiotic therapy was required to target and eradicated the planktonic population during evaluation of the quadrivalent vaccine in rabbits. The disparity between the high titer levels and the limited clearance potential of the anti-0688 antibodies may be the direct consequence of heterogeneity of protein expression within the biofilm. Indeed, confocal microscopy of in vitro MRSA M2 biofilms using anti-0688 antibodies has shown that 0688 expression is restricted to distinct pockets of microcolonies within the biofilm (41). These data demonstrate the importance of the multivalent vaccine approach to provide enhanced coverage of the biofilm.

In general, we measured low antibody titers in response to the conserved hypothetical protein 0037 and the lipoprotein 0119. Since our research group previously detected a 34% reduction in *S. aureus* bacterial burden in a rabbit model of osteomyelitis using a multivalent vaccine of four upregulated biofilm antigens (glucoaminidase, 0688, 0037 and 0486) (43), we propose that 0119 elicited the critical immune response required for bacterial clearance observed in this study. Despite the low titers elicited in response to 0119, the affinity of these antibodies may compensate for the reduced titer levels and target the bacteria for destruction by the immune system. On the other hand, it is feasible

that the humoral response to these vaccine antigens is not the critical protective factor. Current research suggests that the T-cell response (TH1/Th17) plays a more important role in protection against *S. aureus* infection than the humoral response (53, 54). Those studies would support the fact that measurement of the antibody response against our 5 antigens doesn't provide evidence for protection.

We have demonstrated that our multicomponent vaccine developed against chronic infections protects mice against mortality and persistent infection when challenged with *S. aureus.* Overall, the strength of this vaccine study is that we replicated the same rate of mortality and infection prevention in experimental trials. Since the measured humoral response widely varied between the five vaccinated antigens, the individual importance of each antigen needs to be established with further studies. Newer publications also suggest that cell-mediated immunity (Th17/IL-17) plays a more important role than previously assumed for protection against *S. aureus* infections (22, 23, 54). The role of Th17/IL-17 in the protective response mediated by the multicomponent vaccine also need to the evaluated with future studies. Our data demonstrate important criteria for vaccine design established by our laboratory that both the biofilm and planktonic phenotypes must be taken into consideration during antigen selection. While we have demonstrated complete clearance in this study, we do recognize that this interpretation is based on a single murine model. Further studies should explore our multicomponent vaccine in other animal models of infection to confirm the efficacy of the five antigens and/or the multicomponent vaccine approach. In other models of disease, alternate antigens may be required to elicit protection during different disease states or virulence mechanisms.

Table 1. Composition and characteristics of our used pentavalent vaccine antigens

Protein identity[a] (size)	Gene ID	GI number	VERSION	Source of identification [b] (39, 41)	Structure/ function	Up-regulation	Performed vaccine studies
GLUCO: Glucosamine functional subunit of autolysin (SACOL1062) (1,443 bp)	3236133	57650246	AAW36526.1	T and P	Glucosaminidase /Hydrolysis of peptidoglycan, cell attachment (55, 56)	Biofilm	*In vitro* (39, 41), rabbits (43), Humoral response in patients with bacteremia (52)
SACOL0486 (683 bp)	3236972	57651327	YP_185376.1	T	Uncharacterized lipoprotein/unknown	Biofilm	*In vitro* (39, 41, 57), rabbits (43), Humoral response in patients with bacteremia (52)
SACOL0037 (519 bp)	3237004	57652407	YP_184948.1	T	Conserved Hypothetical protein/unknown	Biofilm	In vitro (39, 41), rabbits (43)
Lipoprotein (ABC) (860 bp)	3236721	57651472	YP_185570.1	P and T	ABC transporter binding protein/Putative iron-regulated ABC transporter	Biofilm	*In vitro* (39, 41), rabbits (43), humoral response in patients with bacteremia (52)
SACOL0119 (726 bp)	3236827	57652482	YP_185023.1	T	Cell-wall anchor domain protein/unknown (56)	Planktonic	*In vitro* (56)

[a] Protein identities standardized to *S. aureus* COL genome

[b] In the proteomic study, the immunoreactive proteins were identified by MALDI-TOF analysis and the Profound search engine (Genomic solution's Knexus software). Protein identity corresponds to the GI number of ***S. aureus* Mu50** strain (clinical MRSA strain isolated in 1996 from a Japanese patient with infection of a surgical incision site which was resistance to vancomycin therapy). [b] The proteins identified in the transcriptomic study were identified with microarray methods using the ***S. aureus* COL** (one of the first methicillin resistant MRSA strain isolated in the early 1960s from the operating theatre in a hospital in Colindale, England) genome in the database.

[c] The recombinant proteins are MRSA M2 specific proteins with primer design from *S. aureus* COL genome. Therefore our proteins have homology to the nucleotide sequence of the SA COL genome.

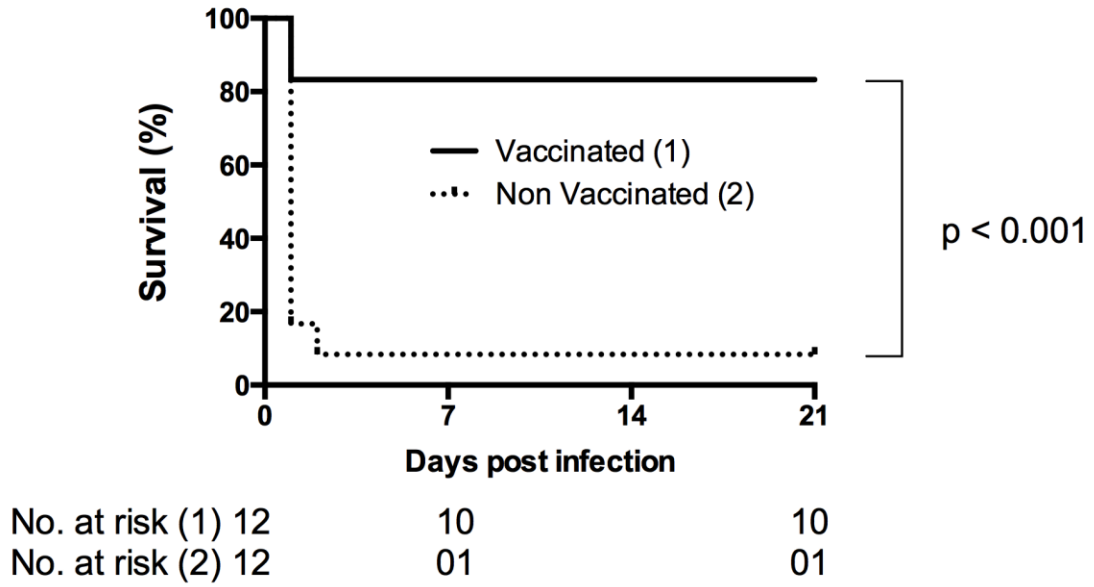

Fig. 1: The survival rates of 12 vaccinated and 12 non-vaccinated mice over 21 days illustrated with a Kaplan Meier Curve. Mice (n=24) were challenged by intraperitoneal injection with 3 x 10^8 CFUs of *S. aureus* M2. Statistical significance was calculated by the log-rank test ($p < 0.001$) between vaccinated and non-vaccinated mice.

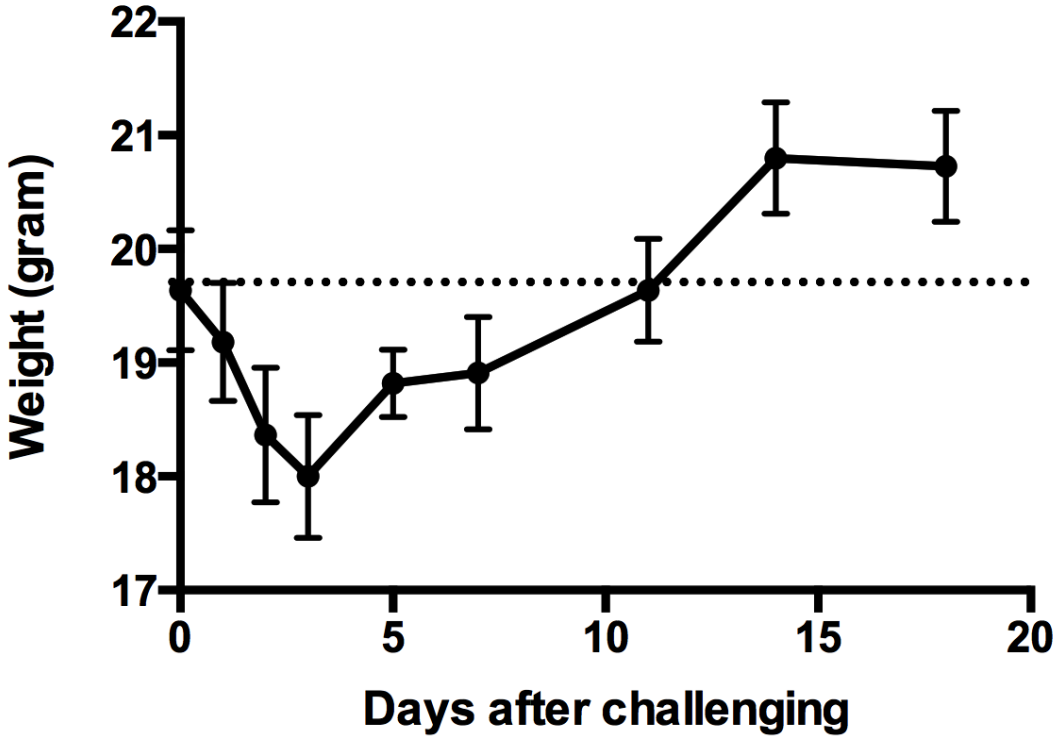

Fig. 2: Weight (in gram) of mice (n=11) after peritoneal challenge with 3 x 10^8 CFUs of *S. aureus* M2. The gram of weight is plotted, with the mean +/- SEM. The dashed line indicates the mean weight before challenge, which was crossed at day 11.

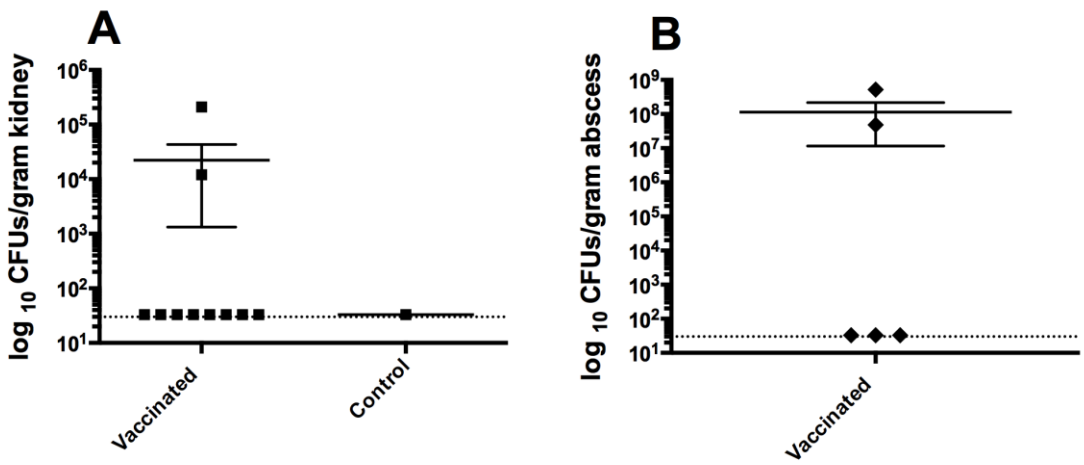

Fig. 3: Bacterial burdens found in the kidneys (A) or peritoneal abscesses (B) of surviving animals at 21 days after intraperitoneal challenge with *S. aureus* M2. Vaccinated and non-vaccinated (control) animals initially received 3 x 10^8 CFUs of *S. aureus* M2. The bacterial burden is plotted as CFUs per gram of kidneys (■) (10 vaccinated, 1 non-vaccinated) or gram of abscess (◆) (5 of 10 vaccinated, 0 of 1 non-vaccinated) with the mean +/- SEM indicated for each group. The dashed line represents the limit of detection. Based on this detection limit, 80% (8 of 10) of the surviving, vaccinated mice cleared the infection.

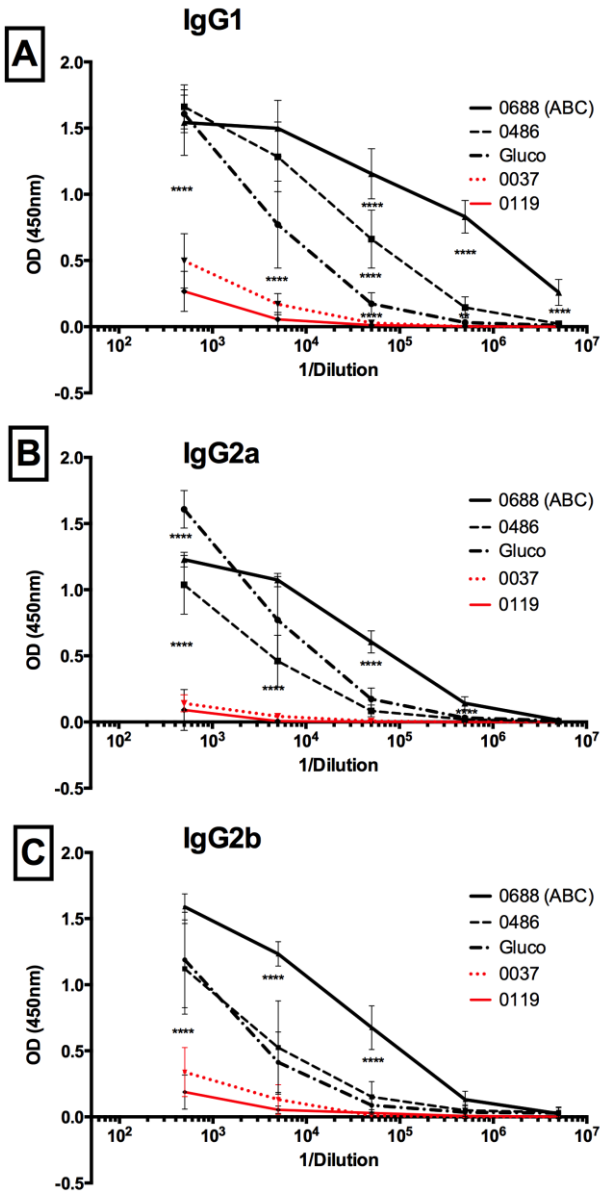

Fig. 4: Effect of pentavalent vaccine (GLUCO, 0486, 0688, 0037 and 0119) on the activation of adaptive immune parameters measured by IgG1 (A), IgG2a (B), and IgG2b (C). Antibody isotype titers were measured in surviving, vaccinated mice at 3 weeks post-challenge with *S. aureus* M2. Serum harvested at (1:500 dilutions). Titer results are presented as the mean value ± SD (n = 9 mice). The graph represents the combined results from both experiments (*p <0.05, ** p< 0.01, *** p < 0.001, p < 0.0001).

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