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An agency of Industry Canada

CA 2865620 A1 2013/09/12

(21) 2 865 620

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2013/03/05
(87) Date publication PCT/PCT Publication Date: 2013/09/12
(85) Entrée phase nationale/National Entry: 2014/08/26
(86) N° demande PCT/PCT Application No.: US 2013/029053
(87) N° publication PCT/PCT Publication No.: 2013/134225
(30) Priorité/Priority: 2012/03/05 (US61/606,750)

(51) Cl.Int./Int.Cl. *A61K 39/085* (2006.01), *A61K 39/02* (2006.01), *A61P 31/00* (2006.01), *A61P 31/12* (2006.01)

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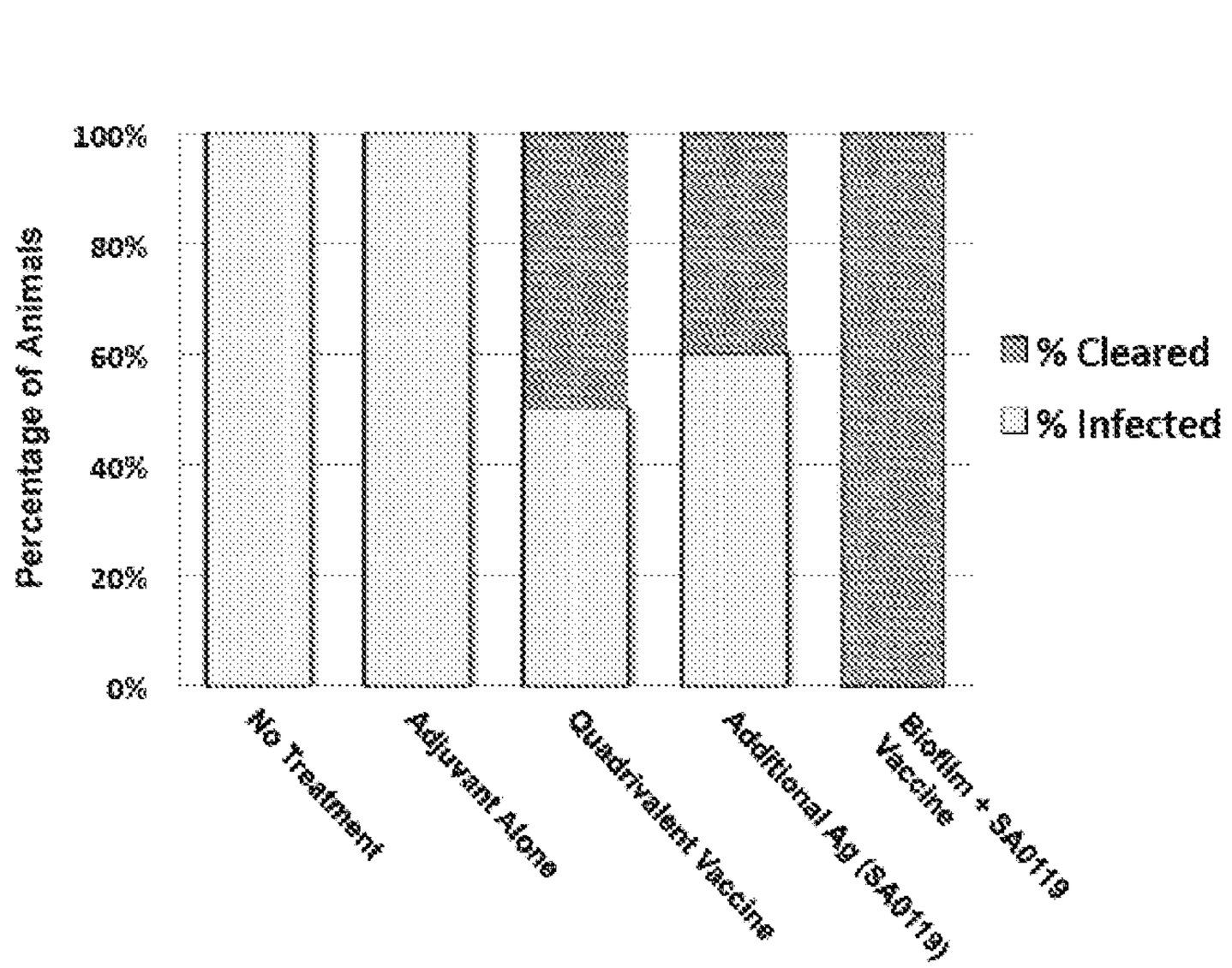
(54) Titre : VACCIN MULTIVALENT PROTEGEANT DE L'INFECTION A STAPHYLOCOCCUS AUREUS
(54) Title: MULTIVALENT VACCINE PROTECTION FROM STAPHYLOCOCCUS AUREUS INFECTION

Figure 3



(57) Abrégé/Abstract:
Vaccine formulations effective against Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus (MRSA) are disclosed, as well as methods of using the vaccine formulations in the treatment and prevention of Staphylococcus aureus infections in a subject.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 September 2013 (12.09.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/134225 A1

(51) International Patent Classification:
A61K 39/085 (2006.01) *A61P 31/12* (2006.01)
A61K 39/02 (2006.01) *A61P 31/00* (2006.01)

(21) International Application Number:
PCT/US2013/029053

(22) International Filing Date:
5 March 2013 (05.03.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/606,750 5 March 2012 (05.03.2012) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

[Continued on next page]

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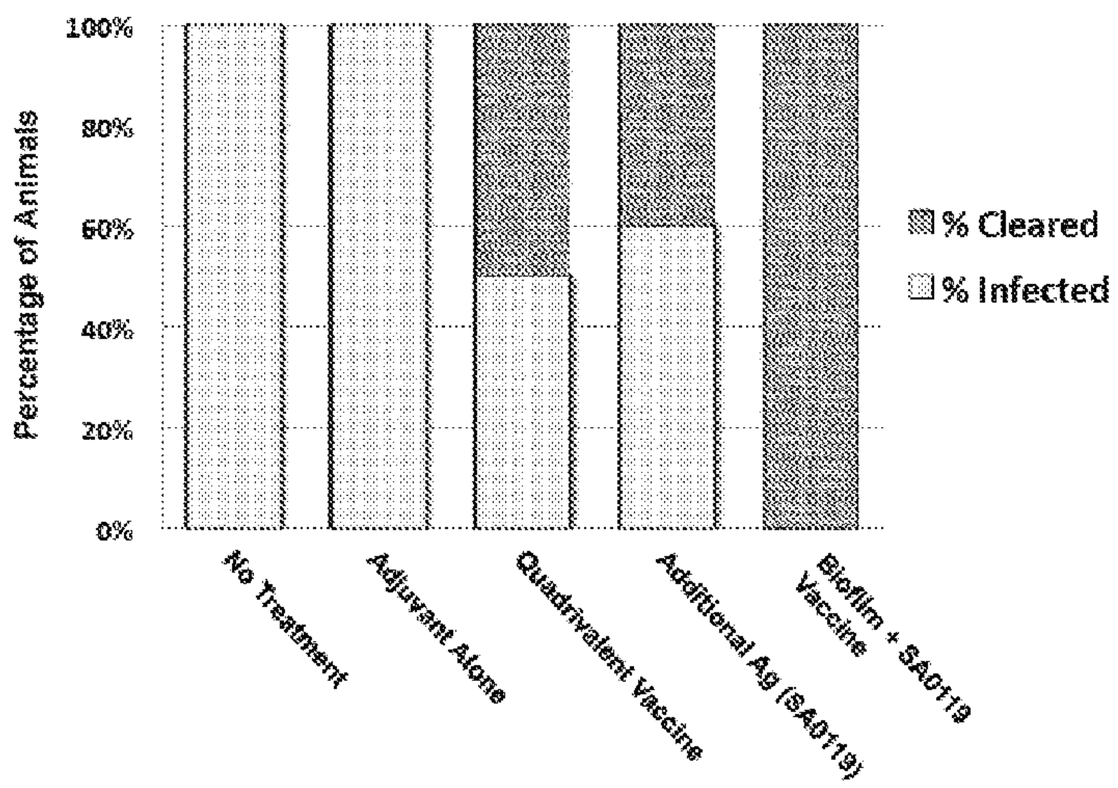

WO 2013/134225 A1

WO 2013/134225 A1

— *with sequence listing part of description (Rule 5.2(a))*

MULTIVALENT VACCINE PROTECTION FROM *STAPHYLOCOCCUS AUREUS* INFECTION

STATEMENT OF GOVERNMENTAL INTEREST

[0001] This invention was made with government support under Grant Number AI069568 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

Field of the Invention

[0002] The present invention relates to multivalent vaccine formulations effective against *Staphylococcus aureus*, including both biofilm and planktonic types of bacterial infections, and to methods of using the formulations in the treatment and prevention of *S. aureus* infections in subjects.

Related Art

[0003] One of the most common and costly problems for the U.S. healthcare system is nosocomial infections (26), with *S. aureus* being the second-leading cause of such infections (4). 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 (36). Community-associated *S. aureus* strains may also acquire methicillin-resistance (CA-MRSA) and the modern emergence of such strains is of great concern (24, 31, 64).

[0004] Recent studies indicate that *S. aureus* is also the major mediator of prosthetic implant infection (1, 54). 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 receiving significant attention.

[0005] Treating prosthetic implant infections is a complicated process, and a number of staphylococcal defense mechanisms may be responsible for this difficulty as well as the capacity of *S. aureus* to evade clearance by the host immune response. One of the most important mechanisms utilized by *S. aureus* to thwart the host immune response and develop

into a persistent infection is through the formation of a highly-developed biofilm. A biofilm is defined as a microbe-derived community in which bacterial cells are attached to a hydrated surface and embedded in a polysaccharide matrix (13). Bacteria in a biofilm exhibit an altered phenotype in their growth, gene expression, and protein production (17), and prosthetic medical devices are often a site of chronic infection, because they present a suitable substrate for bacterial adherence, colonization, and biofilm formation. Biofilm formation by *S. aureus* during prosthetic implant infection makes eradication of this bacteria extremely difficult, due in part to the dramatically increased resistance of bacteria in a biofilm to host defenses (21) and to antibiotics (46, 51), compared to their planktonic counterparts.

[0006] Previous vaccine studies have evaluated the efficacy of bacterial polysaccharides, e.g. polysaccharide capsules, exopolysaccharide, and peptidoglycan (10, 20, 38, 41), as well as recombinant protein subunit vaccines (2, 8, 9, 27, 29, 30, 33, 57, 65) against *S. aureus* infection, but none have demonstrated complete eradication of *S. aureus* in experimental animal models (2, 8, 9, 27, 29, 30, 33, 57, 65) or passed the rigors of phase III clinical testing (56, 59). Most vaccines evaluated to date do not account for biological redundancy of *S. aureus* virulence factors, differential protein expression during different modes of growth (exponential growth versus stationary) or type of infection (planktonic versus biofilm), and the lack of antigen conservation amongst relevant clinical isolates. Indeed, a polysaccharide vaccine (StaphVAX) developed using the *S. aureus* capsular polysaccharide 5 (CP5) and capsular polysaccharide 8 (CP8) conjugated to the *Pseudomonas aeruginosa* exotoxoid A failed to provide protection in phase III clinical trials against *S. aureus*-mediated bacteremia in two different cohorts of 1804 and 3600 hemodialysis patients (59). Factors contributing to this failure are the existence of non-encapsulated strains (CP5 and CP8 strains account for 75-80% of isolates) (12) and differential expression as extrapolated from in vitro data indicating that capsular polysaccharide expression is limited to the stationary mode of growth and the absence of CP5 expression in *S. aureus* bound to endothelial cells (48). The efficacy of the StaphVAX vaccine would, therefore, be limited to planktonic-type infections and ineffective at targeting the humoral response to a *S. aureus* biofilm.

[0007] Similar to the findings with the CP5 / CP8 vaccine (20), subunit vaccines developed against the clumping factor A (ClfA) (2, 27), clumping factor B (ClfB) (57), fibronectin binding protein (FnBP) (65), α-Hemolysin (9, 29), Panton-Valentine leukocidin (PVL) (8),

and the iron-regulated surface determinant B (IsdB) (30, 33) mediate partial protection in experimental animal models. These subunit vaccines did not provide complete protection, despite the candidate proteins being highly immunogenic *in vivo* (25, 33, 57) and the resultant antibodies promoting opsonic killing of *S. aureus* (65). One deficiency of these approaches was relying on a monovalent vaccine to promote protection against the pathogen. *S. aureus* has nearly 70 virulence factors and functional redundancy amongst these factors may abrogate the effect of neutralizing one factor. Arguably, *S. aureus* expresses multiple iron acquisition systems: sidephores staphyloferrin A and B transport transferrin to receptors HtsA and SirA (14, 43), an ABC transporter Fhu imports Fe^{3+} hydroxamates (58), and iron-regulated surface determinant (Isd) B and IsdH receptors that bind hemoglobin/haptoglobin complexes (18, 62), therefore the overall effectiveness of anti-IsdB antibodies that block IsdB-mediated hemoglobin binding may be only a modest effect on iron uptake and the organism's pathogenicity (30). The validity of this argument is exemplified by the cessation of phase III clinical trials of Merck's IsdB vaccine (V710) that failed to provide complete protection (16), despite promising immunogenicity and opsonic killing data from phase II trials (25, 52).

[0008] Efficacy of a monovalent vaccine can also be compromised by differential expression of the targeted protein during the course of infection. While *S. aureus* initiates colonization by binding host extracellular ligands using its adhesin proteins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), including the fibronectin-binding protein (FnBP), these factors are mostly down-regulated as the sessile bacteria encapsulate themselves in an extracellular polysaccharide matrix, or biofilm (44, 55). Hence, vaccines designed to target a MSCRAMM will be ineffective at clearance after the bacteria transition into the biofilm phenotype. Evaluation of the MSCRAMM FnBP vaccine demonstrated it provided partial protection against *S. aureus* in a murine model of sepsis, but the study failed to enumerate bacteria in the blood and/or kidneys to verify bacterial clearance. It is feasible that *S. aureus* can subvert the humoral response to FnBP, form a sessile biofilm and down-regulate FnBP, and become completely recalcitrant the host response.

[0009] A vaccine strategy that circumvents the incomplete protection of monovalent vaccines caused by protein redundancy, differential protein expression, or isolate-specific genetic divergence is the generation of a multifactorial assault using a multivalent subunit vaccine.

Stranger-Jones *et al.* demonstrated a quadrivalent vaccine comprised of surface-exposed proteins: iron-regulated surface determinant A (IsdA), IsdB, and serine aspartate repeat protein D (SdrD), and SdrE increased survival rates against *S. aureus*-mediated lethal challenge compared to protection afforded by each monovalent variant (61). Although the authors stressed the survival rates after lethal challenge, they omitted enumeration of *S. aureus* in the kidneys and survival rates beyond 7 days post-infection from the data analysis. These omissions preclude a conclusion to be reached on the vaccine's ability to promote complete bacterial clearance and prevent future complications due to *S. aureus* persistence via biofilm formation. Overall, the multivalent vaccine had limited efficacy, providing complete protection against only two of five clinical *S. aureus* isolates tested (61). In addition, comparative analysis of multiple *S. aureus* genomes found a lack of conservation amongst some surface proteins, including SdrD and SdrE (39), which indicates the limited efficacy of the IsdA/IsdB/SdrD/SdrE vaccine formulation may extend beyond the clinical isolates tested by Stranger-Jones.

[0010] Vaccine studies have predominately focused on protection against planktonic-mediated infection by examining sepsis (20, 27, 33, 38, 41, 61, 65) or pneumonia (9), while few studies have incidentally evaluated protection, mediated by popular vaccine candidates, against biofilm infection with experimental endocarditis (2), skin (8, 22, 29), or abscess models (20, 61). As a departure from previous *S. aureus* vaccine strategies, Brady *et al.* focused on identifying biofilm upregulated proteins that are immunogenic (4) and established that a multivalent biofilm-based vaccine when coupled with vancomycin treatment could eradicate a biofilm infection, which is traditionally recalcitrant to clearance by either antibiotic treatment or immune response (5). Previous attempts to target the biofilm phenotype, most notably against the staphylococcal intercellular adhesion (PIA) composed of poly-*N*-acetyl-β-1,6-glucosamine (PNAG) (38, 40, 41), were directed towards the biofilm matrix encapsulating the bacteria versus cell wall-associated proteins. The polysaccharide PNAG vaccine elicited a response that reduced bacterial counts (40), but polysaccharides tend to be weak immunogens and induce antibodies with low opsonic killing activity. In addition, PNAG molecules tend to be loosely associated with the bacterial surface and the acetylated PNAG form is released into suspension (11). Efforts to improve efficacy of the PNAG vaccine have evaluated the deacetylated form of PNAG (dPNAG), which may be retained on the cell surface, conjugated to diphtheria toxoid or a synthetic 9-mer of β-(1→6)-D-glucosamine ($GlcNH_2$) conjugated to tetanus toxoid, but partial protection against multiple

S. aureus strains was observed despite improved immunogenicity (22, 38). PIA is generated by enzymes encoded on the *ica*ABDC locus (28), but the presence of the *ica*ABDC locus does not directly correlate to biofilm formation in vitro (32) and the *ica*ABDC locus in *S. aureus* was dispensable in a subset of in vivo orthopedic prosthesis-associated and catheter-associated infections, which are identified as biofilm-mediated infections (53). While the efficacy of the PNAG vaccine against *S.aureus* biofilms requires further evaluation, the dispensability of the *ica*ABDC locus in some *S. aureus* strains isolated from clinical infections suggests that the PNAG vaccine would provide limited protection against *S. aureus* biofilm infections.

[0011] Another consideration for vaccine development is the type of response elicited by the host immune system and the ability of the pathogen to subvert immune mediators using immunoavoidance factors, which may have varied outcomes depending on the host environment. The immune response elicited in vitro against *S. aureus* or its virulence factors, specifically staphylococcal enterotoxin A or B and the alpha toxin, is a pro-inflammatory Th1-response (3, 7, 15, 42). Indeed, comparison of *S. aureus* bacteremia outcomes in mice with different genetic backgrounds found that Th1-biased C57BL/6J mice were resistant and Th2-biased BALB/c mice were susceptible to this acute form of *S. aureus* infection (63). In contrast, a robust Th-1 response was elicited against a *S. aureus* implant infection in C57BL/6J mice, but the mice were susceptible and developed a chronic infection with 10^7 CFU/tibia at 49 days post-infection (45). The *S. aureus* biofilm appears to be recalcitrant to the pro-inflammatory response, which damages host tissue at the infection site generating devitalized sites for *S. aureus* to colonize. Subsequent evaluation found that Th-2 biased BALB/c mice were resistant to the *S. aureus* implant infection, and ablation of interleukin-4 or the depletion of Treg cells abrogated the protection against *S. aureus* in BALB/c mice (46). Th2-mediated resistance to bacterial infection was also revealed for subcutaneous infections with *S. aureus,* where higher bacterial loads were observed in C57BL/6J mice versus BALB/c mice (45). Increased CXCL-2 expression in the C57BL/6J mice correlated with the susceptibility to subcutaneous infection (45), and may halt the killing activity of polymorphonuclear neutrophils (PMNs) after influx and internalization of *S. aureus* (23). This differential immune response against *S. aureus,* which was observed with chronic infections (implant or subcutaneous) versus acute (sepsis), indicates that the choice of mouse strain may impact the outcome of vaccine studies. Most vaccine studies have examined protection against *S. aureus* using experimental models developed in BALB/c mice (2, 8, 33,

61, 65), while few studies have evaluated vaccine efficacy in C57BL/6J mice (9, 29). Emphasis on BALB/c experimental models to evaluate *S. aureus* vaccines may yield insight about efficacy against acute or planktonic infections, but these models will be poor evaluators of chronic, biofilm infections and do not represent the immune response bias in humans.

[0012] Additional vaccine formulations would add to the arsenal of means used to treat and/or prevent *S. aureus* infections.

SUMMARY

[0013] *Staphylococcus aureus* has re-emerged as a major human pathogen and there are presently no vaccines that afford consistent, long-term protection against *S. aureus* infections. While infections, particularly those with MRSA, are often nosocomial in origin, community acquired infections associated with this microbial species have reached epidemic levels. One of the ways in which *S. aureus* is able to persist in the host and remain recalcitrant to clearance by the immune system or antimicrobial agents is through a biofilm mode of growth. Therefore, an effective vaccine and/or treatment modality that could prevent the establishment of biofilm-mediated chronic infections by *S. aureus* is needed.

[0014] The present invention demonstrates protection against biofilm-associated *S. aureus* infection through the use of a multi-component vaccine, alone or in combination with subsequent antimicrobial agent therapy. Complete protection was demonstrated in a murine tibial implant model using a biofilm- and planktonic-specific pentavalent vaccine, with 100% clearance of *S. aureus*.

[0015] The vaccine formulations of the present invention hold significant promise for those with identified risk factors for *S. aureus* biofilm infection. Even in patients that acquire a *S. aureus* infection, an anti-biofilm vaccine could allow these previously untreatable infections to be halted or cured without the need for surgical intervention. The present invention thus provides new means to limit and eradiate *S. aureus* biofilm infections that could help to prevent the onset of chronic disease, saving patients from significant morbidity and mortality.

[0016] The present invention is directed to the following embodiments of vaccine formulations.

[0017] In a first embodiment the present invention is directed to a vaccine formulation comprising five different polypeptides of a strain of *S. aureus* (a first, second, third, fourth

and fifth polypeptide of a strain of *S. aureus*), or portions thereof, or variants thereof, or combinations thereof, and a pharmaceutically acceptable carrier or diluent. The strain of *S. aureus* may be a methicillin-resistant or a methicillin-sensitive strain of *S. aureus*.

[0018] In one aspect, at least one of the *S. aureus* polypeptides is a polypeptide expressed by a planktonic form of the bacteria and at least one of the *S. aureus* polypeptides is a polypeptide expressed by a biofilm form of the bacteria. In a related aspect, one of the *S. aureus* polypeptides is a polypeptide expressed by a planktonic form of the bacteria and four of the *S. aureus* polypeptides are polypeptides expressed by a biofilm form of the bacteria.

[0019] In another aspect, the first, second, third, fourth and fifth polypeptides are *S. aureus* polypeptide SA0037 set forth in SEQ ID NO:13, *S. aureus* polypeptide SA0119 set forth in SEQ ID NO:14, *S. aureus* polypeptide SA0486 set forth in SEQ ID NO:15, *S. aureus* polypeptide SA0688 set forth in SEQ ID NO:16, and *S. aureus* glucosaminidase set forth in SEQ ID NO:17.

[0020] In further aspects, the vaccine formulations comprise one or more portions of one or more of the *S. aureus* polypeptides, wherein the portions individually encompass at least about 20 contiguous amino acids of the full length polypeptide. In the same or aspects, the vaccine formulations comprise one or more variants of one or more of the *S. aureus* polypeptides or portions thereof, wherein the variants individually have at least about 95% identity to a *S. aureus* polypeptide or portion thereof.

[0021] In a particular aspect, the present invention is directed to a vaccine formulation comprising five different, full-length polypeptides of a strain of *S. aureus*. In one example, the five polypeptides are *S. aureus* polypeptide SA0037 set forth in SEQ ID NO:13, *S. aureus* polypeptide SA0119 set forth in SEQ ID NO:14, *S. aureus* polypeptide SA0486 set forth in SEQ ID NO:15, *S. aureus* polypeptide SA0688 set forth in SEQ ID NO:16, and *S. aureus* glucosaminidase set forth in SEQ ID NO:17.

[0022] The present invention is also directed to the following embodiments of methods of using the vaccine formulations of the invention. Thus, in a second embodiment, the present invention is directed to methods of generating an immune response in a subject comprising administering an immunologically effective amount of a vaccine formulation of the present invention to a subject, thereby generating an immune response in a subject. In one aspect, the immune response is a protective immune response.

[0023] In a third embodiment the present invention is directed to methods for treating a *S. aureus* infection in a subject, comprising administering a therapeutically effective amount of a vaccine formulation of the present invention to a subject having a *S. aureus* infection, thereby treating a *S. aureus* infection in a subject.

[0024] In a fourth embodiment the present invention is directed to methods of inhibiting a *S. aureus* infection in a subject, comprising administering a therapeutically effective amount of a vaccine formulation of the present invention to a subject at risk of developing a *S. aureus* infection, thereby inhibiting a *S. aureus* infection in a subject.

[0025] In related embodiments, the methods for treating or inhibiting a *S. aureus* infection may further comprise administering one or more antimicrobial agents to a subject having a *S. aureus* infection or at risk of developing a *S. aureus* infection, wherein the antimicrobial agent is administered prior to, concurrent with or after the vaccine formulation. In these embodiments the antimicrobial agent(s) may be selected from the group that includes, but is not limited to, an Aminoglycoside, such as Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Streptomycin, Tobramycin or Paromomycin; a Carbacephem, such as Loracarbef; a Carbapenem, such as Ertapenem, Doripenem, Imipenem/Cilastatin or Meropenem; a Cephalosporin, such as Cefadroxil, Cefazolin, Cefalotin, Cefalexin, Cefaclor, Cefamandole, Cefoxitin, Cefprozil, Cefuroxime, Cefixime, Cefdinir, Cefditoren, Cefoperazone, Cefotaxime, Cefpodoxime, Ceftazidime, Ceftibuten, Ceftizoxime, Ceftriaxone, Cefepime or Ceftobiprole; a Glycopeptide, such as Teicoplanin or Vancomycin; a Macrolide, such as Azithromycin, Clarithromycin, Dirithromycin, Erythromycin, Erythroped, Roxithromycin, Troleandomycin, Telithromycin or Spectinomycin; a Monobactam, such as Aztreonam; a Penicillin, such as Amoxicillin, Ampicillin, Azlocillin, Carbenicillin, Cloxacillin, Dicloxacillin, Flucloxacillin, Mezlocillin, Meticillin, Nafcillin, Oxacillin, Penicillin, Piperacillin or Ticarcillin; a Polypeptide, such as Bacitracin, Colistin or Polymyxin B; a Quinolone, such as Ciprofloxacin, Enoxacin, Gatifloxacin, Levofloxacin, Lomefloxacin, Moxifloxacin, Norfloxacin, Ofloxacin or Trovafloxacin; a Sulfonamide, such as Mafenide, Prontosil (archaic), Sulfacetamide, Sulfamethizole, Sulfanilimide (archaic), Sulfasalazine, Sulfisoxazole, Trimethoprim or Trimethoprim-Sulfamethoxazole (Cotrimoxazole) (TMP-SMX); a Tetracycline, such as Demeclocycline, Doxycycline, Minocycline, Oxytetracycline or Tetracycline; as well as Chloramphenicol, Clindamycin, Lincomycin, Fusidic acid,

Furazolidone, Linezolid, Metronidazole, Mupirocin, Nitrofurantoin, Macrobid, Platensimycin, Quinupristin/Dalfopristin, Rifampin or Rifampicin.

[0026] In the embodiments directed to methods of treatment and inhibition, the *S. aureus* infection may be any *S. aureus* infection of a subject, including, for example, one or more of a *S. aureus* biofilm infection, a planktonic *S. aureus* infection, a *S. aureus* osteomyelitis infection, a biofilm-associated *S. aureus* osteomyelitis infection, a *S. aureus* indwelling medical device infection, a *S. aureus* endocarditis infection, a *S. aureus* diabetic wound or ulcer infection, a *S. aureus* chronic rhinosinusitis infection, a *S. aureus* ventilator associated pneumonia infection, a *S. aureus* intravenous catheter associated infection, a *S. aureus* skin infection, a *S. aureus* nectrotizing fasciitis, a *S. aureus* keratitis, a *S. aureus* endophthlamitis, a *S. aureus* pyopneumothorax, a *S. aureus* empyema, and a *S. aureus* septicemia.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Figure 1: **(A)** Development of chronic, biofilm-mediated infection that is recalcitrant to antimicrobial therapy. CFU/g bone over time, indicating the development of a chronic infection. Tibiae from infected and uninfected mice were removed at days 4, 7, 14, 21, 28, and 49 days post-infection. No CFUs were found in uninfected mice. Serial dilutions of bone homogenates were plated on blood agar plates. CFU/g bone were calculated and plotted over time (n= 5-8 mice per group, experiments performed in triplicate, * denotes $p<0.05$ compared to controls by Fishers exact test). Bars represent SD. **(B-D)** Confocal scanning laser microscopic images of (B) uninfected pins removed 21 days post-implantation, and *S. aureus* infected pins removed at (C) 7 and (D) 21 days post-implantation. Pins were labeled using a FITC-labeled PNA-FISH probe. Biofilm formation is evident on the pin removed from the infected mouse.

[0028] Figure 2: Vaccination with quadrivalent vaccine and adjunctive vancomycin treatment in a rabbit model of an *S. aureus* osteomyelitis biofilm infection. (A) Animals vaccinated with PBS only (1), PBS and subsequent treatment with vancomycin (2), the quadrivalent vaccine only (3), or the vaccine plus vancomycin (4). The mean +/- SEM for CFU/grams bone is shown for each group. * = significant difference from group 1, PBS control ($P < 0.05$, Student's t test). (B) Animals in each group that were completely cleared of

infection. * = significant difference from group 1, PBS control ($P < 0.05$, Fisher's Exact Test).

[0029] Figure 3: Vaccination with quadrivalent biofilm vaccine, planktonic vaccine, or pentavalent dual phenotype vaccine in a murine model of a *S. aureus* implant infection. Control mice received no treatment (column 1) or unvaccinated with Alum alone (column 2). Experimental mice received a biofilm-directed quadrivalent vaccine (column 3), a planktonic-specific monovalent vaccine (SA0119; column 4), or a combination of the antigens in a pentavalent vaccine (column 5).

DETAILED DESCRIPTION

[0030] Biofilm-embedded bacteria have remarkably different phenotypic and antigenic properties compared to their free-floating, planktonic counterparts. These differences have presented a struggle when designing vaccine formulations for use in treating and preventing both types of bacterial infections. Even individual stages of biofilm growth (from early attached to maturing and fully mature stages) have been shown to be more antigenically distinct from one another than even biofilm versus planktonic bacteria (66).

[0031] Through extensive research into acceptable vaccine candidates, the inventors have identified genes expressed/produced uniquely in biofilm and in planktonic modes of growth via proteomics and transcriptomics techniques. In particular, the inventors found that one must compare multiple stages of biofilm growth (from early attached to maturing and fully mature stages) to multiple stages of planktonic growth (early log, late log, stationary, and post stationary) in order to find those cell wall antigens with up-regulated and sustained expression in all biofilm stages and those with up-regulated and sustained expression in all planktonic growth stages. By combining biofilm and planktonic antigens that are expressed on the membrane or cell wall into a multivalent vaccine, protection of the host against microbial challenge by the specific microbial species can be elicited. This protection can be promoted since bacteria in the host exist in antigenically distinct forms of the planktonic and biofilm modes of growth during an infection and, as a result, a dual immune response against both phenotypes must be produced in the host.

[0032] The vaccine formulations of the present invention include antigens effective at priming the host immune response to clear both detached, free-floating populations of

bacteria as well as bacteria forming a biofilm type of infection. This work is the first to acknowledge, and overcome, the differences of protein expression within different types of infection caused by the same microorganism, and demonstrate (as shown in the Examples) complete clearance in an *S. aureus* animal model of infection instead of only a significant reduction in bacterial populations.

[0033] As discussed above and herein, the present invention relates to vaccine formulations effective against *S. aureus*, including methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA), and to methods of using the vaccines in the treatment and prevention of *S. aureus* infections in a subject.

I. Vaccine Components - Proteins

[0034] The vaccine formulations of the present invention comprise at least a portion of each of five different polypeptides of a strain of *S. aureus* and a pharmaceutically acceptable carrier or diluent. The vaccine formulations are characterized in that they comprise at least one *S. aureus* polypeptide expressed by a planktonic form of the bacteria and at least one *S. aureus* polypeptide expressed by a biofilm form of the bacteria. The vaccine formulations of the present invention may thus comprise one, two, three, or four *S. aureus* polypeptides expressed by a planktonic form of the bacteria, and one, two, three, or four *S. aureus* polypeptides expressed by a biofilm form of the bacteria. In one aspect, the vaccine formulations comprise one *S. aureus* polypeptide expressed by a planktonic form of the bacteria and four *S. aureus* polypeptides expressed by a biofilm form of the bacteria.

[0035] The skilled artisan will understand that the identity, number and size of the different *S. aureus* proteins that can be included in the vaccine formulations of the present invention may vary. For example, the formulations may comprise only full-length versions of the polypeptides. Or the formulations may comprise only portions of the full-length polypeptides. Or the formulations may comprise a combination of portions and full-length polypeptides. Furthermore, combinations include formulations having one, two, three, four, five, six or more different portions of the same *S. aureus* polypeptide in combination with one or more portions of other polypeptides and/or full-length polypeptides and/or both portions and full-length versions of the same polypeptide. However, each of the formulations comprises at least one portion of each of five different polypeptides of a strain of *S. aureus*.

[0036] The identity of the planktonic- and biofilm-expressed polypeptides included in the vaccine formulations of the present invention is not particularly limited but each is a polypeptide from a strain of *S. aureus*. However, because the primary purpose of the vaccine formulations is to prime and activate the immune system of the subject receiving the vaccine formulation, the use of polypeptides exposed on the surface of the bacteria is particularly preferred. For example, the polypeptides may be cell wall and cell wall-associated polypeptides of *S. aureus*. Examples of such polypeptides include the *S. aureus* polypeptides SA0037 (SEQ ID NO:13), SA0119 (SEQ ID NO:14), SA0486 (SEQ ID NO:15), SA0688 (SEQ ID NO:16), and glucosaminidase (SEQ ID NO:17).

[0037] Additional *S. aureus* polypeptides that may be used in the vaccine formulations of the present invention include the polypeptides of Table 1.

Table 1

Biofilm Expressed Polypeptides	
SACOL0405	MATE efflux family protein (SEQ ID NO:18)
SACOL0379	bacteriophage L54a, M23/M37 peptidase domain protein (SEQ ID NO:19)
SACOL2658	arginine repressor (SEQ ID NO:20)
SACOL1041	hypothetical protein (SEQ ID NO:21)
SACOL0048	conserved hypothetical protein (SEQ ID NO:22)
SACOL2292	Na+/H+ antiporter (SEQ ID NO:23)
SACOL0204	formate acetyltransferase (SEQ ID NO:24)
SACOL2729	integrase/recombinase, core domain family (SEQ ID NO:25)
SACOL2424	6-carboxyhexanoate--CoA ligase (SEQ ID NO:26)
SACOL1183	membrane protein, putative (SEQ ID NO:27)
SACOL2446	epimerase/dehydratase, putative (SEQ ID NO:28)
SACOL0386	bacteriophage L54a, hypothetical protein (SEQ ID NO:29)
Planktonic Expressed Polypeptides	
SACOL0633	conserved hypothetical protein (SEQ ID NO:30)
SACOL1664	conserved hypothetical protein TIGR00370 (SEQ ID NO:31)
SACOL0541	stage V sporulation protein G spoVG (SEQ ID NO:32)
SACOL1138	29-kDa cell surface protein, putative sasJ (SEQ ID NO:33)
SACOL0117	polysaccharide extrusion protein (SEQ ID NO:34)
SACOL1659	conserved hypothetical protein (SEQ ID NO:35)
SACOL2150	mrp protein sasB (SEQ ID NO:36)

[0038] When only a portion(s) of a polypeptide is used in a vaccine formulation, the size of the peptide is only limited by its ability to be recognized by the immune system of the subject to which the vaccine is administered. In general, the peptides included in the formulations should be about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more contiguous amino acids of the full-length protein. The preferred size of the peptides is between about 20 amino acids and 3000 amino acids in length, more preferably between about 40 amino acids and 1500 amino acids in length, even more preferably between about 150 amino acids and 1300 amino acids in length. In other aspects, the peptides may 5%, 10%,

15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the size of the full-length protein.

[0039] As indicated above, the polypeptides and portions thereof used in the formulations of the present invention are from strains of *S. aureus*. There is no limitation on the different strains of *S. aureus* that might be used. As an example only, polypeptides from medically important strains of *S. aureus*, such methicillin-resistant *S. aureus* (either community-associated or hospital-acquired strains) and methicillin-sensitive *S. aureus*, may be used to constitute the vaccine formulations of the present invention. Therefore, the vaccine formulations of the present invention include the use of variants of the *S. aureus* polypeptides and portions thereof defined herein and having at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity over their entire length to *S. aureus* polypeptides and portions thereof. Sequence identity is determined by aligning the amino acid sequence of two peptides or proteins and calculating the number of amino acid differences over the entire length of the alignment. The skilled artisan will understand that there are a number of commercially available sequence manipulation programs for use in making such calculations, including the website of the National Center for Biotechnology Information.

[0040] The polypeptides, portions, and variants thereof (collectively, termed "proteins") used in the vaccine formulations may be obtained through any of the many well-established means known in the art. The skilled artisan will understand that the proteins can possess the native glycosylation of polypeptide as it is produced by the corresponding strain of *S. aureus*, or they can lack such glycosylation, or they can have altered glycosylation.

II. Vaccine Components – Carriers and Excipients

[0041] The pharmaceutically acceptable carrier, diluent or excipient included in the vaccine formulations will vary based on the identity of the proteins in the formulation, the means used to administer the formulation, the site of administration and the dosing schedule used. Suitable examples of carriers and diluents are well known to those skilled in the art and include water-for-injection, saline, buffered saline, dextrose, water, glycerol, ethanol, propylene glycol, polysorbate 80 (Tween-80™), poly(ethylene)glycol 300 and 400 (PEG 300 and 400), PEGylated castor oil (e.g. Cremophor EL), poloxamer 407 and 188, hydrophilic and hydrophobic carriers, and combinations thereof. Hydrophobic carriers include, for example, fat emulsions, lipids, PEGylated phospholipids, polymer matrices, biocompatible

polymers, lipospheres, vesicles, particles, and liposomes. The terms specifically exclude cell culture medium. Additional carriers include cornstarch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, alginic acid, croscarmellose sodium, and sodium starch glycolate.

[0042] Excipients included in a formulation have different purposes depending, for example on the nature of the vaccine formulation and the mode of administration. Examples of generally used excipients include, without limitation: stabilizing agents, solubilizing agents and surfactants, buffers, antioxidants and preservatives, tonicity agents, bulking agents, lubricating agents, emulsifiers, suspending or viscosity agents, inert diluents, fillers, disintegrating agents, binding agents, wetting agents, lubricating agents, antibacterials, chelating agents, sweetners, perfuming agents, flavouring agents, coloring agents, administration aids, and combinations thereof.

[0043] As a specific example, intramuscular preparations can be prepared and administered in a pharmaceutically acceptable diluent such as Water-for-Injection, 0.9% saline, or 5% glucose solution.

[0044] In one embodiment of the present invention, the vaccine formulations exist as atomized dispersions for delivery by inhalation. The atomized dispersion of the vaccine formulation typically contains carriers common for atomized or aerosolized dispersions, such as buffered saline and/or other compounds well known to those of skill in the art. The delivery of the vaccine formulations via inhalation has the effect of rapidly dispersing the vaccine formulation to a large area of mucosal tissues as well as quick absorption by the blood for circulation. One example of a method of preparing an atomized dispersion is described in U.S. Patent No. 6,187,344, entitled, "Powdered Pharmaceutical Formulations Having Improved Dispersibility," which is hereby incorporated by reference in its entirety.

[0045] Additionally, the vaccines and vaccine formulations may also be administered in a liquid form. The liquid can be for oral dosage, for ophthalmic or nasal dosage as drops, or for use as an enema or douche. When the vaccine formulation is formulated as a liquid, the liquid can be either a solution or a suspension of the vaccine formulation. There are a variety of suitable formulations for the solution or suspension of the vaccine formulation that are well know to those of skill in the art, depending on the intended use thereof. Liquid formulations for oral administration prepared in water or other aqueous vehicles may contain various suspending agents such as methylcellulose, alginates, tragacanth, pectin, kelgin, carrageenan,

acacia, polyvinylpyrrolidone, and polyvinyl alcohol. The liquid formulations may also include solutions, emulsions, syrups and elixirs containing, together with the active compound(s), wetting agents, sweeteners, and coloring and flavoring agents. Various liquid and powder formulations can be prepared by conventional methods for inhalation into the lungs of the mammal to be treated.

[0046] The vaccine formulations of the present invention may also include an adjuvant. Suitable adjuvants include Freund's Complete and Incomplete Adjuvant, Titermax, Oil in Water Adjuvants, as well as Aluminum compounds where antigens, normally proteins, are physically precipitated with hydrated insoluble salts of aluminum hydroxide or aluminum phosphate. Other adjuvants include liposome-type adjuvants comprising spheres having phospholipid bilayers that form an aqueous compartment containing the vaccine candidate and protecting it from rapid degradation, and that provide a depot effect for sustained release. Surface active agents may also be used as adjuvants and include lipoteichoic acid of gram-positive organisms, lipid A, and TDM. Quil A and QS-21 (saponin-type adjuvants), monophosphoryl lipid A, and lipophilic MDP derivatives are suitable adjuvants that have hydrophilic and hydrophobic domains from which their surface-active properties arise. Compounds normally found in the body such as vitamin A and E, and lysolecithin may also be used as surface-active agents. Other classes of adjuvants include glycan analog, coenzyme Q, amphotericin B, dimethyldioctadecylammonium bromide (DDA), levamisole, and benzimidazole compounds. The immunostimulation provided by a surface active agent may also be accomplished by either developing a fusion protein with non-active portions of the cholera toxin, exotoxin A, or the heat labile toxin from *E. coli*. Immunomodulation through the use of anti-IL-17, anti IFN-γ, anti-IL-12, IL-2, IL-10, or IL-4 may also be used to promote a strong Th2 or antibody mediated response to the vaccine formulation.

III. Methods of Generating an Immune Response

[0047] The present invention is also directed to methods of generating an immune response in a subject to a vaccine formulation of the present invention. In one embodiment, the present invention is directed to methods of generating an immune response in a subject, comprising administering an immunologically effective amount of a vaccine formulation of the present invention to a subject, thereby generating an immune response in a subject. In each of the methods of generating an immune response of the present invention, the immune response is preferably a protective immune response.

[0048] An "immunologically effective amount" of a vaccine formulation is one that is sufficient to induce an immune response to vaccine components in the subject to which the vaccine formulation is administered. A "protective immune response" is one that confers on the subject to which the vaccine formulation is administered protective immunity against *S. aureus*. The protective immunity may be partial or complete immunity.

IV. Methods of Treatment and Prevention

[0049] The present invention is also directed to methods of treating a *S. aureus* infection in a subject using the vaccine formulations of the present invention. In one embodiment, the present invention is directed to methods of treating a *S. aureus* infection in a subject, comprising administering a therapeutically effective amount of a vaccine formulation of the present invention to a subject having a *S. aureus* infection, thereby treating a *S. aureus* infection in a subject. In certain aspects, the method further comprises administering an antimicrobial agent to the subject having a *S. aureus* infection in conjunction with the administration of the vaccine formulation.

[0050] The vaccine formulations of the present invention may also be used in methods of inhibiting a *S. aureus* infection in a subject. Such methods comprise administering a therapeutically effective amount of a vaccine formulation of the present invention to a subject at risk of developing a *S. aureus* infection, thereby inhibiting a *S. aureus* infection in a subject. In certain aspects, the method further comprises administering an antimicrobial agent to the subject at risk of developing a *S. aureus* infection in conjunction with the administration of the vaccine formulation.

[0051] A "therapeutically effective amount" of a vaccine formulation is one that is sufficient to provide at least some reduction in the symptoms of a *S. aureus* infection in a subject to which the vaccine formulation is administered, or one that is sufficient to achieve the goal of the method.

[0052] As used herein, the terms "treating" and "treatment" have their ordinary and customary meanings, and include one or more of, ameliorating a symptom of a *S. aureus* infection in a subject, blocking or ameliorating a recurrence of a symptom of a *S. aureus* infection in a subject, decreasing in severity and/or frequency a symptom of a *S. aureus* infection in a subject, as stasis, decreasing, or inhibiting growth of *S. aureus* in a subject. Treatment means ameliorating, blocking, reducing, decreasing or inhibiting by about 1% to

about 100% versus a subject to which the vaccine formulation of the present invention has not been administered (with or without the additional administration of the antimicrobial agent). Preferably, the ameliorating, blocking, reducing, decreasing or inhibiting is 100%, 99%, 98%, 97%, 96%, 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or 1%. The treatment may begin prior to, concurrent with, or after the onset of clinical symptoms of the infection. The results of the treatment may be permanent, such as where the *S. aureus* infection is completely cleared from the subject, or may be for a period of days (such as 1, 2, 3, 4, 5, 6 or 7 days), weeks (such as 1, 2, 3 or 4 weeks) or months (such as 1, 2, 3, 4, 5, 6 or more months).

[0053] As used herein, the terms "inhibit", "inhibiting" and "inhibition" have their ordinary and customary meanings, and include one or more of inhibiting colonization of *S. aureus*, inhibiting growth of *S. aureus* (all forms, including planktonic and biofilm) and inhibiting propagation of *S. aureus*. Such inhibition is an inhibition of about 1% to about 100% versus a subject to which the vaccine formulation of the present invention has not been administered (with or without the additional administration of the antimicrobial agent). Preferably, the inhibition is an inhibition of 100%, 99%, 98%, 97%, 96%, 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or 1%. As used herein, the inhibition lasts at least a period of days, weeks, months or years upon completing of the dosing schedule. Preferably the inhibition is for the lifespan of the subject.

[0054] The methods for treating or inhibiting a *S. aureus* infection may further comprise administering one or more antimicrobial agents to a subject having a *S. aureus* infection or at risk of developing a *S. aureus* infection. When an antimicrobial agent is included in the methods of the present invention, the antimicrobial agent may be administered prior to, concurrent with or after the vaccine formulation is administered to the subject. Where the antimicrobial agent is administered prior to or after the vaccine formulation, the period of time between when the antimicrobial agent and the vaccine formulation are administered may be a period of hours (such as 6, 12, 18 or 24 hours), days (such as 1, 2, 3, 4, 5, 6 or 7 days), weeks (such as 1, 2, 3 or 4 weeks) or months (such as 1, 2, 3, 4, 5, 6 or more months). The antimicrobial agent may be any that is effective in the treatment of a *S. aureus* infection and may include, but is not limited to, an Aminoglycoside, such as Amikacin, Gentamicin, Kanamycin, Neomycin, Netilmicin, Streptomycin, Tobramycin or Paromomycin; a Carbacephem, such as Loracarbef; a Carbapenem, such as Ertapenem, Doripenem,

Imipenem/Cilastatin or Meropenem; a Cephalosporin, such as Cefadroxil, Cefazolin, Cefalotin, Cefalexin, Cefaclor, Cefamandole, Cefoxitin, Cefprozil, Cefuroxime, Cefixime, Cefdinir, Cefditoren, Cefoperazone, Cefotaxime, Cefpodoxime, Ceftazidime, Ceftibuten, Ceftizoxime, Ceftriaxone, Cefepime or Ceftobiprole; a Glycopeptide, such as Teicoplanin or Vancomycin; a Macrolide, such as Azithromycin, Clarithromycin, Dirithromycin, Erythromycin, Erythroped, Roxithromycin, Troleandomycin, Telithromycin or Spectinomycin; a Monobactam, such as Aztreonam; a Penicillin, such as Amoxicillin, Ampicillin, Azlocillin, Carbenicillin, Cloxacillin, Dicloxacillin, Flucloxacillin, Mezlocillin, Meticillin, Nafcillin, Oxacillin, Penicillin, Piperacillin or Ticarcillin; a Polypeptide, such as Bacitracin, Colistin or Polymyxin B; a Quinolone, such as Ciprofloxacin, Enoxacin, Gatifloxacin, Levofloxacin, Lomefloxacin, Moxifloxacin, Norfloxacin, Ofloxacin or Trovafloxacin; a Sulfonamide, such as Mafenide, Prontosil (archaic), Sulfacetamide, Sulfamethizole, Sulfanilimide (archaic), Sulfasalazine, Sulfisoxazole, Trimethoprim or Trimethoprim-Sulfamethoxazole (Cotrimoxazole) (TMP-SMX); a Tetracycline, such as Demeclocycline, Doxycycline, Minocycline, Oxytetracycline or Tetracycline; as well as Chloramphenicol, Clindamycin, Lincomycin, Fusidic acid, Furazolidone, Linezolid, Metronidazole, Mupirocin, Nitrofurantoin, Macrobid, Platensimycin, Quinupristin/Dalfopristin, Rifampin or Rifampicin.

[0055] In each of the methods of the present invention the vaccine formulations are administered in a pharmaceutically acceptable form and in substantially non-toxic quantities. The vaccine formulations may be administered to a subject using different dosing schedules, depending on the particular use to which the formulations are put (e.g., administration to the subject pre- or post-exposure to *S. aureus*), the age and size of the subject, and the general health of the subject, to name only a few factors to be considered. In general, the vaccine formulations may be administered once, or twice, three times, four times, five times, six times or more, over a dosing schedule. The timing between each dose in a dosing schedule may range between a few hours, six, 12, or 18 hours, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more days. The same quantity of protein in the formulation may be administered in each dose of the dosing schedule, or the amounts in each dose may vary. The identity of the particular peptides and polypeptides in the formulation may also vary or remain the same in each dose in a dosing schedule.

[0056] The amount of protein administered to a subject in a dose when the methods of the present invention are practiced will vary based on the particular methods being practiced (e.g., prevention versus treatment of a *S. aureus* infection), the means and formulation of administration, the age and size of the subject, and the general health of the subject, to name only a few factors to be considered. In general, however, the amount of *S. aureus* protein administered to a subject in a dose will be sufficient to induce or boost an immune response in a subject to the components of the vaccine. For example, the vaccines formulations may contain between about 1 to about 1000 ug of total *S. aureus* protein per kg of body weight of the subject to which the dose of the vaccine formulation will be administered, more preferably between about 10 to about 200 ug, even more preferably between about 15 to about 100 ug.

[0057] Appropriate doses and dosing schedules can readily be determined by techniques well known to those of ordinary skill in the art without undue experimentation. Such a determination will be based, in part, on the tolerability and efficacy of a particular dose.

[0058] Administration of the vaccine formulations may be via any of the means commonly known in the art of vaccine delivery. Such routes include intravenous, intraperitoneal, intramuscular, subcutaneous and intradermal routes of administration, as well as nasal application, by inhalation, ophthalmically, orally, rectally, vaginally, or by any other mode that results in the vaccine formulation contacting mucosal tissues.

[0059] As used herein, the *S. aureus* infection may be any *S. aureus* infection of a subject, including, for example, one or more of a *S. aureus* biofilm infection, a planktonic *S. aureus* infection, a *S. aureus* osteomyelitis infection, a biofilm-associated *S. aureus* osteomyelitis infection, a *S. aureus* indwelling medical device infection, a *S. aureus* endocarditis infection, a *S. aureus* diabetic wound or ulcer infection, a *S. aureus* chronic rhinosinusitis infection, a *S. aureus* ventilator associated pneumonia infection, a *S. aureus* intravenous catheter associated infection, a *S. aureus* skin infection, a *S. aureus* nectrotizing fasciitis, a *S. aureus* keratitis, a *S. aureus* endophthlamitis, a *S. aureus* pyopneumothorax, a *S. aureus* empyema, and a *S. aureus* septicemia.

[0060] The term “subject” is intended to mean an animal, such birds or mammals, including humans and animals of veterinary or agricultural importance, such as dogs, cats, horses, sheep, goats, and cattle.

[0061] A kit comprising the necessary components of a vaccine formulation that elicits an immune response to a strain of *S. aureus* and instructions for its use is also within the purview of the present invention.

[0062] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLES

Materials and Methods

[0063] Unless stated otherwise, the following experimental details pertain to each of the examples provided in the specific Examples set forth and discussed below.

[0064] *Mice.* Inbred C57BL/6 (6-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained under micro-isolator conditions in the animal facility at the University of Maryland Dental School (Baltimore, MD), in accordance with protocols reviewed and approved by the *Institutional Animal Care and Use Committee (IACUC).*

[0065] *Bacterial strain and preparation of infectious inocula.* The strain of *S. aureus* used in these experiments, MRSA-M2, is a clinical isolate obtained from an osteomyelitis patient undergoing treatment at the University of Texas Medical Branch (Galveston, TX) and has been used in previous biofilm molecular analyses and animal infection models (5, 34, 37, 60) (6, 47, 49, 50). An overnight *S. aureus* Tryptic Soy Broth (TSB) culture grown at 37°C with 250 rpm shaking was diluted 1:100 in fresh, prewarmed TSB and incubated for 2 h at 37°C with 250 rpm shaking. Cells were centrifuged, rinsed with PBS, counted via a Petroff Hausser counter, and diluted to 1 x 10^6 CFU/ml.

[0066] *Cloning, expression, and purification of proteins.* Candidate antigens selected from Brady *et al.* (5) were amplified using the primers listed in Table 2.

Table 2. Primers utilized in this study (all products amplified from S. aureus M2 MRSA strain).

Primer name	***Sequence (5' – 3'); SEQ ID NO:***	***Product, size***
5' SA0037 SEQ ID NO:1	ATGAATACAATCAAAACTACGAAA	Conserved hypothetical protein, 519 bp
3' SA0037 SEQ ID NO:2	CTTCTCATCGTCATCTGATTTCAAAATCCATTTTTGA	
5' Lipase SEQ ID NO:3	ACTCTAGGTCTCACTCCCATCTGAAACAACATTATGACCAAAT	Lipase, 966 bp
3' Lipase SEQ ID NO:4	ATGGTAGGTCTCATATCATAAAGGATTTAACGGTAATTCATTACT	
5' SA0688 SEQ ID NO:5	ATGGTAGGTCTCACTCCGATAAGTCAAATGGCAAACTAAAAGT	ABC trans. lipoprotein, 860 bp
3' SA0688 SEQ ID NO:6	ATGGTAGGTCTCATATCATTTCATGCTTCCGTGTACAGTT	
5' Glucosaminidase SEQ ID NO:7	ATGGTAGGTCTCACTCCGCTTATACTGTTACTAAACCACAAAC	Glucosaminidase, 1443 bp
3' Glucosaminidase SEQ ID NO:8	ATGGTAGGTCTCATATCATTTATATTGTGGGATGTCGAAGTATT	
5' SA0486 SEQ ID NO:9	ACTCTAGGTCTCACTCCAAAGAAGATTCAAAAGAAGAACAAAT	Hypothetical lipoprotein, 683 bp
3' SA0486 SEQ ID NO:10	ATGGTAGGTCTCATATCAGCTATCTTCATCAGACGGCCCA	
5' SA0119 SEQ ID NO:11	CATGCCATGGACACGACTTCAATGAATG	Putative uncharacterized protein, 726 bp
3' SA0119 SEQ ID NO:12	AGCTTTGTTTAAACTCAATGATGATGATGATGATGAACTTTTTTGTTACTTTGGTTC	

BsaI sites are underlined in primers.

[0067] The PCR products were cloned into pBAD-Thio/TOPO (SACOL0037 and SACOL0119) or pASK-IBA14 (SACOL0486, SACOL0688, and glucosaminidase), transformed into TOP10 *E. coli*, and sequenced. Details regarding the plasmids are provided in Table 3.

Table 3. Plasmids utilized in this study.

Plasmid	***Genotype or Characteristics***	***Source***
pBAD-Thio/TOPO	4454 bp pUC ori, Amp^R, pBAD promoter, for arabinose-inducible expression of PCR product	Invitrogen Life Technologies
pASK-IBA14	3001 bp pUC ori, Amp^R, tetA promoter, for tetracycline-inducible expression of PCR product	IBA, Göttingen, Germany

[0068] The clones were then expressed using either arabinose induction (SACOL0037 and SACOL0119) or anhydrotetracycline induction (all others). SACOL0037 and SACOL0119 were purified via ProBond cobalt affinity chromatography (Invitrogen, Life technologies, Carlsbad, CA), while all other antigens were purified using Strep-Tactin Superflow Columns (IBA, Göttingen, Germany). Purity was confirmed by resolving each protein on 10-20% SDS-PAGE and quantities were determined by BCA (Pierce, Rockford IL). Desalting and buffer exchange to phosphate-buffered saline (PBS) was performed for SACOL0486, SACOL0688, and glucoaminidase using 30 kDa molecular weight cut-off (MWCO) Amicon filtration units (Millipore, Billerica, MA) per the manufacturer's instructions. Desalting and buffer exchange to PBS was performed for SACOL0119 using 10 kDa MWCO Amicon filtration units (Millipore, Billerica, MA). Desalting of SACOL0037 into Nano-pure water was achieved using desalting PD-10 columns (GE Healthcare, Waukesha, WI) following the manufacturer's procedure. Subsequently, SACOL0037 was lyophilized using a Virtis freezer dryer (SP Scientific, Warminster, PA) and the protein particulate was reconstituted in PBS. Protein quantities were determined by BCA (Pierce, Rockland, IL) and confirmed by resolving the proteins on 10-20% SDS-PAGE.

[0069] *Surgical implantation of pins*. Four to eight mice per experimental group (performed in duplicate) were either non-vaccinated with alum adjuvant alone or vaccinated with the quadrivalent biofilm vaccine, the single additional antigen (SA0119), or the combination of all tested antigens (pentavalent vaccine) at 12.5 μg/antigen in alum adjuvant. Vaccines were administered by intraperitoneal (IP) injection. Animals were boosted 14 days later with a non-vaccinated treatment of PBS or vaccinated treatment with the above vaccine compositions suspended in straight PBS. 14 days following boost, mice were anesthetized via IP injection of 100 mg/kg ketamine (Ketaset® - Fort Dodge Laboratories, Inc., Fort Dodge,

Iowa) and 10 mg/kg xylazine (Rugby Laboratories, Inc., Rockville Center, NY). The left leg of each mouse was cleansed with povidone iodine and rinsed with 70% ethanol before surgical implantation of an sterile 0.25-mm insect pins (Fine Science Tools, Foster City, CA) according to the methods previously described (35, 49). Following implantation, 1 µl of the 1 x 10^6 CFU/ml *S. aureus* suspension prepared above was directly inoculated onto the pin implant followed by incision closure. Since 100 CFUs of *S. aureus* are capable of causing chronic infection in this model (data not shown) and in foreign body infections in humans (19), this infectious dose is at least ten times that required to cause infection. All mice did not undergo any additional treatments after surgery until sacrifice. All animal experiments were performed in accordance to protocols reviewed and approved by the *Institutional Animal Care and Use Committee (*IACUC) at the University of Maryland School of Medicine (Baltimore, MD).

[0070] *Bone Cultures*. In order to demonstrate animal model efficacy, At 4, 7, 14, 21, 28, and 49 days post-implantation, infected and uninfected mice were euthanized, left tibiae were removed, and all soft tissue was dissected from the bone. Using sterile scissors, tibiae were cut into small pieces and placed in 300 µl of sterile 0.85% saline per 100 µg of bone. Bones were homogenized using a Polytron PT 1200 handheld homogenizer (Kinematica, Bohemia, NY) and serial 10-fold dilutions of bone homogenates were plated on tryptic soy blood agar plates to enumerate viable *S. aureus* per g bone and CHROMagar MRSA plates (CHROMagar, Paris, France) to verify a monomicrobial *S. aureus* infection. In addition, non-vaccinated mice (alum alone) and mice vaccinated with the quadrivalent vaccine, the single additional antigen SA0119, or all antigens combined were euthanized at 21 days post infection and tibial colony-forming units (CFUs) were determined as described above. Dissemination of the *S. aureus* infection was monitored by homogenizing kidneys and plating the homogenates as described above.

[0071] *PNA-FISH Biofilm Detection on Explanted Pins*. In order to demonstrate biofilms on infected pins in the tibia of mice, the pins from infected and uninfected mice were carefully removed from the tibiae to prevent perturbation of biofilm mass at 7 and 21 days post-implantation. Pins were fixed in 2% paraformaldehyde in PBS before PNA-FISH hybridization with a FITC-labeled *S. aureus* probe and a rhodamine-labeled universal eukaryotic cell probe, as per manufacturer's instructions (Advandx, Woburn, MA). Each pin was then examined with a Zeiss LSM 510 confocal scanning laser microscope (Carl Zeiss,

Thornwood, NY) for both green and red fluorescence using a FITC/Texas Red dual-band filter and a 63X objective.

[0072] *Statistical Analysis*. Mean and SD were calculated and analyzed using Student's *t*-test with a *P* value of <0.05 to determine statistical significance. Experiments determining the percentage of mice still infected after vancomycin or PBS treatment were analyzed using Fishers Exact test with a *p* value of <0.05 for statistical significance.

Results

[0073] *S. aureus implant infection results in chronic infection.* Tibiae from mice with pins infected with *S. aureus* and control tibiae with non-infected pins were harvested and processed at 4, 7, 14, 21, 28, and 49 days post-implantation. CFUs were enumerated from homogenized bone to determine the development of chronic infection and bacterial loads in the tibia. Results demonstrate that viable *S. aureus* were cultured from the *S. aureus* infected pin and surrounding bone at all time points tested, as far out as 49 days post-infection (Fig. 1). Bacterial loads initially increased to over 3 logs of the infecting dose to > 10^8 CFU/tibia but then decreased between 4 and 7 days post-infection. However, at day 7 and beyond, bacterial loads were consistent. Biofilm formation was evident on implanted pins from infected (see Fig. 1B) but not uninfected mice (see Fig. 1C,D) by confocal scanning laser microscopy.

[0074] *Vaccination with biofilm-upregulated antigens coupled with antibiotic therapy promotes clearance of a S. aureus osteomyelitis infection.* In previous work, Brady *et al.* identified candidate proteins that were upregulated during the biofilm mode of growth and highly immunogenic in rabbits to formulate a multivalent vaccine against *S. aureus* biofilm-mediated infections (4). In an initial vaccination trial, a quadrivalent vaccine composed of SACOL0486, SACOL0688, SACOL0037, and glucoaminidase (10 µg per recombinant protein) was injected into rabbits at 20 and 10 days prior to challenge using a *S. aureus* tibial osteomyelitis infection. Vaccinated rabbits had a slight reduction in bacterial load at 14 days post-infection compared to control animals, but bacterial clearance was not achieved (data not shown / Brady 2011). While the quadrivalent vaccine targets the *S. aureus* biofilm, its components do not activate an effective humoral response against *S. aureus* planktonic cells and these bacteria persist at day 14 post-infection due to the expression of immunoavoidance

factors. Hence, the vaccination strategy was adapted by adding a 10 day vancomycin treatment course starting 14 days post-challenge to eradicate the antibiotic-sensitive, planktonic bacteria dispersed from the biofilm. To evaluate the efficacy of the dual therapy, *S. aureus* enumeration (Figure 2A) and clearance rates (Figure 2B) in rabbits of the dual therapy group were compared with those in unvaccinated and untreated, unvaccinated but treated, and vaccinated but untreated groups. Significant reductions in both bacterial counts and infection rate were observed with the dual therapy (column 4), which establishes that targeting the planktonic phenotype of *S. aureus* is critical to eradicate a biofilm-mediated infection. Overall, a 99.9% reduction in the bacterial population was observed in vaccinated animals compared to control animals.

[0075] *Vaccination with a pentavalent vaccine composed of biofilm-upregulated and planktonic-specific antigens promotes clearance of a S. aureus tibial implant infection.* As an extension of the vaccine study in the rabbit tibial osteomyelitis model, we targeted the planktonic phenotype of a *S. aureus* infection with the addition of a planktonic-specific antigen, SACOL0119, to the biofilm-directed quadrivalent vaccine (SACOL0486, SACOL0688, SACOL0037, and glucoaminidase). The efficacy of this pentavalent vaccine against *S. aureus* infection was evaluated using a murine tibial implant model, which is a critical evaluation of the vaccine against another biofilm-mediated infection besides osteomyelitis. The pentavalent vaccine, which was composed of 12.5 μg of each recombinant antigen, was administered at 28 and 14 days prior to *S. aureus* challenge using the tibial implant model. At 21 days post-challenge, CFUs in the tibiae from mice vaccinated with the pentavalent vaccine were enumerated and compared to counts from mice vaccinated with either the quadrivalent vaccine or monovalent SACOL0119 vaccine and unvaccinated mice. Kidney homogenates were also examined for bacterial counts. We did not observed *S. aureus* in the kidneys of any control or experimental animals, which confirms that the infections were localized and did not disseminate from the tibia. In the unvaccinated mice, we observed a 100% infection rate (Figure 3) and the development of an involucrum around the implant insertion site (data not shown). The quadrivalent vaccine and the SACOL0119 vaccine provided partial protection against *S. aureus* infection with bacterial clearance observed in 50% and 40% of the animals, respectively (Figure 3). In the quadrivalent and SACOL0119 vaccinated mice, the presence of an involucrum on the tibia corresponded with the presence of *S. aureus* at the implant site. Since vaccination with either the biofilm-upregulated antigens or the planktonic-specific antigen alone provide approximately equivalent

protection, we surmised that a combination of the antigens would have a synergistic effect and provide complete clearance of *S. aureus* in the tibial implant model. The addition of this planktonic antigen would substitute for the use an adjunctive antibiotic therapy to eradicate persisting *S. aureus* as previously demonstrated by our lab. Indeed, the pentavalent vaccine provided complete protection against *S. aureus* with 100% clearance in all mice within this vaccine subgroup (Figure 3). Additionally, tibiae from the pentavalent vaccinated mice resembled uninfected tibiae with no signs of infection. Therefore, the incorporation of the single planktonic antigen to the multivalent biofilm-directed vaccine enhanced the vaccine efficacy from 50% to 100% prevention of a biofilm-mediated, implant infection in C57BL/6J mice. Here, we achieved complete bacterial clearance of *S. aureus,* which is an accomplishment that has never been attained with other vaccine formulations including those that advanced into clinical trials, using a vaccination strategy that targeted both the planktonic and biofilm phenotypes of the pathogen.

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[0076] All documents, papers and published materials referenced herein, including books, journal articles, manuals, patent applications, published patent applications and patents, are expressly incorporated herein by reference in their entireties.

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What is claimed is:

1. A vaccine formulation comprising:

(a) five *Staphylococcus aureus* polypeptides, or portions thereof, or variants thereof, or combinations thereof, wherein the *S. aureus* polypeptides are (i) *S. aureus* polypeptide SA0037 set forth in SEQ ID NO:13, (ii) *S. aureus* polypeptide SA0119 set forth in SEQ ID NO:14, (iii) *S. aureus* polypeptide SA0486 set forth in SEQ ID NO:15, (iv) *S. aureus* polypeptide SA0688 set forth in SEQ ID NO:16, and (v) *S. aureus* glucosaminidase set forth in SEQ ID NO:17, and

(b) a pharmaceutically acceptable carrier or diluent.

2. The vaccine formulation of claim 1, wherein the formulation comprises a portion of at least one of the *S. aureus* polypeptides and wherein the portion comprises at least about 20 contiguous amino acids of the full-length polypeptide.

3. The vaccine formulation of claim 1, wherein the formulation comprises a variant of at least one of the *S. aureus* polypeptides and wherein the variant has at least about 95% sequence identity with the full-length polypeptide.

4. The vaccine formulation of claim 1, wherein the formulation comprises full-length versions of the five *S. aureus* polypeptides.

5. A vaccine formulation consisting of:

(a) *S. aureus* polypeptide SA0037 set forth in SEQ ID NO:13, (ii) *S. aureus* polypeptide SA0119 set forth in SEQ ID NO:14, (iii) *S. aureus* polypeptide SA0486 set forth in SEQ ID NO:15, (iv) *S. aureus* polypeptide SA0688 set forth in SEQ ID NO:16, and (v) *S. aureus* glucosaminidase set forth in SEQ ID NO:17, and

(b) a pharmaceutically acceptable carrier or diluent.

6. A method of generating an immune response in a subject comprising administering an immunologically effective amount of a vaccine formulation of any one of claims 1-5 to a subject, thereby generating an immune response in a subject.

7. A method of generating a protective immune response in a subject comprising administering an immunologically effective amount of a vaccine formulation of any one of claims 1-5 to a subject, thereby generating a protective immune response in a subject.

8. A method of inhibiting a *S. aureus* infection in a subject, comprising administering a therapeutically effective amount of a vaccine formulation of any one of claims 1-5 to a subject at risk of developing a *S. aureus* infection, thereby inhibiting a *S. aureus* infection in a subject.

9. A method of treating a *S. aureus* infection in a subject, comprising administering a therapeutically effective amount of a vaccine formulation of any one of claims 1-5 to a subject having a *S. aureus* infection, thereby treating a *S. aureus* infection in a subject.

10. The method of claim 8, further comprising administering an antimicrobial agent to the subject, wherein the antimicrobial agent is administered concurrent with, prior to. or after the vaccine formulation.

11. The method of claim 9, further comprising administering an antimicrobial agent to the subject, wherein the antimicrobial agent is administered concurrent with, prior to. or after the vaccine formulation.

12. The method of claim 10, wherein the antimicrobial agent is selected from the group consisting of an aminoglycoside, a carbacephem, a carbapenem, a cephalosporin, a glycopeptide, a macrolide, a monobactam, a penicillin, a polypeptide, a quinolone, a sulfonamide, a tetracycline, chloramphenicol, clindamycin, lincomycin, fusidic acid, furazolidone, linezolid, metronidazole, mupirocin, nitrofurantoin, macrobid, platensimycin, quinupristin/dalfopristin, rifampin and rifampicin.

13. The method of claim 11, wherein the antimicrobial agent is selected from the group consisting of an aminoglycoside, a carbacephem, a carbapenem, a cephalosporin, a glycopeptide, a macrolide, a monobactam, a penicillin, a polypeptide, a quinolone, a sulfonamide, a tetracycline, chloramphenicol, clindamycin, lincomycin, fusidic acid, furazolidone, linezolid, metronidazole, mupirocin, nitrofurantoin, macrobid, platensimycin, quinupristin/dalfopristin, rifampin and rifampicin.

14. The method of claim 8, wherein the *S. aureus* infection is a *S. aureus* biofilm infection.

15. The method of claim 9, wherein the *S. aureus* infection is a *S. aureus* biofilm infection.

Figure 1

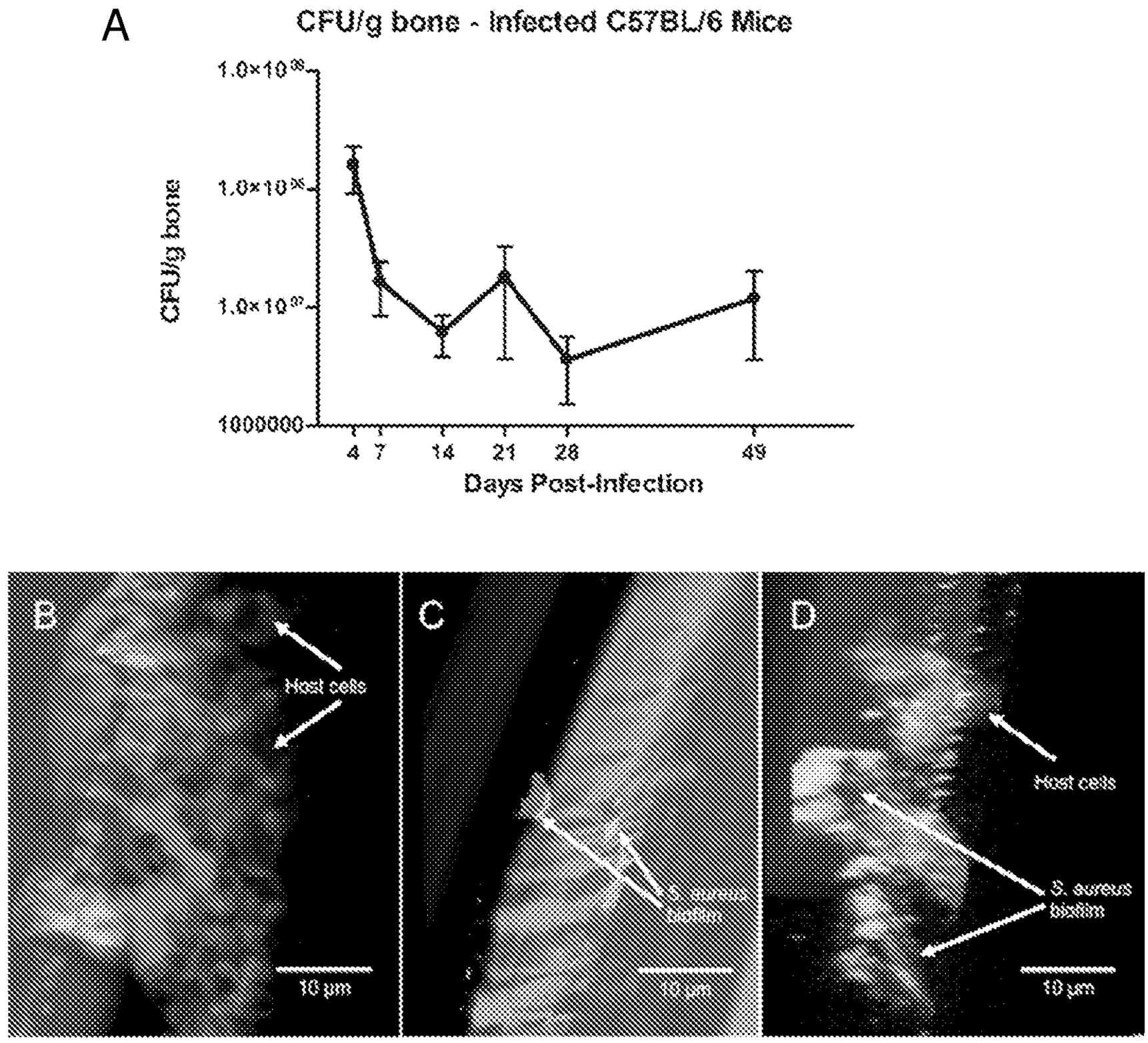

A
CFU/g bone - Infected C57BL/6 Mice
1.0×10^8
1.0×10^6
1.0×10^4
1000000
CFU/g bone
4
7
14
21
28
49
Days Post-Infection
B
Host cells
10 µm
C
S. aureus biofilm
10 µm
D
Host cells
S. aureus biofilm
10 µm

Figure 2

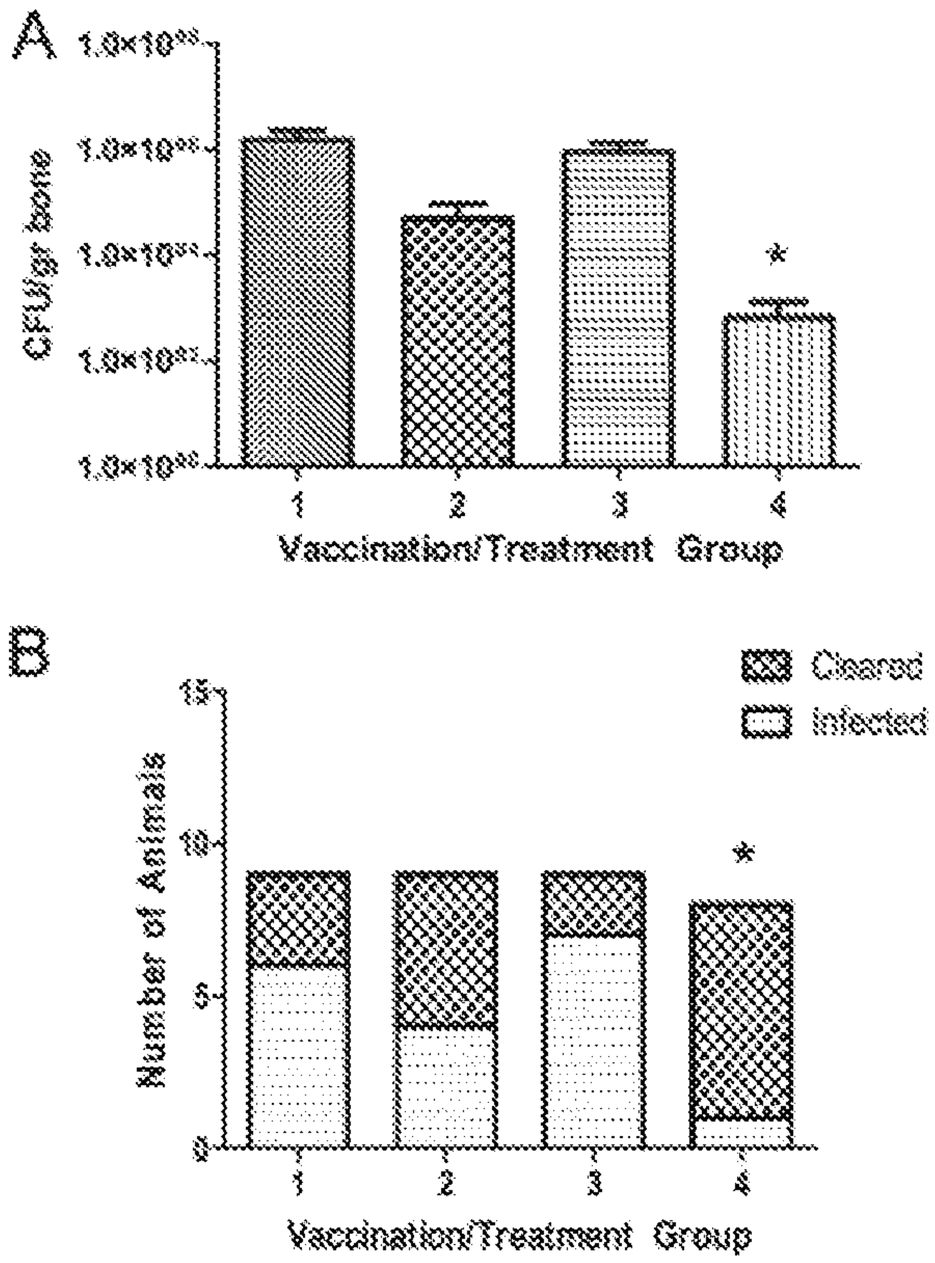

A
1.0×10^8
1.0×10^7
1.0×10^6
1.0×10^5
1.0×10^4
CFU/gr bone
*
1
2
3
4
Vaccination/Treatment Group
B
Cleared
Infected
Number of Animals
15
10
5
0
*
1
2
3
4
Vaccination/Treatment Group

Figure 3

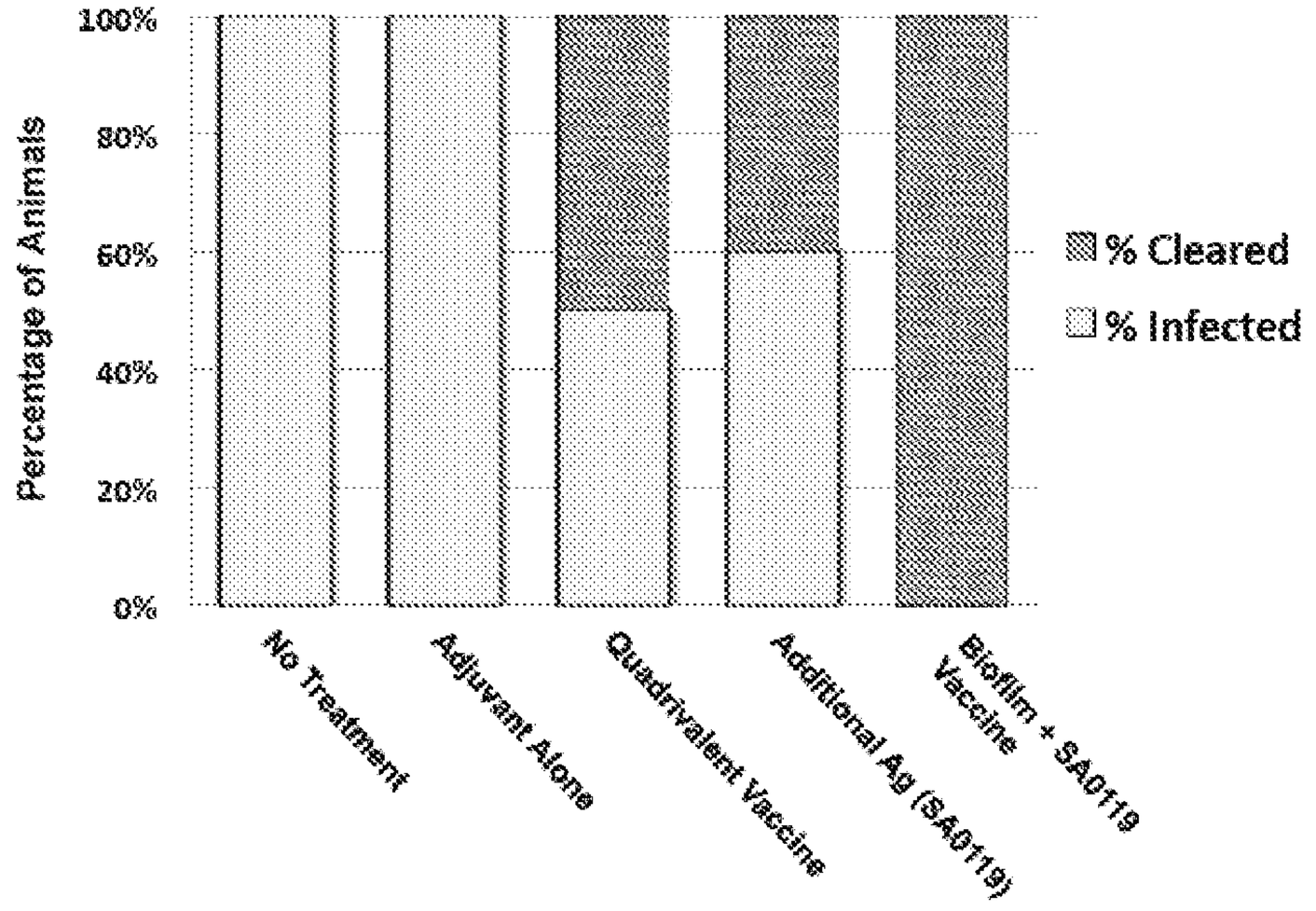
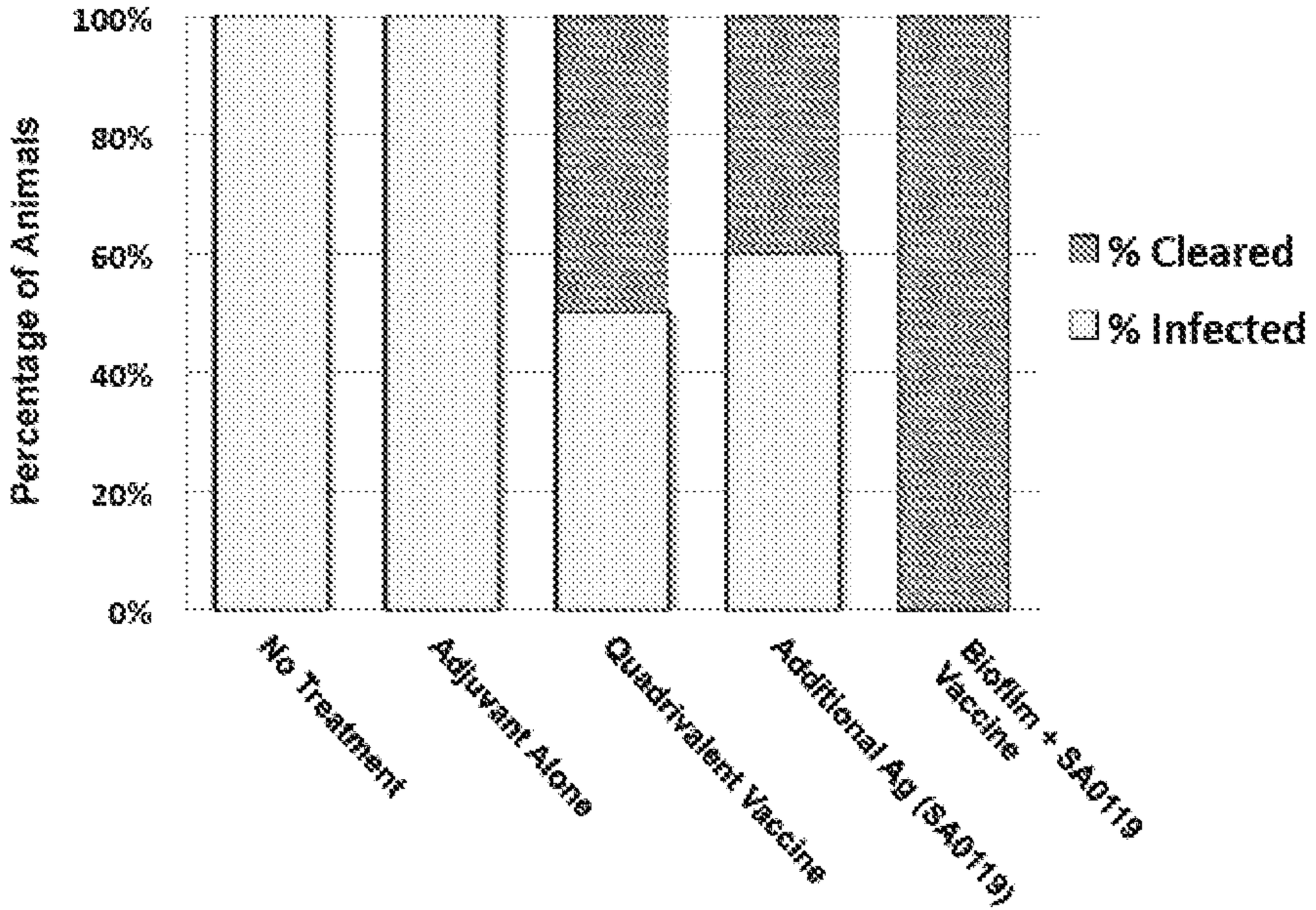

100%
80%
60%
40%
20%
0%
Percentage of Animals
% Cleared
% Infected
No Treatment
Adjuvant Alone
Quadrivalent Vaccine
Additional Ag (SA0119)
Biofilm + SA0119 Vaccine

Figure 3


Percentage of Animals
100%
80%
60%
40%
20%
0%
% Cleared
% Infected
No Treatment
Adjuvant Alone
Quadrivalent Vaccine
Additional Ag (SA0119)
Biofilm + SA0119 Vaccine