

Comparative analysis of PIA and rSesC mixture, arisen antibodies against the biofilm forming *Staphylococcus aureus*.

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Abstract

Background: *Staphylococcus aureus* as a causative agent of hospital-acquired infections, has been considered as the primary concern in biomaterial-related infections (BAIs). Following the purification of polysaccharide intercellular adhesion (PIA) as an efficient macromolecule in biofilm formation in the native condition, recombinant *S. epidermidis* surface exposed rSesC protein, with the most homology to clumping factor A (ClfA) in *S. aureus* was cloned and expressed in a prokaryotic host as well. Fourier transform infrared spectrometry (FTIR) and Western blotting procedure analyzed purified PIA and protein, respectively. Then, the immune response was evaluated by measuring total IgG titers. Moreover, the capacity of Anti-biofilm forming activity of arisen antibodies to a biofilm forming *S. aureus* strains was assessed by semi-quantitative micro-plate procedure.

Results: Data showed that the total IgGs was boosted in mice immunized sera. By performing inhibition assay, biofilm inhibitory effect of secreted antibodies to test strain was observed. Arisen antibodies against the mixture significantly were more potent than PIA and rSesC, when comparing them in a biofilm inhibition assay.

Conclusion: Immunization of mice with mentioned antigens especially a mixture of them, could eliminate the biofilm formation process in *S. aureus*. Hopefully, this study corresponds the suggestion that, the immunization of mice with PIA and rSesC candidate vaccine could protect against *S. aureus* infection.

Background

Staphylococci are opportunistic pathogens and determined as the most common causes of infections related to implanted medical devices, infect both hospitalized patients and immunocompromised individuals [1]. Considering diverse virulence factors, such as capsule and cell wall-bound adhesion molecules, surface proteins, toxins, antibiotic resistance, and biofilm formation cause infections in human and animal. [2]. *S. aureus* is an etiological agent of the mild to severe infections in hospitalized patients, such as bacteremia due to endocarditis, pneumonia, and metastatic infections. Finding revealed that, majority of adults are either permanently or transiently susceptible to colonization by *S. aureus*. [3] Up to 20–30% of humans, asymptotically are colonized by *S. aureus* and 50–60% of those are intermittently colonized [4, 5] Since the 1960s, first (MRSA) strains were detected and with worldwide spreading, becoming a global major challenge. Despite the importance of this bacterium in human and animal infections, there are no available vaccines to prevent *S. aureus* infections yet [6]. Contamination of medical devices with *S. aureus* during insertion might be remarkably depended on the patient health care personal. Although there are some similarity in biofilm-associated infections with *S. aureus* and *S. epidermidis*, but more intensive care is needed for the involvement of *S. aureus*. *S. aureus* biofilm-associated infections are more difficult to be treated by antibiotic therapy, and the devices need to get replaced more frequently [7]. The *ica* operon of *staphylococcus* spp, encodes the production of polysaccharide intercellular adhesin (PIA), also known as poly-N-acetyl glucosamine (PNAG) [8]. The PIA-dependent mechanism is the best understood mechanism of biofilm formation. Cerca et al.,

demonstrated that rabbit anti-PIA antibodies protect against infections with planktonic cells of PIA-positive *S. aureus* and *S. epidermidis* [9]. Based on a study by Maira-Litran et al., anti-dPIA antibodies are mediated opsonic killing and protected against *S. aureus* infection [10]. Recent studies indicate presence of the other proteinaceous mechanisms of biofilm formation. Shahrooei revealed that monoclonal antibodies against *S. epidermidis* surface-exposed Ses proteins can significantly reduce the accumulation phase [11]. Clumping factor A (ClfA) is a fibrinogen (Fg) -binding microbial surface molecule recognizing adhesive matrix molecules (MSCRAMM) of *S. aureus*; 65.1% similarity has been shown between SesC and a 341-aa fragment of ClfA [12].

Owing to the vital biofilm forming capacity role in the chronic staphylococcal disease development, evaluation of the biofilm inhibitory effect of PIA and recombinant SesC antibodies against a biofilm forming *S. aureus*, separately and in mixture form was targeted as the main goal of this study.

Results

Purification of macromolecules:

Based on the cloning and expression procedure, 1399 bp encoding gene of 55KD recombinant protein representative of rSesC was cloned in pET11C vector and transformed to the BL21 (DE3) as a prokaryotic host. The expressed protein was purified taking advantage of affinity chromatography procedure as well. Representative rSesC protein was confirmed based on the size by SDS- PAGE procedure. Then presence of a C-terminal hexa-His- tagged residue in the protein was determined by a Western blot Following the Bradford assay procedure. Amount of the purified protein was assessed about 5mg. Fig.1.

Purification of PIA

PIA as a main compartment of biofilm forming macromolecule in aggregation phase was purified based on the native condition procedure using size exclusion chromatography procedure. To isolate PIA (100 KD), size-exclusion chromatography was performed by Sephacryl S-100. PIA was purified at a flow rate of 0.300 ml min⁻¹ and fractions of 1.5 ml were collected every 5 min in a fraction collector. Contaminated PIA by proteins and nucleic acid (RNA and DNA) was cleaned by enzymatic digestion too. Chemical analysis of purified PIA indicated negligible contamination (<2 % protein and <0.4 % nucleic acid). Subsequently based on the colorimetric assay procedure by targeting the N-acetyl Glucosamine residue in the compartment, data showed that the purified PIA contained 65% hexosamine (5700 µg ml⁻¹), uronic acid (29 µg ml⁻¹) and ketose (170 µg ml⁻¹). The composition and structure of the PIA were confirmed by using FTIR procedure. Fig.2- 4.

Endotoxin contents and general safety

According to the Limulus amoebocyte lysate test to purified macromolecules, the content of endotoxin was determined as 4.5 EU ml⁻¹. Pyrogenicity and toxicity were not observed following *in vivo* challenge.

Anti-antigens humoral response

Using antigen mediated ELISA in order to evaluate the total IgG antibody response against PIA in the antigen mixture, mice sera antibodies titers were determined ($\leq 1: 200$). Following the first immunization by rSesC and PIA ($P = 0.0057$), low level IgGs production comparing to the control group was observed and this amount determined as not significant to rSesC immunized sera. An increase in IgG titres to PIA ($P = 0.0004$) and rSesC ($P = 0.0025$) was observed following the first booster. The third group of mice received a mixture of PIA and rSesC and an increase in anti-PIA antibodies was observed following the second immunization ($P < 0.0001$). Fig. 5.

Biofilm inhibition assay by microtiter plate

The biofilm inhibition assay was performed by comparing pre-post immune sera by semi-quantitative procedure. Experiments were observed with sera from mice that were boosted after specific time spans (14, 28 and 42 days) and results were obtained by comparing the immunized and non-immunized sera, respectively. (Fig. 6). The data showed that those immunized mice sera by PIA antigen provide significant ($P > 0.05$) inhibition after first booster dosage. The biofilm inhibitory effects of immunized sera after first booster dosage by rSesC ($P > 0.0314$) and PIA/rSesC mixture, ($P > 0.0015$) and second booster dosage statistically were significant. The obtained data showed that, vaccinated mice sera by PIA and rSesC antigens compared to the control group have insignificant biofilm inhibitory effect after the first immunization (14 days). However, this effect for immunized sera by PIA/rSesC mixture ($P = 0.0004$) was significant when compared by the control sera. Detailed OD average listed in the Table 1.

Table 1. Average of mean optical density of representative sera to biofilm inhibition assay (595nm).

PC, Positive control (biofilm forming *S. aureus* wild type), NC; Negative control (*S. epidermidis* ATCC 12228), PBS; sodium phosphate buffer, OD; optical density in 595 nm.

Discussion

S. aureus as a main human pathogen because of its ability to biofilm formation and antibiotic resistance pattern has been assigned as an essential concern in worldwide healthcare system [13]. Staphylococcus species specially *S. aureus* and *S. epidermidis* despite being a part of human normal flora, with the ability to surface attachment of medical devices and developing into recalcitrant community multilayered structures, referred to as "biofilm", makes them problematic [14]. Biofilm forming ability is a surveillance factor in Staphylococcus spp., mediating the adherence of bacterial cells to biomaterials and helps organisms to avert the host immune defense [15]. Staphylococcal ability to biofilm formation, is considered as the most important factor triggered in the pathogenesis, and its colonization on medical devices, makes it increasingly resistant not only to multiple antibiotics but also to host defenses. There is an essential need to change the medical devices after *S. epidermidis* biofilm infection, and experimental studies on biofilm-preventing vaccines are necessary [16]. The role of PIA and proteins in biofilm formation has been clearly demonstrated. Targeting of the SesC protein and PIA macromolecules involved in attachment and accumulation of biofilm forming phase and antibodies that arise in response to them may be suitable options for antibodies-dependent treatment of biofilms [15]. In the current study,

the evaluation of arisen antibodies against PIA and rSesC protein, efficacy of antibodies to biofilm inhibition process and putative vaccine candidate activity against the mentioned antigens of *S. aureus* have been listed as the main purposes. Taking advantage of cloning procedure, rSesC protein was generated and precisely confirmed by Western blotting method. The DNA sequence of the truncated protein representative of the anchor site on the SesC protein was inserted into cloning vector and transformed into the prokaryotic host. The 55KD protein representative of rSesC was previously purified [12, 17]. Our results were confirmed when compared by other studies. The specific function of the SesC proteins hadn't describe. closest homologous protein to SesC, is a 341 aa fragment nominated Clumping factor A in *S. aureus*) with specific function (Identity 26.6% and Homology 65.11%) [12]. Because of this homology, immunological aspects of SesC protein was targeted to biofilm forming *S. aureus* too. The published data demonstrate that the arisen antibody to immunized animals with conjugated deacetylated PNAG ($\leq 15\%$ acetyl) to diphtheria toxoid (DT) as a carrier protein showing a killing activity against three strains of *S. aureus* and a PIA dependent biofilm forming *S. epidermidis* M187 [14].

Because of this homology, biofilm inhibitory effect of the arisen antibodies to rSesC was targeted to biofilm forming *S. aureus*. Based on the results, rSesC protein has been determined as the suitable biofilm inhibitory candidate vaccine following the injection of 25 mg/ml of the mentioned antigens in mice. This protein not only led to the rise of the specific antibodies after the first booster, but also biofilm inhibitory effect of raised antibodies was confirmed. Though the occurrence of the mentioned antibodies were not significant when compared to the first immunization (14/28 days) but the amount of antibodies compared to the second booster was significantly increased. Raised antibodies against rSesC protein, as a *S. aureus* clumping factor A protein homolog, successfully decreased biofilm formation in a biofilm forming wild type *S. aureus* comparing the collected sera after injection times. Our results showed that rSesC, due to the biofilm inhibitory effect, separately could be a vaccine candidate against biofilm forming *S. aureus*. Taking advantage of reported findings [25], although rSesC and PIA were described as two effective vaccine candidates in biofilm forming *S. epidermidis*, the conjugation of PIA to rSesC will enhance the opsonic activity of secreted antibodies, too. In this study, a mixture of rSesC and PIA was prepared and occurrence of antibodies against the biofilm forming wild type *S. aureus* were assessed. Findings support the hypothesis that rSesC could be considered as a suitable candidate vaccine against biofilm forming *S. aureus*, too.

PIA as the main component of bacterial accumulation during biofilm formation, was extracted from wild-type strain 1457 based on the native purification process [18]. The composition of purified PIA confirmed by FTIR revealed that our obtained data correspond with the other relevant studies findings [15, 18]. We tried to evaluate the PIA and rSesC efficacies as immunoprophylaxis and immunotherapy against biofilm-forming *S. aureus*. According to the studies, the purity of native purified PIA can elicit an antibody response against the mentioned antigen [14, 19–21]. Though the antigenicity of most polysaccharides is poor, but the increased antibodies to PIA have shown a biofilm inhibitory effect. Corresponding antigens had previously been analyzed as a useful candidate vaccine against biofilm-forming *S. epidermidis*, but the opsonic rate of the rSesC protein was lower in purified IgGs [15].

In this study, 6–8 weeks female mice were immunized by PIA, rSesC and mixture of them in four categorized. Control group received PBS as basic solvent of antigens. All mice were boosted by each specific antigen two weeks after first immunization. Immunized sera were collected in 14, 28 and 42 days after the first immunization. According to the obtained results in ELISA, evaluation of the total IgGs by checker board method, and diluted sera in 1: 200 determined as titers for all of the antigens. PIA and rSesC Immunized sera were not statistically significant when compared by the control 14 days after the first immunization. After the first booster dosage, increasing of the PIA and rSesC antibodies titers were observed, while titers of antibodies in 28 ($P = 0.0025$) and 42 ($P = 0.0001$) collected immune sera were statistically significant. Antibodies to mixture of PIA and rSesC in all immunized sera were statistically significant. Similar results were observed in the study based on the antigenicity of mentioned macromolecules [15].

Reports indicated that the presence of amine groups in the PIA structure increase titers in the first and second boosters is justifiable. The alum as an adjuvant could stimulate antibodies secretion and induction of Th2 immunity as well.

Since one of the most important characteristics of vaccine candidates is the *in vitro* and *in vivo* effects of them, in this study, the features of polyclonal secreted antibodies analyzed on biofilm formation under laboratory conditions. Data showed that, immune sera after 14 days had biofilm inhibitory effect, however after the first and second booster, the effect of biofilm inhibition in the mixture group and other groups showed a significant difference compared with the control group in the last booster. Efficacy of antibodies in immunized sera showed that, at the first immunization for PIA ($P = 0.3466$), rSesC ($P = 0.371$), biofilm inhibitory effect were not statistically significant when compared with the control group. Immunized sera by mixture of PIA and rSesC in two week after the first immunization showed the biofilm inhibitory effect statistically significant ($P = 0.0004$). Biofilm inhibitory effect of antigens comparing with the different immunization era (14/28 days) showed that the immunized sera by PIA, rSesC ($P = 0.0314$) and mixture of them ($P = 0.0003$) were statistically significant as well.

Reports indicated that the mixture of mentioned macromolecules had a good biofilm inhibitory effect. In our previous study using a mixture of PIA and rSesC, the opsonic activity of arisen antibodies enhanced against biofilm forming *S. epidermidis* [25]. In this study, biofilm inhibition capability to PIA/rSesC mixture antibodies against biofilm forming *S. aureus* was evaluated. Although opsonic activity and *in vivo* challenge for survival not checked at the current research but biofilm inhibition assay demonstrated that mixture of PIA and rSesC, because of suitable decreasing of the biofilm against a wild type biofilm forming *S. aureus*, could be considered as a good candidate vaccine regarding inhibition of biofilm formation in both *S. epidermidis* and *S. aureus*.

Conclusions

We found that a PIA/rSesC mixture vaccine could inhibit biofilm-formation process in *S. aureus* by eliciting high titer anti-PIA antibodies. It seems that, this mixture could be employed in patients against

the colonization and biofilm formation of this bacterium. We found that immunization of high-risk patients with mixture of vaccine candidate or for treatment using monoclonal antibodies such as IgG2a could help eradicate bacterial biofilms. The preparation and purification of specific anti-PIA IgG2a are a possible means to inhibit medical device infections caused by *S. epidermidis* and *S. aureus*.

Methods

Bacterial strains:

Purification of the PIA and related procedure were accomplished applying two biofilm-forming *S. epidermidis* strains, ATCC 14990 and 35984, also known as 1457 and RP62A, a transmutant strain, 1457-M10 and a wild type biofilm forming *S. aureus* strain. Moreover, *Escherichia coli* strain BL21 (DE3) was served as prokaryotic host to DNA manipulation and recombinant protein production.

Construction and purification of His-tagged rSesC protein

Based on the reported procedure [12] a 1,359-bp fragment of SesC encoding a 459-aa extracellular part of SesC containing a six-His-tag at the C-terminus was amplified. In short, following the cloning and expression of the representative sequence in a prokaryotic host, 55KD rSesC protein was determined by SDS-PAGE and Coomassie Brilliant Blue staining. Protein was expressed in *Escherichia coli* BL21 DE3 following the induction by 1mM imidazole in optical density 0.7–0.9 in a shaker incubator (150 rpm at 37 °C for 3 hours). Purifying of the recombinant protein was done using the commercial affinity chromatography Kit (GE health care, Sweden) according to the manufacture's recommendation followed by extraction of soluble protein from induced host using sonication (4 times for 30 s on ice) with an additional centrifugation (12 000 r.p.m. for 20 min at 4 °C) to the clarification of the targeted protein. The purity of the recombinant protein was determined by Coomassie blue staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel then analyzed by western blotting. The expression of the protein was done as follows: *E. coli* BL21 (DE3) cells were transformed with the vector. Cells were grown with shaking at 250 rpm at 37 °C in Luria-Bertani broth with 100 g/ml ampicillin to an optical density at 600 nm of 0.6 to 0.8. Isopropyl-D-thiogalactopyranoside (IPTG) in 1 mM as a final concentration was added on media to induction of protein expression. Following cooling on ice, cells were collected by centrifugation at 8,000 rpm for 5 min at 4 °C, resuspended in imidazole buffer, and frozen at –20 °C. Harvested pellets was sonicated three times for 30 s on ice. The expressed protein was determined by SDS-PAGE and coomassie brilliant blue staining (Fig. 1).

Extraction and purification of native PIA

PIA was purified as previously described [18, 22]. Briefly, the cells were harvested from incubated (37 °C for 24 h with shaking at 40–50 rpm/min) 2 liters of trypticase soy broth (TSB) by centrifugation (4,500 rpm for 20 min at 4 °C) and were re-suspended in 20 ml of 50 mM sodium phosphate buffer (pH 7.5). After that, the suspended colonies were sonicated four times for 30 s on ice. The extracts were cleaned up by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was dialyzed overnight in a 12 KD

dialysis bag against the same buffer and concentrated using Centriprep 10 (Amicon, Witten, Germany). The soluble proteins were eliminated by proteinase-K, then sample was directly loaded onto an equilibrated 1.6 x 100 cm Sephacryl S-100 column (Pharmacia LKB GmbH, Freiburg, Germany) with 50 mM sodium phosphate. At the end, purified PIA was stored at -20 °C in Amicon cell (Fig. 2). The concentration of the purified PIA was assessed via the amount of hexosamine, taking advantage of the 3-methyl-2-benzothiazolone hydra-zine hydrochloride method with N-acetyl glucosamine as standard (18).

Biochemical analysis

The amount of protein content in the purified rSesC was assessed by the Bradford assay (17). The constitution and structure of the purified native PIA was analyzed using Fourier transform infrared spectroscopy (FTIR) and colorimetric assays (Fig. 3, 4).

Pyrogenicity test and general safety

Taking advantage of the previous procedure, the pyrogenicity and toxicity of the antigens were checked [23]. The amount of endotoxin in the prepared antigens was measured by a commercial Limulus amoebocyte lysate kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

Immunization of mice

6–8-week-old female 18±2 g BALB/c inbred mice (purchased from the Research Institution of Pasteur Karaj, IR Iran) were divided into four groups of six mice each for the vaccination experiments. The mice were housed in standard stainless cages at 23–25 °C and 60–70 % humidity, with a 12 h light/dark cycle, for a week before the experience. The mice were given free access to a standard diet and water. Each mouse in the specific group was immunized thrice subcutaneously with the respective lyophilized antigens (PIA, rSesC, PIA/ rSesC, and PBS) in 1 % alum (Brentag, Denmark) dissolved in PBS (filtered at 0.22 µm pore diameter). The quantity of candidate vaccines were adjusted to a concentration of PIA and protein in the mixture then an immunogenicity of candidate vaccines was compared to control. After two weeks of each immunization (immunization days were 0, 7, 14 and 28) 500 µl of peripheral blood was collected from the tail vein of five mice in each group. Collected sera were stored at -20 °C followed by centrifugation (3000 r.p.m. for 5 min) of peripheral blood [24].

The experimental groups were as follows:

G-I: PIA (100 µg);

G-II: rSesC (25 µg);

G-III: PIA/rSesC mixture (75/25 µg);

G-V: PBS.

Ethics statement

All animal experiments were performed in accordance with the enlarged ethical statement and approved by Mazandaran University of Medical Sciences ethics committee. All performed on the enlarged ethical statement IR.MAZUMS.REC.1397.55 meeting number in Mazandaran University of Medical Sciences. Inbred mice were purchased from the Research Institution of Pasteur, Karaj, Iran.

Euthanasia method:

Using the halogenated ether procedure as an inhalant anesthetic, mice were euthanized as well. In short, mice encountered to an Inhalant ether over dose (up to 2–5% to effect).

Enzyme-linked immunosorbent assay (ELISA)

Anti-PIA antibodies were introduced into the immunized mice sera by applying a commercial enzyme-linked immunosorbent assay after each immunization. Briefly, 96-well plates (Extra gene, USA) were coated overnight with 100 μ l of PIA (1 μ g/well) in PBS at 4 °C. Then, the plates were washed three times with washing buffer [0.05 % (v/v) Tween 20 in PBS], followed by blocking with PBS/Tween 20 containing 5% bovine serum albumin (BSA) for 2 h at 37 °C. Fling blocking and washing, the mouse sera were (1: 2 to 1: 1024) in blocking buffer and 100 μ l of the samples was added to the wells in duplicate. The plates were incubated for 2 h at 37 °C, washed three times and incubated with HRP- conjugated anti-mouse IgG (Sigma, USA) diluted to 1: 10 000 (as a secondary antibody) at 37 °C for 2 h. The plates were washed as described above; enzymatic activity was measured by adding 100 μ l of tetramethylbenzidine (TMB) substrate.

After 30 min subjoining 100 μ l of 2 N H₂SO₄ the reaction was stopped. (Fig. 5).

In vitro biofilm inhibition assay

Biofilm inhibitory effect of pre- and post-immune IgGs against injected antigens on *in vitro* biofilm formation was analyzed using a semi-quantitative microtiter plate method²⁵, (Fig. 6). In short, 5×10^5 c.f.u. ml⁻¹ of an overnight culture of *S. aureus* grown in BHI for the initial attachment in fresh sterile trypticase soy supplemented by 1 % glucose (TSBg) broth was prepared.

A while later, in a polystyrene microtiter plate (Corning Jointed Life Sciences, Lowell, MA, USA), a mixture of 200 μ l of diluted bacterial cultures and 50 μ l of twofold diluted post and pre-immunized mouse sera were inoculated into three parallel wells. After incubation at 37 °C for 20 h, respectively, the attached cells and biofilm were stained by crystal violet as previously described [26]. The OD at 595 (OD₅₉₅) of the dissolved stain in 160 μ l of 30 % (vol/vol) acetic acid was measured in a multipurpose UV/VIS plate reader. A negative control of sterile TSBg without bacteria was included and the assay was repeated independently three times. A previously described *in vitro* biofilm formation assay was also performed[26].

The percentage inhibition of biofilm formation was calculated using the following formula:

$(A_{595, \text{positive}} - A_{595, \text{antibody}}) / (A_{595, \text{positive}} - A_{595, \text{negative}}) \times 100$ [26].

Statistical analysis

Utilizing multiple- group analysis of variance (ANOVA), statistical analysis of results was accomplished and a P-value<0.05 was considered significant.

Declarations

Abbreviations:

Not applicable

Ethics approval and consent to participate:

This study was approved by Mazandaran University of Medical Sciences ethics committee. All performed on the enlarged ethical statement *IR.MAZUMS.REC.1397.55* meeting number in Mazandaran University of Medical Sciences. In this study, all ethics including Ethics and Consent to participate from their parents have been collected in the research.

Consent for publication:

Not applicable

Availability of data and material:

All the results of this study have been classified and maintained by the dissertation in the Pasteur Institute of Iran. We have indeed provided all raw data on which our study is based.

Competing Interests:

The authors declare no competing interests.

Authors' contributions:

all authors have read and approved the manuscript. Contributions of the authors in this study was as follow:

Bahman Mirzaei: Designing the study, interpretation of results and writing the manuscript.

Reyhaneh Babaei: Performing laboratory tests.

Fatemeh Mohammadi: preparation of manuscript

Hamid Reza Goli: Performing laboratory tests

Sanaz Amir Gholami: Biofilm inhibition assay and PIA purification

Mohammad Reza Haghshenas: Interpretation of results.

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Conflict of Interest:

The authors announce that they have no conflict of interest.

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Tables

Due to technical limitations, Table 1 is only available as a download in the supplemental files section.

Figures

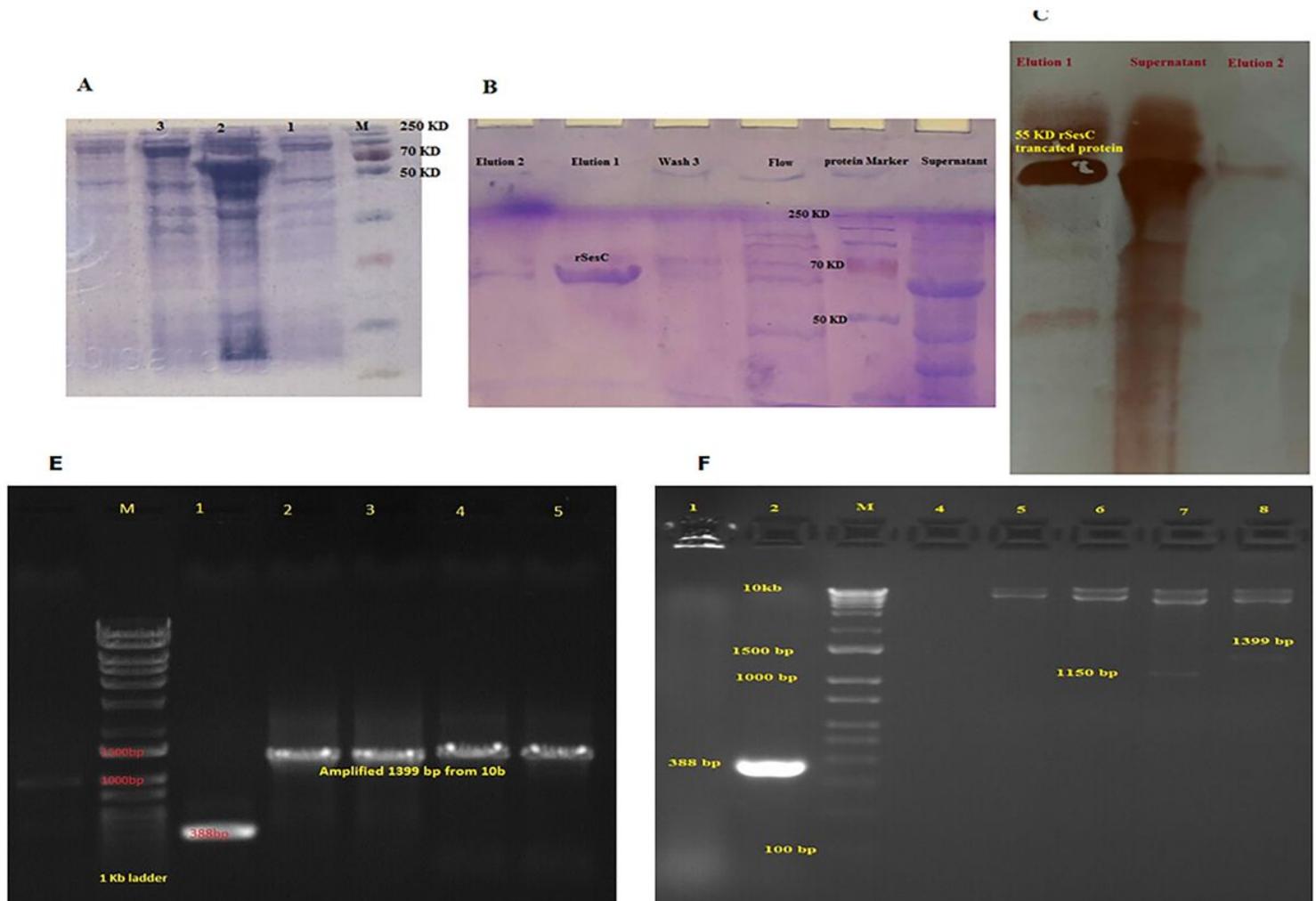


Figure 1

Construction and evaluation of rSesC protein. A) Confirmation of expressing protein after 3hrs by 1mM IPTG induction. 55KD band representation of rSesC protein expressed in BL21 (DE3) after induction. B) Purification of recombinant protein based on the Ni- affinity process. Soluble 55KD protein was purified by affinity chromatography. Target protein eluted using 300 mM imidazole as an elution buffer followed by washing the non-specific proteins by 30- 100mM imidazole as washing buffers. C) Reconfirmation of purified protein by specific antibodies to hexahistidin tail. E) Amplification of a 1399 bp sequence encoded rSesC protein, DNA ladder; 1: *S. epidermidis* ATCC 12228 confirmed by SesC primers and amplicon size is 388 bp; 2- 5: *S. epidermidis* wild type 10b. F) Digestion of the responsible gene for protein synthesis (1359 bp plus 40 bp restriction sites and hexa his sequence) by restriction enzymes in expression vector pET11C (M, DNA ladder; 5, purified expression vector; 6, single digestion by BamH1

enzyme: 4, single digested vector by Nhe1 Enzyme: 7, double digested expression vector by BamH1 and Nhe1 restriction enzymes, 8, single digestion by HindIII restriction enzyme.

PIA purification chromatogram

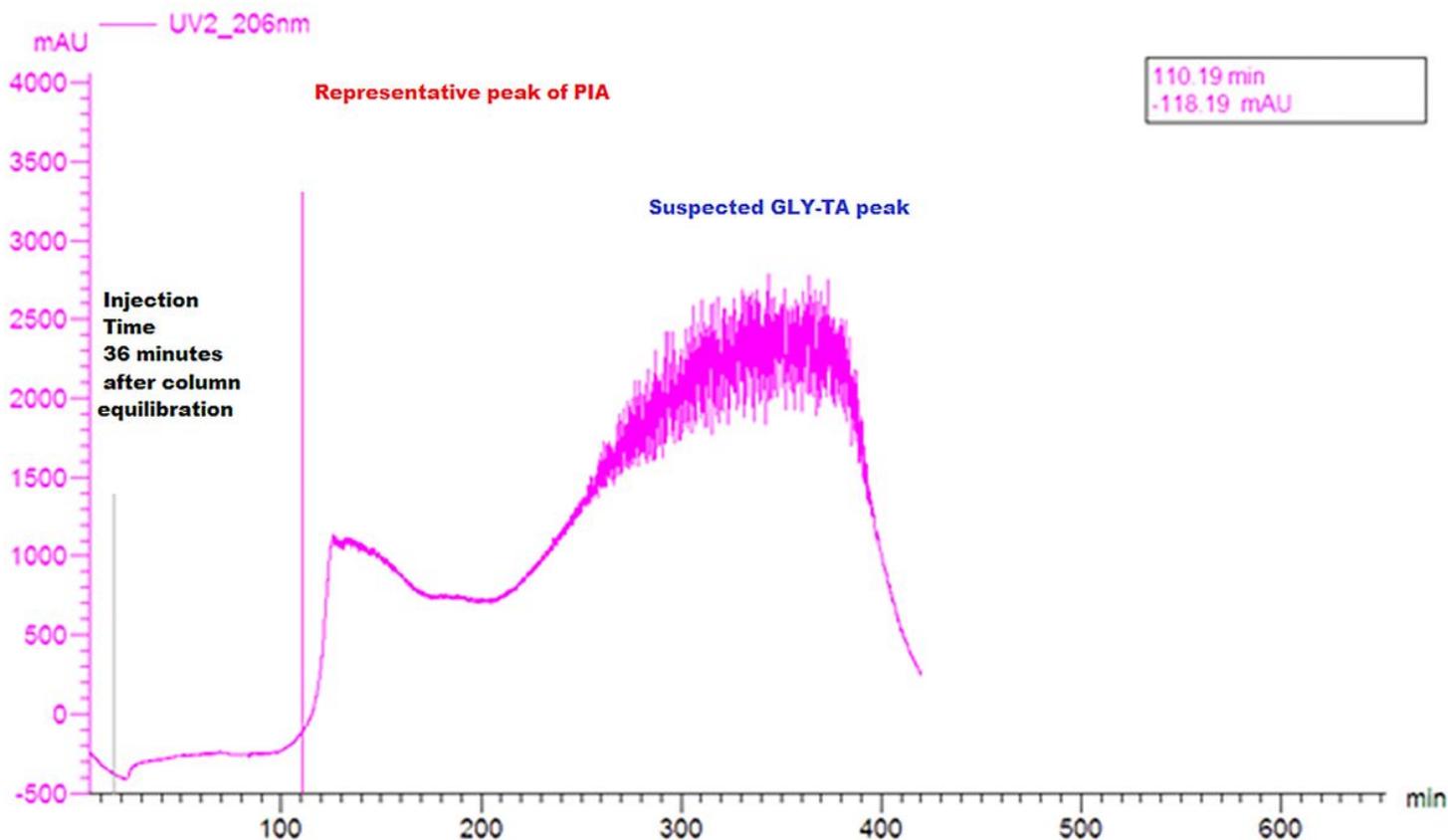


Figure 2

Purification of PIA. Representative Fast protein liquid chromatography (FPLC) chromatogram for native PIA. Sample in 1ml final volume was injected after 36 minutes column equilibration. PIA was eluted near the void volume following the 110 minutes after sample injection by using 0.3ml/min flowrate. Polysaccharide was identified at a wavelength of 206 nm and protein was identified at 280 nm.

Hexosamine assay standard curve

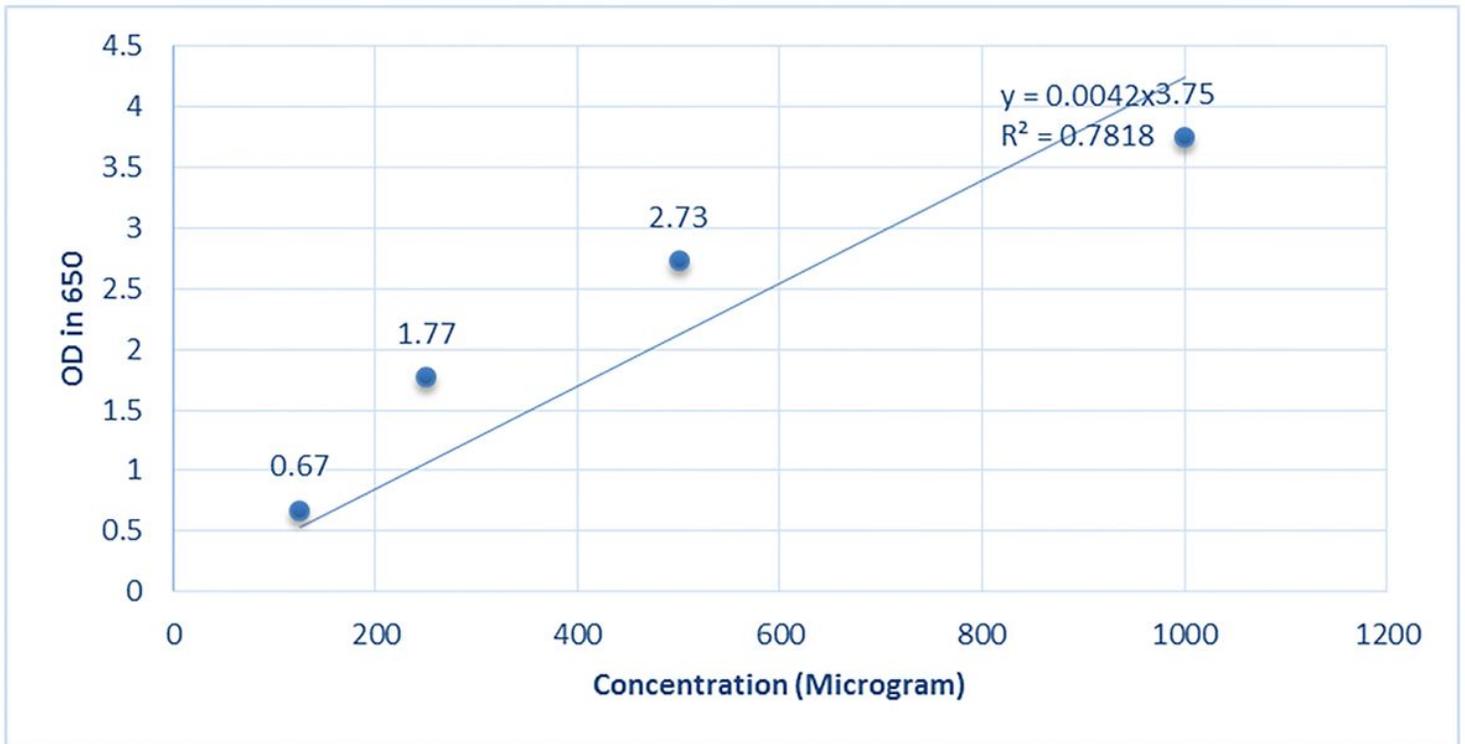


Figure 3

Standard curve to hexosamine assay. According to the procedure, three concentration ($\mu\text{g/ml}$) of N-acetylglucosamine as standard were used and optical density of these concentrations evaluated in 650 nm.

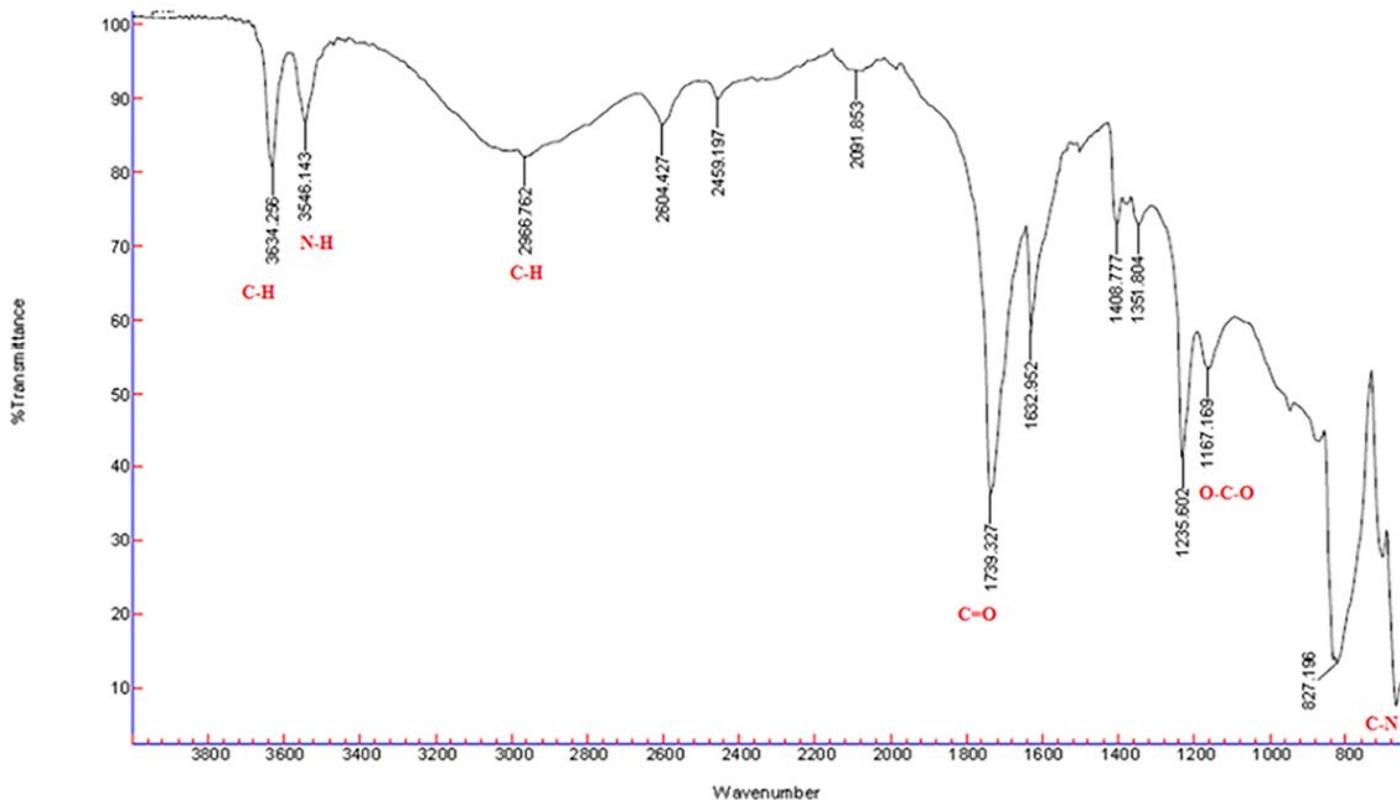


Figure 4

IR spectra of Purified PIA in the 4000–500 cm^{-1} range and the result of this deconvolution. Infrared spectroscopy of purified polysaccharide was investigated using the regularized method of deconvolution. According to the composition of the PIA molecules C=O groups in 1739.327 of the native polysaccharide in the FTIR pattern, was detected.

Booster effect

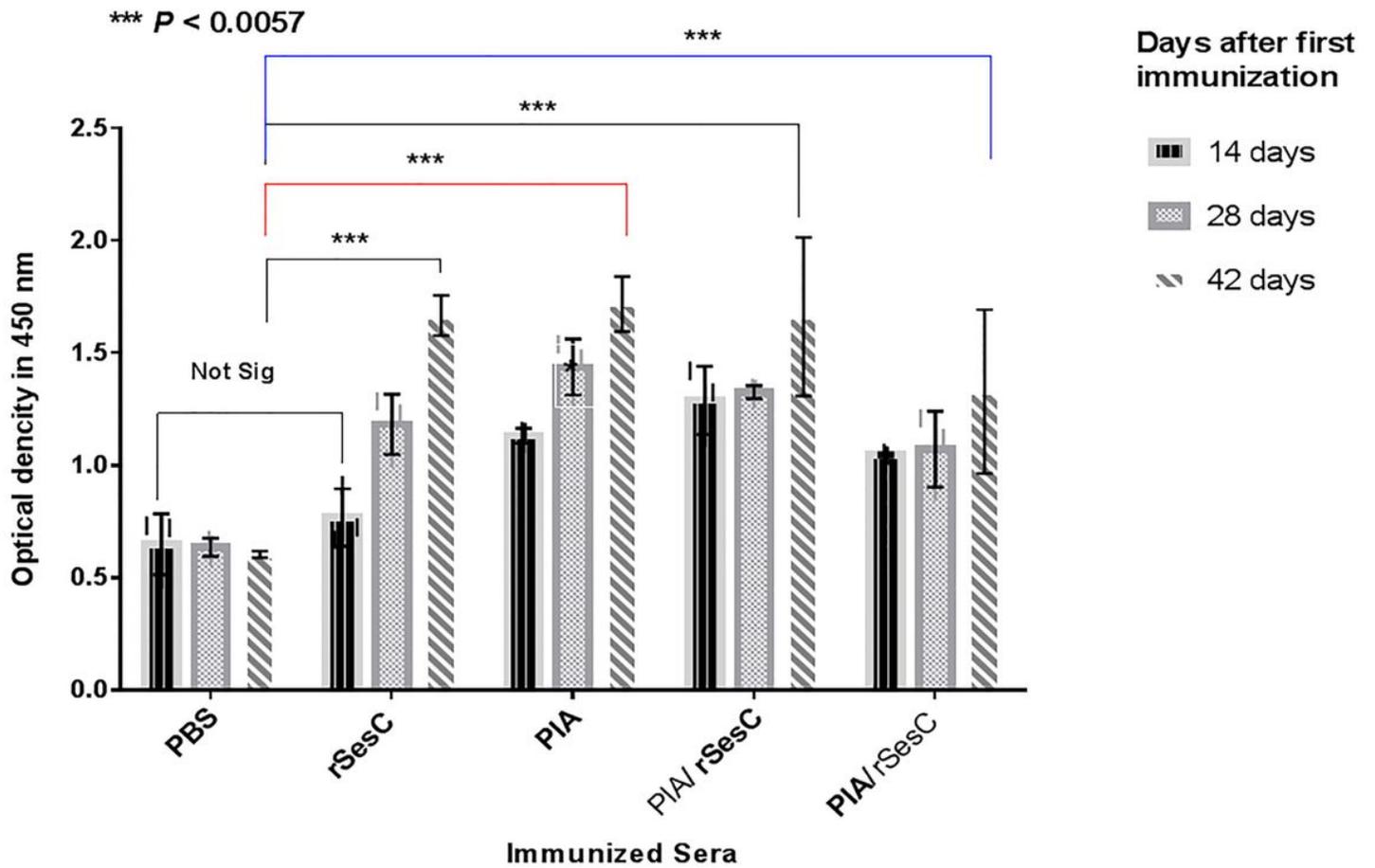


Figure 5

Total arisen antibodies to antigens. The booster effect of the assessed antigens at different times. ELISA was performed by coating the native PIA and rSesC. The titers of the antibodies was assessed for immunized sera compared to the controls. Significant effects were observed from use of the mixture and conjugate booster. Six weeks after the first injection, the titres of antibodies had increased. Antibody titration was assessed in 1: 200 titer, the error bar is representative of the mean \pm sd (n=3).

Biofilm inhibition assay

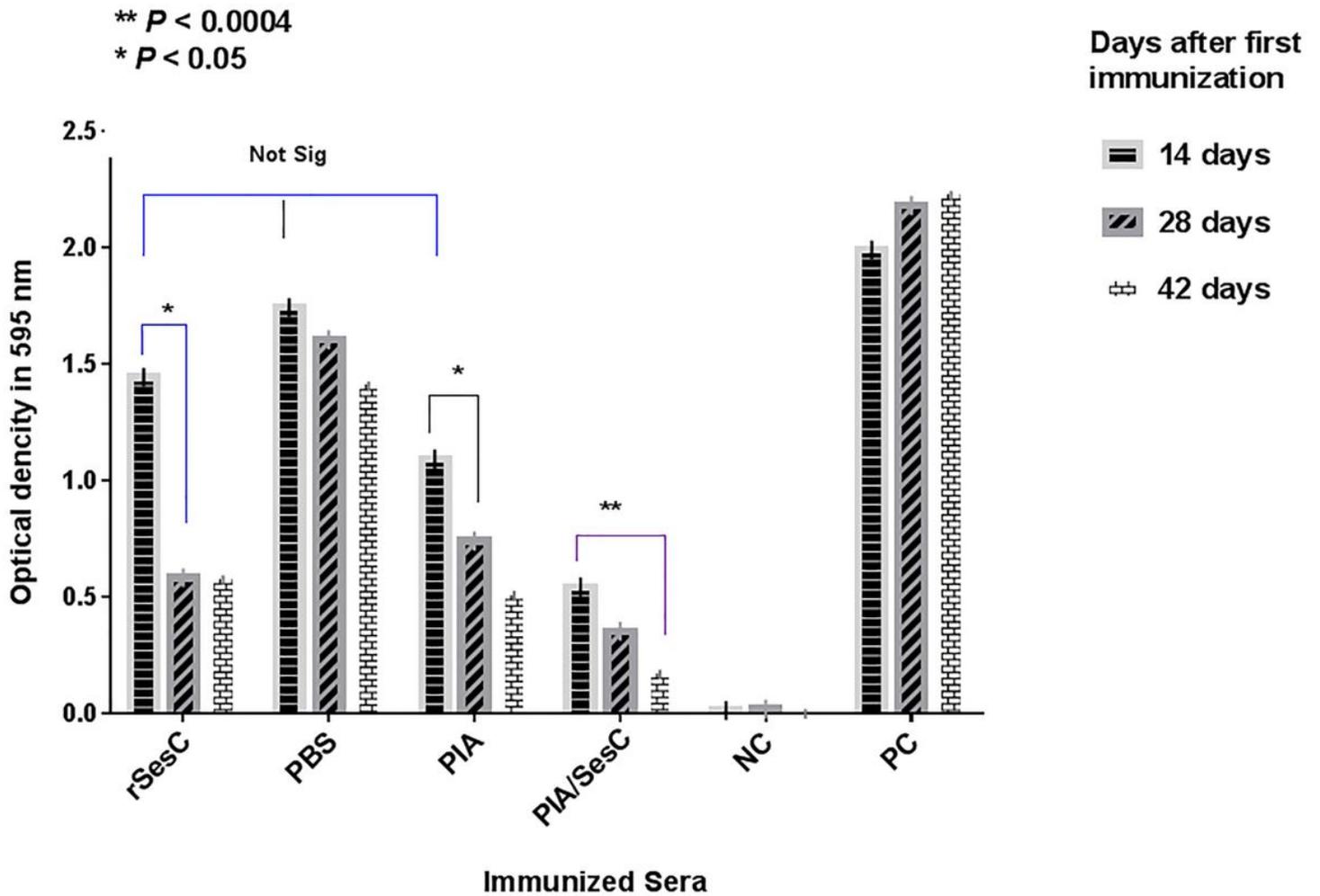


Figure 6

Biofilm inhibition assay by comparing diluted sera. The biofilm-inhibitory effects of increasing antibodies (after each shot) were determined by comparing the positive and negative controls using the mentioned formula. The error bar is representative of the mean \pm sd (n=3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [NC3RsARRIVEGuidelinesChecklist.docx](#)
- [Table1.jpg](#)