ISSN:2521-0807 (Print)
ISSN:2521-0424 (Online)

Contents List available at RAZI Publishing
Matrix Science Medica (MSM)
Journal Homepage: http://www.razipublishing.com/journals/matrix-science-medica-msm/

DETECTION OF BIOFILM PRODUCTION BY *STAPHYLOCOCCUS AUREUS* AND *IN-VITRO* INHIBITION OF BIOFILM USING SERUM ANTIBODIES

Ali Raza[1*], Abdul Ghaffar Qamar[1], Asad Manzoor[1], Misbah Ijaz[1], Khizar Hayat[2], Tayyab Rehmanand[2], Ghulam. Muhammad[1]

[1]*Department of Clinical Medicine & Surgery, University of Agriculture, Faisalabad, Pakistan*
[2]*Department of Anatomy, University of Agriculture, Faisalabad, Pakistan*
**Corresponding author email: aliraza@uaf.edu.pk*

ARTICLE DETAILS

Article History:

Received 16 July2017
Accepted 14 September 2017
Available online 1 November 2017

Keywords:

Biofilm production, *Staphylococcus aureus*, Biofilm inhibition assay.

ABSTRACT

The biofilm producing ability of *Staphylococcus aureus* has been implicated for development of different diseases in animals and humans. The presence of biofilms makes the bacterial population resistant to a wide range of antibiotics. Biofilms are also antigenic structures of the bacterial population. Therefore, the present study was planned to evaluate the biofilm-producing ability of *S. aureus* and *in-vitro* inhibition of biofilm production using serum samples from rabbits immunized with biofilm-producing *S. aureus* bacterin-toxoid. A total of 64 *S. aureus* isolates were harvested from lactating cows and buffaloes suffering from clinical and sub-clinical mastitis. The biofilm-producing ability of *S. aureus* isolates was determined by tube method and micro-plate assays. The rabbits were vaccinated using strong biofilm producing *S. aureus* bacterin-toxoid. The ability of serum antibodies to inhibit *in-vitro* biofilm production was assessed by performing a biofilm inhibition assay. The results showed that 71.87% and 56.25% of the isolates were positive for biofilm production in tube method and micro-plate assays, respectively. The serum samples collected at days 30, 45 and 60 post-immunization completely inhibited the biofilm production by *S. aureus*. Our results suggested that both micro-plate assay and tube method can be used for determination of biofilm production, however micro-plate assay is quantitative, more accurate and reliable. The study also concluded that the antibodies produced against biofilm producing *S. aureus*bacterin-toxoid seems helpful to avert the attachment of bacteria, which may further implicate the better control of chronic infections.

1. INTRODUCTION

Staphylococcus aureus is one of the major pathogenic microorganisms in human and veterinary medicine. This contagious pathogen is one of the most challenging pathogens owing to its ability to develop antibiotic resistance [1,2]. Generally, bacteria exist as two forms of life during growth and proliferation. Sometimes the bacteria are present as single independent cells (planktonic), whereas the counterparts are organized into sessile aggregates, which are termed as biofilm [3]. It has been realized that 99% of all the bacteria in the world exist as biofilms. According to the Centers for Disease Control and Prevention (USA), 65% of the human bacterial infections are caused by biofilm producing microorganisms [4]. Biofilm production endows adherence to inert and free-living surfaces which provides protection to growing bacteria in the environment [5]. The ability of biofilm production by *S. aureus* has been implicated for disease production in animals and human beings. The biofilm production implicates for reduced efficiency of antibiotics to treat infections, which ultimately results into chronic infections [3]. In dairy animals, the biofilm production has been associated with establishment of clinical and sub-clinical udder infections. The problems due to biofilms are of major concerns due to specific characteristics of biofilms including resistance to UV light, antibiotics, increased rate of genetic changes, altered biodegradability of drugs and increased secondary metabolites production [6].

Biofilm production is one of the major virulence factors of *S. aureus*and other bacteria such as *Pseudomonas aeruginosa* [7]. *Staphylococcus aureus* is one of the major pathogens associated with mastitis in dairy animals [8]. It is now well established that *S. aureus* produces biofilm in the udder of infected cows which not only impair the immune system of the host but also resist to different antimicrobials thus results in persistent infection [9]. Biofilms also act as immunogenic structures and elicit a protective immune response against the invading bacteria. Active immunization with exopolysaccharides and bacterins extracted from a highly adherent *S. aureus* isolate (*i.e.* strong biofilm producer) has been shown to trigger antibodies production against this pathogen in sheep and cattle [10,11].

Recently, it has been reported that the immunization of rabbits using bacterin-toxoid of strong biofilm producing *S. aureus* showed increased survival rate in a challenge protection assay and elevated serum antibody titer in vaccinated rabbits as compared to control group [2]. These studies suggest that biofilm production is a good immunogenic factor that can elicit a protective immune response in host.

Since the infections due to biofilm producing microorganisms are difficult to treat once established, it was hypothesized that if antibodies against biofilm producing *S. aureus* bacterin-toxoid inhibit bacterial adhesion, this may enhance the protection against establishment of chronic infections due to resistant bacteria. This research aimed to evaluate the biofilm production trait of *S. aureus* and the ability of serum antibodies to biofilm producing *S. aureus* bacterin-toxoidin inhibiting the *in-vitro* production of biofilm.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

A total of 64 Staphylococcal isolates were recovered by screening of 117 lactating cows and buffaloes suffering from clinical (n=12) and sub-clinical (n=105) mastitis. All the isolates were initially identified by standard microbiological techniques. *Staphylococcus epidermidis* ATCC 35984 (high slime producer) and *S. epidermidis* ATCC 12228 (non-slime producer), obtained from American type culture collection (ATCC, Rockville, Md.) were used as reference strains for evaluation of biofilm production. However, we were unable to use a medium slime producer isolate due to unavailability.

2.2 Evaluation of biofilm production by Staphylococcal isolates

The biofilm production trait of *S. aureus* was evaluated by using tube method and micro-plate assay [12,13].

2.2.1 Tube Method

Qualitative assessment of biofilm production by Staphylococci (n=64) was evaluated by tube method (TM) as described earlier [13]. Briefly, a loop of fresh growth of the isolates from blood agar plates was inoculated into 5mL of Tryptone Soy Broth (Oxoid, UK), additionally supplemented with 1% glucose (TSB_{glu}). The tubes were incubated in vertical position for 24 hr at 37 ^{0}C tolet the bacteria to growing in static conditions. After incubation, the tubes were decanted carefully, washed with phosphate buffered saline (pH 7.2) and then air-dried. After drying, the tubes were stained with 1% safranin. The tubes were washed with deionized water to remove the excess stain and observed for biofilm production. The production of visible films at the walls and bottom of the tubes after staining with safranin was interpreted as positive biofilm producer. Ring formation at the liquid interface was not considered as biofilms. The intensity of biofilm production was subjectively assessed on a score of 0 to 3*viz.*0, 1, 2 and 3 represented none, weak, moderate and strong biofilm production, respectively.

2.2.2. Micro-plate assay

Quantitative evaluation of biofilm production by *Staphylococcal* isolates (n=64) was performed with micro-plate assay (MP) using flat bottom 96 well ELISA plates (Greiner®, Germany) following the modified method as described by Mathur et al. (2006). Individual wells were filled with 200 µL of diluted cultures of *S. aureus* (5 µL in 100 µL) in fresh TSB_{glu}. For negative control, 200 µL of broth, sodium acetate and water were poured into three wells to check the non-specific binding of the media, fixative and stain, respectively. The reference strains were used in positive control wells. The plates were incubated at 37^0 C for 18-20 hrs.

After incubation, the contents of the plates were gently removed by tapping the plates over the filter paper pad. The wells were washed twice with 200 µL of PBS (pH 7.2) to remove planktonic bacteria. The attached biofilms were fixed using sodium acetate (2%) and stained with crystal violet (0.1% w/v) for 10 minutes. Excess stain was removed by washing with deionized water. The plates were read by micro ELISA auto reader (Bio-Tek Instruments, Highland Park, USA) at wavelength of 570 nm ($OD_{570\ nm}$) to determine the optical density (OD). The OD values were used as an index for bacterial adherence to the wells surface (biofilm formation). Experiment with each isolate was performed in duplicate and the mean OD values were calculated. The mean OD values for negative control, fixative and stain (crystal violet 0.1%) were averaged and deducted from all the test values. The classification criteria for biofilm production given in table 1 were set after initial experimental trials during standardization of the protocol.

2.3 Immunization of rabbits

Twenty adult healthy rabbits were divided into two groups: R_1(Placebo control) and R_2 (bacterin-toxoid group). The rabbits of R_1received2 doses (0.2 mL per dose) of placebo (composition same as vaccine except bacterin-toxoid) in the thigh region through intra-muscular route 15 days apart. The rabbits of R_2 group were administered 2 shots (0.2 mL per dose; 1x 10^9 cells mL^{-1}) of aluminum hydroxide adjuvanted bacterin-toxoid prepared from strong biofilm producing *S. aureus* at days0 and 15 [2]. The serum samples were collected from rabbits of each group fortnightly for a period of two months post-vaccination (0, 15, 30, 45 and 60 days). The trial was conducted under the guidelines of Animal Ethics Committee, University of Agriculture, Faisalabad, Pakistan.

2.4 Biofilm inhibition assay

The *S. aureus*isolate (MRL-V1) used to prepare bacterin-toxoid was cultured overnight in SB_{glu} at 37^{o}C in a screw-capped tube. Freshly prepared TSB_{glu} (500 uL) was poured into each well of the 24 well tissue culture plates (Greiner®, Germany) followed by the addition of 500 µL of serum samples in first well of each row. Afterwards, the serum samples were serially diluted using 2-fold serial dilutions from first well to the second well and so on up to the well six. The overnight-cultured TSB_{glu} broth (50 µL) was added into each well. In row A of each plate, three wells were poured with media, water and sodium acetate while the other three wells received the same isolate of *S. aureus* to make sure the bacteria were producing biofilm. The pre-immune serum samples (control group) were poured in the row B of 24 well plates. Similarly, the serum samples collected from vaccinated rabbits at days 1, 15, 30, 45 and 60, were dispensed in each row using two-fold serial dilution.

After 24 hr incubation at 37^0 C, the plates were processed as previously described in micro-plate assay for biofilm determination [12]. The wells of the plates were examined visually and microscopically (10 X) for the presence or absence of biofilm production. The wells were interpreted on the basis of adhered stained layer at the bottom (biofilm production), while absence of stain adherence indicated the inhibition of biofilm production. The plates were also read by micro ELISA auto reader (Bio-Tek Instruments, Highland Park, USA) at wavelength of 570 nm ($OD_{570\ nm}$) to determine the optical density values.

2.5 Statistical analysis

The results of MP and tube methods were analyzed by calculating percentages of the mean values whereas data from the biofilm inhibition assays were analyzed by using Graph Pad Prism software Inc. (USA, version 6.0) and MS Excel program.

3. RESULTS

3.1 *Staphylococcus aureus*isolates

The *S. aureus* isolates were identified as Gram-positive cocci, β hemolytic on blood agar, catalase positive and tube coagulase positive at 4 hr. The isolates were bio-typed using API® Staph (bio Merieux, France) and Staphytect plus kit® (Oxoid, UK). For the candidate isolate only, a seven-digit numeric profile (6716153) was generated based on the biochemical reactions on API® Staph kit. The isolate also showed positive result for Protein A and clumping factor when tested using Staphytect plus kit® in latex agglutination test. These biochemical tests confirmed the isolate as *S. aureus.*

3.2 Biofilm Production

3.2.1. Tube method

The findings of the tube method showed that 28.12% (18/64) of the isolates were non-biofilm producers, while 46 (71.87%) isolates were positive for biofilm production. The scoring for intensity of biofilm production represented 23 (35.94%), 17 (26.56%), and 6 (9.37%) isolates as weak, moderate, and strong biofilm producers, respectively (Figure 1).

3.2.2. Micro-plate assay

In MP assay, 56.25% (36 of 64) isolates were positive, whereas 43.75% (28 of 60) isolates were negative for biofilm production. It was observed that MP assay determined 4 (6.25%), 14 (21.87%)and 18 (28.12%) isolates as strong, moderate and weak biofilm producers, respectively (Figure 1).

3.3 Biofilm inhibition

The negative control wells showed clear transparent bottom indicating no biofilm production while a visible dense violet color adhered to the bottom of positive control wells clearly suggested biofilm adhered to the surface. The pre-immune (control group) and day 1 serum samples showed similar type of stained bottom as seen in positive control wells (dense visible violet color; denoted by +++ as scored 3 for TM). Serum samples collected at day 15 showed a light color that reflected some inhibition of biofilm production (++ or TM score2) in first well while rest of the wells displayed a dense violet color (+++ or TM score 3).

The serum samples collected at days 30, 45 and 60 resulted in complete inhibition of biofilm production (X or TM score 0) at initial serum dilutions (1:2 and 1:8) as showed by clear transparent bottoms. The wells with 1:16 and 1:32 serum dilutions had very light color that indicated mild biofilm production (denoted by + or as TM score1) and the last wells of all three rows (1:64 dilution) showed moderate adhesion of stain, which indicated intermediate biofilm production (TM score2) by *S. aureus* (Table 2; Plate I)

The biofilm inhibitory effects of serum samples were quantified by measuring the OD values using micro ELISA auto reader at wavelength of 570 nm ($OD_{570\ nm}$). The pre-immune (control group) serum samples and samples collected at days 1 and 15 showed increased OD values suggest the production of biofilms in the wells. The serum samples collected at days 30, 45 and 60 resulted in decreased OD values, which showed inhibition of biofilm production when evaluated at a scale described earlier for determination of biofilm production by micro-plate assay (Figure 2).

4. DISCUSSION

The present study was conducted to evaluate the ability of biofilm production bylocal isolates of *S. aureus*and *in-vitro* inhibition of biofilm production using serum antibodies produced against the strong biofilm producing *S. aureus*. The *S. aureus* biofilm production was determined

bytube method (TM) and micro-plate assay (MPA) that have been reported in previous studies [12-14].

Tube method is a qualitative method for the detection of biofilm production as it was difficult to differentiate between moderate and weak biofilm producers [15]. The results were correlated with previous studies [12,13]. In terms of biofilm presence or absence, 18 (28.12%) isolates were non-biofilm producers while 46 (71.87%) isolates were positive for biofilm production. The results demonstrated that tube method is easy to perform while interpretation of the results is quite complicated particularly with the novice observer, so it cannot be recommended as general screening test for determination of biofilm production [16,15]. In addition, micro-plate assay showed that 56.25% of the isolates were positive for biofilm production. Previously, it has been showed that MP assay is more specific than that of tube method with more variations among presence and/or absence of biofilm production [17]. Furthermore, it was reported that the rate of slime production by Staphylococcal spp. was 55.5% when determined by MP assay [14]. We observed that MP assay clearly differentiated between weak and non-biofilm producers as was obvious from the comparison of two methods which has also been reported earlier [12,18]. These findings suggest thatMP assay is more sensitive, accurate and reproducible screening method to detect biofilm production by Staphylococci. The tube method correlates well with the MPassay for the detection of strong biofilm producing isolates but it was hard to single out between weak and intermediate biofilm producing isolates due to the variability in recording the observations. Therefore, high variability was observed and categorization of biofilm production for weak and intermediate biofilm producers was difficult by the tube method. In accord with the previous reports, tube method cannot be recommended as general screening test to identify biofilm-producing isolates.

Biofilm production has been documented in the strains of *Staphylococcus* spp. which because infections associated with biomedical devices in humans and chronic udder infections in animals [16,19]. The biofilm results in increased resistance to a wide range of antibiotics, disinfectants and also opposes the clearance of bacteria by the host immune system [9]. The increased use of antibiotics against mastitis in dairy animals results in drug residues in milk that causes different health related problems and is a matter of concern for human being as consumers. Therefore, the need of local vaccination and/or alternate ways to control this infection further increases.

Our study also showed that biofilm production ability of the bacteria could be interfered by the antibodies produced against the biofilm-producing bacteria. The study findings demonstrated that serum samples from vaccinated rabbits provide the evidence of *in-vitro* inhibition of biofilm production by *S. aureus.* The serum samples collected at days 30, 45 and 60 showed complete inhibition of biofilm production at lower serum dilutions (1:2 to 1:8). The microscopic visual results of biofilm inhibition assay were also supported by the decreased OD values obtained by spectrophotometer. Compounds that hinder the growth of bacteria have routinely been used to manipulate the biofilm production ability of microbes. However, the use of such compounds like aminoglycosides may select for the resistant strains and the use of these agents at sub-inhibitory levels may cause stimulation of biofilm production by microorganisms [20-22]. Therefore, inhibitors should interfere with biofilm production without stimulating this trait. Certain herbal extracts including garlic and ginger have been reported to inhibit the biofilm formation by *Pseudomonas aeruginosa* [23,24]. Quorum sensing is a phenomenon that controls coordinated bacterial behaviors in response to the bacterial population and is closely related to the biofilm formation by bacterial cells. Quorum sensing inhibition is the most widely used approach to study the interference with biofilm production. Various chemicals have been known that inhibit quorum sensing in Gram-negative bacteria by competing with quorum sensing signal molecules or suppressing the genes that code the respective proteins [25,26]. The mechanism through which biofilm production was inhibited in this study is speculative, but one possible explanation for this lies in the ability of the serum antibodies to directly neutralize the biofilm proteins which then reduces the bacterial attachment. Although, effects of antibodies on bacteria were not negated in this study, that might also be a factor in suppressing the bacterial population. Similarly, the previously reported biofilm inhibitors (ginger and garlic) also affect the growth of bacteria.

5. CONCLUSION

The study clearly suggested that the antibodies produced against bacterin-toxoid prepared from a strong biofilm producing isolate of *S. aureus* were able to inhibit the *in-vitro* biofilm production. This might be useful to avert the attachment of bacteria with living tissue that could further implicate the better control of chronic infections due to biofilm producing *S. aureus*. However, this needs further studies to observe the immune response against biofilm contents excluding the whole bacterial cells.

REFERENCES

[1] Fagiolo, A. 2007. Mastitis in buffalo. Italian Journal of Animal Sciences6, 200-206.

[2] Raza, A., Muhammad, G., Rahman, S.U., Rashid, I., Hanif, K., Atta, A., and Sharif, S. 2015. Evaluation of humoral immunity and protective efficacy of biofilm producing Staphylococcus aureus bacterin-toxoid prepared from a bovine mastitis isolate in rabbit. Iranian Journal of Veterinary Research, 16, 69-74.

[3] Bjarnsholt, T. 2013. The role of bacterial biofilms in chronic infections. APMIS, 121, 1-58.

[4] Prakash, B., Veeregowda, B.M., and Krishnappa, G. 2003. Biofilms: A survival strategy of bacteria. Current Science, 85, 1299-1307.

[5] Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J. 1987. Bacterial biofilms in nature and disease. Annual Review of Microbiology, 41, 435-464.

[6] Costerton, J.W., Stewart, P.S., Greenberg, E.P. 1999. Bacterial biofilms: a common cause of persistent infections. Science, 284, 1318-1322.

[7] Dhanawade, N.B., Kalorey, D.R., Srinivasan, R., Barbuddhe, S.B., Kurkure, N.V. 2010. Detection of intercellular adhesion genes and biofilm production in Staphylococcus aureus isolated from bovine subclinical mastitis. Veterinary Research Communications, 34, 81-89.

[8] Raza, A., Muhammad, S.S., and Atta, A. 2013. Biofilm Producing Staphylococcus aureus and Bovine Mastitis: A Review. Journal of Molecular Microbiology Research, 3, 1-8.

[9] Melchior, M.B., Fink-Gremmels, J., Gaastra, W. 2007. Extended antimicrobial susceptibility assay for Staphylococcus aureus isolates from bovine mastitis growing in biofilms. Veterinary Microbiology, 125, 141-149.

[10] Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., Penades, J.R. 2001. Bap, a Staphylococcus aureus surface protein involved in biofilm formation. Journal of Bacteriology, 183, 2888-2896.

[11] Perez, M.M., Prenafeta, A., Valle, J., Penades, J., Rota, C., Solano, C., Marco, J., Grillo, M.J., Lasa, I., Irache, J.M., Maira-Litran, T., Jimenez-Barbero, J., Costa, L., Pier, G.B., de Andres, D., Amorena, B. 2009. Protection from Staphylococcus aureus mastitis associated with poly-N-acetyl beta-1,6 glucosamine specific antibody production using biofilm-embedded bacteria. Vaccine, 27, 2379-2386.

[12] Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., Rattan, A. 2006. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. Indian Journal of Medical Microbiology, 24, 25-29.

[13] Christensen, G.D., Simpson, W.A., Bisno, A.L., Beachey, E.H. 1982. Adherence of slime-producing strains of Staphylococcus epidermidis to smooth surfaces. Infection and Immunity, 37, 318-326.

[14] Turkyilmaz, S., and Eskiizmirliler, S. 2006. Detection of slime factor production and antibiotic resistance in staphylococcus strains isolated from various animal clinical samples. Turkish Journal of Veterinary and Animal Sciences, 30, 201-206.

[15] Deighton, M.A., Franklin, J.C., Spicer, W.J., Balkau, B. 1988. Species identification, antibiotic sensitivity and slime production of coagulase-negative staphylococci isolated from clinical specimens. Epidemiology and Infection, 101, 99-113.

[16] Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M., Beachey, E.H. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. Journal of Clinical Microbiology, 22, 996-1006.

[17] Baselga, R., Albizu, I., De La Cruz, M., Del Cacho, E., Barberan, M., Amorena, B. 1993. Phase variation of slime production in Staphylococcus

aureus: implications in colonization and virulence. Infection and Immunity, 61, 4857-4862.

[18] Vanhaecke, E., and Pijck, J. 1988. Bioluminescence Assay for Measuring the Number of Bacteria Adhering to the Hydrocarbon Phase in the BATH Test. Applied and Environmental Microbiology, 54, 1436-1439.

[19] O'Gara, J.P., and Humphreys, H. 2001. Staphylococcus epidermidis biofilms: importance and implications. Journal of Medical Microbiology, 50, 582-587.

[20] Anderson, G.G., O'Toole, G.A. 2008. Innate and induced resistance mechanisms of bacterial biofilms. Current Topics in Microbiology and Immunology, 322, 85-105.

[21] Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., Miller, S.I. 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature, 436, 1171-1175.

[22] Lewis, K. 2008. Multidrug tolerance of biofilms and persister cells. Current Topics in Microbiology and Immunology, 322, 107-131.

[23] Kim, H.S., Park, H.D. 2013. Ginger extract inhibits biofilm formation by Pseudomonas aeruginosa PA14. PLoS One, 8, e76106.

[24] Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristoffersen, P., Kote, M., Nielsen, J., Eberl, L., and Givskov, M. 2005. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. Journal of Bacteriology, 187, 1799-1814.

[25] Hentzer, M., Riedel, K., Rasmussen, T.B., Heydorn, A., Andersen, J.B., Parsek, M.R., Rice, S.A., Eberl, L., Molin, S., Hoiby, N., Kjelleberg, S., Givskov, M. 2002. Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound. Microbiology, 148, 87-102.

[26] Muh, U., Schuster, M., Heim, R., Singh, A., Olson, E.R., and Greenberg, E.P. 2006. Novel Pseudomonas aeruginosa quorum-sensing inhibitors identified in an ultra-high-throughput screen. Antimicrobial Agents and Chemotherapy, 50, 3674-3679.

Table 1: Classification of bacterial adherence in micro-plate assay

*Mean OD values	Score	Adherence	Biofilm production
<0.10	0	Non-adherent	Non biofilm production
0.10- 0.15	1	Mild	Weak
0.16- 0.25	2	Moderate	Moderate/ Intermediate
> 0.25	3	Strong	Strong

* Mean OD values were determined at 570nm

Table 2: Interpretation of biofilm inhibition assays obtained by using microscope (10X)

	Biofilm inhibition at different serum dilutions					
Days of serum collection	1:2	1:4	1:8	1:16	1:32	1:64
Pre-immune serum	+++	+++	+++	+++	+++	+++
Day 1	+++	+++	+++	+++	+++	+++
Day 15	++	+++	+++	+++	+++	+++
Day 30	x	x	x	+	+	++
Day 45	x	x	x	+	+	++
Day 60	x	x	x	+	+	++

Where

x = 0 no biofilm formation.

+ = 1 mild biofilm formation as portrayed by light color stained bottoms.

++ = 2 moderate formation of biofilm revealed by moderately violet colored bottoms

+++ = 3 strong biofilm formations as reflected by dense violet colored bottoms

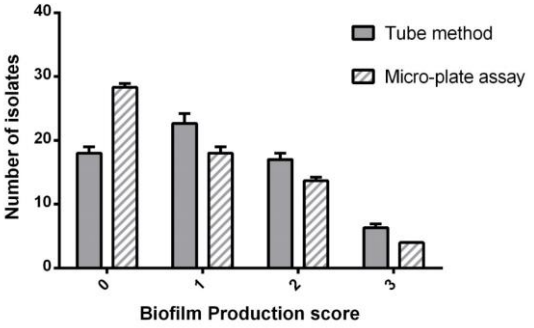

Figure 1: Screening of 64 *Staphylococcus aureus* isolates for biofilm production by tube method (filled) and micro-plate assay (shaded). The tubes were stained with 1% safraninand micro-plate wells with 0.1% crystal violet. Tubes were scored manually while micro-plates were read through micro ELISA auto reader (Bio-Tek Instruments, Highland Park, USA) at OD 570 nm. Each data point represents mean ± S.D, n=3 assays.Scoring criteria:**0** = No biofilm production, **1** = Weak biofilm producers, **2** = Moderate biofilm producers and **3** = Strong biofilm producers

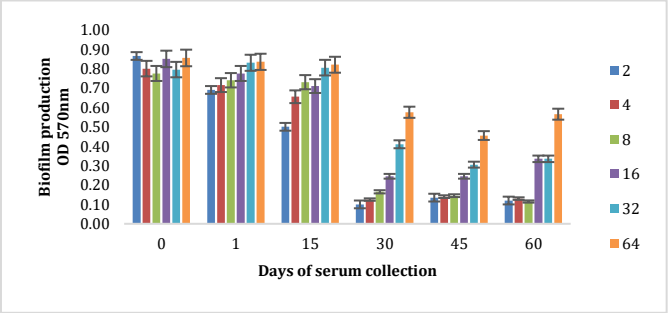

Figure 2: Effects of serum antibodies on inhibition of biofilm production at various serum dilutions (OD values) and different time intervals post immunization. The serum dilutions were added to the bacterial culture for overnight incubation. Day 0 represents the control group (Pre-immune serum samples). Each data point represents mean ± S.D, n=2 assays (pooled data from two separate experiments). Key describes the two folds serial dilutions of serum samples used in biofilm inhibition assays.

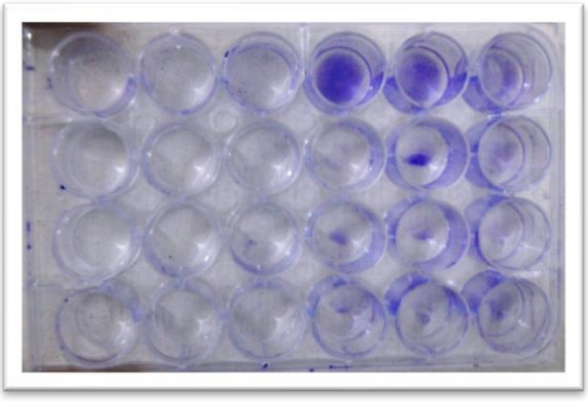

Plate I: Biofilm inhibition assay:adhered violet color at bottom of wells indicated biofilm formation while clear wells showed no biofilm production, light and moderate violet color indicated mild and moderate biofilm formation. First three wells of row A served negative controls and last three wells were positive control inoculated with *Staphylococcus epidermidis* ATCC 35984 (high slime producer). Serum samples from days 30, 45 and 60 were loaded in rows B, C and D, at different serum dilutions (1:2, 1:4, 1:8, 1:16, 1:32 and 1:64), respectively.