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Preparation of Vaccine from Biofilm Proteins of *Staphylococcus aureus* and Incorporate it in Liposomes for Induction of CXCL-5 Production in Albino Rats

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Abstract

In this study biofilm matrix proteins (BMP) were isolated from bacterial biofilm and incorporated in a delivery system (liposome) to stimulate an immune response against virulent pathogens. The specimens were cultured and identified depending on morphological and biochemical tests . Around 67.74% of isolates were found to be biofilm formers. The immunomodulatory activity revealed that BMP-liposome vaccine and BMP vaccine significantly increased chemokine CXCL5 levels in sera of rats in first and second doses. The concentration of total WBCs count, neutrophils, and lymphocytes also increased. In rats groups that had received BMP-liposome vaccine, the challenge test revealed high immunogenicity and effective protection. It can, therefore, be concluded that BMP-liposome are a promising nanomedicine for regulating biofilm hyper-reaction. They may, however, cause tissue inflammation. On the other hand, it appeared to be helpful in activating the immune response at moderate levels, allowing infection to be eradicated without tissue damage.

Keywords: Biofilm matrix protein, Liposome, *S. aureus*, Immunomodulator activity, CXCL5.

تحضير لقاح من بروتينات الأغشية الحيوية للمكورات العنقودية الذهبية ودمجها في الجسيمات الشحمية لتحفيز إنتاج *CXCL-5* في الجرذان البيضاء

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الخلاصة

في هذه الدراسة تم عزل بروتينات الأغشية الحيوية للبكتريا ودمجها في نظام توصيل (الجسيم الشحمي) لتحفيز الاستجابة المناعية ضد مسببات الأمراض الفتاكة. تم زرع العينات ، و تم تحديد البكتريا اعتمادًا على الاختبارات الشكلية والكيموحيوية. كانت 67.74٪ من العزلات مكونة للغشاء الحيوي. أظهر النشاط المناعي أنللقاح م بروتينات البايوفيلم–ليبوسوم و لقاح بروتينات الاغشية الحيوية زاد بشكل ملحوظ من مستويات البروتين CXCL5 في مصل الفئران في الجرعتين الأولى والثانية. كما أدى إلى زيادة تركيز إجمالي عدد كرات الدم البيضاء ، العدلة ، والخلايا الليمفاوية. في مجاميع الجرذان التي لقحت بلقاح بروتينات الغشاء الحيوي–الجسميات الدهنية، كشف اختبار التحدي عن تمنيع عالي وحماية فعالة ، لذا فإن لقاح بروتينات البايوفيلم–ليبوسوم هو دواء نانو واعد لتنظيم التفاعلات المرتفعة ضد الغشاء الحيوي ؛ اذ قد يتسبب هذا في التهاب الأنسجة ، ولكن يبدو أنه مفيد في تنشيط الاستجابة المناعية بمستويات معتدلة ، مما يسمح بالقضاء على العدوى دون تلف الأنسجة.

1. Introduction

One of the more challenging nosocomial organisms that hospitals deal with is multidrug-resistant *Staphylococcus aureus* which is related to a considerable prevalence of community-acquired infections (CAIs) and health care-associated infections (HAIs) in patients who are hospitalized and immunocompromised [1]. It has a distinctive capacity to evade innate immune responses and survive in blood and other tissues during persistent infections [2].

The major virulence factor for *S. aureus* is their ability to produce biofilm which protects it from host immune system along with antibacterial agents [3]. A prominent feature of biofilms is the production of an extracellular polymeric substance (EPS), a complex polymer matrix that consists of proteins, carbohydrates, nucleic acids and others on the outside of the cell membrane protecting it from host immune response and antibiotic treatment, making it difficult to eradicate once staphylococcal infections occur [4].

Antigenic materials extracted from pathogenic bacteria to stimulate immunity against diseases caused by pathogens are one of the most promising approaches against infections.

The ability of EPS to induce an immune response by activating T-cell and production of cytokines such as TNFα, IL-6, IL-8, IL-12, and INF-γ, also induces macrophage to phagocytosis and increases neutrophil activation [5]. Liposomes are a small, spherical nano carrier (bubble) made of cholesterol and natural non-toxic phospholipids that can transport a wide range of biologically active substances to cells and tissues, both *in vitro* and *in vivo*. They have also been used as an immunological adjuvant to modify immune responses, particularly cell mediated immunity to various antigens [6]. The study aimed to detect the immunomodulation capacity of BMP-liposome and BMP isolated from *S. aureus* to prevent infection.

2. Materials and Methods

2.1. Isolation and Identification of Bacteria

A total of 100 clinical samples, including burn swabs, tonsils swab, and urine-UTI, were collected during April - June 2020 from Al-Hussein General Teaching Hospital in Karbala province. The specimens were inoculated on mannitol salt agar and incubated for 24 hours at 37°C. Mannitol-fermenters colonies were picked up and re-inoculated on new mannitol salt

agar and blood agar. All isolates were identified based on their microscopical and morphological characteristics, including bacterial cells shapes and arrangement, colony size, shape, color, pigment type, edge, elevation translucency texture, and specific biochemical tests (catalase test, DNase test, coagulase test, blood hemolysis test, mannitol fermentation test, urease test, citrate test, Voges-Proskauer test, methyl red test, and oxidase test) [7].

2.2. Biofilm Production and Detecting Efficient Isolate

The efficient isolate was selected based on the capacity to form biofilm by 69 wells plate method. A colony of *S. aureus* was isolated from a fresh agar plate and inoculated in 2 ml of trypticase soy broth. The broth was incubated overnight at 37°C. The culture was then diluted to 1:100 with fresh medium. A sterile plate with 96 flat-bottom polystyrene wells was filled with 200 µl of the diluted culture and then incubated at 37°C for 24 hours. After incubation, the contents of each well were removed by gentle tapping. The wells were washed with 200 µl of phosphate buffer saline (pH 7.3) to remove free-floating bacteria. Biofilms formed by bacteria adherent to the wells were fixed by 99% methanol and stained with 0.1% crystal violet (CV) for 10 min. Excess stain was washed gently by using deionized water and the plate was left to dry. The crystal violet bound to the biofilm was extracted later with 200 µl of 95% ethanol and agitated. The optical density of the stained adherent biofilm was measured using a micro-ELISA auto-reader at a wavelength of 570 nm. The experiment was performed in triplicate and, hence, repeated three times. Optical density (OD) value of each isolate was detected as 0.120-0.240 moderate, > 0.240 strong and < 0.120 weak biofilm formation [8].

2.3. Extraction of Extracellular Polymeric Substances (EPS)

The efficient isolate was inoculated in TSB-glucose 2% medium and incubated for 48 h. at 37ºC. Bacterial culture was centrifuged at (3700 xg,4ºC, 15 min.) after the development of mature biofilm. Pellet was used for extraction of EPS by ethanol method [9]. Biofilm solution was then purified by dialysis for 2 days against phosphate buffer saline solution (pH 7.3) by using dialysis membranes MW. 3500 dalton, (Spectra/Por company-USA) wide flat 34 mm [10].

2.4. Partial Purification and Precipitation of Biofilm Matrix Protein (BMP)

Protein purification was done by using batch wise ion exchange chromatography method [11]. Protein precipitation was conducted by protein extraction and precipitation kit, according to instructions of manufacturer (Biomedical/China). The protein concentration was estimated by Bradford method [12]. Partially purified BME was then mixed with liposome to prepare BMP-liposome immunogen.

The heat killed *S. aureus* immunogen was prepared by culturing the isolate in brain heart broth at 37°C for 24 h, and centrifuged 3000 rpm for 15 min and later washed with phosphate–buffered saline, was adjusted to McFarland standard tube No. 0.5 to concentration 1×10^8 which eventually killed *S. aureus* after being heated for one hour in a 80°C water bath [13].

Each immunogenic material's sterility was confirmed by inoculating it on nutrient and blood agar. Three rats were given 1 ml of each vaccine intraperitoneally for one week and monitored for safety. Normal histological appearance in rats indicated the safety of immunogens.

2.5. Laboratory Animals

A total of 194 albino rats, secured from animals house in College of Pharmacy, Karbala University, weighed between 150-200 g and were about 4 weeks old. They were held in an

air-conditioned room 30±5°C, dark/light periods 10-14 h/day. They were left for 7 days to get acclimatized to laboratory conditions before commencing the experiment.

2.6. Determination of LD50 of Immunogens

Five groups of male rats with five replicates for each group, were injected intraperitoneally with 1ml of bacterial suspension in one of 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}; and 10^6, 10^7, 10^8, 10^9,10^{10},10^{11} CFU/ml for live, and heat killed bacterial suspension respectively. Control group was injected with PBS [14]. For LD50 of BMP, rats groups were intraperitoneally injected with 400, 500, 600, 700, 800 and 900 μg/ml [15].

2.7. Immunization of Rats

CXCl5 level, total WBCs and differential count were detected for each BMP immunogen, BMP-liposome, heat killed *S. aureus* suspension, live bacterial suspension, and liposome in albino rats by the following design:

Procured rats were divided into five groups (6 rats per group), immunized (I.P.) with 0.1 LD_{50}% of each group. The control group was fed normal saline as (1^{st} & post dose) with 14-day intervals. At the end of each period 3 rats were sacrificed and 4 ml of blood was collected by heart puncture. Two ml was put in EDTA tube for total WBCs and differential leukocyte count using automated hematological analyzer (Ruby -U.S.A.). The remaining 2 ml was placed in gel tube for separation sera for CXCL5 chemokine level by ELISA (Elabscience, USA).

2.8. Challenge Test.

After two weeks of post dose of immunization for BMP, BMP-liposome and HK bacteria 6 rats from each group were challenged by intraperitoneally injection with 0.1 ml of 100 LD_{50} of a virulent *S. aureus* suspension. The number of rats who survived 7 days after being injected were then used to evaluate the relative level of protection provided by immunogen. The rats were then sacrificed, and blood was immediately collected for hematological and serological analysis.

2.9. Ethical Considerations

The present study was approved by the Institution Ethics Committee for Human Studies (Ref: 11/05/2020/643). In addition, a written informed consent was obtained from all the subjects before the study.

2.10. Statistical Analysis.

Data was analyzed with one- and two-way ANOVA using the Statistical Package for the Social Sciences (SPSS), Version26. The results were expressed as Mean ± S.D. *P*-values below 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Isolation and Identification of Bacteria

Total of 31 (31%) of *S. aureus* were recovered which were distributed as following: 16 (44.44%) isolates from tonsils (Tonsillitis), 12 (30%) from urine (UTI) and 3 (12.5%) from burn infections.

The study revealed that *S. aureus* was most common infectious agent recovered from tonsils and urine specimens. *S. aureus* isolates from the surface of tonsils were 44.44% and this percentage is relatively similar to the results obtained in a local study by Najim *et al.*

[16] in Basra. They showed that *S. pyogenes* and *S. aureus* isolated from chronic tonsillitis patients, were the most prevalent gram-positive bacteria.

Staphylococcus aureus isolates recovered from UTI patients (30%) were almost similar to the results obtained by Mollick *et al.* [17] who revealed that *S. aureus* is the most common organism (28%) obtained from urine sample of patients with UTI. The high incidence of *S. aureus* in UTI infections is due to its ability to overcome body defense mechanisms and antibiotic resistance and poses several virulence genes such as encodes an active urease enzyme. *S. aureus* isolates from burns (12.5%) were relatively similar to the results (8.89%) obtained by Aljanaby and
Aljanaby [18] in Najaf, Iraq.

3.2. Detection of the Efficient Bacterial Isolates for Biofilm Production

Among 31 isolates, 9 (29.03%) were strong biofilm formers and 12 (38.7%) moderate biofilm producers (Table 1). The results also indicated that S_{20} was the best biofilm producing isolate among others that gave highest absorbance value of 0.409 and showed multidrug resistance (data not shown). It was therefore chosen to complete the experiment.

Table 1: The distribution of *S. aureus* according to biofilm formation

Standard Range of Optical Density	Biofilm	*S. aureus*	
		No	%
< 0.12	Non/Weak	10	32.25
0.12-0.240	Moderate	12	38.7
> 0.240	High	9	29.03
Total		31	100

3.3. Extraction of biofilm matrix protein (BMP)

The results of precipitation protein after extraction of (biofilm) EPS by ethanol method revealed that protein was yellowish smooth aggregate in appearance and was quickly soluble. The protein concentration detected by Bradford method after purification method (Batchwise ion exchange chromatography), was 912 µg/ml.

The results of biofilm extraction demonstrated that the present method yielded higher values of protein content because the ethanol method had the highest (biofilm) EPS yield as shown by Gong *et al.*[9] who demonstrated that ethanol method is the best and efficient method that gives higher protein yield. The chemical analysis of BMP showed that the concentration of protein was 912 µg/ml.

3.4. Estimation of Lethal Dose LD_{50} of Heat Killed S. aureus.

According to the results, the LD_{50} was $3.1x10^8$ CFU/ml. An estimation was made by counting the number of alive and dead rats for each group after five days (Table 2).

Proportionate distance = 50- mortality below 50%/ mortality above 50% –mortality below 50%

= (50-33.3) / (66.6-33.3) =0.501

$LD50 = 10^8+0.501$

$= 3.1 x10^8$ CFU/ml

Table 2: LD50 of heat killed *S. aureus*.

Concentration of Bacteria	No. of Rats	No. of Survived Rats	No. of Died Rats	Accumulated Number of Survived and Dead Rats				
				S	D	D+S	D/D+S	Mortality %
$1*10^{11}$	5	0	5	0	15	15	1	100
$1*10^{10}$	5	1	4	1	10	11	0.909	90.9
$1*10^{9}$	5	2	3	3	6	9	0.666	66.6
$1*10^{8}$	5	3	2	6	3	9	0.333	33.3
$1*10^{7}$	5	4	1	10	1	11	0.090	9
$1* 10^{6}$	5	5	0	15	0	15	0	0

S: Accumulated number of surviving rats, D: Accumulated number of dead rats.

For live- bacterial suspension the results revealed that LD_{50} was 6.8 x 10^6 CFU/ml.

3.5. Estimation of Lethal Dose LD_{50} for Biofilm Matrix Protein (BMP) Immunogen

The results demonstrated that the LD_{50} was 590 µg /ml (Table 3).

LD50 = Biggest lethal dose – (Σ (a*b / n))

LD50 = 900- (1550/ 5) = 590 µg /ml

Table 3: LD50 of *S. aureus* BMP immunogen

Group	Dose (µg/ml)	Dose Difference a	No. of Dead Rats	Mean b	Product a * b
1	900		5		
2	800	100	5	5	500
3	700	100	4	4.5	450
4	600	100	2	3	300
5	500	100	2	2	200
6	400	100	0	1	100
7	Control	0	0	0	
		Total			T=1550

The same outcomes were demonstrated for immunogen BMP and BMP-liposome.

The results of LD_{50} for BMP extracted from *S. aureus* was 590 µg/ml, while for live and HK bacteria it was 6.8 x 10^6 ,3.1x10^8 CFU/ml respectively. This results were similar to the results obtained by Tao *et al.* [19] who isolated *S. aureus* from sputum of patients suffering from chronic tonsillitis and detected the LD 50 for live bacteria which recorded 6 × 10^6 CFU of live *S. aureus* strain and 1 × 10^8 for heat killed and formalin killed *S. aureus.* Senna *et al.* [20] found that the LD_{50} for methicillin resistance *S. aureus* which was injected intraperitoneally (IP) was 1.2x10^6 CFU/ml and the results suggested by Saganuwan [21] found that the lethal dose of live *S. aureus* was 1.75 x 10^{10} CFU/ml in rats injected intraperitoneally. Also Van Den Berg [22] found that the 50% lethal dose for *S. aureus* was 1.2 x 10^5 CFU and that 5.8 x 10^5 CFU was 100% a lethal dose.

The causes for the differences in LD50 in different research may be traced back to the animal species and strain virulence, pathogenesis strain used in the experiment, and isolated location, as well as the immune system's ability to target the animal utilized in the experiment.

3.6. Concentration of $CXCL_5$ in Rats Serum

The production of an inflammatory mediator chemokine CXCL5 significantly increased ($P < 0.05$) in rats injected with BMP- liposome, and BMP in the first and second doses (32.63±1.53, 51.0±0.98; 29.24±2.52, 38.93±1.04) pg/ml respectively, followed by live, and heat killed bacterial suspension as compared to control (20.2±1.44 pg/mL). No significant differences were observed in rats injected with liposome only (Figure 1)

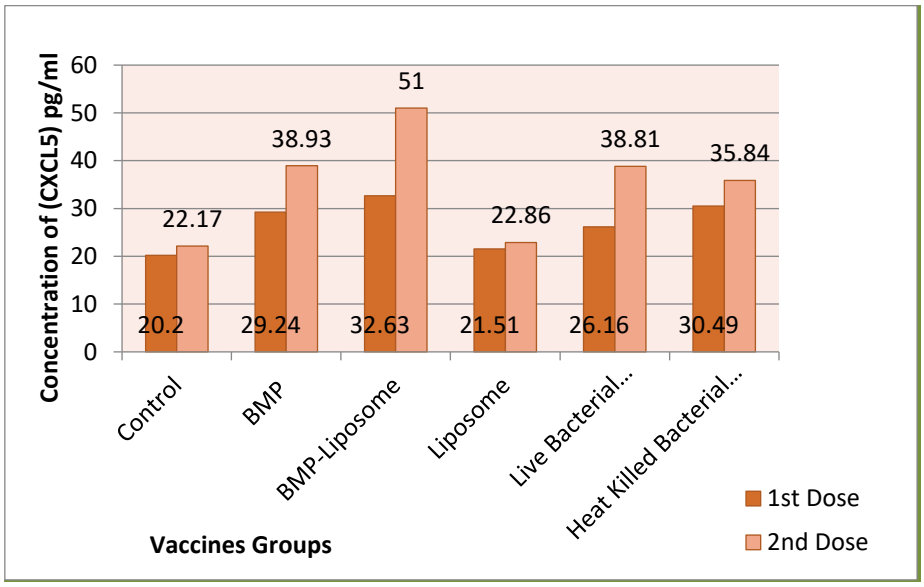

Figure 1: The contribution of several vaccines to the enhancement of CXCL5 production in treated rats.

The results of CXCL5 level in the present study were similar to the results obtained by Adhikary *et al.*[23] that the injection mice with killed *S. aureus* bacterial suspension 3×10^{10} CFU/ml elevated in the concentration of CXCL5 in blood as compared with control group. CXCL5 chemokine plays specific role in regulating the neutrophil response during *S. aureus* infection [24]. While S. Jeyaseelan *et al.* [25] found elevated serum level of $CXCL_5$ in murine and rats injected with live bacterial suspension from *E. coli*, *K. pneumonia* and *S .aureus*. Gil *et al.* [13] explained that immunization in mice with biofilm exoprotiens induces phagocytosis and attracts chemokine and provides protection against *S. aureus* biofilm infection, since multicomponent vaccine ties together cell-mediated immunity and a humoral response where opsonic antibodies play a supportive role to eradicate the biofilm infection. A previous study found that superantigen dependent mechanism of T cell activation by *S. aureus* to activate CD4 T cells, production of IFN at the sites of infection and inducing secretion of CXCL-5 to attract neutrophils [26]. In same line, A. O. Tzianabos *et al.* [27] found that *S. aureus* has a variety of cell-associated polymers that contain a zwitterionic charge motif, providing the capacity to induce chemokine production like CXC-2 , CXC-5 and activate T cells.

3.7. Effect of Vaccines on Total and Differential WBCs Account in Rats

The result clarifies a significant increase ($P < 0.05$) in total WBCs and differential WBCs count in rats treated with different vaccines groups in the first and second doses. BMP-liposome, BMP immunogen and live bacterial suspension gave maximum total WBCs count in first and second doses (10.9±0.173, 14±1.252; 9.87±0.141, 13.46±0.057; 12.43±0.321, 13.76±0.513) $\times10^3$cells/µL respectively), followed by HK bacterial suspension (12.13±0.05, 12.5±0.0$\times10^3$cells/µl respectively). Lymphocytes significantly elevated ($P < 0.05$) in first and second dose in rats injected with BMP, BMP-liposome, and live bacterial suspension. Also, BMP-liposome significantly increased ($P < 0.05$) the count of neutrophil (24.73±0.32) in the

second dose as compared with the first dose (25.7±0.43). On another hand, the count of monocytes in present study significantly elevated ($P < 0.05$) in rats injected with BMP-liposome, BMP, and live bacterial suspension in first and second doses (2.54±0.5, 3.63±0.24; 2.64±0.38, 3.57±0.39; 2.87±0.7, 3.69±0.27 respectively) (Table 4).

Table 4: Effects of immunogens and doses on account of complete WBCs, and differential leucocytes.

Immunogen	Dose (µg/ml)	Total WBCs Count ($\times 10^3$/µl)	Monocytes (%)	Lymphocytes (%)	Neutrophils (%)
		Mean± SD			
Control	1	7.05±0.095	2.55±0.05 (b)	70.83±0.56	20.63±0.11
	2	7.06±0.05	2.98±0.5 (a)	71.96±0.68	21.03±0.7
BMP	1	9.87±0.14(b)	2.64±0.38 (b)	73.00±0.78 (b)	23.63±0.7(b)
	2	13.46±0.05(a)	3.57±0.39 (a)	74.66±0.32 (a)	25.70±0.75 (a)
BMP-liposome	1	10.9±0.173(b)	2.54±0.50 (b)	73.76±0.05 (b)	24.73±0.32(b)
	2	14±1.252(a)	3.63±0.24 (a)	76.13±0.25 (a)	25.70±0.43 (a)
Liposome	1	9.89±0.08(b)	2.30±0.38	72.70±0.17 (b)	23.36±0.40(b)
	2	10.3±0.17(a)	2.02±0.03	73.47±0.74(a)	23.80±0.58 (a)
Live B.S.	1	12.433±0.32(b)	2.87±0.70 (b)	72.76±1.50 (b)	21.36±2.74(b)
	2	13.76±0.51 (a)	3.69±0.27 (a)	74±.26±0.76 (a)	23.63±2.98 (a)
Heat Killed B.S.	1	12.13±0.05 (b)	2.21±0.33 (b)	72.10±0.85(b)	20.63±0.75(b)
	2	12.5±0.00 (a)	2.95±0.00 (a)	74.90±1.00 (a)	22.06±0.20 (a)
$L.S.D_{0.05}$		0.398	0.62	2.333	2.844

The different small letters indicate significant differences between the mean doses within the immunogens under a significant level (0.05). * Similar letters indicate that there were no significant differences between doses or between immunogens.

The leukocytes count increased in rats injected with BMP-liposome which indicated the enhanced role of these components to stimulate immune system that are able to respond to biofilms. These responses included accumulation, penetration, phagocytosis and killing of the biofilm bacteria. This result agrees with those of Philippe Fouchard [28] during the treatment with compounds of EPS extracted from bacterial biofilm. De Souza *et al.* [29] showed an increased number of neutrophil in rats after intraperitoneally injections of 10^8 *S. aureus* suspension. On another hand, Molne *et al.* [30] confirmed that *S. aureus* suspension with 10^7 concentration increased the frequency of WBCs, especially neutrophil that phagocytosis and kills bacteria via the release of lytic enzymes stored in granules and induces produced pro-inflammatory cytokines. So, neutrophils represent a multifunctional cell type that plays a main role in the innate immune response against extracellular pathogens and their toxin products by recognition, phagocytosis and killing. The count of monocytes as well as lymphocytes significantly increased in the second dose in rats injected with BMP-liposome, BMP, and live bacterial suspension in first and second doses. The lymphocytes possess the attributes of diversity, specificity, memory and self/non- self-recognition, the hallmark of an immune response. This result agrees with the results obtained by [31]. On the same line Bjarnsholt *et al.* [32] observed intense accumulation of neutrophils at the site of biofilms infection, indicating the enhanced role of these compounds of biofilm to immune system. On the other hand, long-lived mononuclear cells, monocytes and macrophages, are front line soldiers to eradicate obligate intracellular pathogens. However, the overall production of

PMNLs is about 10^9cells/kg/day. However, the number of circulating cells markedly increases during infection and inflammation [33].

3.8. Challenge Test

The results showed that BMP-liposome provided 90% production, BMP provided 80%, and HK provided 70%, while control group lost all tested animals (Table 5**).**

Table 5: Percentage of rats who survived after the challenge dose

Groups	No. of Rats	Number of Survived Rats after Challenge dose	Percentage of Survived Rats %
BMP	10	8	80
BMP+Liposome	10	9	90
Heat Killed Bacterial Suspension	10	7	70
Control	10	0	0

4. Conclusions

This study found that BMP-Liposome and BMP immunogen were the best immunogens that induces a production of CXCL5 chemokine which plays a specific role in regulating the neutrophils response, also increases counts of immune cells to defense against *Staph. aureus* virulent strains, thus reducing colonization and persistence by increased phagocytosis.

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