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PJBS

ISSN 1028-8880

# Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (11): 1888-1892, 2004
ISSN 1028-8880
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# Immunological and Toxicological Studies of Staphylococcin Bac188 (A Bacteriocin/Bacteriocin-like Inhibitory Substance) on Experimental Animals

[1]S. Saeed, [1]S.A. Rasool, [1]S. Ahmad and [2]S.A Ali
[1]Laboratory of Molecular Genetics, Department of Microbiology, University of Karachi,
[2]International Center for Chemical Sciences, HEJ Research Institute of Chemistry,
University of Karachi; Karachi-75270, Pakistan

**Abstract:** Staphylococcin Bac188, a bacteriocin/Bacteriocin-like Inhibitory Substance (BLIS) from *Staphylococcus aureus* AB188 was studied for its immunological and toxicological effects on experimental animals. The antigenicity/immunogenicity of staphylococcin Bac188 was determined by Ouchterlony technique and Enzyme Linked Immunosorbent Assay (ELISA). Accordingly, staphylococcin Bac188 is antigenically poor (in rabbit immune system) as the antibody titer was not found to be significant in Ouchterlony test. However, some immunogenicity was witnessed in ELISA, suggesting that low level of antigen-antibody reaction has occurred. The acute and chronic toxic studies with Bac188 do not show any toxic effects in diseases virgin (domestic rabbits) when injected with different regimen of doses (20, 40, 60 and 80 μg $mL^{-1}$). Hematological studies with staphylococcin Bac188 showed that except for marginal increase in lymphocyte count in the test animal groups, no other significant effect was observed as compared to the control (rabbits injected with plain sterile saline). Further, different doses of staphylococcin Bac188 showed no significant biochemical changes in the normal profile of rabbit blood biochemistry.

**Key words:** Staphylococcin, Bacteriocin-like Inhibitory Substances (BLIS), immunogenicity, hematology, biochemistry

## INTRODUCTION

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria[1]. The term Bacteriocin-like Inhibitory Substances (BLIS) is applied to antagonistic substances which are not fully defined or do not fit the typical criteria of bacteriocin. They have been reported to inhibit a wide range of both gram-positive and gram-negative bacteria. In recent years, interest has been shown on the microbiology, biochemistry and molecular biology of Bacteriocin Like Inhibitory Substances (BLIS) because they are medically, industrially and agriculturally important[2,3]. Staphylococcal bacteriocins have been the subject of much study over the past few decades and several reports describe their spectrum, production, purification and characterization[4-6].

Staphylococcal bacteriocins generally termed as staphylococcin (including both Class I, antibiotics containing modified amino acids and class II, bacteriocins/BLIS which are small heat-stable, non-antibiotics) have been the subject of much study over the past few years. Staphylococcins may vary from monomeric BacR1 from *S. aureus* UT0007[7,8]. Epidermin1580 from *S. epidermidis* 1580[9] and staphylococcin-T from *S. cohnii* T [10], two component C55 from *S. aureus* C55[4,11] and multi-peptide Aureocin A70 from *S. aureus* A70[12].

There is a large and increasing number of therapeutic proteins approved for clinical use and many more are undergoing preclinical studies and clinical trials in humans. Most of them are 'humanized' recombinant molecules. Virtually all therapeutic proteins elicit some level of antibody response, which in some cases can lead to potentially serious side effects. Therefore, immunogenicity of therapeutic proteins is a concern for clinicians, manufacturers and regulatory agencies. In order to assess immunogenicity of these molecules, appropriate detection, quantitation and characterization of antibody response are necessary[13,14]. The interaction between antigen and antibody can be observed in the form of immune precipitates both in solutions as well as in agar matrix[15]. Ouchterlony technique is the classical procedure used to detect the presence of antibodies and determine their specificity by visualization of "lines of identity" (precipitin lines). These precipitin lines

**Corresponding Author:** Dr. Sheikh Ajaz Rasool, Department of Microbiology, University of Karachi, Karachi-75270, Pakistan
Tel: 021 9243582 E-mail: ajazrasool@hotmail.com

(precipitated antigen-antibody complexes) form where the binding concentrations of antigen and antibody are equivalent[16].

The aim of this study was to monitor the effects of staphylococcin Bac188, from *Staphylococcus aureus* AB188 on hematological and biochemical parameters of rabbits when administered intravenous (IV) up to one month.

## MATERIALS AND METHODS

**Partial purification of Bac188:** For the production of Bac188, *S. aureus* AB188 was grown in Brain Heart Infusion broth at 37°C for 24 h. The bacterial cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was filter sterilized by passing it through 0.45 μ diameter filter, (Millipore MA, USA). Ammonium sulfate was added slowly to the supernatant of 1 L of culture broth with constant stirring at 4°C till the level of 80% concentration to attain the optimum precipitation of protein. The system was held overnight and the precipitate was recovered by centrifugation at 15,000 rpm for 30 min at 4°C. The resulting pellet was dissolved in 200 mL 50 mM sodium phosphate buffer pH 7.0 and designated as crude preparation. Ammonium sulfate precipitate was preliminary fractionated by ultrafiltration using Stirred Cell (Millipore MA, USA) with the membrane pore sizes of 10 kDa. Fractions of maximum activity (>10 kDa) obtained after ultracentrifugation were concentrated in a minimal volume by rotary evaporator and diluted to a known protein concentration in 10 mM sodium phosphate buffer, pH 7.0 and designated as "staphylococcin Bac188". The antibacterial activity was assayed using *S. aureus* SS-1 and antimicrobial titer was determined in terms of arbitrary units/mL[5]. The protein concentration at each step was measured by the method of Bradford[17] using BSA as the standard protein.

**Immunogenic potential of staphylococcin Bac188:** Healthy rabbits of either sex were used. The animals were housed in a well ventilated experimental section of the animal house, at room temperature. They were kept on standard diet under standard conditions. Both the experimental and control animals had free access to both food and drinking water during the experimental period.

**Ouchterlony:** Plate Ouchterlony was performed in 1% agarose in Sodium barbital buffer (pH.7.0). Control wells contained BSA and anti-BSA. Test wells: Central well contain first dose of antigen, while side wells contain pre-immune serum and first, second and third sera, respectively. Same set was repeated for second and third doses, respectively[18].

**Enzyme Linked-immunosorbent Assay (ELISA):** The wells of 96-microtitre well plate were coated with 100 μL of diluted antigens in coating buffer [0.05 M $CO_3HCO_3$ buffer ($Na_2CO_3$, 1.59 g/5000 mL; $NaHCO_3$, 2.93 g/500 mL)] of pH 9.6. The plate was refrigerated at 4°C for overnight and washed with washing buffer [0.05 mL, Tween 20 in 100 mL saline (0.85% NaCl)]. Then 100 μL of the serum dilution was added to the corresponding wells and incubated at 37°C for 1 h. The plate was again washed five times with washing buffer and 100 μL of diluted anti-human rabbit IgG-HRP-/IgM-HRP was added in each well and incubated at 37°C for 1 h. Washing is repeated with washing buffer. 100 μL of 10 μg $mL^{-1}$ substrate [(OPD+$H_2O_2$) [10 mg OPD in 25 mL Citrate buffer + 25 μL 30% $H_2O_2$] was added to each well. The color change was observed after 15, 30 and 60 minutes. The reaction was stopped by adding 50 μL of 20% $H_2O_2$ to each well. Optical density was read in ELISA reader[16,19].

**Toxic studies in rabbits:** The animals were randomly divided into 5 groups of 4 animals each. Group 1 animals served as control and received normal saline (0.4 mL). Group 2, 3 and 4, animals were treated with intravenous injections of 20, 40, 60 and 80 μg $mL^{-1}$ of Staphylococcin Bac188 $kg^{-1}$ body weight of the animals. Blood samples were drawn from each member of all groups[20]. Toxic manifestations like abnormal motor activity, alteration in water or food intake, respiration, body temperature, diarrhea and mortality were observed for (1, 2, 6 and 24 h) for acute toxic and (1 month) for chronic toxic studies[21]. Before the first and the last treatment, blood samples were collected from each animal in syringes containing sodium citrate as anticoagulant and hematological and biochemical tests were performed[22,23].

**Hematological and biochemical studies of rabbit blood:** The hematological and biochemical test were also performed at the beginning and end of experiment[24].

## RESULTS AND DISCUSSION

Staphylococcin Bac188, a Bacteriocin Like Inhibitory Substances (BLIS) produced by *Staphylococcus aureus* AB188[6] has been investigated for its immunological and toxicological response on rabbit as experimental animals. Staphylococcin Bac 188 (80% ammonium sulfate precipitated and fractionated by ultrafiltration through 10 KDa cut-off membrane (designated as staphylococcin Bac188) was checked for its antibacterial activity against *S. aureus* SS-1 (Fig. 1). Figure 2 shows the preliminary fractionation of Bac188. Where by staphylococcin Bac 188 was found to be strongly active and showed zone of 30 mm zone of inhibition.

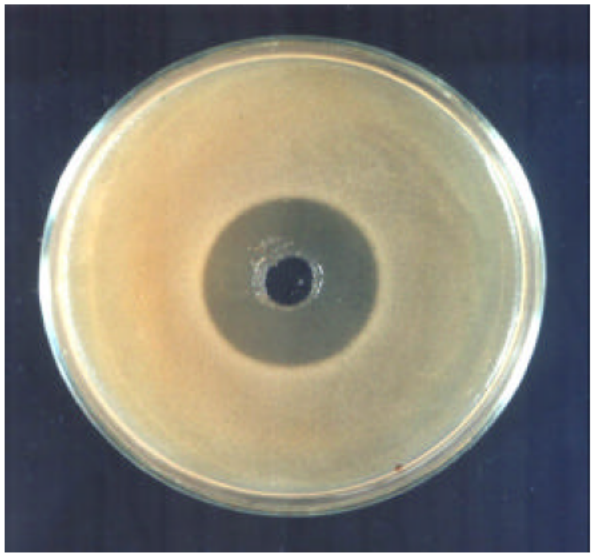

Fig. 1: Antimicrobial activity of staphylococcin Bac188 against *S. aureus* SS-1 by agar-well diffusion method

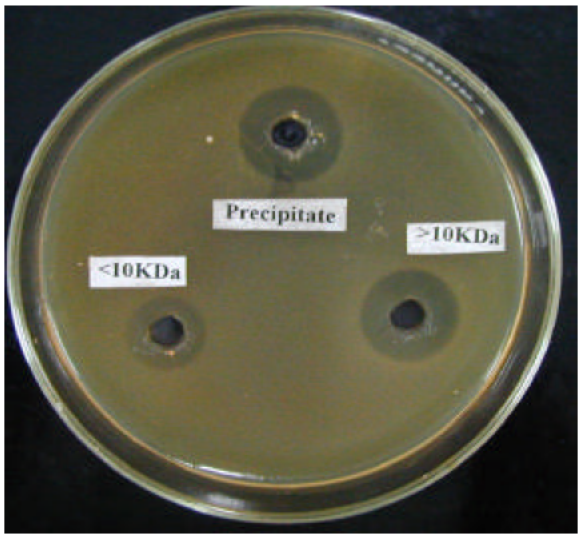


Fig. 2: Antimicrobial activity of staphylococcin Bac188 showing the preliminary fractionation of Bac188. All analysis were carried out in BHI agar plates, seeded with 2 x10 $^{8}$ cells of *S. aureus* SS-1

Table 1: *In vivo* immunization schedule of rabbit

| | | Staphylococcin 188 | | |
|---|---|---|---|---|
| Day | Control saline Animal-1 | First dose Animal-2 (undiluted) | Second dose Animal-3 (1:10) | Third dose Animal-4 (1:100) |
| First day | Pre-immune bleeding performed on first day | | | |
| 1st Day | 0.5 mL | 0.5 mL | 0.5 mL | 0.5 mL |
| 3rd Day | 0.5 mL | 0.5 mL | 0.5 mL | 0.5 mL |
| 6th Day | 0.5 mL | 0.5 mL | 0.5 mL | 0.5 mL |
| 13th Day | First bleeding Performed | | | |
| 13th Day | 0.5 mL | 0.5 mL | 0.5 mL | 0.5 mL |
| 23rd Day | Second bleeding performed | | | |
| 23rd Day | 0.5 mL | 0.5 mL | 0.5 mL | 0.5 mL |
| 31st Day | Third bleeding performed | | | |

Animal models are use to predict the immunogenicity of therapeutic proteins for humans reliably. In non-human primate studies, variety of responses is noted including from little to no antibody response, to a strong neutralizing response, or even a cross-reactive antibody response[13]. These have generally not correlated well with the immune response seen in humans. The route of administration, duration and schedule of administration, the cumulative dosage of the protein, the pharmacological properties of the protein, as well as the purity of the clinical material can influence the immunogenicity[15]. Ouchterlony test of Bac 188 was performed to check its antigenicity, where by no line(s) of precipitation were observed when staphylococcin Bac188 was allowed to react with the rabbit antisera taken after immunization with different doses of Bac188 (Table 1). However, normal precipitations were prominent in the control (when BSA and anti-BSA were used). More precise and sensitive ELISA[25] was also performed (in 96-microtitre well plate). According to which, the preparation seems to be of low grade (poor) antigenic/immunogenic (data not shown).

For the toxicological studies of Bac 188 rabbits were injected with varying concentration (20, 40, 60 and 80 μg mL$^{-1}$) of staphylococcin Bac188. No abnormal effects i.e. abnormal motor activity, sedation, hypnosis, changes in respiration and body weight was found in both control and test groups. Food and water intake was also found normal with no diarrhea for 2 and 6 h nor any mortality within 24 h and through 30 days.

The biochemical and hematological studies of rabbit blood (at the beginning and end of injections) were also performed[22] (Table 2 and 3). Accordingly, the

Table 2: Blood biochemistry of rabbits injected with different concentrations of staphylococcin Bac188

| | | Biochemical tests performed | | | |
|---|---|---|---|---|---|
| Group | Dosage | Blood Glucose (BG) | Total Lipid (TL) | Total Protein (TP) | A/G ratio |
| Control | 0.4 mL saline | 86 mg mL$^{-1}$ | 180 mg mL$^{-1}$ | 6.0 G dL$^{-1}$ | 3.6 |
| Group 1 | 0.1 mL Bac188 | 126 mg mL$^{-1}$ | 180 mg mL$^{-1}$ | 6.5 G dL$^{-1}$ | 3.6 |
| Group 2 | 0.2 mL Bac188 | 130 mg mL$^{-1}$ | 162 mg mL$^{-1}$ | 6.0 G dL$^{-1}$ | 2.8 |
| Group 3 | 0.3 mL Bac188 | 135 mg mL$^{-1}$ | 172 mg mL$^{-1}$ | 6.8 G dL$^{-1}$ | 2.5 |
| Group 4 | 0.4 mL Bac188 | 120 mg mL$^{-1}$ | 250 mg mL$^{-1}$ | 6.1 G dL$^{-1}$ | 2.4 |

Table 3: Blood picture of rabbits injected with different concentrations of staphylococcin Bac188

| | | Hematological tests performed | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | Dosage | Hb | ESR | PC | PCV | LC | EC | WBC | MC |
| Control | 0.4 mL saline | 10.7 | 5.04 | 342 | 31.1 | 32 | 2 | 7.0 | 2 |
| Group 1 | 0.1 mL Bac188 | 11.7 | 5.01 | 345 | 35.0 | 34 | 4 | 8.8 | 4 |
| Group 2 | 0.2 mL Bac188 | 13.0 | 5.70 | 300 | 37.0 | 50 | 1 | 5.5 | 1 |
| Group 3 | 0.3 mL Bac188 | 11.0 | 4.70 | 320 | 35.0 | 65 | 5 | 6.7 | 2 |
| Group 4 | 0.4 mL Bac188 | 10.0 | 4.20 | 310 | 30.0 | 62 | 8 | 6.6 | 2 |

Key: Hb: Hemoglobin, ESR: Erythrocyte sedimentation rate, PC: Platelet count, PCV: Packed cell volume, LC: Lymphocyte count, EC: Eosinophile count, WBC: White blood cells, MC: Monocyte count

concentration of staphylococcin Bac188 used to inject rabbits has not shown any adverse reactions on different components of rabbit blood like hemoglobin, packed cell volume, red blood cells, white blood cells, lymphocytes, eosinophiles and monocytes. However, the lymphocytes count was found little a higher than the normal range in a dose dependent manner. Parameters which were used for biochemical effects are blood glucose, total protein and total lipids. Present results indicate that doses of staphylococcin Bac188 used in this experiment did not show any adverse effects on the normal biochemistry of rabbit blood as the values of blood glucose (random), total protein and total lipid were found normal and similar in control and in test group.

Hence this study demonstrates the first evidence of the toxicological, immunological and biochemical studies of bacteriocin or bacteriocin-like inhibitory substances from *Staphylococcus aureus*. Concluded, staphylococcin Bac188 did not show any toxicological effects on rabbits and no adverse alterations in biochemical and hematological parameters were observed like blood glucose, blood protein and blood lipids. On that ground it seems plausible that the putative/hypothetical staphylococcin Bac 188 could act as potential reservoir for therapeutic interventions. Further, studies are in progress and will be reported later.

## ACKNOWLEDGMENT

This project is supported from Pakistan Science Foundation (PSF-S-KU/CHEM-363) to S.A. Rasool.

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