

IMMUNIZATION MODEL OF RATS TREATED WITH TWO ANTIGENS FROM MRSA BACTERIA ISOLATED FROM BURN PATIENTS

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ABSTRACT The present study aimed to investigate the efficiency of alpha hemolysin (Hla) and heat inactivate bacteria suspension (HIBS) antigens prepared from Methicillin Resistant *Staphylococcus aureus* (MRSA) bacteria isolated from burn wound in rats after immunization. Both antigens examined after the period of immunization to determine the total IgG concentration, IFN- γ and TNF- α levels in serum. The results indicated that Hla antigen had the ability to modulate and induce the humoral and cellular immune response since that there was a significant elevation in serum total IgG and IFN- γ at ($p \leq 0.05$) in comparison with the HIBS antigen. Resent data also indicated to a significant decreasing in TNF- α concentration in sera of rats immunized with two studied antigens Hla (44.54) pg/ml and HIBS (16.5) pg/ml.

Keywords: MRSA, Alpha Hemolysin, HIBS, IgG, TNF- α , IFN- γ

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* strains are isolated in more than half of all community and hospital infections (Klevens *et al.*, 2007). The virulence factors of *Staphylococcus* include surface components that associated with pathogenesis of this bacteria, such as the capsule, peptidoglycans, various toxins as alpha, beta, gamma and delta hemolysins (Kuroda *et al.*, 2007 and Vasconcelos & Cunha, 2010). Alpha toxin (Hla) is released by 95% of *S. aureus* strains was pore-forming and pro-inflammatory properties (Xiong *et al.*, 2006). Alpha-toxin is a secreted pore-forming toxin that has cytolytic activity toward a variety of host cell types, including human keratinocytes, epithelial cells, platelets, monocytes, T lymphocytes and fibroblasts (Bubeck Wardenburg *et al.*, 2008). Staphylococcal haemolysin that contribute for bacterial invasion and to escape from the host immune response, alpha-haemolysin considered to be a main pathogenicity factor because of its haemolytic, dermonecrotic and neurotoxic effects (Da Silva *et al.*, 2005).

Humoral immunity is the principal protective immune response against extracellular bacteria such as *S. aureus*. The functional antibodies that facilitate the clearing of staphylococci from the site of an infection, which is through uptake and killing by professional phagocytes, especially neutrophils, or functional antibodies that neutralize virulence factors, are absent in the majority of the human population (Krishna & Miller, 2012). The protein antigens of extracellular bacteria activate CD4⁺ T helper cells. These cells produce cytokines which stimulate antibody production, induce local inflammation and enhance the phagocytic and microbicidal activities of macrophage (Abbas & Lichtman, 2003). Staphylococcal infections in humans result in a transient increase in anti-staphylococcal antibody levels (Jacobsson *et al.*, 2010). Clearly, immunization with defined antigens can elicit protection in animal models of *S. aureus* disease, suggesting that during infection antibody

levels may simply be too low to be protective (Cheng *et al.*, 2011). Immunization with inactive α -hemolysin or pharmacologic inhibition of α -hemolysin can prevent or reduce the severity of *S. aureus* pneumonia in mice (Ragle *et al.*, 2010).

S. aureus molecules such as peptidoglycan and lipoteichoic acid are potential stimulators of cytokine production (e.g. TNF- α , IL-1 β , IL-6, IL-4, IL-8, IFN- γ and IL-12), in response to infection but unregulated cytokine production may contribute to *S. aureus* pathogenesis (Wang *et al.*, 2000). The production of chemokines stimulates migration of lymphocytes to the site of inflammation and switches initial immune reactions to the antigen-specific mechanisms of the cellular immune response (Tikhonov *et al.*, 2001). Secretion of IFN- γ is a hallmark of the Th1-type response, and IFN- γ induces opsonizing antibodies and activates macrophages to kill intracellular bacteria and therefore likely plays an important role in immunity to *S. aureus* (Gómez *et al.*, 2002). So recent work directed to evaluate the humoral and cellular response after immunization with crude alpha hemolysin and HIBS antigens.

MATERIALS AND METHODS

Bacterial isolates and Culture Media

In this study, *S. aureus* isolated from burn patients in burn unit of AL- Hussain Teaching Hospital, in AL- Thi-Qar province, Iraq. *S. aureus* subjected to diagnostic tests, such as culturing on mannitol salt agar, blood agar, biochemical tests such as catalase test, coagulase tube test and DNase production test (MacFaddin, 2000), and API staph system, and all isolates identified by Staphylo Monotec test kit Plus as serodiagnosis, then *S. aureus* isolates identified as MRSA according to method of Bauer *et al.*, (1966) against methicillin antibiotic disc (5 μ g/disc) (Bioanalyse, Turkey).

Preparation of crude alpha hemolysin antigen

Bacterial isolate: MRSA isolate was selected for preparation of crude alpha hemolysin, expressed *hla* gene, that detected by PCR (Mehrotra *et al.*, 2000), and it was given alpha hemolysis on blood agar, this antigen was prepared according to method of (Siritool & Makonkawkeyoon, 1978) as the follows:

- 1- The medium was prepared by dissolving 42.4 grams of BHI broth powder (LAB/ United Kingdom) in 100 ml of D.W and the viscous solution was dialysed overnight against 1.100 ml of D.W.
- 2- Three-tenth percent agar (Himedia/ India) was then added to the dialysate and the media appropriately dispensed in flasks was sterilized.
- 3- The semisolid BHI medium was inoculated with 5 hours, old BHI dialysate broth culture of selected hemolytic colonies of the organisms.
- 4- One milliliter of this inoculum was used for inoculating each 100 ml of the media that dispensed in petri plates and incubated in 15% CO₂ in air.
- 5- Following incubation, the culture were pooled and frozen at -20°C overnight then thawed at room temperature.

6- The culture was mixed and distributed in glass centrifuge tubes and supernatant fluid was collected by centrifugation at 2500 rpm for 40 min.

7- The pooled supernatant was filtered through a 0.22 μ membrane filters.

8- This cell free filtrate constituted the crude alpha toxin.

Heat-inactivated bacterial suspensions antigen of MRSA

Bacterial isolate: MRSA isolate was selected for preparation this antigen had *sea* gene that detected by PCR (Betley and Mekalanos, 1988) with modification, and this antigen prepared by using the method of (Lawrence *et al.*, 2012) as bellow:

- 1- One MRSA isolate was used to prepare HIBS antigen for immunization. Bacteria were cultured overnight from frozen stocks in Tryptic Soy Broth (TSB) (LAB/ United Kingdom) at 37°C with shaking (250 rpm) in a shaking incubator.
- 2- Bacterial culture were diluted 1:200 in fresh TSB the following, and then cultured to the early stationary phase of growth (optical density at 600nm[OD600] of 2.0).
- 3- To prepare inactivated suspensions, bacteria were harvested by centrifugation at 8,000g for 10 min and washed three times with phosphate buffered saline (PBS, pH=7.2), to remove secreted proteins, and resuspended in PBS.
- 4- Serial ten-fold dilutions in PBS were then plated on Tryptic soy agar (LAB/ United Kingdom) plates to determine the number of CFU.
- 5- Bacterial suspensions were heat inactivated at 60°C for 1 h.

Estimation of protein concentration for prepared antigens

Protein concentration of prepared antigens were estimated by Bradford kit (Sigma/ USA). A serial dilution of bovine serum albumin protein BSA was used to obtain the standard curve (Fig 1).

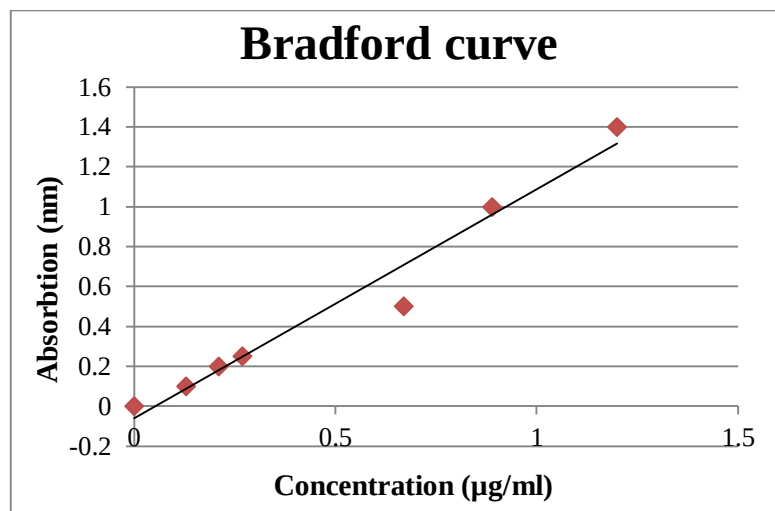


Fig (1): Standard Bradford curve for protein concentration determination.

Cytotoxicity Test

The cytotoxicity activity of the both antigens was determined against human red blood cells using the method described by (Nair *et al.*, 1989).

Efficiency evaluation of antigens in immunization

For evaluation of Hla and HIBS antigens efficiency in immunization. Thirty white albino female rats (170-230 g) were obtained from the animal house at the Science college in Thi-Qar university, Iraq. The rats were housed in standard metal cages (5 rats/cage). The rats were divided into three groups comprising ten animals in each group (20 of them were immunized of each antigen and 10 were treated as control group).

Antigens emulsified with an equal volume (v/v) of complete Freund's adjuvant and three booster injection performed for rats by using the method of (Wang *et al.*, 2015) with modification, as follows:

First injection: 0.25 ml of each antigen with a concentration of 200 and 160 µg / ml mixed with 0.25 ml of complete Freund's adjuvant and injected subcutaneously at first day.

Second injection: after seven days later, rats were injected intradermally in limbs with 0.5 ml of each antigen mixed with 0.5 ml of incomplete Freund's adjuvant.

Third injection: after fourteen days after the second injection, rats were injected intradermally in limbs with 1 ml of each antigens directly.

Control group: were injected with PBS only in same periods and volumes described in immunization schedule of immunized rats .

Seven days after the last injection all rats were bled and sera were stored at (-20C°) until used.

Diagnostic efficiency evaluation of prepared antigens with total immunoglobulin G (IgG) by ELISA

The ELISA technique for the detection of specific total IgG in sera of immunized and control rats with two prepared antigens was performed according to Senna *et al.*, (2003) with modification as follows:

- Serial dilution of antigens were prepared as 1:1, 1:2 and 1:3.
- Microtitration polystyrene plate was coated by overnight incubation at (4 C°) with 100µl/well of antigens, each antigen was coated in separated plate.
- After incubation period, the coating solution was discarded and plates then were washed three times with phosphate-buffered saline (PBS) pH 7.4 and 0.05% Tween 20 (PBS-T) (washing buffer).
- The plates were blocked for 1h at 37C° with 100 µl/well of blocking buffer (PBS containing 3% bovine serum albumin) and then washed three times with washing buffer.
- The serum samples were diluted to 1:10, 1:20, 1:30 in blocking buffer.

- Sera were added (100 μ l/well) to each coated plate and the plates were incubated for 1h at 37C°.
- Washing buffer was used as blank.
- Then the plates were washed three time, the secondary antibody (horse radish peroxidase-labeled anti-rat immunoglobulin G (HRP-IgG) (Koma Biotech Inc./ Korea) was added (100 μ l/well) at a 1:2000, 1:2000 and 1:4000 dilution in PBS, and the plates were incubated for 1h at 37C°.
- After washing, (100 μ l/well) of a substrate solution containing H₂O₂ and 2,2-azino-bis 3-ethylbenz-thiazoline-6-sulfonic acid was added in 50 mM citrate buffer, pH 4.0, and incubated in the dark for 15 min at 37C°.
- The reaction was stopped with 50 μ l/well of 3 N sulfuric acid.
- Absorbance was read at 490 nm in ELISA reader .

Estimation of serum TNF- α and IFN- γ concentration in immunized and control rats

Sera collected from immunized and control rats was used for measuring serum TNF- α and IFN- γ concentration using ELISA kit (Elabscience/china).

Serial dilutions of the standard (5000, 2500, 1250, 625, 312.5, 156.25, 78.12 and 0 pg/ml) and (2000,1000, 500, 250, 125, 62.5, 31.25 and 0 pg/ml) were prepared with standard diluent consecutively from the original standard with standard diluent for TNF- α and IFN- γ respectively. The optical density (OD value) of each well was determined at once, using a micro-plate reader set to 450 nm. The TNF- α and IFN- γ concentration of unknown samples and control groups were calculated from the standard curve (Fig 2 and 3) for TNF- α and IFN- γ respectively.

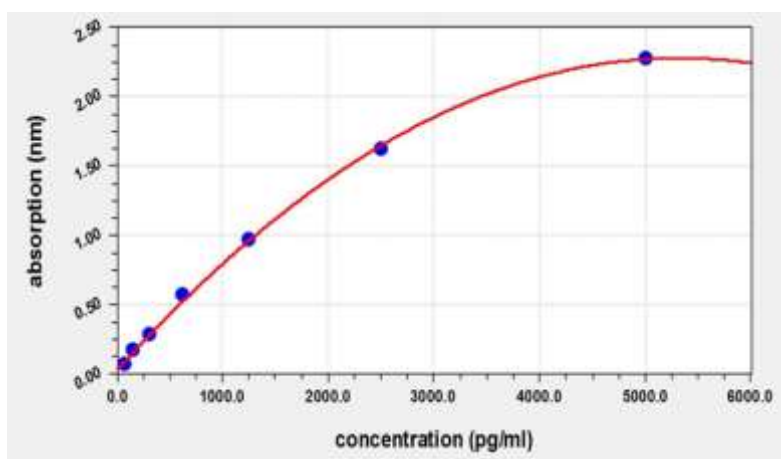


Fig (2): Standard curve of TNF- α concentration.

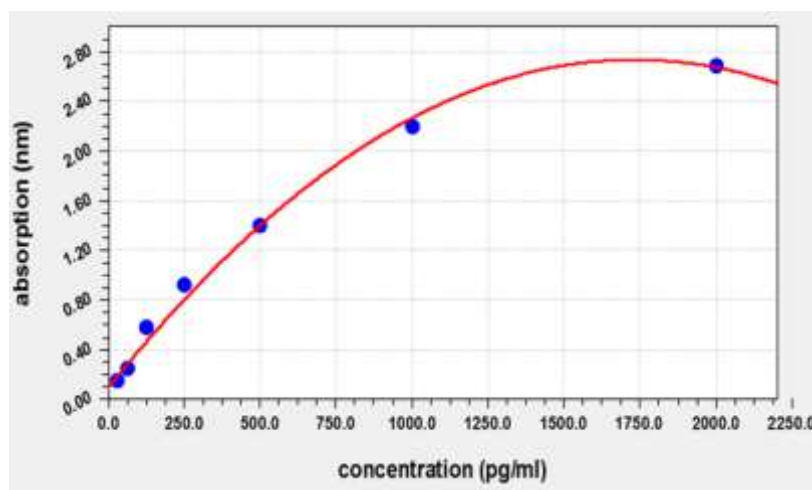


Fig (3): Standard curve of IFN- γ concentration.

Statistical analysis

The results of present study were statistically analyzed by using SPSS statistics program version 16 at ($P \leq 0.05$) were considered statistically significant (Al-Raw & Khalafallah, 1989).

RESULTS AND DISCUSSION

The concentrations of two prepared antigens were determined as followed:

Hla: 160 $\mu\text{g/ml}$ HIBS: 200 $\mu\text{g/ml}$.

Cytotoxicity test

The results of cytotoxicity test, showed that all concentrations of both antigens had no lysis human RBCs, and revealed that these cells were not susceptible to effective of those antigens.

The non-susceptibility of human RBCs may be associated with erythrocyte types of diverse animal species. One remarkable finding related to the variation in susceptibility to lysis exhibited by erythrocytes of different animal species for example, an approximately 400-fold-higher concentration of alpha-toxin was required to lyse human erythrocytes compared with rabbit erythrocytes (McCartney & Arbuthnott, 1978).

The intrinsic resistance of human erythrocytes against toxin action fostered the impression that a pathogenic role for this agent might be confined to certain animal species only. Also they indicated that rabbit, but not human, erythrocytes express a limited number of surface receptors for alpha-toxin. Binding was optimal at 24°C and appeared irreversible, and the presence of these receptors was responsible for the high sensitivity of rabbit erythrocytes. They concluded that at high concentrations of alpha toxin could bind via another, unspecific interaction to resistant cells such as human erythrocytes (Bhakdil & Tranum-Jensen, 1991).

Efficiency evaluation of antigens in immunization

The specific antibody production was measured in sera of immunized and control groups after immunization by an indirect ELISA checkerboard titration. The results revealed that the IgG

level in immunized group with Hla was higher than those of immunized group with HIBS antigen as shown in table (1). The data represented in table (2) revealed that the optimal concentration of Hla and HIBS antigens were (20 and 50 µg/ml) respectively, while the optimal dilution of sera and conjugate were 1:10 and 1:2000 for both antigens.

Table (1): IgG level in immunized and control groups.

Antigen	Group	No	Mean	Range	SD	SE
Hla	Immunized	10	1.05	0.73-1.2	0.148	0.022
	Control	10	0.43	0.3-0.51	0.07	0.01
HIBS	Immunized	10	0.86	0.64-1	0.067	0.01
	Control	10	0.39	0.27-0.48	0.01	0.01

Table (2): Best concentration of antigen, sera, conjugate in ELSA test.

Antigen	Ag concentration	Sera dilution	Conjugate dilution
Hla	1:3 (20µg/ml)	1:10	1:2000
HIBS	1:2 (50µg/ml)	1:10	1:2000

Recorded data in table (1) showed an elevation in IgG level which referred to humoral response against antigens, and Hla was best than HIBS antigen. Diverse proteins from *S. aureus* were identified as promising candidate antigens that used in immunization such as alpha-toxin mutant H35L, capsular polysaccharides and staphylococcal enterotoxin was able to induce specific antibodies and cellular immune responses, resulting in reduced bacterial loads and inflammation reaction (Zhang *et al.*, 2015).

The induction to production of specific IgG in immunized rats incorporated with results of studies like Clarke *et al.*, (2006) whom recorded a significantly higher titers of reactive IgG against α -hemolysin was detected in serum samples from individuals with confirmed disease, similarly the study done by Goëmez *et al.*, (1998) documented that the levels of anti-alpha toxin, anti-lipoteichoic acids and anti-total proteins of *S. aureus* IgG in the serum of mice immunized were significantly higher than those detected in control mice.

The production of IgG antibody against Hla antigen was identical with results of study by Uppalapati *et al.*, (2014) showed that immunization mice with recombinant α -toxin of *Clostridium perfringens* and *S. aureus* (r- α CS) was effective in inducing high titers of serum anti- (r- α CS) antibody after three administration.

Present study recorded that there was a significant elevation of IFN- γ and a significant decreasing in TNF- α comparing with control group in response to both antigens of MRSA after 21 days of immunization as shown in tables (3 and 4). The mean concentrations of TNF- α in immunized group with Hla and HIBS antigens were (44.54 and 16.5 pg/ml), respectively lower than those of control group (102.7pg/ml). On the other hand, the mean concentrations of IFN- γ in immunized group were (21.8 and 7.15 pg/ml) for Hla and HIBS respectively was higher than those of control group (2.88pg/ml).

The production of IFN- γ was key indicator of cellular response, these results were agreed with the study done by Gómez *et al.*, (2002) whom recorded that the production of IFN- γ as indicator of cellular response induced by immunization in rats, and showed a significant increase in the levels of IFN- γ produced by lymphocytes from immunized mice after stimulation with heat-killed *S. aureus* or total *S. aureus* proteins when compared with control mice.

The current results were in agreement with Bubeck Wardenburg & Schneewind, (2008) whom showed an increased releasing of IFN- γ and blunted secretion of IL-1 β in immunized animals 24 h after infection with *S. aureus* Newman (animals receiving anti-Hla). IFN- γ , a cytokine that promotes phagocytic uptake and killing of *S. aureus* by immune cells (Zhao *et al.*, 1998). Secretion of IFN- γ is a hallmark of the Th1-type response, and IFN- γ induces opsonizing antibodies and activates macrophages to kill intracellular bacteria and therefore likely plays an important role in immunity to *S. aureus* (Gómez *et al.*, 2002).

Other study recorded that different antigens of *S. aureus* can induce high level of IFN- γ , while others cause low level of this cytokine such as Lawrence *et al.*, (2012) whom showed the best antigens, putative lipoprotein, and alkaline shock protein, induced high levels of IFN- γ in culture, ranging from (206-429 U/ml), while (foldase protein, putative lipoprotein, γ -Hemolysin A, γ -Hemolysin C, and antigen A) antigens induced generally lower levels of IFN- γ , ranging from (44-219 U/ml).

The present data recorded a significant decreasing in TNF- α level in immunized rats comparing with control rats after immunization with both antigens. Both Gram-negative (lipopolysaccharide) and Gram-positive (lipoteichoic acid) were a signature molecules cause upregulation of proinflammatory cytokines through processes that are suppressed by cationic peptides (Bloch-Shilderman *et al.*, 2001).

The decreasing level of TNF- α that documented in present study were alike with results of Uppalapati *et al.*, (2014) recorded that level of IL-10 increased while TNF- α was found to be down regulated in the r- α CS induce splenocytes after immunization with recombinant α toxin of *Clostridium perfringens* and *S. aureus* (r- α CS), and this down regulation of TNF- α can be an important sign for protection against α C toxicity. The recent results were not identical with results of other studies documented that several antigens of *S. aureus* cause production of TNF- α such as Dauwalder *et al.*, (2006) showed SEA prompts a strong Th1 response in vitro, with related production of TNF- α and MIP-1 α , also Wang *et al.*, (2000) recorded that TNF- α , IL-6, and IL-10 release induced by peptidoglycan and lipoteichoic was dose dependent, and only peptidoglycan was a potent inducer of TNF- α secretion, and high concentrations of lipoteichoic caused low levels of TNF- α in plasma, thus, lipoteichoic is probably not a significant trigger for TNF- α release *in vivo*.

Thus, recent results revealed a novel opportunities for development of Hla vaccines or immunotherapies.

Table (3): Serum TNF- α level in immunized and control groups.

Antigen	Group	No	Mean (pg/ml)	Range	SD	SE
Hla	Immunized	10	44.54	27-90	26.21998	8.29148
	Control	10	102.7	97-115	9.12627	2.88598
HIBS	Immunized	10	16.5	2-40	15.07205	4.76620
	Control	10	102.7	97-115	9.12627	2.88598

Table (4): Serum IFN- γ level in immunized and control groups.

Antigen	Group	No	Mean (pg/ml)	Range	SD	SE
Hla	Immunized	10	21.80	8-43	11.92383	3.77065
	Control	10	2.88	1-8	2.35834	.74577
HIBS	Immunized	10	7.10	2-17	5.04442	1.59519
	Control	10	2.88	1-8	2.35834	.74577

CONCLUSION

According to the results of present work, the Hla antigen is the best in induction humoral and cellular immune response related with increasing the total IgG level and in IFN- γ and decreasing in TNF- α comparison with control rats.

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