

## Some immunological responses in rats injected with prepared bacterin toxoid of local methicillin resistant *Staphylococcus aureus*

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### Abstract

This study was aimed to prepare bacterin toxoid from local isolate of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from bovine mastitis in Basrah province. Isolated MRSA was detected and confirmed using polymerase chain reaction. Then inactivated bacterin toxoid was prepared. A total of 45 male albino rats were used in 3 mainly groups to assess the bacterin toxoid. For immunological response, the concentrations of IL-4, CD4+, and CD8+ T-cells were estimated using enzyme linked immunosorbent assay. The results showed increase in the concentrations of IL-4, CD4+ and CD8+ T-cells in vaccinated group comparing with non-vaccinated control group. Increasing concentrations of IL-4 enhance humoral immune response by stimulation of Th2 that effect directly on B-cells differentiating them to plasma cells responsible for production of specific antibodies against MRSA. Increased CD4+T-cells also enhance humoral immune response as a result of interaction between them and antigen-presenting cells which presented major histocompatibility complex (MHC) type II on their surface, while increased CD8+T-cells enhance cellular immune response as a result of interaction between them and somatic effected cells presented MHC type I on their surface leading to differentiation them to Cytotoxic T lymphocytes (CTLs) responsible for the killing of effected cells. It was concluded that locally prepared bacterin toxoid proved their efficacy to stimulate both humoral and cell mediated immunity in rats as an experimental animal model.

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### Introduction

Bovine mastitis one of the serious diseases that considered a challenge to the economy of dairy animals and milk production, among the important causative agent of this disease *Staphylococcus aureus* is one of the more problematic pathogens that affect dairy farms and causes both acute and chronic mastitis (1). Many virulent factors exhibited by *Staph. aureus* such as excreting a wide range of enterotoxins and exotoxins to it is living environment in addition to specific mechanism attributed by it such as biofilm formation, antibiotic resistance, help it to colonization and develop to skin and mammary gland infection of cow causing mastitis (2). In addition to ability of

MRSA to transmission between animals and man (3). Many strategies were used for controlling mastitis such as improved nutrition, environmental sanitation, use of teat sealants (4), cleaning of teats with antiseptic before and after each milking, contentious cleaning of milking machines, culling of chronically diseased animals (5). Treatment of mastitis by antibiotics may be of value in some cases, but the presence of MRSA, as pathogenic organism, is a major problem. Because it is resistance against wide range of antibiotics that used in the field (6). Using of antibiotics by hazard may lead to transmission antibiotic resistance factors between microorganisms (7). For these reasons vaccination still the better method for the control bovine mastitis caused by MRSA (1). There are many types of vaccines have been

used to prevent much more serious diseases both in man and animals around the world, these vaccines included, killed or inactivated vaccines, live attenuated vaccines, sub unit vaccines, nucleic acid-based vaccines which included DNA and RNA vaccines (8), finally toxoid or bacterin toxoid which means inactivation of toxins produced by bacteria that cause damage of the tissue and produce disease in the host, these pathogenic toxins can be inactivated or detoxified by heating or adding chemical substances such as formaldehyde or using both (8). Therefore, the aims of this study were to prepare inactivated bacterin toxoid from a local isolate of methicillin-resistant *Staphylococcus aureus* and evaluate the immunological response in rats as an experimental animal model.

## Materials and methods

### Bacterial strain

Strong biofilm producing MRSA strain, isolated from bovine mastitis, was obtained from central research laboratory, College of Veterinary Medicine, University of Basrah. All the requirements for conducting the search were done in College of Veterinary Medicine, University of Mosul.

### Molecularly detection of the local MRSA strain

The MRSA strain was detected by PCR for each of the following genes: *nuc* gene: a universal gene for *Staph. aureus*, *mec A* gene: antibiotic resistance gene to methicillin (9), *ica A* and *ica D* genes: biofilm producing genes (10).

### Bacterin toxoid preparation

The bacterin toxoid has been prepared according to Giraudo *et al.* (11) with modification as: the strong biofilm-producing MRSA strain was firstly activated on nutrient agar. A single colony from growing MRSA in mannitol salt agar was elected for the production of the vaccine which was cultured on ten plates of blood agar at 37°C for 48 hours, the growing bacterial cultures were harvested then to 50 ml of modified nutrient broth which was composed of 90% nutrient broth with 10% of Iraqi buffalo whey, the benefit of whey in this modified medium was to stimulate MRSA and facilitate of pseudocapsule formation. Then, it was incubated at 37°C for 48 hours to allow for maximum encapsulation of the bacteria. After that 50 ml of 0.8% formalin was added (V/V) and incubated at 37°C for 24 hours to kill the bacterial growth mass. Sixty-five milliliters of it have been centrifuged at cold centrifuge 4°C for 60 minutes at 4100 xg, the supernatant has been discarded while the pellet was re-suspended in PBS and has been washed three times. Finally, the pellet was suspended in PBS and adjusted by spectrophotometer at OD 600 to become  $1.2 \times 10^{10}$  CFU/ml. On other hand, remained 35 ml has been entered into the autoclave for 20 min at 121°C and 15 bar then it has been centrifuged at 4100 xg for 60 minutes. The supernatant has been kept at - 80°C as crude toxoid for further use later while

the pellet has been discarded. The prepared bacterin toxoid has been filled and sealed in 5 ml capacity test tubes after adding each of the following: 3.5 ml of pellet suspension with 1.5 ml of crude toxoid with preservatives that have been added before sealing it which were included each of the following: 0.0005 gm of sodium azide with 0.0005 gm of thiomersal with 20 microliters of 0.4% formalin. After that each tube has been sealed and kept under deep freeze - 80°C to be used later in the vaccination of the experimental animals.

### Sterility, safety and purity tests

The prepared bacterin toxoid was examined for each sterility test, by culturing of the prepared vaccine on every of nutrient agar, blood agar, mannitol salt agar, brain heart infusion agar and also of nutrient broth, brain heart infusion broth which were kept under 37°C for 48 hours to detect any intact bacterial growth. The safety test was done by injecting ten of male albino rats with 0.3 ml of prepared bacterin toxoid subcutaneously and they were followed up for one week to detect any possible undesirable side effect (post vaccinal reactions). Finally, purity test was carried out by preparing the smears from bacterin toxoid and stained using Gram's stain to detect the presence of any intact bacteria.

### Study design

A total of 45 male Wistar albino rats at 8 weeks' age and 200-220 grams' body weight/animal, were used in 3 mainly groups and each main group was divided into three sub-groups which they were used as follow: the first group was composed of 15 animals divided into three sub-groups by 5 animals for each sub-group used to show the effect of locally prepared bacterin toxoid. All animals in three sub-groups were vaccinated S/C with prime vaccination dose at 0 day with 0.3 ml of a mixture of prepared bacterin toxoid with Complete Freund Adjuvant (CFA). After 5 days first sub-group animals were killed according to animal ethics committees after blood was collected from them and serum was separated to detect each of IL-4, CD4+ T-cells and CD8+ T-cells concentrations by ELISA. At 14 days, remained two sub-groups of animals were vaccinated S/C with first booster dose 0.25 ml of a mixture of prepared bacterin toxoid with Incomplete Freund Adjuvant (IFA). At 19 days the second subgroup animals were killed after blood were collected from them and serum separated to follow up of IL-4, CD4+ T-cells and CD8+ T-cells concentrations. At 28 days, the final remained sub-group were vaccinated S/C with a second booster dose with 0.2 ml of a mixture of prepared bacterin toxoid with IFA and at 33 days they were killed after blood was collected from them and serum separated to follow up of IL-4, CD4+ T-cells and CD8+ T-cells concentrations. The same program was used for each of the second control positive group which were treated with an inactivated commercial vaccine MASTIVAC® from OVEJERO company/Spain inactivated polyvalent vaccine

and the third control negative group which was treated with PBS only.

### DNA extraction

The MRSA DNA was extracted using DNA extraction kit (Presto™ Mini g DNA Bacterial kit under Lot No. FF14110-G, Geneaid company) (12).

### Primers and PCR program

Four sets of primers were used and provided by Alpha DNA company, Montreal (Table 1).

Table 1: Sets and size of primers of *Staph. aureus* genes

Primer		Sequence of the primers (5' to 3')	Product size /bp	Reference
<i>nuc gene</i>	F	GCGATTGATGGTGATACGGTT	279 bp	(13)
	R	AGCCAAGCCTTGACGAACCTAAAGC		
<i>ica A gene</i>	F	GAGGTAAAGCCACGCACCTC	151 bp	(14)
	R	CCTGTAAACCGCACCAAGTTT		
<i>ica D gene</i>	F	ACCCAACGCTAAAATCATCG	211 bp	(14)
	R	GCGAAAATGCCCATAGTTTC		
<i>mec A gene</i>	F	AAAATCGATGGTAAAGGTTGGC	533 bp	(15)
	R	AGTTCTGCAGTACCGGATTTGC		

Table 2: PCR program for each primer

Program	°C/minutes - Cycle					Reference
	Primary denaturation	Denaturation	Annealing	Extension	Final extension	
<i>nuc</i>	94/5-1	94/1-34	55/1-34	72/1-34	72/10-1	(13)
<i>ica A + D</i>	95/5-1	95/0.3-40	60/0.3-40	72/0.3-40	72/3-1	(14)
<i>mec A</i>	95/5-1	94/0.5-40	55/0.5-40	72/1-40	72/5-1	(15)

### Gel electrophoresis

PCR product was electrophoresed in 2% of agarose gel that stained with ethidium bromide and DNA ladder with 11 fragments 100-1000 bp plus a band at 1500 bp in size. The electrophoresis apparatus was optimized at 50V for 75 min. After that DNA bands have been read by transilluminator (Biometra/Germany).

### ELISA assay

ELISA to determine the concentrations of IL-4, CD4+ and CD8+ T-cells (Elabscience®/USA).

### Statistical analysis

SPSS version 23 one-way analysis of variance ANOVA with LSD and Duncan test were used at P≤0.05.

### Results

#### Molecular detection of MRSA local isolate

Specific primers with 279 bp, 533 bp, 151 bp and 211 bp sizes which were used for amplification of *nuc gene*, *mec A gene*, *ica A gene* and *ica D genes* respectively (specific genes for MRSA). These genes were successfully amplified by PCR

### polymerase chain reaction (PCR)

It has been done as follow: 4 µl of the extracted DNA of MRSA bacteria has been used as DNA template in a mini-Eppendorf tube 200 µl capacity. 1 µl of each forward and reverse primers of the target gene has been added to the tube. 12.5 µl of master mix has been added to the tube. 6.5 µl of nuclease-free water PROMEGA / USA, has been added to the tube. All the components of the tube have been mixed well by a mini centrifuge. Finally, the tube has been entered into the PCR thermocycler type machine according to the specific program for every gene (Table 2).

from the extracted DNA of MRSA which mean the local isolate was positive for each of *nuc gene*, *mec A gene*, *ica A* and *ica D genes* indicating that the local isolate was biofilm producing methicillin resistant *Staph. aureus* strain (Figure 1).

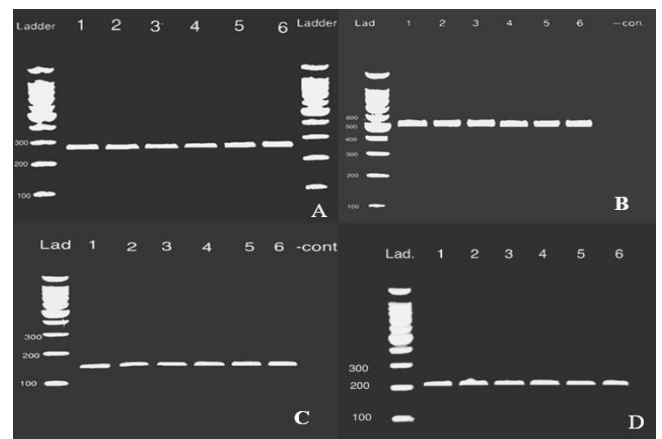


Figure 1: Amplification of: A- *nuc gene* with 279 bp in size, B- *mec A gene* with 533 bp in size, C- *ica A gene* with 151

bp in size, D- *ica D gene* with 211 bp in size electrophoresed in 2% of agarose gel stained with ethidium bromide and using DNA ladder of 1500 bp in size.

#### Sterility safety, and purity tests

No growing bacteria in cultured media with prepared bacterin toxoid. All of the injected animals were followed up for one week indicating the safety of vaccine, there was no shown any undesirable side effect due to post vaccination reaction or effects. Finally, the purity test was also negative for intact bacteria in Gram's stained smears.

#### IL-4 concentration

Significance increase in the IL-4 concentrations in vaccinated group with prepared bacterin toxoid in compare with non-vaccinated control group at each of 5, 19, 33 days. in comparing of control to vaccinated group with an inactivated commercial vaccine there was significant increases only in first 5 days with no any other increase in 19 and 33 days. Also, in comparison between each of vaccinated group with prepared bacterin toxoid and group vaccinated with an inactivated commercial vaccine the results showed no any significance between them at 5 days but there was a significant decrease in IL-4 concentration in the at 19 and 33 days (Table 3).

Table 3: The IL-4 concentration (pg/ml) at 5, 19 and 33 days in vaccinated and control groups

Groups	5 days	19 days	33days
Control	392.92 ± 61.22	370.78 ± 18.30	383.26 ± 23.80
Bacterin toxoid	837.75 ± 125.05*	765.25 ± 115.87*	879.43 ± 118.10*
Inactivated commercial vaccine	736.64 ± 190.79*	543.93 ± 250.06**	511.54 ± 178.48**

\*mean presence significance between vaccinated and non-vaccinated control groups at the same column. \*\*mean presence significance between vaccinated groups with prepared and inactivated commercial vaccine each to other at the same column. P<0.05.

Table 4: The CD4+ T-cells concentration (pg/ml) at 5, 19 and 33 days in vaccinated and control groups

Groups	5 days	19 days	33days
Control	742.89 ± 86.83	815.31 ± 112.35	767.84 ± 63.25
Bacterin toxoid	1387.64 ± 463.12*	1665.44 ± 373.66*	1691.84 ± 196.14*
Inactivated commercial vaccine	1941.43 ± 130.28* **	1540.39 ± 374.72*	1825.09 ± 390.27*

\*mean presence significance between vaccinated and non-vaccinated control groups at the same column. \*\*mean presence significance between vaccinated groups with prepared and inactivated commercial vaccine each to other at the same column. P<0.05.

Table 5: The CD8+ T-cells concentration (pg/ml) at 5, 19 and 33 days in vaccinated and control groups

Groups	5 days	19 days	33days
Control	44.17 ± 11.33	43.23 ± 9.29	42.12 ± 9.24
Bacterin toxoid	79.88 ± 0.06*	66.93 ± 9.93*	58.42 ± 18.82a
Inactivated commercial vaccine	66.44 ± 14.33*	53.42 ± 20.99	59.85 ± 23.26

\*mean presence significance between vaccinated and non-vaccinated control groups at the same column. Small litters mean presence significance inside the same group with time progression at the same row.

#### CD4+ T-cells concentration

A significant increase in the concentration of CD4+ T-cells in both vaccinated groups with each of prepared bacterin toxoid and inactivated commercial vaccine in compare with non-vaccinated control group at 5, 19 and 33 days respectively. Also, the results showed significant increase in CD4+ T-cells concentration in group vaccinated with an inactivated commercial vaccine comparing with group vaccinated with prepared bacterin toxoid at 5 days only with no any significance between them in in 19 and 33 days respectively (Table 4).

#### CD8+ T-cell concentrations

Significant increase in the CD8+ T-cells between vaccinated groups with each of prepared bacterin toxoid and inactivated commercial vaccine respectively in compare with non-vaccinated control group at 5 day, while at 19 day a significant increase in CD8+ T-cells only in vaccinated group with prepared bacterin toxoid in compare with non-vaccinated control group and no change in CD8+T-cells in vaccinated group with an inactivated commercial vaccine . Also, the results clarified change in time progression within the vaccinated group with prepared bacterin toxoid by significant decrease in concentration of CD8+ T-cells at 33 days in comparing with 5 and 19 days (Table 5).



## Discussion

Vaccination against MRSA considered the best suitable method for controlling of mastitis in dairy cows due to possession of MRSA many of virulent factors such as antibiotic resistant, excretion of enteric and exotoxins in addition to it is ability to formation of biofilm that complex the problem of infection by changing the infection by it to chronic form (2). So, vaccination considered easy and suitable method for controlling of bovine mastitis caused by MRSA. Our results showed significant increase in IL-4 concentrations in vaccinated animals with prepared bacterin toxoid and this may indicate for humoral immune response enhancement. Our result agree with Misra *et al.* (16) who demonstrated elevation in IL-4 level as a result of vaccination. IL-4 level elevation in vaccination process may due to several reasons, T-helper cells can differentiate mainly in to Th1 and Th2 cells, at first time IL-4 effect on naïve CD4+ T-cells or TH0 (naïve T-helper) leading to differentiation them into Th2 cells but not Th1 cells, Th2 cells lead to enhancement and produce additional amount of IL-4 by feedback loop mechanism and this process was called position feedback loop (17). Also, IL-4 effect directly on a subset of T-cells which was called T-helper CD4+T-cells producing IL-4 that finally enhances IL-4 itself production later (18). Naïve CD4+ was able to the production of IL-4 at the prime, later this produced IL-4 lead to activation and differentiation of these cells in to effector T-cells responsible for further IL-4 production (19). One of the important function of IL-4, the ability to promote further production of IL-4 from Th2 cells that effect directly on B-cells leading to differentiation of these cells to plasma cells which were responsible for the specific antibody production against target antigen so it was called sometimes B-cell growth factor (20), another function of IL-4, it plays an important role in the expression of MHC II molecules that enhance the humoral immune response by acting it directly on CD4+T-cells (21), so IL-4 play an important role in humoral immunity but not cell-mediated immunity. Our results showed presence significant increase in the concentrations of CD4+T-cells in vaccinated group with prepared bacterin toxoid in comparison with non-vaccinated control group at each of 5, 19 and 33 days respectively and this indicate that the locally prepared vaccine succeeded in the stimulation of potent humoral immune response against prepared vaccine which was used in the study. Our result agreed with Misra *et al.* (16) that indicated presence significant increase in CD4+ levels due to vaccination against MRSA, also agreed with Zuo *et al.* (22) that vaccination with chimeric bivalent vaccine caused strong T-cell response to vaccine. Also, our result showed a significant increase in CD8+ T-cells that agreed with at each of 5 and 19 days then it decreased significantly at 33 days in a vaccinated group with locally prepared bacterin toxoid also there was a significant increase in CD8+ T-cells at 5 days only in the vaccinated group with an inactivated commercial

vaccine, these results may agree with Misra *et al.* (16), Zuo *et al.* (22) which they revealed increase T-cell response to vaccination. In addition, it may have indicated that MRSA may act as intracellular pathogen sometimes and this may lead to increase concentrations of CD8+T-cells that were activated throughout vaccination and differentiated to cytotoxic T-lymphocytes CTLs which they were responsible for cell-mediated immunity by killing these cells and elimination of the intracellular pathogen. Rollin *et al.* (23) referred that MRSA bacteria can be lived inside endothelial cells which were responsible for relapsed infection after full treatment with antibiotics. *Staph. aureus* bacteria may be considered now a day non-classical facultative intracellular bacterium giving them the chance to behave as intracellular and extracellular pathogen in the host at the same time (24). One of an important factor that helps *Staph. aureus* bacteria to live as an intracellular pathogen is sigma factor B which was important operon allows *Staph aureus* to cause silent chronic infection later (25). So, the ability of *Staph aureus* to live intracellular pathogen gives the chance for CD8+ T-cells to differentiate to cytotoxic T-lymphocyte that kill these cells and eliminate these intracellular pathogens.

## Conclusion

It was concluded that locally prepared bacterin toxoid proved their efficacy to stimulate both humoral and cell mediated immunity in rats as an experimental animal model.

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## Conflict of Interest

The authors declare that there is no conflict of interest.

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## بعض الاستجابات المناعية في الفئران المحقونة بالذوفان الجرثومي المحضر من المكورات العنقودية الذهبية المقاومة للميثيسيلين

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### الخلاصة

استهدفت هذه الدراسة إمكانية تحضير الذوفان الجرثومي المعطل من العزلة المحلية لبكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلين والمعزولة من حالة التهاب الضرع البقري في محافظة البصرة. تم الكشف عن بكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلين وتأكيدها باستخدام تفاعل البلمرة المتسلسل وبعد ذلك تم تحضير الذوفان الجرثومي منها. تم استخدام مجموع خمس وأربعون جرذاً من ذكور الجرذان البيضاء مقسمة في ثلاثة مجاميع رئيسية لتقييم الذوفان الجرثومي. للاستجابة المناعية، تم تقدير تراكيز الانترلوكين الرابع والخلايا التائية بنوعها CD4+ و CD8+ باستخدام الاليزا. أظهرت النتائج زيادة في تراكيز الانترلوكين الرابع والخلايا التائية بنوعيه CD4+ و CD8+ في المجموعة الملقحة مقارنة مع مجموعة السيطرة غير الملقحة. أن زيادة تراكيز الانترلوكين أدى إلى تحفيز الخلايا التائية المساعدة من النوع الثاني والتي أثرت بشكل مباشر على الخلايا البائية مؤدية تمايزها إلى خلايا البلازما المسؤولة عن إنتاج الأجسام المضادة المتخصصة ضد العزلة من بكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلين. الزيادة في تراكيز الخلايا التائية نوع CD4+ قد عززت الاستجابة المناعية الخلوية أيضاً نتيجة للتفاعل بينها وبين الخلايا المناعية المقدمة للمستضدات والتي تحمل على سطحها معقد التوافق النسيجي الكبير من النوع الثاني بينما الزيادة في تراكيز الخلايا التائية نوع CD8+ عززت الاستجابة المناعية الخلوية نتيجة للتفاعل بينها وبين الخلايا الجسمية المتأثرة والمقدمة للمستضدات والتي تحمل على سطحها معقد التوافق النسيجي الكبير من النوع الأول مؤدية في النهاية تمايزها إلى الخلايا التائية السامة والمسؤولة عن قتل الخلايا المصابة.