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Inactivated Vaccine Against Staphylococcus Aureus: Its Effect on the Nature of the Immuno-Inflammatory Response (Based on the Dynamics of the Level of Cytokines IL6, IL4 and IgG) and Therapeutic and Prophylactic Reliability of Use (Experimental Study)

Conflict of interest: nothing to declare.
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Abstract

Introduction. Staphylococcus aureus is classified as a dangerous pathogenic organism that causes several disorders in both humans and animals. The antibiotic resistance is a serious public health problem. Immunization still the best way to prevent and cure the S. aureus infection.
Purpose. Study of the impact of the created inactivated vaccine against S. aureus on the formation of an immuno-inflammatory response in the body of experimental animals by changes in the blood levels of pro- and anti-inflammatory cytokines IL6 and IL4, total immunoglobulin G and assessment of biological reliability.
Materials and methods. Bacterial inactivated vaccine technology prepared in this study by treated the bacterial cells with NaOH to obtained whole surface antigens. The prepared vaccine was injected in rats with 1×10^6 cells/ml to investigate the immunity response. The treated bacterial cells were examined by using scanning electron microscope (SEM). The experimental animal divided in to four groups which include the non-treated group (CT) control, group were injected with 300µl PBS. Intramuscular immunized (IM) group, intravenous immunized (IV) group and subcutaneous immunized (SC) group were injected with 300 µl of inactivated S. aureus vaccine at zero day and with 250µl booster dose at 14 and 28 days.
Results. In all vaccinated groups, a significant increase in the levels of IL6 and IL4 was noted in comparison with similar test indicators in the group of unvaccinated animals ($p<0.05$). The identified changes were accompanied by an increase in the total IgG content as a result of the use of an inactivated vaccine in all "therapeutic" (with animal vaccination) groups ($p<0.05$). The results showed that the developed vaccine induced immunity and provided reliable protection in a bacterial challenge test.

Conclusion. The inactivated S. aureus whole surface antigens vaccine give the protection to the experimental animals 100% against methicillin resistant S. aureus (MRSA) homologous challenge which considered big issue for humans and animals and threat the public health.
Keywords: staphylococcus aureus, surface antigens, rats, ELISA, IL4, IL6

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Инактивированная вакцина против Staphylococcus aureus: ее влияние на характер иммуновоспалительного ответа (по динамике уровня цитокинов IL6, IL4 и IgG) и лечебно-профилактическая надежность применения (экспериментальное исследование)

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Резюме

Введение. Золотистый стафилококк (Staphylococcus aureus) входит в группу наиболее опасных патогенных микроорганизмов, вызывающих ряд заболеваний как у людей, так и у животных. Его устойчивость к антибактериальным препаратам представляет собой серьезную проблему для общественного здравоохранения. Наиболее эффективным способом профилактики и лечения инфекций, вызванных S. aureus, по-прежнему является вакцинация.
Цель. Исследование воздействия созданной инактивированной вакцины против S. aureus на формирование иммуновоспалительного ответа в организме экспериментальных животных, выражающегося в изменении содержания в крови про- и противовоспалительных цитокинов IL6 и IL4 и общего иммуноглобулина G, и оценка ее биологической надежности.
Материалы и методы. Технология приготовления бактериальной инактивированной вакцины в данном исследовании включала обработку бактериальных клеток

раствором едкого натра (NaOH) для получения поверхностных цельноклеточных антигенов. Приготовленную вакцину вводили крысам в объеме 1×10^6 клеток/мл для изучения иммуновоспалительного ответа. Обработанные бактериальные клетки исследовали с помощью сканирующего электронного микроскопа (СЭМ). Экспериментальных животных разделили на четыре группы, включая контрольную (без введения в организм вакцины, СТ). Животным контрольной группы вводили 300 мкл физиологического раствора с фосфатным буфером (PBS). Животным групп внутримышечной вакцинации (IM), внутривенной вакцинации (IV) и подкожной вакцинации (SC) вводили 300 мкл инактивированной вакцины S. aureus в нулевой день и 250 мкл бустерной дозы в 14-й и 28-й дни исследования.

Результаты. Во всех вакцинированных группах отмечено достоверное повышение уровней IL6 и IL4 в сравнении с аналогичными показателями тестов в группе невакцинированных животных ($p<0,05$). Выявленные изменения сопровождались увеличением общего содержания IgG в результате применения инактивированной вакцины во всех «лечебных» (с вакцинацией животных) группах ($p<0,05$). Полученные результаты показали, что разработанная вакцина индуцировала иммунитет и обеспечила надежную защиту при проведении теста с бактериальным заражением.

Заключение. Инактивированная цельноклеточная (цельновирионная) вакцина с поверхностными антигенами S. aureus обеспечивала 100%-ную защиту экспериментальных животных от гомологического заражения метициллин-резистентным S. aureus (MRSA), представляющего собой серьезную угрозу здоровью людей и животных и проблему для общественного здравоохранения.

Ключевые слова: staphylococcus aureus, поверхностные антигены, крысы, ИФА (ELISA), IL4, IL6

■ INTRODUCTION

Staphylococcus aureus is an opportunistic human and animal pathogen that contaminates food and causes a wide range of infections, from skin and soft tissue infections to life-threatening illnesses [1], include toxic shock syndrome, osteomyelitis, septicemia, endocarditis, and pneumonia [2]. Drug resistance in S. aureus has been increasing steadily in recent years as a result of the abuse of antibiotics such as using them without a prescription, in large dosages, or for no reason [3], which caused bacterial evolution [4]. Methicillin-resistant often known as methicillin-resistant Staphylococcus aureus (MRSA) is resistant to most Beta lactam antibiotics as a result of the penicillin-binding proteins (PBPs) alteration that are repressed by antibiotics being replaced by PBP (PBP2a) function with limited affinity for most Beta lactam antibiotic resistant to methicillin in animals [5].The threat of Staphylococcus aureus (LA-MRSA) to human and animal health is significant [6]. This bacterial strain causes a number of illnesses that range from mild skin infections to serious, sometimes lethal disorders [7]. Up to 40% of instances of mastitis in dairy cows are caused by a variety of bacteria, with Staphylococcus aureus being the predominant pathogen among them [8]. Immunization is still the best way to prevent and cure S. aureus infections, even if there aren't many viable therapies for multidrug-resistant S. aureus strains [9].

The creation of innovative vaccine production technology that is efficient, simple, and effective is crucial to managing bacterial infection [9]. Extracellular vesicles, bacterial cell wall components and toxins are examples of typical biological nano-materials that will be used in vaccines and treatments in the future [10]. Non-living vaccine development provide a promising method for vaccine development. The empty, non-living cell envelope transmembrane structures develop on the cell surface as a result of action of sodium hydroxide (NaOH) leaving the cell envelopes vacant. The resultant of surface antigens of S. aureus benefits immune responses and protection against certain infections in experimental animal models [11]. The minimum inhibitory concentration and minimum growth concentrations of sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), and calcium carbonate were utilized in a novel method for generating multivalent surface antigens [12]. The NaOH mechanism was faster method of vaccine induction nonliving (cell wall surface antigens).

S. aureus peptidoglycan vaccination in experimental animals led to the acquisition of protective immunity against a fatal challenge [13]. Recent research revealed that vaccination of experimental animals with a cocktail of four surface antigens (IsdA, IsdB, serine-aspartate repeat A [SdrA], and SdrE) generated in opsonophagocytic antibodies and substantially protected against clinical S. aureus isolates. Components of the S. aureus cell wall have been reported to both humoral and cell-mediated immunity to be boosted [14].

Immune responses, hematopoiesis, inflammation, wound healing, and trauma all depend on cytokines, which are significant immunoregulators. Cytokines are a group of minor, secreted proteins that regulate a number of aspects of cell development and differentiation in certain cell types [15]. A strong pleiotropic cytokine, IL-4 controls cellular activation, differentiation, and apoptosis rescue and IL-4 causes the activation receptors to be down-regulated in mast cells and encourages cell death [16]. IL-4 plays a variety of biological tasks, including promoting the growth of T and B cells that have been activated as well as plasma cell differentiation in B cells [17]. Interleukin-6 (IL-6) is important for regulating temperature and metabolic activity as well as other components of the inflammatory response [16]. It is still hard to tell exactly how IL-6 plays a function in the pathophysiology of inflammation. In fact, IL-6 has been linked to both pro- and anti-inflammatory actions [18]. In the present study, using the MIC of NaOH, we developed new S. aureus surface antigens vaccine.

■ PURPOSE OF THE STUDY

Study of the impact of the created inactivated vaccine against S. aureus on the formation of an immuno-inflammatory response in the body of experimental animals by changes in the blood levels of pro- and anti-inflammatory cytokines IL6 and IL4, total immunoglobulin G and assessment of biological reliability.

■ MATERIALS AND METHODS

Sample collection

Five suspected Staphylococcus aureus isolated from cows with mastitis condition were obtained using sterile test tubes from Basrah dairy farms. The samples were collected between February 2022 to April 2022, and analysis was done after collection. The laboratory work was completed in Basrah Veterinary College.

Isolation and identification of S. aureus

According to a previous research [6], the milk samples were analysis for the isolation and identification of bacteria. The identification of S. aureus performed by Using the VITEK 2 system for each suspected isolate and the identification was verified using a molecular technique.

Molecular method

The genomic DNA was extracted by using (Bioneer/Korea) kitgenomic DNA from bacterial cells using the Gram-positive bacterial methodology. Conventional PCR amplification of essential nuc gene was used to amplify by using specific primers (Table 1). The PCR program was carried out as in Table 2.

Preparation the inactivated bacterial vaccine

Determination of NaOH MIC

Determination the MIC of NaOH for S. aureus was performed by using broth dilution method [19], bacterial culture of S. aureus was grown in nutrient agar and adjusted to 1×10^8 CFU/ml. The initial concentrations of NaOH were used as 10 mg/dl, 8 mg/dl, 7.5 mg/dl, 6 mg/dl and 5 mg/dl. The solution of NaOH were added to samples of the bacterial culture, and they were incubated at 37°C for 18 h. After incubation, the turbidity of each individual tube was assessed visually and the MIC was determined as the lowest concentration of NaOH that inhibit bacterial growth.

Production of inactivated vaccine

Inactivated S. aureus vaccine was produced by using the MIC of NaOH method as described previously [19]. In briefly, the biomass of 72-h-old S. aureus cells was collected by centrifugation (10,000 g for 10 min at 4°C) and washed three times with phosphate-buffered saline. One milliliter of the MIC of NaOH was added to 2 ml of the bacterial suspension (1×10^8 CFU/ml) and incubated at 37°C for 75 min. To determine the lysis rate, samples of cells treated with the MIC of NaOH and non-treated control cells were collected

Table 1
Nuc gene primers sequences with product size

Primer	Sequences of the primer (5 to 3)		Product size	Reference
nuc gene	Forward	GCCATTGATGGTGATACGGT	279 bp	(Fasihi et al., 2017)
	Reverse	AGCCAAGGTTGACGAACTAAAGC		

Table 2
The specific program of amplification the nuc gene

No	Stage	Temp/C	Time	Cycle
1	Initial denaturation	94	5 min	1
2	Denaturation	94	1 min	34
3	Annealing	55	1 min	34
4	Extension	72	1 min	34
	Final extension	72	10 min	1

at 15-min intervals (15, 30, 45, 60, and 75 min) then spread onto mannitol salt agar plates. After incubation at 37°C for 24 h, viable colonies were enumerated and the numbers of CFU/ml compared with control plate.

Determination of the DNA concentration of prepared vaccine

Samples of bacterial cells treated with the MIC of NaOH and non-treated control cells were collected. Their genomic DNA was extracted by using isolation kit (persto mini DNA extraction kit), according to the manufacturer's instructions. The extracted genomic DNA was analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide.

Sample preparation and examination by Scanning electron microscope examination

Morphological features of inactivated S. aureus vaccine were analyzed by scanning electron microscopy (SEM) [19].

Experimental animals

A total of 125 male Wistar albino rats (8 weeks age and 200–220 grams body weight / animal) were used. 75 of them were used in the vaccination program (Sc, IM and IV) whereas 25 of them were used as a control (CT) to determine the protection level of the prepared vaccine in challenge test at the end of the study, finally 25 of them have been used in the safety test to determine safety of each prepared vaccine in this study. Experiment using vaccination and challenge. A set of 100 rats were separated into four groups and given the names Ct, SC, IM, and IV in order to evaluate the immunogenicity and protective effectiveness of S. aureus inactivated vaccine. Sterilized PBS (300 µl) was administered subcutaneously to Group Ct rats (the non-immunized control group). The vaccine (1×10^6 cells/ml) in sterile PBS was administered intravenously, subcutaneously, and intramuscular to the rats in Group IM, Sc, and IV, respectively. Rats from Sc, IM and IV received three vaccinations at intervals of day 1, 14 and finally day 28. All rats were administered an intravenous with 300 µl of the challenge dose with virulent S. aureus at 2×10^8 CFU/ml of PBS two weeks following the previous inoculation (week 7). Blood samples were obtained from each rat's tail vein to assess the immunological response at several days (day 5, day 19 and day 33) for ELISA assay.

ELISA assay

According to the manufacturers' instructions, ELABSCIENCE®/USA ELISA kits were used to measure the quantities of IL6 and IL4 as well as IgG.

Statistical analysis

SPSS version 24 were used and ANOVA with LSD test used for data analysis, the significant differences between groups measured at P value ≤0.05 with mean Standard deviation.

■ RESULTS

Identification of MRSA

Five suspicious isolates from cows suffering from mastitis were analyzed using cultural methods. The five suspicious isolates were submitted to the Vitek 2 compact system that

GP identity card after performing the culture approach for primary identification. The results of Vitek2 revealed that underwent biochemical identification using a turbidity and colorimetric basis, with 99% portable analytical results.

Molecular Detection

After performing agarose gel electrophoresis, the nuc gene was amplified using specific primers, and the results showed a distinct band with a 279 bp size, indicating that the suspected isolates with positive vitek 2 compact system results also had positive PCR findings for the nuc gene (Fig. 1).

Antibiotics susceptibility testing

The Vitek 2 compact device was used to determine the antibiotics susceptibility of PCR-positive S. aureus isolates. The isolates were tested positive for resistance to more than 80% (20) of drugs from several antibiotics families. According to antibiotics sensitivity testing, all isolates was resist to Oxacillin which refer as all isolates were Methicillin Resistant S. aureus (MRSA) cause the use of cefoxitin as a surrogate marker for detection of mecA gene mediated methicillin resistance.

Minimum inhibitory concentration of NaOH

The results showed the minimum inhibition concentration of NaOH appears minor growth was observed with the concentration of 8.0 mg/ml for 60 min that decrease number of CFU on the nutrient agar as in Fig. 2.

Determination of the DNA concentration of prepared vaccine by Electrophoresis

After DNA extraction of treated isolates and control isolates DNA was run by electrophoresis in 1% agarose gel stained with ethidium bromide, the results showed clear bands of total extracted DNA of control while there were no clear bands for prepared vaccine of treated isolates with NaOH (Fig. 3).

Scanning electron microscope (SEM) examination

After scanning electron microscopy (SEM) examination of the treated cells. The control untreated S. aureus cells (Fig. 4 (A)). The image revealed the formation of transmembrane

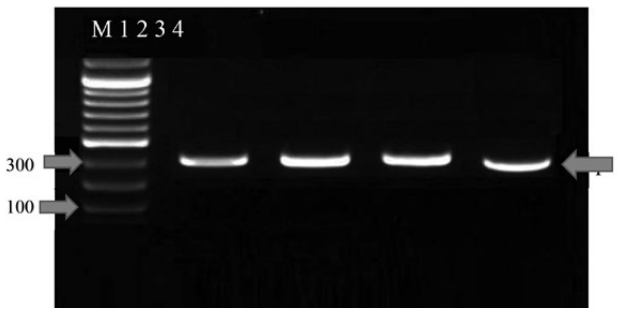

Fig. 1. Amplification results of using nuc gene (lane 1–4) primer with 279 bp, L.M.: marker. Agarose 1% concentration

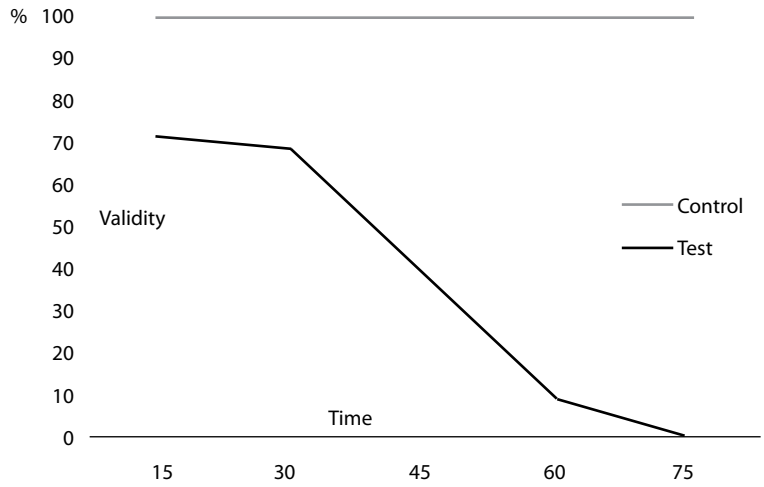

Fig. 2. The Curve of MRSA in CFU/ml different time after treated with NaOH

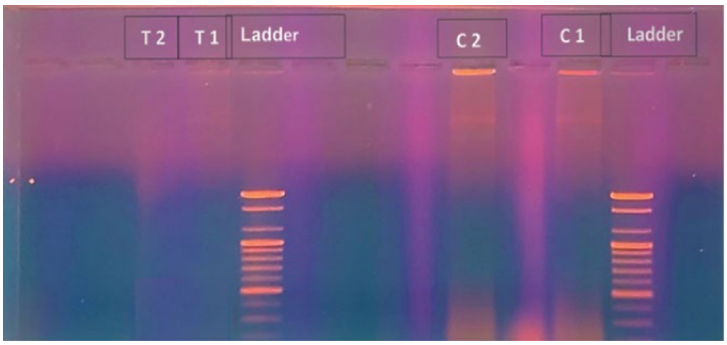

Fig. 3. Electrophoresis showed clear bands for C1 & C2 (control1 and Control2) while there were no band for both T1 and T2 (treated1 and Treated 2)

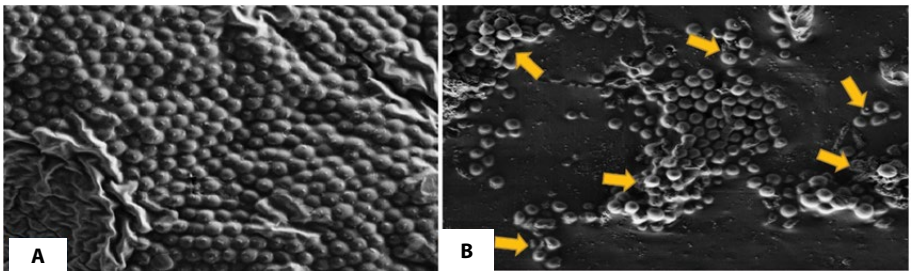

Fig. 4. A – SEM images for control untreated S. aureus cells; B – showed the transmembrane channel formation for treated S. aureus cells and production of Ghost cells

lysis tunnel structures in the treated cells, which are indicated by yellow arrows in the figure below. Additionally, the procedure of treatment with MIC of NaOH did not change the cell's morphology or cause damage to the cells, which is assumed to be a crucial component of surface antigens (Fig. 4 (B)).

ELISA Results

IL6 concentration

Utilizing an ELISA kit to measure IL6, results revealed that all vaccine groups had increasing amounts of the immune ligand 6 (IL6) at days 5, 19, and 33 compared to the non-vaccinated group, with statistically significant differences (P≤0.05) were found. IV group had the highest levels of concentration between all vaccinated groups (Table 3).

IL4 concentration

Using an ELISA kit for measuring IL4, it was revealed that all vaccine groups had elevated concentration of IL4 at days 5, 19, and 33 compared to the non-vaccinated group, with statistically significant differences (P≤0.05) were found. The IM group displayed the highly concentration of all groups (Table 4).

IgG concentration

ELISA IgG test results indicated that all vaccinated groups had increasing levels of IgG than the non-vaccinated group at days 5, 19, and 33, with significant differences. The IM group had the highest concentration (Table 5).

Table 3
The concentration of IL-6 (µg/l) at 5, 19 and 33 day in vaccinated and non-vaccinated groups

Period of observation / Groups of animal	IL-6 (µg/l)		
	Day 5	Day 19	Day 33
Ct	3.1±1.5	3.3±1.6	2.8±1.8
IV	28.5±2.7	54.8±5.9	77.2±11.5
IM	8.4±3.0	25.55±7.75	38.25±10.45
SC	27.15±4.25	54.4±15.9	67.95±12.25

Note: data were presented as mean±SD; Significance value under P≤0.05.

Table 4
The concentration of IL-4 (µg/l) at 5, 19 and 33 day in vaccinated and non-vaccinated groups

Period of observation / Groups of animal	IL-4 (µg/l)		
	Day 5	Day 19	Day 33
Ct	57±9.0	48±12	51±8.5
IV	83.5±14.5	154±66	158±62
IM	78±12	114±30	250±148
SC	178.5 ±71.5	115±17	196.5±44.5

Note: data were presented as mean±SD; significance value under P≤0.05.

Table 5
The concentration of IgG (μg/l) at 5, 19 and 33 day in vaccinated group and non-vaccinated group

Period of observation / Groups of animal	IgG (μg/l)		
	Day 5	Day 19	Day 33
Ct	3.1±1.5	3.3±1.6	2.8±1.8
IV	28.5±2.7	54.8±5.9	77.2±11.5
IM	8.4±3.0	25.55±7.75	38.25±10.45
SC	27.15±4.25	54.4±15.9	67.95±12.25

Note: data were presented as mean±SD; significance value under P≤0.05.

Table 6
Percentage and number of death and a live animals after challenge test

Group	No.	Live	Death	Survival Percentage
Ct	10	3	7	30
SC	10	10	0	100
IM	10	10	0	100
IV	10	10	0	100

Challenge test

The challenge test was conducted on day 42 of the study to evaluate the level of protection provided by the prepared vaccine that elicited an immune response in rats. The test revealed the proportion of animals that survived when compared to control animals that had not received the prepared vaccine. Animal survival rates were 100%, and every animal that had received vaccinations via various injection techniques survived. More than 70% percentage death occurred in the unvaccinated group while displaying the virulent bacterium with a dosage of $5{,}526\times10^{10}$ CFU/ml, which represented LD50 (Table 6).

■ DISCUSSION

Staphylococcus aureus bacteria had a number of essential virulence characteristics that contributed to the grave illness they caused in both humans and animals [20], therefor the vaccination could be a better solution for S. aureus management.

In this study, we observed that the Vitek method produced positive outcomes as a S. aureus with a higher confidence rate (99%) for all suspected isolates that demonstrated a clear growth on mannitol salt agar. Also, when conducted a traditional PCR investigation, all of these positive isolates showed distinct nuc gene bands. The compatibility of the PCR with the Vitek system results was agree with many studies such as [21–25] a which these studies evaluated the performance of Vitek system with other methods. The Vitek system is regarded as one of the more effective and affordable choices for the regular detection of microorganisms.

Due to certain isolates' resistance to cefoxitin, which indicates that all isolates are Methicillin Resistant Staphylococcus aureus (MRSA), cefoxitin is used as a surrogate marker for the identification of mecA- gene-mediated methicillin resistance. The results agreement with [26, 27], which they recorded as a higher percentage of MRSA percentage with S. aureus isolates. The high incidence of multidrug-resistant MRSA in milk samples in

this investigation may be attributed to producers' overuse of antibiotics and unhygienic farming practices. The public health of the owners may suffer as a result.

The development of a reliable inactivated S. aureus vaccine is urgently needed for infection control and prevention. The use of NaOH was easier and less expensive than other approaches like the E-mediated lysis procedure [24]. In this study, established that the development of a new inactivated vaccine may be achieved by rapidly and effectively lysing S. aureus cells using NaOH. The S. aureus cells were treated with 8.0 mg/ml of MIC NaOH, which showed a high lysis ratio after 60 minutes. This inline to the study conducted by [25] in which S. aureus cells were treated with 7.5 mg/ml of NaOH, and the effectiveness of the lysis appeared after 60 minutes, indicating that the chemical approach of NaOH MIC was the most efficient and swift.

Using SEM, we compared the production of chemically inactivated vaccine in treated cells that had been exposed to MIC of NaOH to untreated cells. The photos following the NaOH treatment's lysis period showed the presence of a transmembrane lysis tunnel. The images showed same results of another study [25] which observed the similar morphological alterations in the treated cells due to transmembrane tunnel development, which caused the cells to be evacuated and become empty cells.

In contrast to control cells, which showed a clear band of genomic material after being exposed to MIC of NaOH for 60 min, the exposed cells showed no DNA bands after agarose gel electrophoresis. It was taken into consideration as a major signal that the created inactivated vaccine was missing both DNA and cells' genetic components. The results matching with study of [24] These studies support the hypothesis that DNA free of prepared inactivated vaccines may be effectively induced using the NaOH method.

Our findings show a significant increase in IL-6 levels in rats given the prepared inactivated vaccine, supporting the potential of the vaccine to stimulate the production of proinflammatory and inflammatory cytokines, which play different roles in the immune response [26]. These findings were consistent with [27] that revealed an increase in IL6 in animals following S. aureus vaccination. This finding is in line with descriptions of IL-6 many effects on T cells that have previously been reported, including its ability to promote the growth and differentiation of CD4+ [28]. Further encouraging the growth of peripheral naive and memory T lymphocytes. It has been demonstrated that IL-6 has the power to significantly increase the proliferation of Th1 cells, making them immune to suppression [29].

Our results reveal a considerable rise in IL-4 levels in rats given the produced inactivated vaccine, which may point to an improved humoral immune response. Our findings concur with those of [30] who showed that immunization increased IL-4 levels. T-helper cells may develop primarily into Th1 and Th2 cells, and IL-4 influence on naive CD4+ T-cells or TH0 (naive T-helper) causes their differentiation into Th2 cells when it occurs for the first time during the vaccination process. One of IL-4 key roles is to induce Th2 cells to produce additional IL-4, which directly impacts B cells and causes them to differentiate into plasma cells, which are responsible for producing specialized antibodies [31].

Over the study period, the IgG rate had increased in each group relative to the non-vaccinated group, achieving their peak in the SC group on day 19. These outcomes were in line with a [19] that showed an increase in IgG levels following vaccination with a inactivated S. aureus vaccine. throughout the vaccination period. The IgG antibody levels in the vaccinated groups also increased significantly after each booster injection with

inactivated vaccine, with SC group rats displaying the strongest IgG antibody response. The results confirm that immunization of rats with prepared vaccines can strongly stimulate the production of antibodies and provide protection against virulent strains.

The challenge test findings of the vaccinated group and the non-vaccinated group following injection with a virulent isolate the final results showed that the vaccinated group had 100% more protection than the non-vaccinated group. The vaccinated groups had no a mortality risk, in contrast to the non-vaccinated group, which had a death rate of 70%. We suggested that animals be protected against homologous challenges by receiving full immunity and defense against pathogenic virulence S. aureus strains from the inactivated whole surface antigens vaccine.

■ CONCLUSION

The inactivated S. aureus whole surface antigens vaccine give the protection to the experimental animals 100% against methicillin resistant S. aureus (MRSA) homologous challenge which considered big issue for humans and animals and threat the public health.

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