OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Immunological Evaluation of Methicillin Resistant *Staphylococcus aureus* (MRSA) of *SpA* Vaccine from Cases of Mastitis in Cows

Sabrin Ibraheem Mohsin*, Aida Bara Allawe

Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Iraq.

Abstract | Mastitis is a prevalent and economically significant disease affecting dairy cows globally. The disease is regarded as a significant welfare concern within the dairy business, with the loss of productivity and the premature culling or mortality of affected cows. Losses are attributable to the elevated expenses of veterinary pharmaceuticals and the financial impact of unsalable milk from treated cows. Mastitis may result from either infectious or environmental diseases, both of which are more effectively prevented than treated. The predominant pathogen isolated from infected udders is *Staphylococcus aureus* (*S.aureus)*. Consequently, there exists a worldwide requirement for the advancement of innovative therapeutic procedures. *S. aureus* exhibits several virulence factors. *Protein A* (*SpA*) is a significant contender for immunization. In this case, *SpA* gene was cloned in to PET28a plasmid for purification with six Histag.100 mg of *SpA* recombinant protein was synthesized once the recombinant *SpA* protein was obtained Forty_ eight Female lactating BALB/C mice were divided into four groups: Group 1 (G1) was injected intramammary with phosphate buffer saline, Group 2 (G2) was injected with intra mammary with *S. aureus* only, Group 3 (G3) was administered 20 µg of *SpA* protein subcutaneous only, and Group 4 (G4) was subcutaneously injected with 20 µg of *SpA* protein mixed with an equal volume of incomplete Freund's adjuvant. A booster dose of the vaccine was given after 14 days. At 21 days, the mice were challenged with 5 × 10^8 *S.aureus*, and serum samples were collected at 7 days post-challenge.enzyme linked immunosobant assay (ELISA) was utilized to evaluate interleukin 12 (IL12) and interferon gamma (IFN-γ) in sera samples. The serum samples exambed by Eliza showed there was significant differences ($p < 0.05$) in IL12 , IFN-γ between all vaccinated groups and the control group.The mice immunized with *SpA* vaccine and adjuvant showed a significant increase of IL12 , IFN-γ as compared with the control group (<0.05) . Also, The all vaccinated groups had lower bacterial count in their mammary glands as compared to group was given *S. aureus* only.The histopathological examination of the spleen in all vaccinated groups revealed reactive hyperplasia of lymphoid follicles were moderate to mild in white pulp of spleen with parafollicular and focal aggregations of mononuclear cells (histiocytes and lymphocytes), "eosinophilic amyloid-like material in dilated red blood sinuses." cuffing and parafollicular infiltration of lymphocytes and diffuse infiltration of lymphocytes as compared to others groups.

Keywords | *SpA vaccine*, Incomplete freund's adjuvant, IL12, IFN-γ, Enzyme linked immunosorbent assay

Received | March 21, 2025; **Accepted** | April 08, 2025; **Published** | June 02, 2025
***Correspondence** | Sabrin Ibraheem Mohsin, Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Iraq; **Email:** sabrin.i@covm.uobaghdad.edu.iq
Citation | Mohsin SI, Allawe AB (2025). Immunological evaluation of methicillin resistant *Staphylococcus aureus* (MRSA) of *SpA* vaccine from cases of mastitis in cows. Adv. Anim. Vet. Sci. 13(7): 1435-1444.
DOI | https://dx.doi.org/10.17582/journal.aavs/2025/13.7.1435.1444
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

Mastitis usually denotes inflammation of the breast gland, nearly invariably caused by bacterial or fungal diseases. The condition is marked by physical, chemical, and typically bacteriological alterations in the milk, alongside pathological modifications in the glandular tissue (Ismail 2017; Mahmoud and Yassein 2024). Mastitis arises when leucocytes, or white blood cells, are activated within the mammary gland, usually in response to bacterial invasion of the teat canal. Bacterial toxins damage the tissue that secretes milk and the many channels inside the mammary gland. The most common cause of mastitis in dairy cattle is bacteria. The main route of infection between infected and uninfected people is infectious bacteria, which are usually found on the udder or teat surface of infected cows. (Pérez *et al*., 2009; Ahmed and Yousif, 2021). The bovine mammary gland possesses multiple defense systems to thwart bacterial invasion and consequent illness. The defenses are intricate and interconnected, comprising anatomical barriers of the teat canal, cellular components such as macrophages, neutrophils, and natural killer (NK) cells, as well as soluble defense molecules including cytokines, antibodies, and complement (Ferreira *et al*., 2013; Saleem *et al*., 2021). The primary response is followed by the more specialized adaptive immunity, which includes B and T lymphocytes (Fournier and Philpott, 2005). B lymphocytes are seen in the mammary gland during *S. aureus* intramammary infection, largely generating antibodies specific to the pathogen, while also engaging in phagocytosis and antigen presentation.

T lymphocytes are also located in the mammary gland. During this phase, the cluster of differentiation 4 (CD4+) T: cluster of differentiation 8 (CD8+)T cell ratios are reversed from the normally high CD4+ levels seen in healthy mammary glands. Park and colleagues demonstrated that activated CD8+ T lymphocytes dominate the T lymphocyte population during *S. aureus* mastitis, suggesting a potentially critical effector role for these cells (Park *et al*., 1993). The categorization of CD8+ T lymphocytes in the mammary gland after chronic *S. aureus* infection as either CD8+ cytotoxic or CD8+ suppressor subsets is unresolved, dependent on the cytokines they produce. CD8+ cytotoxic clones are recognized by their production of IFN-γ, "a T-helper 1 (Th1) cytokine (Fong and Mosmann, 1990), while suppressor clones are reported to produce interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin10 (IL-10), which are T-helper 2 (Th2) cytokines (Mahmoud and Yassein, 2024).

"T-helper cells (Th cells), also known as CD4+ cells" are divided into two subtypes: Th1 and Th2. These subtypes are responsible for cell-mediated immune responses and antibody-mediated immune responses, respectively." (Al Dujaily and Mahmood, 2021; Ahmed *et al*., 2023). It is widely acknowledged that the Thl pathway, which is controlled by the cytokines IFN-γ, IL-12, and interleukin 2 (IL-2), plays a critical role in the defense mechanism against intracellular infections (Kidd, 2003). The Th2 pathway is alternatively stimulated by the cytokines IL-4, IL-5, interleukin 6 (IL-6), and IL-10 (Masso-Welch *et al*., 2012). Type 1 cytokines are associated with reactions that are assisted by macrophages, cytotoxic T cells, and neutrophils. On the other hand, type 2 cytokines enhance B cell differentiation, proliferation, and antibody generation, in addition to limiting the activity of macrophages (Ferens and Bohach, 2000; Ibraheim *et al*., 2023).

Numerous therapeutic and preventive measures for mastitis have been implemented throughout the years to enhance the overall health, welfare, and productivity of dairy cow (Sharun *et al*., 2021). Vaccine development against common udder pathogens has been advancing in the past few decades. The subunit vaccinations, which combine an adjuvant and multiple *S. aureus* surface proteins, are the most promising experimental vaccines.

"Protein A (*SpA*) is a major virulence factor of *S. aureus*" (Mandelli *et al*., 2024). In this study, evaluated the recombinant protein (*SpA* protein) encoding by *S.aureus* in order to use as a possible candidate for immunization against mastitis caused by *S..aureus*. Then, evaluated the IFN-γ and IL12 in the sera of mastitic BALB/C mice model.

MATERIALS AND METHODS

Bacterial Strains and *SpA*- Protein Vector

Luria-Bertani broth (LB) was employed to isolate and identify clinical mastitis bovine MRSA bacteria at 37°C until preparation for PCR amplification. *Escherichia coli* Rosetta (DE3) from Novagen, USA, was utilized in the cloning procedure to generate the recombinant proteins. The plasmid pET-24a (Novagen, USA) served as the expression vector (Sabrin and Aida, 2025, under press).

The cloning procedure of *SpA* gene was involved the following steps:

Amplification and cloning of *SpA* gene: The GenEluteTM plasmid miniprep kit (Merk,Argentina) was used for DNA extraction. A 750 bp fragment of the *SpA* gene was amplified using NotI-tailed forward (TTG CGG CCG CG AAG CTC AAC AAA ATG CT) and XhoI-tailed reverse (GCC GTC TTC TTT ACC AGG TTC TCG AGA A) primers. The PCR technique started with an initial denaturation of 5 minutes at 95 ˚C, followed by 33 cycles, including denaturation at 95 ˚C for 30 seconds, annealing at 61 ˚C for 55 seconds, extension at 72 ˚C for 55 seconds, and a final extension at 72 ˚C for 10 minutes.

PCR reaction (50 µl) 25 µl of 2X PCR Master Mix, 3 µl of MRSA DNA, 2 µl of each forward and reverse primer, with the total volume adjusted to 50 µl using deionized water. Restriction enzymes were used to cleave the purified *SpA* fragment prior to its ligation into the pET-24a vector, which contains six its residues at the C-terminus to facilitate the purification of the resultant protein. The recombinant vector pET24a-*SpA* was introduced into competent *E. coli* BL21(DE3) strains using heat shock transformation. Figure 1 demonstrates that colony PCR, restriction endonuclease digestion, and sequencing were used to verify the integrity of the recovered plasmid (Khateb, 2014; Vahdani *et al*., 2021).

Expression of *SpA* protein: A competent BL21(DE3) was created by transforming the pET24a/SpA construct to overexpress the protein. LB agar supplemented with 50 µg/ml of kanamycin was used to cultivate cultures. The colonies harboring pET24a/*SpA* were cultivated at 37 ◦C in 5 ml of LB medium that was treated with kanamycin.

The T7 promoter was activated in the vector that was expressing the recombinant protein by injecting IPTG (Sigma, USA) at a final dose of 1 mM. This was done during the early exponential phase of growth, which was measured at an optical density of 600 nm of 0.6. After stimulating the expression of the protein for 4 hours with 1.0 Mm IPTG, the cell pellets were subjected to protein expression analysis using 12% SDS-PAGE at 30 ◦C (Khateb 2014; Vahdani *et al*., 2021).

Purification of 6xHis-tagged *SpA* protein: The ideal expression parameters (temperature, IPTG concentration, and duration) were determined at the flask level, which included 50 milliliters of bacterial culture. The parameters were then adjusted to reflect one liter of culture. Following the collection of recombinant protein-expressing bacteria, their pellets were resuspended in a lysis solution containing protease inhibitors.

The lysis solution had a pH of 8.0 and included 8 M urea, 0.1 m NaH2PO4, and 0.01 M Tris. The cell suspension was centrifuged for 20 minutes at 10,000 revolutions / min and underwent sonication. Ni-nitrilotriacetic acid (NiNTA) affinity chromatography was used to purify recombinant proteins (about 35.8 kDa) from the supernatant under denaturing conditions, using His-tag interactions post-centrifugation. G Bioscience, according to the manufacturer's guidelines. An elution buffer with a pH of 4.5 was used to elute fractions of pure *SpA* protein after washing steps using a wash buffer composed of 100 mM NaH2PO4, 10 mM Tris-HCl, and 8 M urea at a pH of 6.4. The Bradford protein assay and Nanodrop spectrophotometer measured the protein concentration in the output fractions, which were then analyzed using SDS-PAGE. After dialysis with 0.1 m phosphate-buffered saline (PBS, pH = 7.4) for 72 hours to eliminate urea, the *SpA* solutions were filtered (0.22 µm) and preserved at −70 ◦C (Khateb, 2014; Vahdani *et al*., 2021).

Bacterial Preparation for Challenge

S. aureus was prepared as following *S.aureus* was injected in two mice for increased pathogenicity after grown at 37 ◦C in brain–heart infusion and the bacteria were extracted by centrifugation at 3000× g for 15 minutes at 4 degrees Celsius. Phosphate-buffered saline (1X PBS, pH 7.2) was used to wash the cell pellet after it had been prepared. As previously disclosed, the L4 and R4 mammary glands of mice were infected with a volume of 50 microliters containing 5 × 10 ^{8} colony-forming units per milliliter (cfu/ml) of *S. aureus* (Garc|èa *et al*.,1996; Johnzon *et al*., 2016).

Animals and Immunization

The present investigation was conducted at the Animal House of the College of Veterinary Medicine, University of Baghdad. Female lactating BALB/c mice, weighing 20–25 g, were housed in normal cages and had unrestricted access to food and water for one week prior to the commencement of the experiment. Mice categorized into four types.

G1 was injected with phosphate buffer saline, G2 Injected with *S.aureus* only, G3: injected with *SpA* protein subcutaneously and G4 were injected subcutaneously with *SpA* protein 20µg with equal volume of incomplete Freund's adjuvant. Abooster dose of vaccine after 14 days was admistrated. A dose-response curve established to ensure this dose would induce mastitis without causing excessive mortality by using several dilutions of a number of doses each dilution was tested on a group of mice and it was found that 5 × 10^{8} cfu/ml the appropriate one to cause mastitis. At 21 days mice challenged with 5 × 10^{8} cfu/ml *S..aureus* according to (Garc|èa *et al*.,1996; Johnzon *et al*., 2016).

Subsequently, blood samples were obtained via cardiac puncture on days 7, 9, and 11 of the challenge, and permitted to coagulate at ambient temperature for 10 to 20 minutes before centrifugation. Centrifuged at 2000-3000 RPM for 20 minutes. The serum samples were preserved at −20 ˚C until utilized.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was used to evaluate the quantity of IFN-γ and IL12 in sera samples of mice. at 7 ,9,11 days of challenge which were considered earlier time points to capture the full cytokine response using Kit (BT LAB, China) according to the manufacturer's recommendations. (IL12 , IFN-γ are secreted at the onset of inflammation in the early stages of innate immunity by phagocytic cells. And these cy-

tokines were considered as mediators for adaptive immune responses). In order to perform ELISA, the 96-well polystyrene plates were coated with mouse IL12, IFN-γ.

Concentration of IL12

IL-12 in the samples binds to antibodies coated on the wells, followed by the addition of biotinylated mouse IL12 antibodies, which bound to the IL12 present in the samples. Subsequently, streptavidin-HRP is introduced to bind with the biotinylated IL-12 antibody.

Following incubation, unbound streptavidin- horse radish peroxidase (HRP) is removed during a washing phase. The substrate solution is subsequently added, resulting in color development proportional to the quantity of mouse IL-12 present. The reaction was halted by the addition of an acidic stop solution, and absorbance was measured at 450 nm. The cytokine quantities were quantified using the standard curve for each individual cytokine.

Concentration of IFN-г

IFN-γ was detected in samples that adhered to antibodies coated on the wells; subsequently, biotinylated mouse IFN-γ antibodies were introduced and bound to the IFN-γ present in the samples. After that, streptavidin-HRP is employed in order to establish a binding relationship with the biotinylated IFN-γ antibody. During the washing phase, this unbound streptavidin-HRP is removed once the incubation period has been completed. The third step involves the addition of the substrate solution, which ultimately leads to the production of color that is proportionate to the amount of mouse IFN-γ.After the addition of an acidic stop solution, the process was brought to a halt, and the absorbance was measured at 450 times the wavelength. In order to determine the quantities of each specific cytokine, the standard curve was utilized for the quantification process.

Enumeration of *Staphylococcus aureus* Count

The spread plate count method was utilized in order to carry out microbiological counts of *S. aureus* on mannitol salt agar (MSA). After seven days from the challenge, the mice were put down. Each mammary gland was collected in an aseptic manner, homogenized, and then diluted in phosphate-buffered saline (PBS) to the necessary concentration. Following the preparation of tenfold serial dilutions from the initial homogenate, twenty microliters of each diluted sample were carefully placed onto blood agar plates. These plates were then incubated at 37 degrees Celsius for twenty-four hours before the bacterial colonies were counted. *S. aureus* count was determined by identifying golden yellow colonies on blood agar with catalase and coagulase-positive isolates, complete hemolysis on blood agar, and the number of colony-forming units per gram of the test sample was determined by the following formula, with some modifications. Presumptive colonies were subjected to a confirmatory test (Harley *et al*., 2002; Da Silva *et al*., 2018).

$$CFU\ per\ gram\ of\ sample = Cd \times vd \times vC$$

where CC is the number of colonies, dd is the dilution factor, and vv is the volume of infected material (in mL).

Histopathology

All animals were euthanized at the conclusion of the studies, and spleen samples were obtained. We preserved the tissues in a 10% formaldehyde solution and subsequently processed them using a histokinete as per standard protocol. Paraffin blocks containing implanted tissue slices were sectioned using a microtome and stained with Hematoxylin and eosin, subsequently examined under a light microscope (Luna, 1968).

Statistical Analysis

Data statistical analysis was conducted utilizing SAS (Statistical Analysis System - version 9.1). two-way ANOVA because I have two variables groups and periods. and the Least Significant Difference (LSD) post hoc test were conducted to evaluate significant differences among means. A P value of less than 0.05 is deemed statistically significant.

RESULTS

IL12 Concentration

It was observed that there were notable variations in IL-12 levels ($p < 0.05$) between all of the groups that were vaccinated and the control group. According to the data presented in Table 1 and Figure 1, the mice that were inoculated with vaccine protein A and adjuvant exhibited a noteworthy rise in IL-12 levels when compared to the control group (p-value 0.001).

Table 1: Means±SE of IL-12 concentration between vaccinated and non vaccinated groups (groups x periods interaction) by using ELISA.

Groups	7 days	9 days	11 days
Control (-)	A62.04±1.63d	A62.04±1.63d	A62.04±1.63d
Control (+)	A115.68±2.04c	A112.87±2.85c	A110.68±4.03c
SpA protein	A221.31±4.01b	A214.12±3.67b	B188.50±3.26b
SpA protein plus Incomplete Freund's adjuvant	A249.43±3.67a	A240.68±4.02a	B200.68±4.01a
LSD	8.76		

A statistically significant difference (P<0.05) exists between the means of the same column that include a different tiny letter.

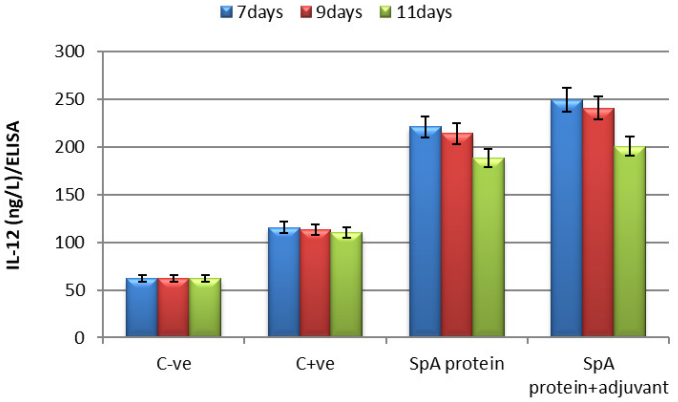

Figure 1: Comparison of IL12 concentration between experimental groups.

IFN-γ Concentration

The levels of IFN-γ for all of the vaccinated groups and the control group were found to be significantly different ($p < 0.05$). A significant increase in IFN-γ was observed in the mice that were inoculated with vaccine protein A and adjuvant, as compared to the control group (p-value < 0.05), as demonstrated in Table 2 and Figure 2.

Table 2: Means±SE of IFN-γ concentration between vaccinated and non vaccinated groups (groups x periods interaction) by using ELISA.

Groups	7days	9days	11days
Control (-)	A43.00±1.91c	A43.00±1.91c	A43.00±1.91d
Control (+)	A89.00±3.67b	B62.21±2.65b	B56.14±3.03c
*Sp*A protein	A139.35±2.76a	C70.29±3.41b	B117.92±4.22b
SpA protein plus Incomplete Freund's adjuvant	C44.47±1.21c	A176.50±3.54a	B131.61±3.83a
LSD	8.27		

A statistically significant difference (P<0.05) exists between the means of the same column that include a different tiny letter.

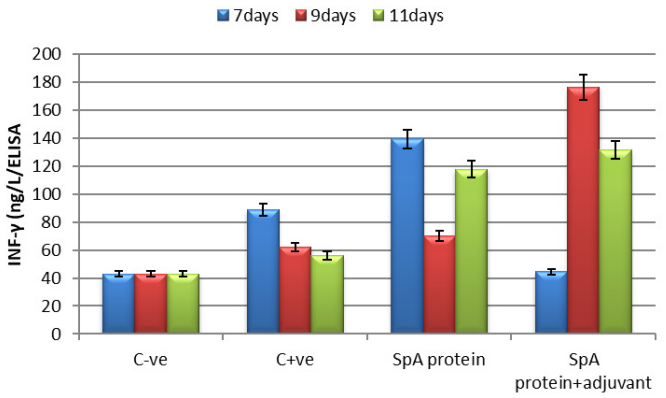

Figure 2: Comparison of IFN-γ concentration between experimental groups.

Bacterial Load

there were significant differences (P<0.05) in bacterial count between all vaccinated groups and the control positive group. The all vaccinated groups had lower bacterial count in their mammary glands as compared to group given *S. aureus* only, also the group was given *SpA* protein plus Incomplete Freund's adjuvant had lower bacterial count in their mammary glands with mean 0.36±0.07c log10 cfu /g as compared to control group (Table 3).

Table 3: Detection of bacterial load in the mammary glands of mice immunized with Protein A.

Groups	Log10 average number of bacteria (CFU) recovered from mammary glands ± SE	Anti $\log_{10}$ CFU
Control (-)	-	0.00
Control (+)	2.11±0.08a	128.82
SpA protein	1.59±0.07b	38.90
SpA protein plus Incomplete Freund's adjuvant	0.36±0.07c	2.29
LSD	0.22	

Histopathological Analysis

The tissue sections of spleen from groups injected with vaccine *SpA* recombinant protein then challenged with virulent *Staphylococcus aureus* ; their showed reactive hyperplasia of lymphoid follicles were moderate to mild in white pulp of spleen with parafollicular and focal aggregations of mononuclear cells (histiocytes and lymphocytes) in dilated sinusoids of red pulp (Figure 3A, 3B and 3C) also there was precipitation of eosinophilic amyloid-like material (Figure 3D) in dilated red blood sinuses also there focally around lymphoid follicles (Figure 3E).

The tissues of spleen from *SpA* vaccine plus incomplete Freund's adjuvant group; appeared activation of lymphocytes in white pulp also there are perivascular (sinuses of red pulp) cuffing and parafollicular infiltration of lymphocytes (Figure 3F and 3G), activation of lymphocytes also seen in interstitial of red pulp (Figure 3H).

The hyperplasia of lymphoid follicle in tissue sections from spleen in positive group was prominent as in (Figure 3I) with fusion of proliferated lymphoid follicles (Figure 3J). Other section showed dilation of blood vessels sinuses in red pulp contains mononuclear cells (histiocytes) (Figure 3K), as compared with negative group in (Figure 3L).

DISCUSSION

The immune response against intracellular bacterial pathogens is complex and regulated by various signaling molecules, including cytokines.It has been demonstrated in the past that the release of IL-12 by macrophages or dendritic cells (DCs) is essential for the optimum cell-mediated im-

mune responses against a wide range of intracellular infections (Al-Hamdani *et al*., 2023).

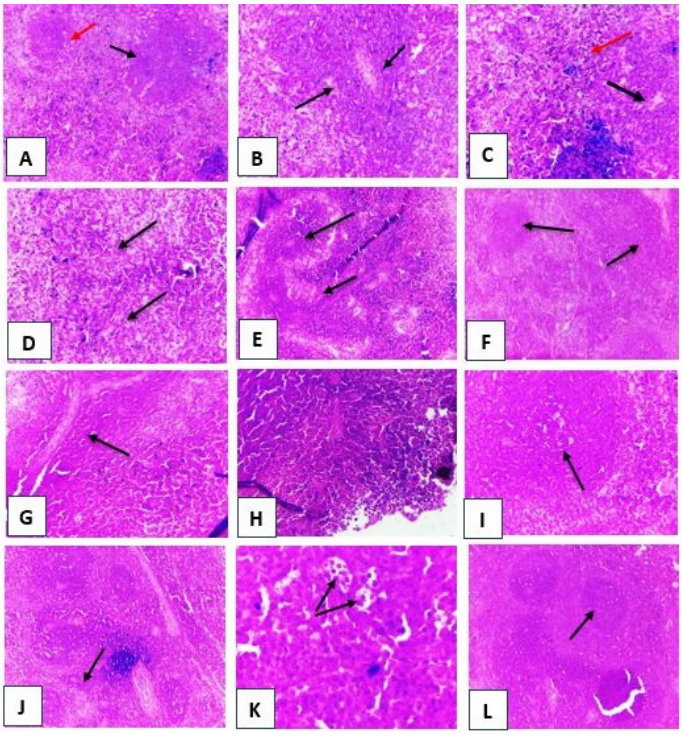

Figure 3: Histopathological section in spleen tissue across experimental groups shows; **A:** moderate follicular hyperplasia (black arrow) and focal aggregation of lymphocytes in dilated red pulp sinuses (red arrow); **B:** elongation of hypertrophied eccentric arteriole (black arrow) in reactive lymphoid follicle; **C:** congestion of blood vessel sinuses of red pulp (black arrow) contain histiocytic cells with focal aggregation (red arrow); **D:** dilatation of blood vessel sinuses of red pulp precipitated with amyloid-like material (arrow); **E:** focal precipitation of amyloid-like material (arrow). (H and E stain, 200X); **F:** activation of lymphoid follicles (arrow). (H and E stain, 200X); **G:** parafollicular infiltration of lymphocytes (arrow). (H and E stain, 200X); **H:** activation of white palp. (H and E stain, 400X); **I:** hyperplasia of lymphoid follicle (arrow). (H and E stain, (200X); **J:** hyperplasia of lymphoid follicle (arrow). (H and E stain, (200X); **K:** dilated red pulp blood vessels sinuses (arrow) contains mononuclear cells (histiocytes). (H and E stain, (400X); **L:** negative group shows normal lymphoid follicle in white pulp (arrow) and red pulp. (H and E stain, (100X). **Panel A, B, C, D, E:** Vaccine group; **Panel F, G, H:** Vaccine + adjuvant group; Panel I, J, K: positive control group; **Panel K:** negative control group.

In the mammary gland, leukocytes, including neutrophils, macrophages, and lymphocytes, are the predominant cells of innate immunity. DCs and mammary epithelial cells should be included among the other cell types in leucocytes. The letters are situated at the interface between the body and invading microbes, constituting a crucial element of an effective immune response (Fakhry *et al*., 2022).

When injected *SpA* protein IL-12 is produced by DCs, macrophages, neutrophils. Additionally, it has been demonstrated that non-immune cells, such as infected keratinocytes and osteoblasts, as well as epithelial and endothelial cells, are capable of producing a certain quantity of this cytokine (Aragane *et al*., 1994; Bost *et al*.,1999).

Macrophages make use of proteases and reactive oxygen species to eliminate the germs during the phagocytosis. Within the presence of opsonic antibodies, these cells have the potential to exhibit an increase in their phagocytic activity.

Macrophages have the ability to release chemicals that encourage the migration of neutrophils as well as their ability to kill bacteria (Andrés *et al*., 2022). The activation of macrophages causes them to secrete prostaglandins, leukotrienes, and cytokines, all of which have the potential to amplify the inflammatory processes that occur locally (Abdulkhaleq *et al*., 2018).

Neutrophils serve as the secondary defense mechanism of the cow mammary glands innate immune system. They are drawn into the milk by chemotactic stimuli where they phagocytose pathogens, hence playing a vital role in the eradication of invading microorganisms (Rainard and Riollet, 2006).

Macrophages, neutrophils, and DCs produce IL-12 through toll like receptors (TLRs) and other receptors, reacting to both membrane-bound and soluble signals from activated T cells and NK cells, in addition to components of the inflammatory extracellular matrix via cluster of differentiation 44 (CD44) and TLRs (Hamza *et al*., 2010).

In the immune system, lymphocytes are specialized cells that are distinguished by membrane receptors that are able to identify antigens on the cell surface. It is believed that the Thl pathway, which is driven by the cytokine IL-12, plays a significant role in giving protection against germs (Ferens and Bohach, 2000). There was a discernible rise in the number of Th1 cells in the mammary gland that occurred concurrently with the development of mastitis. The findings of this investigation were consistent with those of the study carried out by Zhao *et al*. (2015).

Protein A (*SpA*) is the most significant virulence factor of *S. aureus* because it has the potential to impede phagocytosis (Lichota *et al*., 2024).

In order to investigate the immune response of T cells against *S. aureus*, the virulence factor protein A (*SpA*) was chosen as the target due to its surface expression and well-established immunogenicity. In order to produce interferon alph (IFN-α), the *SpA* vaccine activated CD4

cells, CD8 cells, and natural killer cells. The IFN-α is a crucial mediator of inflammation. Their phagocytic activity is increased as a result of their activation and recruitment of neutrophils.

According to Oviedo-Boyso *et al.* (2007), IFN-γ is responsible for inducing the synthesis of IL-12, which is a crucial mediator that links the innate immune response with the acquired immunological response in the mammary gland because it regulates Th1 and Th2.This study was in agreement with the findings of the study that Chen *et al.*, (2022).

The establishment of a powerful memory response by T cells against specific pathogens is the primary objective of vaccination. This event appears to take place within the first week following immunization or infection (Chand *et al.*, 2023) In comparison to crude inactivated microbe vaccines, the vaccines of the new generation, particularly those based on recombinant proteins or synthetic peptides, are not only safer but also less aggressive to the immune system.

In comparison to crude inactivated microbe vaccines, the vaccines of the new generation, particularly those based on recombinant proteins or synthetic peptides, are not only safer but also less aggressive to the immune system.

The immunogenicity of these vaccinations can be improved by employing adjuvants that are appropriate for the situation (Hassan *et al.*, 2024; Al-Rubaee *et al.*, 2020) Research has shown that adjuvants have the ability to significantly boost the immune responses to vaccines. It was underlined by Korsholm *et al.* (2009) that adjuvants have an effect on various cell populations at the injection site, which results in a rapid recruitment of selected cells.

Incomplete water-in-oil emulsion Freund's adjuvant (IFA) has been utilized as an adjuvant in both prophylactic and therapeutic vaccinations since its inception. Incomplete-Freund's adjuvant is suitable for the work of *S. aureus* because the bacteria are extracellular, and this is suitable for the composition of Incomplete Freund's Adjuvant and its work. but not intracellular to choose complete Freund's adjuvant for example. Also,incomplete Freund's adjuvant is widely used in veterinary vaccines, it has no side effects when used in animal experiments in the field, it is not expensive and is widely available in Iraq.

All vaccine adjuvants effectively facilitated cell migration to the location without causing tissue harm. Furthermore, they prompted the selective migration of neutrophils, macrophages, and lymphocytes.This study aligns with the research conducted by Vitoriano-Souza *et al.* (2012).

When the lactating female BALB/c mice were sacrificed each mammary glands were harvested aseptically, homogenized, and diluted in PBS and dropped on blood agar the results showed significant differences ((P<0.05) in bacterial count between all vaccinated groups and the control positive group.The all vaccinated groups had lower bacterial count in their mammary glands as compared to group was given *S. aureus* only,also the group was given *SpA* protein plus Incomplete Freund's adjuvant had lower bacterial count in their mammary glands with mean 0.36±0.07c log10 cfu /g as compared to control group.this study was agreed with study conducted by (Gogoi-Tiwari *et al.*, 2016).

From the pathological results various changes in the spleen were be observed. it is clear that immunization with the *SpA* vaccine induced a higher protective effect by induction reactive hyperplasia of lymphoid follicles with moderate to mild in white pulp of spleen with parafollicular and focal aggregations of mononuclear cells (histiocytes and lymphocytes) in dilated sinusoids of red pulp, also there was precipitation of eosinophilic amyloid-like material in dilated red blood sinuses also there focally around lymphoid follicles. The immunization with the *SpA* vaccine conferred extensive protection when mice were challenged with pathogenic *S. aureus* in vivo, and this immunization also elicited a Th1/T helper 17 (Th17) polarized cellular response, which may be pivotal in immunoprotection. Immunization with a non-toxigenic *SpA* vaccine combined with an adjuvant in an animal model induced protective immunity against *S. aureus* infections. The outcomes were agreed upon (Dayan *et al.*, 2016) which were indicated Th1 responses.

The tissues of spleen from *SpA* vaccine plus incomplete Freund's adjuvant group; appeared activation of lymphocytes in white pulp also there are perivascular (sinuses of red pulp) cuffing and parafollicular infiltration of lymphocytes, activation of lymphocytes also seen in interstitial of red pulp .The outcomes were agreed upon (Vitoriano-Souza *et al.*, 2012) that assumed that all vaccine adjuvants effectively facilitated cell migration to the location without causing tissue harm.

Several pathological changes in spleen of positive group assumed by the hyperplasia of lymphoid follicle in tissue sections from spleen was prominent with fusion of proliferated lymphoid follicles. Other section showed dilation of blood vessels sinuses in red pulp contains mononuclear cells (histiocytes) as compared with negative group. The study was agreed with study conducted by (Liao *et al.*, 2021).

CONCLUSIONS AND RECOMMENDATIONS

Experimental results from the present investigation demonstrated that the *SpA* vaccination is non toxic to mas-

titic mice, with the effects being amplified in the presence of IL12 and IFN-γ concentration, low in bacterial count also induce low pathological lesion in the spleen as compared to infected group. Therefore, the next steps in translating these findings are trial of the *SpA* vaccine on cows with field mastitis caused by *S.aureus* (MRSA) to reduce mastitis caused by this bacterium.

Also, There are plans to conduct field trials on dairy cows infected with mastitis in Iraq, as the disease is endemic in Iraq and is causing economic losses to our livestock.

ACKNOWLEDGEMENTS

The authors express gratitude to the head of the College of Veterinary Medicine at the University of Baghdad for their support during the project.

NOVELTY STATEMENT

Insufficient vaccination against *S. aureus* for mastitic cows, hence the current study attempted to identify potential effects of *SpA* vaccine in lactating female BALB/C mice challenged with *S. aureus*.

AUTHOR'S CONTRIBUTION

These authors each contributed equally.

Conflict of Interest

The authors have declared no conflict of interest.

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