Assessment of irradiated *Staphylococcus aureus* isolates as a vaccine

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A B S T R A C T

Studies were initiated in an attempt to produce an inactivated vaccine of high potency with low toxicity. In this study structural integrity of bacteria can be preserved when different radiation doses of gamma rays were used to inactivate *S. aureus*. The results revealed that the 7 KGy of γ- radiation was the most suitable dose for attenuation *S. aureus*. Moreover, this study demonstrated that exposure of *S. aureus* to γ- radiation at a dose of 7 KGy inactivated bacterial cells growth with low DNA damage and did not alter bacteria protein profile. Meanwhile, higher radiation doses caused an obvious DNA damage and alteration in the bacterial protein profile. Furthermore, the effect of gamma irradiated *S. aureus* on the innate and adaptive immune components after vaccination was demonstrated. All the measured immunological parameters (TLR2, IL10, IL1β, iNOs, TGFβ, MAPK and PI3K) were increased when compared to the control group. This approach could expedite vaccine production for emerging and established pathogens for which no protective vaccines exist.

Keywords: *Staphylococcus aureus*, vaccine, gamma radiation

1. INTRODUCTION

Mastitis is the most devastating worldwide disease of dairy animals and of major economic threat to the dairy farmers (Seegers et al., 2003). It is an inflammatory disease that most commonly results from bacterial infection of the mammary gland (Osman et al., 2014). According to Blood and Radostits (1989), the economic losses from the decline of farm milk productivity due to mastitis, could reach 70%. *Staphylococcus aureus* is the major cause of bovine mastitis when compared to other species of the *Staphylococcus* genus (Vasudevan et al., 2003). It is easily transmissible and responds poorly to treatment. Another facet of *S. aureus* pathogenesis is the organism's ability to maintain cellular homeostasis while enduring environmental challenges, such as changes in host cell temperature or exposure

to phagocyte-mediated reactive temperature species (Voyich et al., 2005). Most of the mastitis was treated by antibiotics application, which is primarily eliminates bacteria. However, the uncontrolled use of antibiotics in the field may leads to new problems of the resistant pathogenic strains to antibiotics and drugs residue in milk (Chotiah, 1993).

Inactivated or attenuated bacterial vaccines are produced by killing the pathogens with chemical agents, heat, or radiation. Ionizing radiation (e.g., gamma rays and X-rays) was introduced for the vaccine development strategy since the early days of vaccinology (Elena et al., 2012).

The inactivation using different chemical compounds eventually leave some residues in the final products, further some are toxic while others cause allergic responses. However, it is possible that some bacteria may escape during the chemical inactivation routes. The bacterial inactivated vaccines using ionizing radiation are more stable, retain most of their antigenicity and safer than live vaccines. Also, the bacterial particles cannot escape from inactivation by gamma irradiation and they can be stored and transported in a freeze-dried form that makes them accessible to people in developing countries (Ho Seong Seo, 2015).

Gamma radiation is electromagnetic radiation of short wavelength emitted by radioactive isotopes as the unstable nucleus breaks up and decays to reach a stable form. Radiation technology has been used in the development of human and animal vaccines, because it can remove chemical contaminants and penetrate pathogens while preserving immunogenicity (Hewitson et al., 2005).

The major advantages of ionizing radiation in vaccine development compared to ultraviolet light or chemical agents are its ability to penetrate through most biological materials, and the fact that it targets both double and single stranded nucleic acids while causing less damage to surface antigenic proteins. Moreover, there is no need to remove any chemical residue after inactivation. Although the argument remains that irradiated vaccines elicit different immune responses than those generated by heat-killed or chemically killed methods, the demand for the application of gamma radiation is increasing for the development of safe vaccines (Magnani et al., 2009).

This work aim at assessment of irradiated Staphylococcus aureus isolates as a vaccine.

2. MATERIAL AND METHODS

2.1. Collection of milk samples:

A total number of 143 samples of bovine mammary secretions including 41 and 102 clinical and subclinical milk samples respectively (according to California Mastitis Test; CMT) were collected from mastitic cows in five different dairy herds from different localities in Egypt.

2.2. Bacterial strains and growing conditions (Isolation, phenotypic and genotypic identification):

Samples were cultured on mannitol salt agar (specific media), blood agar (for detection of haemolysis) and nutrient agar

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(Oxoid media). Isolates were identified biochemically by catalase, oxidase, urease tests, phosphatase test (Quinn et al., 2003), coagulase test (Koneman et al., 2005), sugar fermentation, nitrate reduction test (Cruickshank et al., 1975) and Ornithine decarboxylase test (Kloos et al., 1991). Final identification was done using API-Staph. kit (BioMerieux) according to manufactures instructions. Reference strains used for quality control were *S. aureus* ATCC 25213 and *S. aureus* ATCC 25923.

2.3. PCR Identification of S. aureus isolates by using 16S rRNA gene:

The identification of *S. aureus* isolates was determined using PCR amplification with universal 16S ribosomal RNA primers 8F T- 3' (Monday and Bohach, 1999 and Brakstad *et al.*, 1992) as described by James

et al., (2013). DNA was isolated from pure cultures using ZR Fungal/Bacterial DNA Mini Prep™ kit (ZYMO RESEARCH). Thermal cycling was performed using a Nexus gradient Master cycler (Eppendorf, Germany) as described previously: initial denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 1 min (denaturation), 60°C for 45 s (annealing), 72°C for 1 min (extension), followed by a final extension cycle at 72°C for 4 min, and a final hold at 4°C. Amplimers of 16S rRNA genes were purified using DNA Clean and Concentrator™-25 kits (ZYMO RESEARCH) according to the manufacturer's recommendations and eluted DNA was stored at −20°C until needed. DNA products were analyzed by running on gel electrophoresis.

Table (1): List of primers used in PCR

Primer name	Primer sequence 5'-3' (reference)	product size	Specificity
16SrRNA f 16SrRNAr	5' GTA GGT GGC AAG CGTTAT CC 3' 5' CGC ACA TCA GCG TCA G 3'	228 bp	Staphylococcus genus specific primers
nuc 1 *nuc* 2	5'-GCGATTGATGGT GATACGGTT-3' 5'-AGCCAAGCCTTGACGAACTAAAGC-3'	279 bp	*S. aureus* specific primers

Gene symbol	Primer sequence 3-5	Gene bank accession no.
TLR2	5'-TGTTTGCTCCTGCGAACTCCTA-3' 5'-AGCCTGGTGACATTCCAAGACG-3'	XM_008761102.2
iNOS	5'-TAAAGGGACAGCGTCAGCGA-3' 5'-TGGGGGAACACAGTAATGGC-3'	XM_006246949.3
b-actin	5'-TGTTGTCCCTGTATGCCTCT-3' 3'-TAATGTCACGCACGATTTCC-5'	NM_031144.3

2.4. Gamma radiation facility:

Irradiation treatments of S. aureus grown on TGY agar plates were performed by using Cobalt 60 Indian Gamma cell; The National Center for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt, at room temperature (25 ± 2°C). Samples were irradiated at 4, 5, 6, 7, 8 and 9 KGy in the gamma cell with a dose rate of 1.19 kGy/h.

2.5. Protein determination:

2.5.1. Preparation of S. aureus sample for PAGE:

Bacteria isolates cultured in Brain heart infusion (BHI) broth and subjected to gamma irradiation, Immediately after irradiation 500 ml of each bacterial culture were quickly chilled in an ice ∕ ethanol bath until the temperature dropped below 10°C. Cells were harvested by centrifugation at 10,000g for 10 minutes at 4°C and the resulting pellet was washed in 10 ml of phosphate buffer saline pH 7.4 and diluted by phosphate buffer saline to 1.5ml/v. Cell suspension was sonicated for 3 minutes at level 4, then centrifuged at 11.000 g for 3 minutes. Supernatant obtained was taken and stored at 0-4ºC (Tuasikal et al., 2012).

2.5.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

The cellular protein extracts were denatured and separated in 10% sodium dodecyl sulphate-30% polyacrylamide separating gel electrophoresis according to the method of Laemmli, (1970). Using mini-protein II electrophoresis cell (Bio-Rad) at 50 volts for 4 hrs. The electrophoretic patterns of the structural bacterial proteins were matched with the full-range molecular weight protein marker ranging from 3.5 to 240 KDa (Gene Direx, Cat. No. PM008-0500).

2.6. Single cell gel electrophoresis (Comet Assay):

Single cell gel electrophoresis or Comet assay is a simple, sensitive and rapid method for the detection of DNA damage (Singh et al., 1988). Slides were prepared in duplicate per group and the test was performed for at least 3 different bacterial cells samples from each group. For the cell suspension, approximately 4×10^6 cells were mixed with 80 µl of 0.7% low-melting agarose in phosphate buffered saline (PBS) at 37ºC in a microtube, and then spread over a window microscopic slide. The slides were preloaded with 150 µl of 0.5% low-melting agarose in PBS and were specially designed for this assay. The slides were immediately placed in cold lysis buffer containing 2.5 M sodium chloride (NaCl), 100mM EDTA sodium salt Na EDTA,10mM Tris (pH 10), and 1% Triton X-100, at 4ºC for a minimum of 1 hr. After lysis, the slides were drained and placed in a horizontal gel electrophoresis tank placed in ice and filled with fresh cold electrophoresis buffer (300mM sodium hydroxide (NaOH), 1mM Na EDTA, pH 13). To allow uncoiling of DNA, the slides were kept in the high PH buffer for 20 minutes. Subsequently, electrophoresis was carried out for 20 minutes at 25 V and 300mA. The slides were then drained and flooded slowly with 3 changes of neutralization buffer (0.4M Tris, pH 7.5) for 5 minutes each and then stained with 30 ml of ethidium bromide (20 mg/l) and covered with cover

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slips. All those steps were performed under dimmed light to prevent additional DNA damage caused by visible light. A total of 50 randomly selected cells per slide were analyzed. Imaging was performed with a fluorescence microscope (Zeiss Axiovert L410 Inc., Jena Germany), attached to a digital camera (Olympus Inc., Tokyo, Japan), and equipped with a 549 nm excitation filter, and a 100-W mercury lamp. The percentage of DNA in the comet tail (DNA damage) was automatically calculated using a 'Toolbox' from the IN cell Investigator analysis package (GE Healthcare Life Sciences), Tail moment was calculated as a product of tail length multiplied by tail % damage.

2.7. Mice inoculation:

Forty female mice of 30 ± 5 gm body weight, and 4-6 weeks age were divided into 5 groups (8 mice per each group) and non-infected group considered as a control group. Four groups were inoculated i/p with 0.25 ml/ of irradiated and non-irradiated *S. aureus* (0, 7, 8 and 9 KGy) for three times with one-week interval and the mice were bled 14 days after the third booster (Dane Parker 2017).

2.8. Experimental protocol (Morton et al 1970):

Studies investigating the pathogenesis of *S. aureus* infection have relied heavily on the use of mouse models. Mice have been used to understand the role of virulence factors play during infection as well as the contribution of specific host pathways and factors in the response to *S. aureus*.

After an acclimatization period of 7 days, mice were randomly allocated and divided into five equally sized groups, ten animals each, as follows: Group I, mice, kept as controls, received daily i/p. 1 ml of physiological saline. Group II, mice received non-irradiated *S. aureus* at a dose of 1×10^6 cells. Group III, mice were injected with 0.1 ml of irradiated (7 KGy) *S. aureus* i/p. at a dose of 1×10^6 cell. Group IV, mice were i/p. injected with 0.1 ml of irradiated (8 KGy) *S. aureus* at a dose of 1×10^6 cells and Group V, mice were i/p. injected with 0.1 ml of irradiated (9 KGy) *S. aureus* at a dose of 1×10^6 cell. The mice were injected once /week for 3 times and sacrificed 7 days after the last injection and blood samples were collected from all groups.

2.9. Biochemical assays (Borreham et al., 2000):

The quantitative determinations of mice interleukin -6 (IL-6), interleukin -1β (IL-1β) and Tumor growth factor (TGF-β) concentrations in serum were carried out using mice ELISA kits (R&D Systems and MyBioSource).

2.10. Western blotting of MAPK and Phosphoinositide 3-kinases (pi3K):

Liver tissue proteins were extracted using TRIzol reagent (Invitrogen) according to Chomczynski (1993), and protein concentrations were quantified according to Bradford (1976). The membranes were developed and visualized by chemiluminescence using Amersham detection kit according to the manufacturer's protocols and then exposed to X-ray film. Quantification of MAPK and pi3K proteins was carried out using a scanning laser densitometer analysis (Biomed Instrument Inc., USA).

2.11. RNA extraction and cDNA synthesis:

To investigate the changes in mRNA expression for TLR2 and iNOS genes, total RNA was isolated from 50mg liver using TRIzol reagent (Invitrogen) according to Chomczynski (1993). First-strand complementary DNA (cDNA) synthesis was performed using reverse transcriptase (Invitrogen) throughout template 1 μg RNA.

2.12. Quantitative real-time polymerase chain reaction (qPCR):

RT-PCR was performed in a thermal cycler Step One Plus (Applied Biosystems, USA) using the Sequence Detection Software (PE Biosystems, CA). A reaction mixture of total volume 25 μl consists of 2× SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer (TLR2 and iNOS), and 2 μl of cDNA. PCR thermal cycling conditions included an initial step at 95 °C for 5 min and 40 cycles at 95 °C for 20 s, 60 °C for 30s, and72°Cfor20s. The relative mRNA expression of the studied genes was calculated using the comparative threshold cycle method of Pfaffl (2001).

2.13. Statistical analysis:

All determinations were done in triplicate. Statistical analysis was done using SPSS (version 20) program. Mean and standard error were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples.

3. RESULTS

3.1. Identification of bacteria isolates by multiplex PCR:

Identification of bacterial isolates using m-PCR identified bacteria isolates as *Staphylococcus aureus*. M-PCR results were analyzed using agarose gel electrophoresis showed two bands positive amplification of 228 bp specific for the Genus *Staph*ylococcus, and positive at an amplification of 279 bp specific for *S. aureus*.

3.2. Effect of gamma rays on S. aureus:

Preliminary tests were conducted to determine the lethal doses of irradiation for *S. aureus*. Attenuation of bacterial isolates was achieved by exposure to different doses of gamma radiation (4-9 KGy) to study the effect of gamma rays on bacterial cells viability, protein profile and DNA.

3.3. Effect of gamma rays on S. aureus protein profile:

3.3.1. Electrophoretic protein profile using SDS-PAGE analysis:

Samples of suspended *S. aureus* were exposed to different doses of γ- radiation (4, 5, 6, 7, 8 and 9 KGy). All bacteria samples were inoculated on nutrient agar plates and recorded no bacterial growth at 5 KGy. Extracted bacterial proteins were analyzed using SDS-PAGE analysis. The results showed that protein profile of irradiated *S. aureus* at doses of 4 to 7 GKy showed no alterations in the protein profile in comparison to the control of non-irradiated *S. aureus*. Meanwhile, an obvious alteration in the bacterial protein profile of irradiated *S. aureus* at doses of 8 and 9 GKy was revealed by SDS-PAGE analysis (Table 2) and Fig. (1).
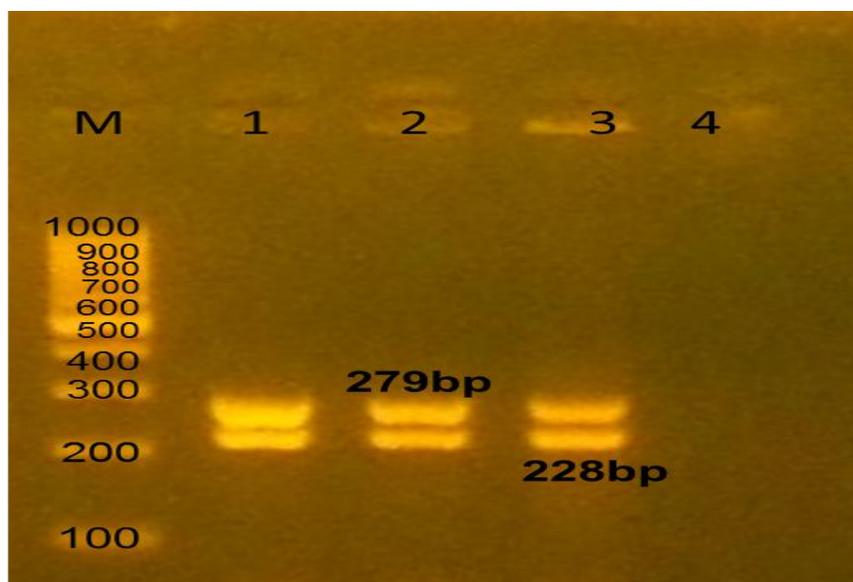
Figure.1 Identification of *S. aureus* isolates by multiplex polymerase chain reaction
Lane M: 100bp marker.
Lane 1: Amplification of 228 and 279 bp fragment of *S. aureus* local strain sample.
Lane 2: Positive control of *S. aureus* (ATCC 25213) DNA.
Lane 3: Positive control of *S. aureus* (ATCC 25923) DNA.
Lane 4: Negative control (without DNA).

Table (2): Electrophoretic protein pattern of γ- irradiated *Staphylococcus aureus*.

Marker KD	Control (0KGy)	Gamma radiation Dose (KGy)											
		4	4	5	5	6	6	7	7	8	8	9	9
240	247	241	241	239	239	241	242	242	242	-	-	-	-
165	203	216	216	216	216	216	216	216	216	216	216	216	216
125	195	195	195	195	195	195	195	195	195	195	195	195	195
92	128	128	128	128	-	128	128	128	128	-	-	-	-
72	122	122	122	122	122	122	122	122	122	122	122	-	-
57	116	116	116	116	116	116	-	116	116	-	-	-	-
42	114	114	114	114	-	114	114	114	114	114	114	-	-
31	84	84	84	84	84	84	-	84	84	84	84	-	-
24	77	77	77	77	77	77	77	77	77	-	-	-	-
18	59	59	59	59	59	59	59	59	59	-	59	-	-
15	55	55	55	55	55	55	55	55	55	55	55	-	-
8	41	41	41	41	41	41	41	41	41	-	-	-	-
3.5	37	37	37	37	37	37	34	34	34	-	-	-	-

3.4. Effect of γ-radiation on S. aureus DNA:

3.4.1. Comet Assay:

The DNA damage of *S. aureus* exposed to different doses of γ-rays was analyzed quantitatively by comet assay. The typical DNA comet for the control *S. aureus* showed almost rounded nuclei Figure (3a). The irradiated samples of *S. aureus* at 7 KGy (Figure 3b) showed nuclei of bright

head with no tail which means there was no DNA damage in *S.aureus* cells. On the other hand, a typical DNA damage was shown for *S. aureus* samples exposed to 9GKy of gamma rays, the cells appeared to have small head with long diffused tail (Figure 3 c). The DNA damage in *S. aureus* cells exposed to different doses of γ- rays was expressed as % of DNA damage, tail moment (TM) and tail length (TL) (Table 3, Fig. 3).

Comet assay results showed that non-irradiated *S.aurues* have small tail length (16.5 ± 6.7) and low percentage of DNA damage (10.8 ± 2.1). Exposing *S.aurues* to different doses of gamma rays induced DNA damage, which increases in proportion to gamma rays doses. The results showed that, changes caused in the DNA by gamma radiation in *S.aureus* cells was moderate at dose of 7 KGy which showed tail length (41.18 ± 17.1) with percentage of damage (49.33 ± 2.3) in comparison to control. While, high percentage of DNA damage (59 ± 4.5) and maximum tail length (37.9 ± 29.3) induced by gamma radiation at a dose of 9 KGy.
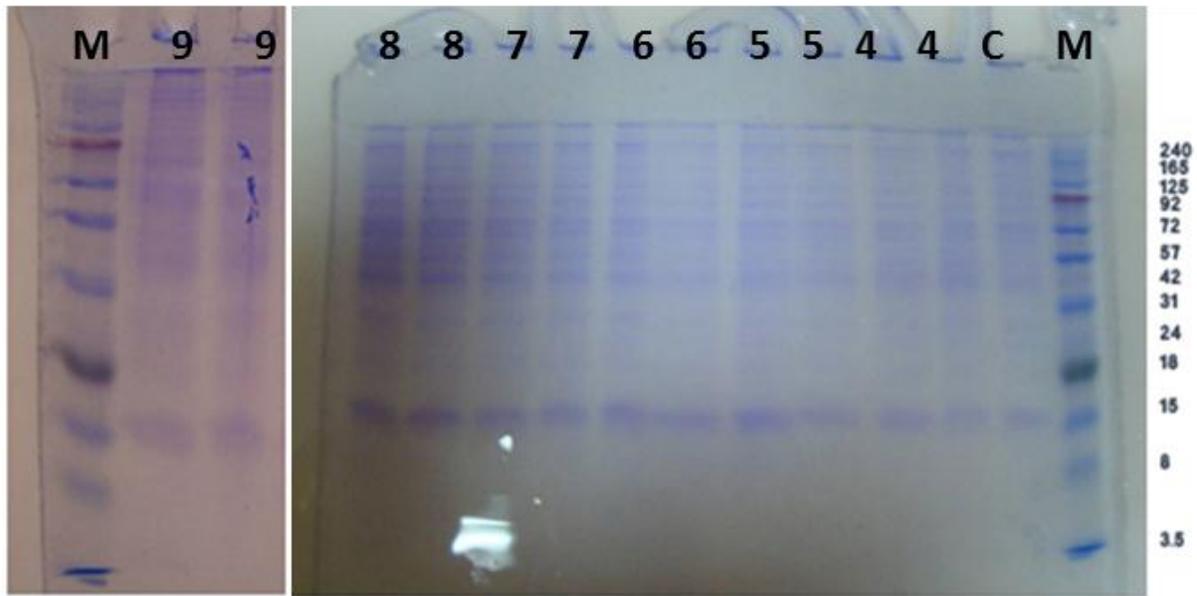
Figure (2): SDS-PAGE electrophoresis of *S. aureus* protein profile pattern after exposure to γ-radiation.
Lane M   : Marker protein molecular weight: 3.5 – 240 kDa.
Lane C   : Control *S. aureus* non-irradiated.
Lane 4   : *S. aureus* exposed to γ- radiation dose of 4 KGy.
Lane 5   : *S. aureus* exposed to γ- radiation dose of 5 KGy.
Lane 6: *S. aureus* exposed to γ- radiation dose of 6 KGy.
Lane 7: *S. aureus* exposed to γ- radiation dose of 7 KGy.
Lane 8: *S. aureus* exposed to γ-radiation dose of 8 KGy.
Lane 9: *S. aureus* exposed to γ- radiation dose of 9 KGy.
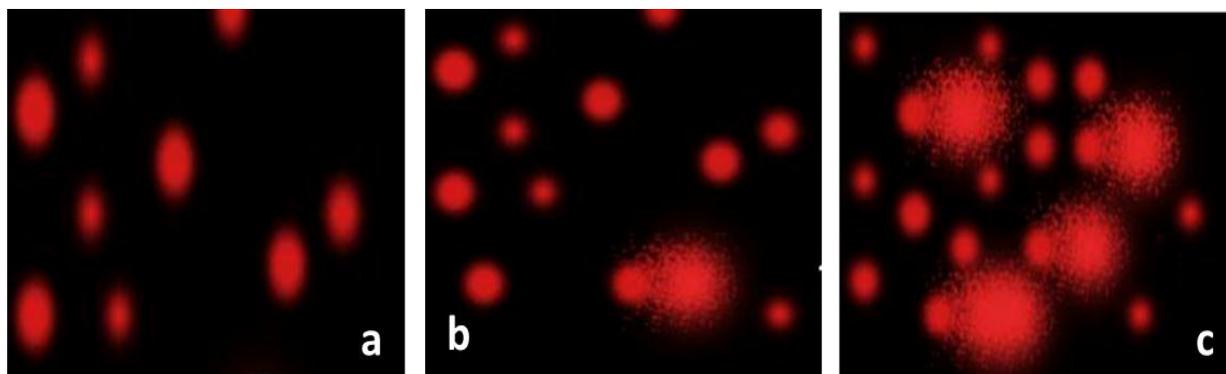
Figure (3). Typical fluorescence microscope images (comets). a: control, non-irradiated (0 KGy) *S. aureus,* b: *S. aureus* exposed to gamma radiation at a dose of 7 KGy, c: *S. aureus* cells exposed to gamma radiation at a dose of 9 KGy

Table (3): Comet analysis of DNA breakage of *S. aureus* cells exposed to γ- radiation.

Gamma radiation dose (KGy)	% of DNA Damage	Tail Moment	Tail Length (µm)
Control (0)	10.8 ± 2.1	1.15 ± 0.03	16.5 ± 6.7
5	48.66 ± 3.2^a	2.77 ± 0.85^a	37.57 ± 15.2^a
6	44.00 ± 1.9^a	2.85 ± 0.67^a	40.58 ± 16.9^a
7	49.33 ± 2.3^a	2.91 ± 0.92^a	41.18 ± 17.1^a
8	56.00 ± 3.6^a	4.33 ± 0.98^a	58.72 ± 23.3^a
9	59.00 ± 4.5^a	5.45 ± 1.20^a	73.90 ± 29.3^a

Results are represented as mean ±SE. [a], significant to control ($p<0.05$).

3.5. Effect of γ--irradiated S. aureus on the immune system of mice:

The innate and adaptive immune responses are key components of host responses after vaccination. Herein the pattern of innate immune response to *S. aureus* inoculation was demonstrated in Fig. (4).

3.6. The effect of S. aureus inoculation on TLR2 and iNOs relative gene expression/β actin:

The TLR2 and iNOs relative gene expression/β actin were analysed in the liver tissue using RTqPCR. Inoculation of mice with *S. aureus markedly* induced transcript level of TLR2 mRNA almost four folds compared to the control group. Mice challenged with non-irradiated, irradiated *S. aureus* at 7, 8 and 9 KGy induced alteration in the transcript level of TLR2 mRNA recording 4.65, 4.61, 6.37 and 8.22 respectively (Table 4 and Fig.4)

In the other hand, iNOs mRNA transcript level was significantly elevated almost six folds in mice inoculated with *S. aureus* compared to the control. The transcript mRNA level of iNOs in mice injected with non-irradiated, irradiated *S. aureus* at 7, 8 and 9 KGy was significantly increased to be 6.49, 6.85, 6.18 and 6.42, respectively (Table 4 and Fig.4)

Table (4): Effect of γ- irradiated Staphylococcus *aureus* inoculation on the immune system.

Group	TLR2 gene expression /β-actin	iNOs gene expression /β-actin	IL-10 (pg/ml)	IL-1β (pg/ml)	TGF-β1 (pg/ml)	MAPK Relative intensity/ β actin	Pi3K Relative intensity/ β actin
Control	1.0± 0.01[b]	1.0± 0.0[b]	133.45 ±10.73[b]	25.03± 1.07[b]	42.85± 4.54[b]	1.0± 0.01[b]	1.0± 0.01[b]
Non-irradiated 0 KGy	4.65± 0.20[a]	6.49± 0.59[a]	91.87 ±9.54[a]	108.47± 9.87[a]	75.47± 8.64[a]	1.11± 0.02[a]	1.27± 0.1a
7 KGy	4.61± 0.20[a]	6.58± 0.32[a]	82.02 ±10.79[a]	108.6± 15.8[a]	111.77± 13.43[ab]	1.26± 0.05[ab]	3.52± 0.2[ab]
8 KGy	6.37± 0.19[ab]	6.18± 0.64[a]	90.97± 8.63[a]	١٢2.2± 10.95[a]	108.07± 10.95[ab]	1.38± 0.06[ab]	5.38± 0.4[ab]
9 KGy	8.22± 0.19[ab]	6.42± 0.80[a]	٨٥.08± 8.89[a]	١٢0.75± 9.87[a]	124.18± 13.54[ab]	1.26± 0.04[ab]	5.8± 0.5[ab]

The results are represented as the mean±SE, where n=6. Where, a: the results are significant ($p<0.05$) compared to control group, b: results are significant ($p<0.05$) compared to group infected with non-irradiated *Staph. aureus*.

TLR2: toll like receptor
IL10: interleukin 10
TGFβ: transforming growth factor Beta
Pi3K: phosphoinositide 3 kinase

iNOs: Inducible nitric oxide synthase
IL1b: lymphocyte activating factor
MAPK: mitogen activated protein kinase
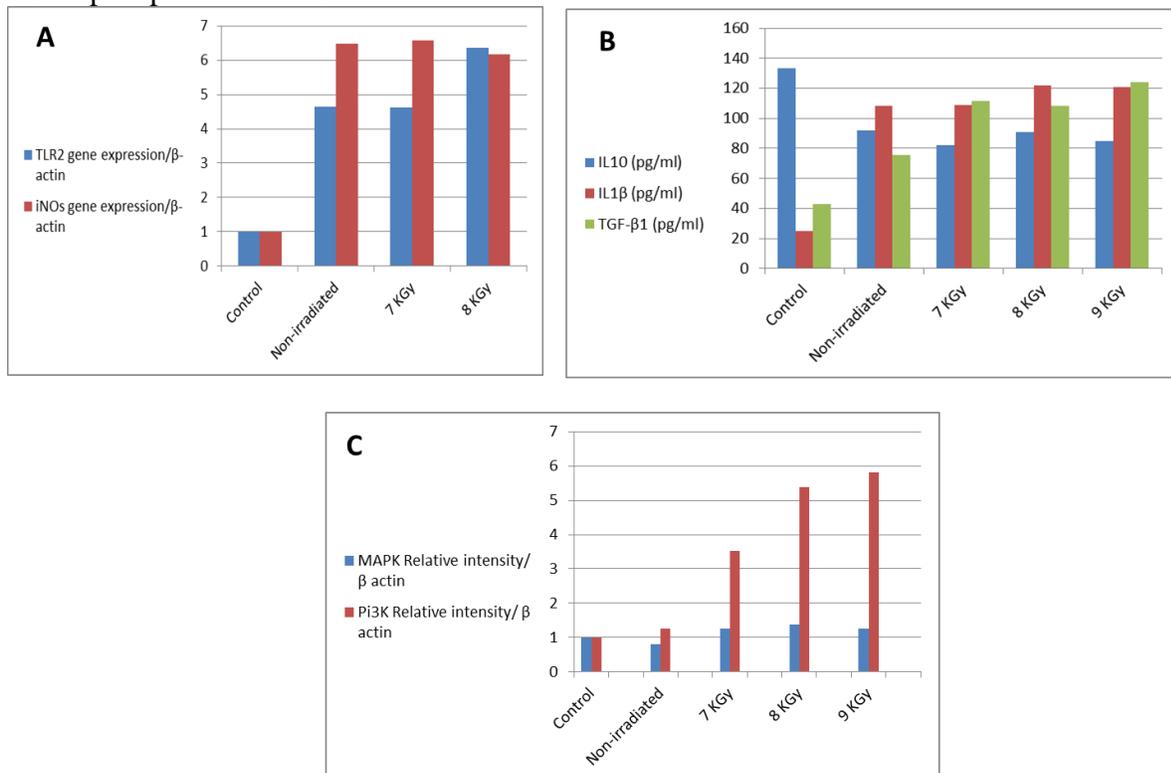
Figure (4): *Staphylococcus aureus* inoculation in female mice activating the immune system.

3.7. The effect of S. aureus inoculation on inflammatory parameters: IL-10 and IL-1β levels:

Disturbance in the pro- and anti-inflammatory cytokines balance eventually results in an exaggerated inflammatory response, multiple organ dysfunction syndrome, and septic shock. Therefore, the cytokine response to *S. aureus* infection in mice was examined.

Cytokine IL-10 is an immunological and anti-inflammatory parameter which was significantly reduced with bacterial infection compared to the control group. IL-10 level in the mice serum was significantly decreased showing a level of 91.87, 82.02, 90.97 and 85.05 pg/ml in challenged mice previously inoculated with *S. aureus* of non-irradiated, irradiated at 7, 8 and 9 KGy, respectively (Table 4 and Fig. 4).

Inoculation of radiation killed *S. aureus* induced IL-1β and TGF-β1 level in mice serum. While, IL-10 concentration was significantly elevated about 4 folds to reach 108.47, 108.60, 122.2 and 120.75 pg/ml of serum in mice previously inoculated with *S. aureus* of non-irradiated, irradiated at 7, 8 and 9 KGy respectively. TGF-β1 level was raised almost double in response to non-irradiated *S. aureus* inoculation. While inoculation with irradiated killed bacterial cells elevated TGF-β1 concentration to 3 folds to be 111.77, 108.07 and 124.18 pg/ml in mice injected with bacterial cells exposed to 7, 8 and 9 KGy respectively.

3.8. Effect of S. aureus inoculation on protein intensity of MAPK, Pi3K relative to β-catenin

After ligation of TLR2 or NOD2 to *S. aureus* pathogenic factors that activates the gene expressions of MAPK signaling pathway including extracellular PI3K/Akt signaling pathway, and accounting for the production of the downstream cytokines

MAPK and Pi3K proteins relative intensity was significantly increased in response to inoculation with *S. aureus* compared to control group. Western blotting analysis of MAPK showed significant increase in mice inoculated with non-irradiated, irradiated at dose of 7, 8 and 9 KGy to be 0.8, 1.26, 1.38 and 1.26 respectively. Pi3K level of protein intensity was significantly elevated with MAPK level. Mice inoculated with non-irradiated, irradiated at a dose of 7.8 and 9 KGy. In the same line Pi3K level was increased from 1.27 in the group infected with non-irradiated bacteria to be 3.52, 5.38 and 5.8 in mice challenged with *S. aureus* irradiated at doses of 7, 8 and 9 KGy respectively (Table 4 and Fig. 4).

A relative level of TLR2 and iNOs mRNA expressions were analyzed by quantitative real-time polymerase chain reaction (RTqPCR); values were rectified by mRNA expression of β-actin for each sample, compared with non-infected mice. B: Quantitative analysis by Eliza of TGF-β1, IL-1β and IL-10. C: relative intensity of PI3K and MAPK protein / β-actin using western blotting (Table 4 and Fig. 4).

4. DISCUSSION

During radiation treatment, DNA molecules are heavily damaged, preventing them from functioning normally. Bacterial cells exposed to radiation will be affected in different ways: arrest cell cycle progression and repair of DNA lesions (Lee *et al*., 2010).

In this study, we have developed an attenuated radio-vaccine of *S. aureus* using gamma radiation at a dose of 7 kGy, which act as an immunostimulator to the infected host without molecular adverse and good safety.

During development of irradiated vaccine, SDS-PAGE of proteins normally reveals information on the electrophoretic pattern of the irradiated samples (Dosselli et al., 2012).

SDS-PAGE showed that minor changes were found in the protein profile of irradiated bacterial strain up to the dose of 7 KGy, and major changes were appeared at higher doses of 8 and 9 KGy, indicating that *S. aureus* was inactivated by 7 KGy irradiation dose by inhibiting its replication capability while keeping its antigenic. Previous studies of reported that gamma radiation, a dose of 5.9 kGy, inhibited *S. aureus* capability of replication.

In conclusion, 7 KGy the electrophoretic protein profile of *S. aureus* and this result came in agreement with Tuasikal et al., (2012), who recommended irradiated producing base material for mastitis vaccine.

Inactivation of microorganisms by irradiation comes from the inhibition of DNA repair mechanism by increased energy demand of homeostasis on the cell (Hong et al., 2008). Resulting in typical morphological and biochemical changes including nuclear chromatin condensation, cell shrinking, blabbing of the cytoplasmic membrane, break-up of DNA and the final cell fragmentation into apoptotic bodies (Wyllie et al., 1984).

In the current, the comet assay appears to be an appropriate tool to estimate DNA base damage in cells exposed to different doses of ionizing radiation produces a variety of DNA damage types and degrees. The comet assay avoids the induction of artefactual DNA oxidation that occurs in most analytical cells. This seems to come in accordance with the study of Pouget et al., (1999), who used this approach in order to measure DNA damage levels in *S. aureus* exposed to γ-radiation and found that the comet assay provides a mean to measure base damage without DNA extraction. The study also recommended using comet assay to measure DNA damage level of irradiated *S. aureus*. The assay allows the simultaneous detection of strand breaks after exposure to different radiation doses.

In order to estimate DNA damage levels within the range of doses compatible with the application of the comet assay (from 0 to 9 kGy), it was found that the DNA percent of change was less than 50% within the used doses (0–7kGy). On the other hand, protein analysis by SDS-PAGE revealed that, radiation doses up to 7 KGy caused this results pointed to 7KGy as the proper irradiation dose to be used for

inactivation of *S. aureus* with minimum DNA and protein damage effect. The DNA resistance to ionizing radiation deleterious effects in the viable cells compared to free (extracted) DNA for the presence of low molecular mass scavengers of free radicals, antioxidant defense system that mop up free radicals in cells, also to physical protection of DNA by packaging in cells, and cellular repair system of damaged DNA (Hall and Giaccia, 2006).

From previous studies, *S. aureus* exposed to 5.9 kGy failed to growth. Irradiated *S. aureus* vaccine elicits anti-*S. aureus* specific antibodies reducing *S. aureus* colonization in the kidneys and skin (Ho Seong Seo, 2015).

In order to evaluate stimulatory effect of attenuated *S. aureus*, different immunological parameters were evaluated in female mice inoculated with irradiated *S. aureus*. Bacterial pathogenicity in the case of *S. aureus* is multifactorial. *S. aureus* possesses a number of potential virulence factors including cell wall-associated and secreted proteins, which especially cause immunogenic and toxic injuries (Parker, 2017).

Our results showed marked activation of the immune system of female mice represented in significant elevation in mRNA levels of TLR-2 and iNOS and the protein intensity of signal transduction MAPK and Pi3K in the liver and serum levels of inflammatory markers IL-1β and TGF -β1 with significant reduction in IL-10 level.

From the presented immunological results, it was obvious that, the level of the

TLR2 showed a remarkable decrease in the challenged mice that were previously inoculated by the non-irradiated *S.aureus*, then it reached to its highest levels in the challenged mice that were previously inoculated with the irradiated *S.aureus,* and this result may support the protection role of the irradiated *S.aureus* in the challenged mice. Meanwhile, TLR2 reached to its highest levels in the challenged mice at a dose of 9 followed by 8 KGy, and the least was at the dose 7 KGy. An explanation for these results was offered by Knuefermann, (2004) who referred it to the protective effect of the *S. aureus* treated by lower dose of radiation but the higher dose of radiation destroyed the whole bacteria leading to infection of the mice when subjected to the challenge dose of the *S. aureus* leading to increasing the level of the TLR2 as it is a key sensor for detecting *S. aureus* invasion.

Interleukins (ILs) are a group of cytokines (secreted proteins and signal molecules) that were first seen to be expressed by white blood cells (leukocytes). The function of the immune system depends in a large part on interleukins. The majority of interleukins are synthesized by helper CD4 T lymphocytes, as well as through monocytes, macrophages, and endothelial cells. They promote the development and differentiation of T and B lymphocytes, and hematopoietic cells (Ben et al., 2011).

IL-10 as it has profound clinical value, especially during vaccinations (Jinhai et al., 2012) and essential for efficient elimination of bacteria and thereby for protection against *S. aureus* infection as it is a factor produced by mouse T helper type 2 cells

which inhibit Th1 cytokine synthesis acting as an anti-inflammatory molecule and subsequently down-regulates cell mediated immune functions to enhance the bacterial clearance (Gjertsson et al., 2002).

In the on-going study, the challenge of mice which are previously inoculated by the irradiated *S. aureus* resulted in more enhancements in IL-10 expression in mice inoculated by 7 KGy gamma rays as it recorded 109.02 in comparison to other groups which means more protection. Similar observations were recorded by Ewa et al., (2018), who assured more protection levels of the IL-10 with innate and adaptive immune responses. At the same time, Leech et al., (2017) reported that *Staphylococcus aureus* has previously been shown to manipulate the IL-10 response as a mechanism of immune evasion during chronic systemic and biofilm models of infection. They referred the detrimental role of IL-10 production to its facilitating effect on the bacterial persistence via the same mechanism of controlling pro-inflammatory T cell responses. Their findings also demonstrated that induction of IL-10 has a major influence on disease outcome after *S. aureus* challenge (i.e. Too little IL-10 may tend toward fatal host-mediated pathology through excessive activation of T cells and associated phagocyte-mediated damage).

Fowler and Proctor, (2014) stated that, IL-1β level served as a biomarker for vaccine efficacy as the protective vaccine can promote its production. They also added that, the low level of IL1β can be used as an indicator for low vaccine potency. These data may come in agreement and give an explanation to the obtained results as when the experimental groups were vaccinated by the irradiated *S. aureus* then challenged by the virulent one, the level of IL-1β was changed in comparison to the control group.

iNOS induced in the spleens and kidneys of *S. aureus*-infected mice for protection of mice against *S. aureus* infection. When mice were infected with a challenge dose of *S. aureus*, the expression of iNOS mRNA in the spleens and kidneys was already induced at 3 h post infection and persisted thereafter. So, higher level of iNOS means higher level of protection against *S. aureus* (Sanae et al., 1998).The obtained results showed that, iNOs recorded its highest levels in challenged mice which are previously inoculated by radiated *S. aureus* at a dose of 7 KGy which indicate that the mice are well protected against *S. aureus* infection but the level of iNOs decreased at the mice inoculated by radiated *S. aureus* at higher dose of radiation (8 and 9 KGy). These results may be nearly similar to that of the pervious study, who found that the high level of iNOS, which is induced by *S. aureus* infection, is important in the protection of mice against death from *S. aureus* infection. They added that, when mice were infected with a lethal dose of *S. aureus,* iNOS expression is induced immediately after *S. aureus* infection.

TGF-β regulateS mammary gland development, as well as mediating mammary gland host innate immune responses to *S. aureus*. (Douglas et al., 2006). In a study of Zhigang et al., (2015), increases in TGF- β levels were detected within 40 h of *S. aureus* vaccination and mediating mammary gland host innate immune responses to *S. aureus*. These data

explain our results as the TGF-β level was increased after challenge of groups inoculated with irradiated *S. aureus* strain recording high level when compared to the group inoculated with Non-irradiated staph aureus strain. .And this may be due to the role of TGF-β in regulating inflammatory processes.

Chen et al., 2013 recorded that, they were released in response to *S. aureus* secreted bacterial exoproducts. In the present study Mitogen activated protein kinase (MAPK) was released in response to *S. aureus* bacterial exoproducts. These results were illustrated by Fuguang et al., (2013) who mentioned that, the MAPK signalling pathways, which regulated the expression of pro-inflammatory cytokines and decreased the susceptibility to mastitis and play a central role during inflammatory responses leading to the release of pro-inflammatory cytokines, such as IL-1β.

(PI3Ks) are a family of signaling enzymes that regulate a variety of important cellular functions, including growth, cell cycle progression, apoptosis, migration, metabolism and vesicular trafficking (Engelman et al., 2006). In our investigation the increased level of phosphoinositide 3 kinase (PI3K) after challenge with virulent *S. aureus* strain indicated prevention of *S. aureus* internalization and powerful vaccination effect (Adam et al., 2015).

The findings in the present study demonstrated that the exposure of *S. aureus* to gamma radiation at a dose of 7 KGy is a suitable method for production of *S. aureus* inactivated safe potent vaccine with low DNA damage, no protein profile alteration

and good innate and adaptive immune responses with immunogenic signaling of immune response components (TLR2, IL10, IL1β, iNOs, TGFβ, MAPK and PI3K). This approach could expedite vaccine production for emerging and established pathogens for which no protective vaccines exist.

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